16

STABILITY: PHYSICAL AND CHEMICAL

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16.1 INTRODUCTION

The pharmaceutical properties of a drug, such as solubility, dissolution, and toxicology, can change dramatically when a drug undergoes chemical or physical degradation. This can severely limit the drug's efficacy and safety. After a compound is synthesized it must undergo several steps before being used by the patient. Some of these steps include formulation into the final product, storage during manufacturing and distribution, and dispensing to the patient. Understanding the stability of a potential drug is a vital component to the pharmaceutical development process because if the drug changes during any one of these steps it may not have the desired pharmaceutical effects. Therefore, the evaluation of drug stability becomes part of the development process as early as candidate selection and continues for the lifetime of the product. For the purposes of this chapter, however, we only consider drug stability up to and through the formulation process.

There are four main reasons to study the stability of drugs [1]:

1. *Quantitation of Drug Loss*. It is essential to know how fast the drug degrades so that an accurate shelf life can be determined.
2. *Identification and Quantitation of Degradation Products*. It is important to know what the degradation products are, as they may be toxic, and to know at what levels they are produced.
3. *Change in Aesthetic Properties Upon Degradation*. If a drug product changes notably in its aesthetic appearance it is presumed adulterated, even if by all other criteria the drug product is perfectly safe for use. This typically occurs if one of the degradation products either has an offensive odor or is highly colored. An example is tetracycline degrading to form epianhydrotetracycline, which is red [2, 3].
4. *Degradation of Drug After Administration*. Even if the drug is stable in its formulation, it is important to know how it will degrade under physiologic conditions; for example, degradation at low pH for an orally administered drug.

There have been numerous articles and books published on the subject of stability. The best way to begin a stability study is to perform a thorough search of the literature to find similar compounds that have been studied previously, which may provide some insight into potential reaction mechanisms and products. In doing this, a foundation is laid for the studies that are to be performed on the new compound.

16.2 SOLUTION STABILITY

Stability studies on new drug candidates are typically performed in the solution state. These studies are used as an aid in candidate selection and are intended to identify compounds that would likely pose a significant challenge during the drug development process. Solution stability is also important as a guide for the formulation process. For example, if a drug is very sensitive to low pH then an enteric-coated tablet may be required for oral drug delivery.

16.2.1 Chemical Stability

Chemical stability is a measure of the susceptibility of a compound to undergo a chemical reaction in which atoms are rearranged via bond breakage and bond formation to produce a different chemical entity. Drugs have a myriad of different chemical structures and are thus subject to a variety of different possible mechanisms of degradation. Most drugs degrade by one or more of the following mechanisms: hydrolysis, dehydration, isomerization, racemization, decarboxylation, elimination, oxidation, or photodegradation [1, 4, 5]. It may also be possible to have reactions between two drug molecules [6] or between a drug molecule and an excipient within a formulation [7].

Hydrolysis is one of the most frequent degradation mechanisms observed in pharmaceuticals for two main reasons. First, drugs are typically dissolved in media primarily composed of water, which results in a relative abundance of water that is available to react. Second, ester and amide functional groups degrade primarily via hydrolysis and these are two very common functional groups in pharmaceuticals.

Proteins and peptides are steadily becoming more popular as pharmaceuticals. These biopharmaceuticals, as they are called, are subject to the same degradation reactions as "traditional" small molecule pharmaceuticals, and in some cases these reactions are given specific names to describe both the reaction type and the amino acid that is involved [8, 9]. For example, the hydrolysis of the amino acid asparagine to form aspartate and isoaspartate is referred to as deamidation. Biopharmaceuticals are inherently more complex than small molecules because of their increased size and higher levels of organization (primary, secondary, tertiary, and quaternary structure), which leads to significantly increased complexity when the stability of these compounds is considered [9–13].

16.2.2 Physical Stability

Physical stability is a measure of the propensity of a compound to undergo changes that do not involve the breaking or formation of covalent bonds. In this case the compound has the exact same chemical structure but the physical state has changed in some way. For small molecules, two potential physical stability problems in solution are precipitation and aggregation [14, 15]; however, these are both relatively rare when compared with chemical degradation mechanisms of small molecules. On the other hand, physical stability is a major concern in the case of biopharmaceuticals. If the protein/peptide denatures (typically through the loss of quaternary and tertiary structure) then it can adsorb to surfaces (such as glass or plastic), aggregate, and precipitate [16]. In the case of biopharmaceuticals, in particular, these physical

changes typically are not easily reversed [10, 17]. One additional mechanism of physical degradation is vaporization. If the drug has a sufficiently high vapor pressure it can evaporate from the formulation whether it is in the solution or solid state. This phenomenon has been observed in nitroglycerin and can result in large variability in the drug content of tablets after being stored in the same container [18].

16.3 SOLID-STATE STABILITY

Pharmaceuticals are typically formulated in the solid state for several reasons. One major advantage is increased patient compliance, because it is easy to dose the pharmaceutical. It is also discreet, meaning that a patient can use it in a social setting and not draw the attention of other people while doing so. Another advantage is that it is typically cheaper than developing and producing a sterile product for parenteral administration. One of the main reasons for formulating a pharmaceutical in the solid state is that typically the compound is more stable in the solid state compared to the solution state. However, the solid state presents its own unique challenges.

16.3.1 Physical Stability

In the solid state a compound can be either amorphous or crystalline. A solid is amorphous if it lacks long-range order in the arrangement of the molecules. Amorphous materials can exist in one of two states: glassy or rubbery. The glassy state (low temperature state) is a more rigid state while the rubbery state (high temperature state) is a much more mobile state and hence is less stable. An amorphous material has a characteristic glass transition temperature (T_g), which is the temperature at which the material changes from the glassy to the rubbery state, or vice versa [19, 20].

If the molecules in the solid state are packed in a regular pattern with long-range order, then the solid is said to be crystalline. The different conformations or orientations in which molecules are able to pack are referred to as crystal forms. If the molecules pack in one crystalline form the material may be called Form I and if the molecules pack in a different crystalline form the material may be called Form II. The naming of these crystal forms is arbitrary and usually reflects the order in which they were discovered. They are usually represented by either Roman numerals (e.g., I, II, III, IV), letters of the Latin alphabet (e.g., A, B, C, D), letters of the Greek alphabet (e.g., α, β, γ, δ), or Arabic numerals (e.g., 1, 2, 3, 4). These different crystalline forms must be chemically identical in order to be called polymorphs. If the solid also contains solvent molecules in the crystal structure then it is referred to as a solvate, and if that solvent is water then it is specifically called a hydrate. The stoichiometry of the water molecules present in the crystal structure is represented by the name (e.g., a monohydrate has one molecule of water per molecule of the compound of interest, a dihydrate has two molecules of water, etc.). On the other hand, if the crystal structure contains no water then it is referred to as an anhydrate. If multiple anhydrous forms of the same compound exist they are said to be polymorphs. Additionally, if multiple monohydrate forms of the same compound exist they are also said to be polymorphs. Other terms (such as solvatomorphs and pseu-

dopolymorphs) have been used to describe solvates, but these are less favorable because their definitions are not as clear. Crystal habits are another phenomenon of crystalline solids, where crystals have the same internal structure (molecular packing) but have different external shapes or morphologies (e.g., needles, plates), which leads to differences in their pharmaceutical properties, including mixing, flowability, and dissolution [21].

The stability of a polymorph is represented by its free energy. The free energy of a polymorph is proportional to its solubility. Since polymorphs have different free energies, they will also have different solubilities [19]. Therefore, the solubility of polymorphs can be used to determine their relative stability. Only the form with lowest free energy at a specified temperature and pressure is said to be stable. All other forms are said to be metastable relative to that form. If at any temperature the relative solubility difference between two polymorphs is maintained, as shown in Fig. 16.1a, then the polymorphs are said to be related monotropically. However, if at some temperature the relative solubility of the polymorphs inverts, as shown in Fig. 16.1b, then they are said to be enantiotropically related. Unfortunately, solubility studies have their drawbacks when it comes to identifying monotropic and enantiotropic systems. One of the major problems is a limited temperature range over which the measurements can be made. While many times the data is linear on a van't Hoff plot (as in Fig. 16.1), the extrapolation of this data outside the experimental range is often prone to large errors [21]. Differential scanning calorimetry (DSC) can be a very useful tool in identifying monotropic and enantiotropic systems, because it provides heats of transition and fusion for polymorphs and polymorphic conversions. These values can then be interpreted using the rules developed by Burger and Ramberger [22, 23]:

1. *Heat* (*or Enthalpy*) *of Transition Rule*. If a solid–solid transition is endothermic then the polymorphs are related enantiotropically; if the transition is exothermic then they are related monotropically.
2. *Heat* (*or Enthalpy*) *of Fusion Rule*. If a polymorph has a lower heat of fusion but a higher melting point than another polymorph, then the two forms are related enantiotropically; if not, the relationship is monotropic.

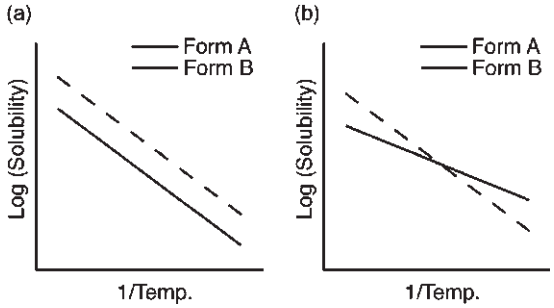


FIGURE 16.1 Theoretical van't Hoff plots showing relative water solubility of two polymorphs related (a) monotropically and (b) enantiotropically.

Figure 16.2a shows the heat of transition rule. In the monotropic system, the metastable Form B converts, at some temperature, to the stable Form A; since Form A has a lower enthalpy (H_A) the conversion is an exothermic process. This also holds true for the enantiotropic system if Form B converts to Form A before the crossover temperature, where Form B becomes the stable form and Form A becomes metastable. On the other hand, if Form A converts to Form B in the enantiotropic system, at a temperature above the crossover temperature, then since Form B has a higher enthalpy (H_B) the conversion will be endothermic. Figure 16.2b shows the heat of fusion rule. The point at which the free-energy curve of each polymorph crosses the liquid line in the energy–temperature diagrams is the temperature at which it melts. In the monotropic system the metastable Form B melts at a lower temperature and has a smaller heat of fusion ($\Delta_F H$) than the stable Form A, which has a higher

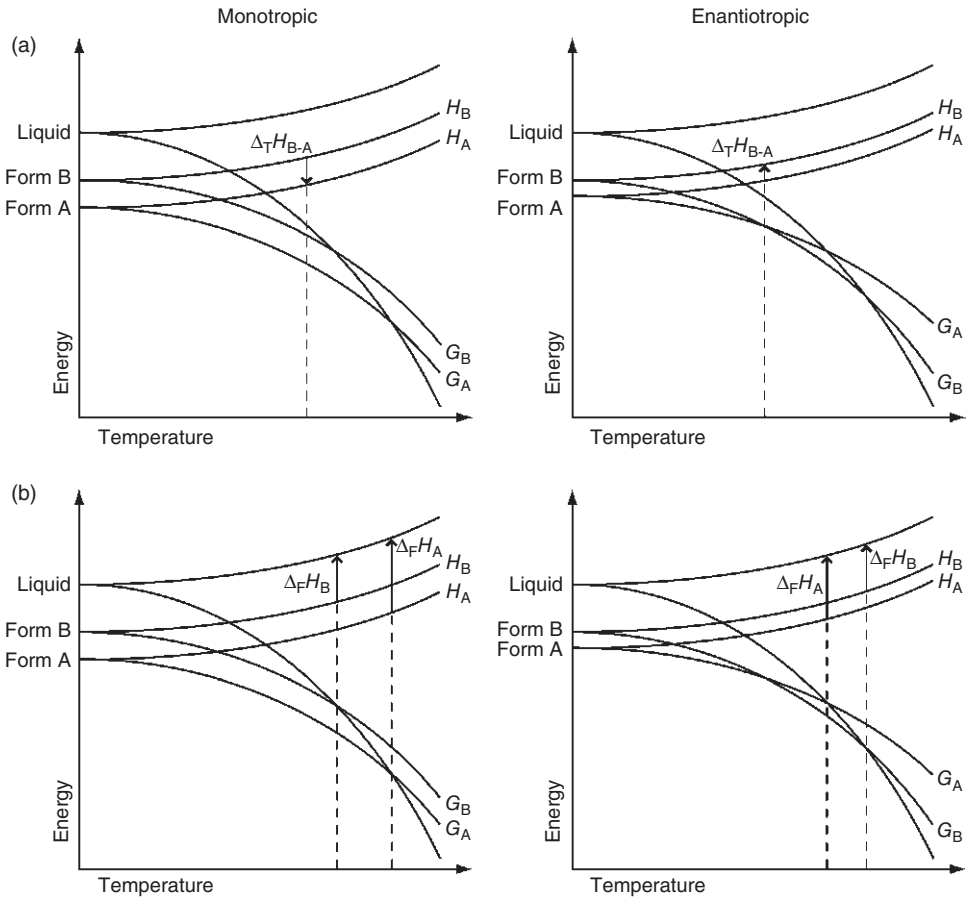


FIGURE 16.2 Theoretical energy–temperature diagrams for two polymorphs—Form A and Form B—showing the enthalpy (H) and free energy (G) of each polymorph as a function of temperature. (a) Energy–temperature diagrams illustrating the heat of transistion ($\Delta_T H$) rule for both a monotropic and an enantiotropic system. (b) Energy–temperature diagrams illustrating the heat of fusion ($\Delta_F H$) rule for both a monotropic and enantiotropic system.

melting temperature and a larger $\Delta_F H$. Conversely, in the enantiotropic system, Form A melts at a lower temperature with a higher $\Delta_F H$ and Form B melts at a higher temperature with a lower $\Delta_F H$. This occurs because the free-energy lines cross but the enthalpy lines do not cross. Additionally, different polymorphs may have different rates of dissolution.

Occasionally, the solubility of the stable form is too low for the drug to reach therapeutic levels when administered to patients. This may require the use of a metastable form with a higher solubility to be used in a formulation. Typically, an amorphous material will have a significantly higher solubility and dissolution rate than any crystalline form of a material and thus is also less stable. Caution must be used when developing a formulation because many of the processing techniques that are used in the pharmaceutical industry can cause polymorphic conversions or other changes to the physical state of a solid, making it vital to identify and control these properties throughout the development process and especially in the final formulated product. One rule of thumb is that if an amorphous material is stored 50 °C below its T_g, then its mobility will be sufficiently low to prevent crystallization or other degradation [19, 20, 24, 25].

One example of the consequences of polymorphic change in a formulation is ritonavir [26], a protease inhibitor, that was discovered in 1992 at Abbott Laboratories and was given FDA approval in 1996. It was formulated and marketed as a semisolid capsule (containing a nearly saturated solution) and a liquid formulation. Despite attempts to produce and identify other crystal forms, only one (Form I) was known to exist. Then in 1998 the semisolid formulation began failing dissolution tests because the drug was precipitating in the formulated product. When the precipitate was analyzed it was found to be in a previously unknown crystal form (Form II) and this new form was found to be more thermodynamically stable and thus less soluble. Form II spread throughout their production facilities and eventually they were only able to produce the new less soluble form.

While small molecules are almost always more stable in a crystalline form, the same is not necessarily true for biopharmaceuticals. Pikal and Rigsbee [27] showed that insulin is actually more stable when it is amorphous.

16.3.2 Chemical Stability

All of the chemical reactions that are possible in the solution state are also possible in the solid state. Reactions in crystalline solids are governed by the topochemical postulate [28], which states that reactions will occur with a minimum of atomic and molecular movement. In the solid state, functional groups on a molecule or neighboring molecules may be held closer or farther away from one another than they are in the solution state, which may change the rates of the reactions or even the degradation products that are formed [29]. Additionally, polymorphs and solvates may have different reactivities because of the differences in their crystal structures.

16.3.3 Identifying True Solid-State Chemical Reactions

Water is often present in the solid state, whether it is atmospheric, in the excipients of the formulation, or in the drug itself (e.g., a hydrate). This raises the question of

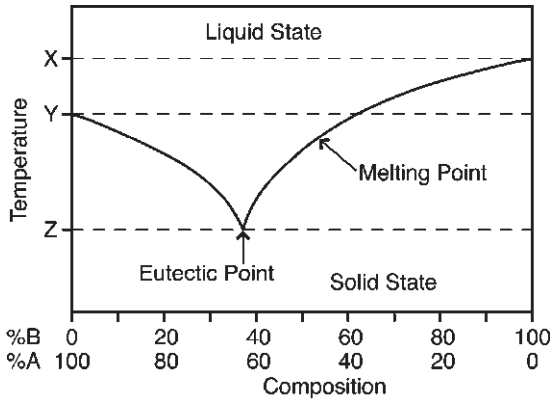


FIGURE 16.3 Phase diagram depicting how the melting point of the material changes as the hypothetical degradation of A proceeds to Form B. (Adapted from Ref. 21.)

whether a reaction actually occurs in the solid state or a solution state, where the molecules actually dissolve in the water, react, and then precipitate back out of solution. Five criteria have been developed to identify true solid-state reactions [21].

A reaction occurs in the solid state if:

1. In solution the reaction is slower or does not occur.
2. Pronounced differences are found in the reactivity of compounds with very similar chemical structures when they are in the solid state.
3. Reaction products are different from those observed in solution.
4. Different crystalline forms of the same compound have different reactivities or yield different reaction products.
5. The reaction occurs at a temperature below the eutectic point of a mixture of the starting material and products.

The fifth criterion is illustrated in Fig. 16.3, where A reacts to form B. A has a melting point of Y and B has a melting point of X. As B is formed it depresses the observed melting point because it acts as an impurity in A. At some critical composition (eutectic point) the melting point begins to rise again because A begins to be the impurity in B. The fifth criterion is fulfilled if the reaction occurs at a temperature below Z.

16.4 KINETICS

In the most general sense drug stability is a very simple concept. A drug reacts via some mechanism, at some rate, and produces a product. However, reactions can become very complex when variables such as temperature, pH, physical state, and so on are taken into account.

16.4.1 Kinetic Models

A chemical reaction can be represented by a rate expression, such as

$$\frac{-d[\mathrm{A}]}{dt} = k[\mathrm{A}]^x[\mathrm{B}]^y[\mathrm{C}]^z \cdots \tag{16.1}$$

The order of the reaction is given by the sum of the powers (x, y, z, . . .) in the rate expression. In the simplest system the rate of the reaction does not depend on the concentration of any of the reactants. This is called a zero-order reaction and is given by

$$\frac{-d[\mathrm{A}]}{dt} = k \tag{16.2}$$

The integrated form of this equation is

$$[\mathrm{A}]_t = [\mathrm{A}]_0 - kt \tag{16.3}$$

Equation 16.4 represents a first-order reaction and Eq. 16.5 gives the integrated form:

$$\frac{-d[\mathrm{A}]}{dt} = k[\mathrm{A}] \tag{16.4}$$

$$[\mathrm{A}]_t = [\mathrm{A}]_0 e^{-kt} \tag{16.5}$$

Equation 16.6 represents one example of a second-order reaction and Eq. 16.7 gives the integrated form:

$$\frac{-d[\mathrm{A}]}{dt} = k[\mathrm{A}]^2 \tag{16.6}$$

$$\frac{1}{[\mathrm{A}]_t} = kt + \frac{1}{[\mathrm{A}]_0} \tag{16.7}$$

The order of a reaction is determined by observing the reaction over time and then fitting it to the models to determine which one fits the observed data. Most reactions will be zero, first, or second order. However, if several reactions are occurring simultaneously the model may become more complex. Once the order of the reaction is determined it is important to verify that the order matches the mechanism. For example, hydrolysis reactions should depend on the concentration of both the drug and the water. However, these reactions often appear to follow first-order kinetics because the media in which they occur are composed primarily of water. The concentration of water is so large that it does not change significantly over the course of the reaction, so the rate of the reaction only depends on the concentration of the drug because there is essentially an infinite supply of water for the reaction.

Since this reaction is second order, but appears to be first order, it is referred to as a pseudo-first-order reaction.

Solid-state reactions often display more complicated kinetics, which leads to difficulties in understanding the kinetics of the system. There are four main steps in a solid-state reaction [21].

1. *Loosening of Molecules*. This step occurs at nucleation/defect sites, which are imperfections in the crystal that typically form during the crystallization process or during processing (e.g., grinding) and allow sufficient mobility for the reaction to occur.
2. *Molecular Change*. This step is the actual reaction of interest and is typically slower than the same reaction in solution due to the restricted motion.
3. *Solid Solution Formation*. Early in the reaction only a small amount of the product has been formed and is randomly mixed with the reactant (this mixture is the solid solution), eventually enough product forms and the reactants and products separate.
4. *Separation of Product*. When the product separates from the reactant it forms a new phase that can be amorphous or crystalline.

It is often difficult to fully understand the kinetics of a reaction in the solid state. Several models are commonly used (shown in Table 16.1) and they can be grouped

TABLE 16.1 Solid-State Kinetic Models

Name	Equation[a]
Prout–Tompkins	$\ln\left(\frac{\alpha}{1-\alpha}\right) = kt + c$
Avrami–Erofe'ev	$[-\ln(1-\alpha)]^n = kt \quad n = \frac{1}{4}, \frac{1}{3}, \frac{1}{2}, \frac{2}{3}$, or 1
One-dimensional phase boundary (zero order)	$1 - \alpha = kt$
Two-dimensional phase boundary (contracting area)	$1 - (1-\alpha)^{1/2} = kt$
Three-dimensional phase boundary (contracting volume)	$1 - (1-\alpha)^{1/3} = kt$
Diffusion-controlled one-dimensional	$\alpha^2 = kt$
Diffusion-controlled two-dimensional	$(1-\alpha)\ln(1-\alpha) + \alpha = kt$
Diffusion-controlled three-dimensional	$1 - \frac{2}{3}\alpha - (1-\alpha)^{2/3} = kt$
Jander equation	$[1 - (1-\alpha)^{1/3}]^2 = kt$
Power law	$a^n = kt \qquad n = \frac{1}{4}, \frac{1}{3}, \frac{1}{2}$, 1, or 2
First order	$\ln(\alpha) = kt$
Second order	$\frac{1}{1-\alpha} = kt$

[a] α = fraction decomposed, k = rate constant, t = time, and c = constant.

into four types: reactions involving nucleation, reactions controlled by phase boundaries, diffusion-controlled reactions, and other reactions [21].

The two models that fall into the nucleation category are the Prout–Tompkins and Avrami–Erofe'ev equations. Prout and Tompkins [30, 31] developed an equation that assumes a solid-state reaction begins at a nucleation site, branches out in chains, and is terminated more rapidly as the number of nuclei increase. This can be likened to a polymer chain reaction in which there is an initiation, propagation, and termination step. The Avrami–Erofe'ev equation [32–34] also assumes that the reaction starts at nuclei; however, it assumes that the nuclei grow and engulf other nuclei and the directionality of this growth is interpreted from the value of n.

If a reaction begins on the surface of a crystal and advances toward the interior, then it falls into the phase boundary category. There are three equations in this group and each represents a different dimensionality of the growth. The one-dimensional or zero-order rate equation (identical to the solution-state zero-order rate equation) assumes that the reaction only moves in one direction across the crystal (e.g., in a rod-shaped crystal the reaction begins at one or both ends and the reaction front travels down the crystal to the opposite end). If the reaction progresses in two dimensions (e.g., in a rod-shaped crystal the reaction initiates on the circumference and then travels radially toward the long axis of the rod) then it is represented by the two-dimensional or contracting area equation [35]. Finally, if the reaction travels in three dimensions (e.g., in a rod-shaped crystal the reaction initiates on both the ends and the circumference and then travels toward the center of the rod) then it can be modeled by the three-dimensional or contracting volume equation [35].

The third group contains the diffusion-controlled reactions and, as the name implies, these equations describe reactions in which two reactants must come together to form the product. As with the phase boundary equations there are three equations that describe the dimensionality of the diffusion: one-, two-, and three-dimensional diffusion-controlled rate equation [35–39]. The Jander equation also falls into this category and it is a simplified form of the three-dimensional diffusion-controlled rate equation.

The final group is composed of all the other equations that are used but do not have any physical interpretation. The power-law equation has been used in solid-state kinetic studies; however, it has no theoretical basis for interpretation except that when $n = 2$ it is identical to the one-dimensional diffusion equation. The first- and second-order rate equations are sometimes used to describe solid-state reactions; however, the order of a reaction in the solid state is not understood or defined as well as it is in the solution state and therefore it has little interpretive value. On the other hand, the zero-order (one-dimensional phase boundary) equation does have a physical meaning in the solid state.

Typically in solid-state kinetic studies the data is fit to each of the equations described earlier, and in the case of the Avrami–Erofe'ev and power-law equations, where n can have multiple values, all of the possible values of n are used to fit the data. This results in approximately 19 different fits of the same set of data and for most fits the correlation will be very good ($R^2 > 0.9$). If information is known about the reaction mechanism, then it can be used to narrow the possible fits to use. For example, if it is known that the reaction does involve two reactants that are not held closely in the crystal structure, then it is likely that one of the diffusion models would

be more representative of the reaction rate. However, typically these distinctions cannot be made and the models with the best fits ($R^2 > 0.98$) are analyzed to see if they can yield some information about the mechanism of the reaction. For example, do the nucleation equations seem to fit better or do the phase boundary equations seem to fit better? One model is then chosen and used to measure the degradation rates in the subsequent stability studies.

16.4.2 Temperature

Drugs must be stable enough to be stored for long periods of time (~2 years); however, when trying to develop a stable formulation it is not desirable to wait for a long period of time to find out if the formulation will be stable enough to survive storage. Therefore, a method for speeding up stability measurements is needed. It is well known that the Arrhenius equation (Eq. 16.8) describes the relationship between the activation energy (E_a) of a reaction and the rate of the reaction (k) at a given temperature (T), where A is a frequency term and R is the gas constant. The Arrhenius equation can also be linearized (Eq. 16.9) to simplify data fitting and analysis.

$$k = A\exp\left(\frac{-E_a}{RT}\right) \tag{16.8}$$

$$\ln(k) = \ln(A) + \left(\frac{-E_a}{RT}\right) \tag{16.9}$$

Therefore, if the rate of degradation is measured at multiple temperatures it is possible to measure E_a for the reaction by plotting $\ln(k)$ versus $1/T$ to produce an Arrhenius plot, and determine E_a from the slope ($-E_a/R$). The stability of the formulation can then be predicted at room temperature or any other storage conditions such as refrigeration (assuming that the reaction mechanism does not change [40]). While E_a and A are typically assumed to be constants, in theory they are not. The Eyring equation (Eq. 16.10) can be used to account for the temperature dependency of both E_a and A, and as with the Arrhenius equation it can be linearized (Eq. 16.11) to simplify data analysis.

$$k = \left[\frac{k_B T}{h}\exp\left(\frac{\Delta S^{\ddagger}}{R}\right)\right]\exp\left(\frac{-\Delta H^{\ddagger}}{RT}\right) \tag{16.10}$$

$$\ln\left(\frac{k}{T}\right) = \left[\ln\left(\frac{k_B}{h}\right) + \left(\frac{\Delta S^{\ddagger}}{R}\right)\right] + \left(\frac{-\Delta H^{\ddagger}}{RT}\right) \tag{16.11}$$

The Eyring plot is produced by plotting $\ln(k/T)$ versus $1/T$, where $\Delta H^{\ddagger}$ can be obtained from the slope and used to predict the stability at lower temperatures (although the Arrhenius plot is much more commonly used for this purpose). The Arrhenius and Eyring equations form the basis for the accelerated stability tests that are used to evaluate and compare potential formulations in the development process (for more information see Section 16.5).

TABLE 16.2 Sensitivity of Rate Constants to Changes in Temperature

	k_2/k_1	
E_a (kcal/mol)	$\Delta T = 1\,°C$	$\Delta T = 0.1\,°C$
10	1.058	1.006
20	1.119	1.011
30	1.184	1.017

When studying stability it is important to maintain a constant temperature because, as shown by the Arrhenius and Eyring equations, if the temperature changes the rate constant will change. The relevant degradation reactions that most pharmaceuticals undergo in the solution state have an E_a between 10 and 30 kcal/mol and Table 16.2 shows how sensitive the rate constant of a reaction with an E_a in this range is to changes in the temperature.

Table 16.2 demonstrates the need for accurate temperature control during stability studies. If the reaction being studied has an E_a of 30 kcal/mol, the measured degradation rate can be off by as much as 18.4% if the temperature is off by only 1 °C. Therefore, ovens that are used for stability studies should have the ability to maintain temperatures as accurately as possible.

In the solid state it is usually assumed that reactions will exhibit Arrhenius kinetics (linear Arrhenius plot); however, this is not always the case. One potential reason for non-Arrhenius kinetics is a change in the mechanism of degradation. Additionally, the physical state of the material may change. For example, an amorphous material may not exhibit Arrhenius kinetics if the temperature range being studied spans the T_g of the material [41], or the material may even crystallize at higher temperatures. Also, a crystalline material may undergo a polymorphic conversion to a different crystalline form at some temperature in the range that is being studied [40].

16.4.3 pH

Hydrogen (H^+) and hydroxide ions (OH^-) can act as catalysts in reactions. Therefore, the observed rate of a reaction (k_{obs}) is actually the sum of the multiple of the rate constants of each reaction mechanism and the concentration (or more accurately the activity) of the corresponding catalyst:

$$k_{obs} = k_{H^+}[H^+] + k_{H_2O} + k_{OH^-}[OH^-] \tag{16.12}$$

The observed rate of degradation can be measured at numerous pH values and then plotted, as in Fig. 16.4, to yield a pH–rate profile. Buffers are typically employed to maintain a constant pH during the stability studies; however, they can dramatically affect the results if not properly accounted for (see Section 16.4.4 on buffers). These profiles can be very simple as they are in Fig. 16.4a–e, or very complex as in Fig. 16.4f. If there is no catalysis ($k_{H^+} << k_{H_2O} >> k_{OH^-}$; Fig. 16.4a) the pH–rate profile should just be a flat line parallel to the x-axis (no change in rate). However, if hydrogen ions catalyze the reaction ($k_{H^+} \geq k_{H_2O} >> k_{OH^-}$; Fig. 16.4b) then the left side

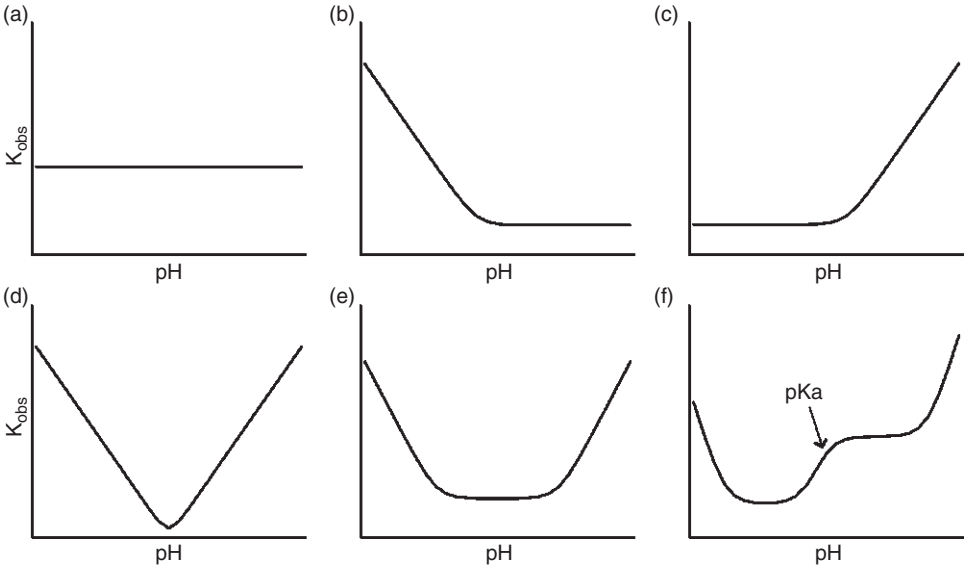


FIGURE 16.4 Theoretical pH–rate profiles showing (a) no acid or base catalysis, (b) only acid catalysis, (c) only base catalysis, (d) acid and base catalysis in V-shaped profile, (e) acid and base catalysis in U-shaped profile, and (f) acid and base catalysis of a weak base.

of the profile should curve up, and if hydroxide ions catalyze the reaction ($k_{H^+} << k_{H_2O} \leq k_{OH^-}$; Fig. 16.4c) then the right side of the profile should curve up. Subsequently, if both ions catalyze the reaction then both ends should curve up, and in this case there are two types of profiles that are possible: the V shaped and the U shaped. In the V shape (Fig. 16.4d) the k_{H^+} and k_{OH^-} are significantly larger than the k_{H_2O} such that the two sloped regions meet at a point and the profile resembles the letter V. However, in the U shape (Fig. 16.4e) all three rate constants are much closer to the same value so that the center of the profile is flattened and the entire profile resembles the letter U. If the molecule that is being studied is a weak acid or a weak base (Fig. 16.4f), the neutral and the ionized forms can have different reactivities and the H^+ and OH^- may have different abilities to catalyze the reactions of the two forms. This results in a much more complex equation to describe k_{obs} (Eq. 16.13) where k is the rate of the reaction of the ionized form and k' is the rate of the neural form and f_{DH^+} and f_D are the fractions of the ionized and neutral forms of a weak base, respectively.

$$k_{obs} = (k_{H^+}[H^+] + k_{H_2O} + k_{OH^-}[OH^-]) f_{DH^+} + (k'_{H^+}[H^+] + k'_{H_2O} + k'_{OH^-}[OH^-]) f_D \quad (16.13)$$

$$f_{DH^+} = \frac{[H^+]}{K_a + [H^+]} \quad (16.14)$$

$$f_D = \frac{K_a}{K_a + [H^+]} \quad (16.15)$$

It is possible to show that $k_{H_2O}f_{DH^+}$ and $k'_{H^+}[H^+]f_D$ are mathematically equivalent and that $k_{OH^-}[OH^-]f_{DH^+}$ and $k'_{H_2O}f_D$ are also mathematically equivalent; therefore, Eq. 16.13 can be simplified to produce Eq. 16.16:

$$k_{obs} = k_{H^+}[H^+]f_{DH^+} + k_{H_2O}f_{DH^+} + k'_{H_2O}f_D + k'_{OH^-}[OH^-]f_D \tag{16.16}$$

When examining a pH–rate profile the regions of acid and base catalysis should have a slope of –1 and +1, respectively. If the slopes are closer to zero then the reaction may be pH dependent but not acid or base catalyzed. The individual rate constants can be determined manually or the pH–rate profile can be fit to an equation, such as Eq. 16.16, that appropriately describes k_{obs} using a software program that is capable of performing nonlinear regression analysis. If a software program is used to fit the profile the data should be weighted because the $[H^+]$ can span as many as 12 orders of magnitude.

When it comes to the solid state, pH has no definition. However, differences have been observed in the solid state when proteins are prepared from solutions of different pH values. Therefore, effective pH or "pH" has been defined as the pH of a solution before it is converted to a solid (typically by lyophilization) or after the solid is reconstituted. While the physical basis of this "pH" is not yet fully understood it can be a very helpful parameter when trying to develop a solid formulation for peptides and proteins [41].

The pH– and "pH"–rate profiles play a very important role in formulation development. It provides an easy mechanism by which an appropriate formulation pH or "pH" can be identified visually and it provides an idea of how vital pH control is. For example, if a drug has a V-shaped pH–rate profile it may be vital to buffer the formulation because a small change in the pH may result in a large change in the degradation rate; however, if the drug has a U-shaped profile the formulation could be prepared near the center of the flat region and it may not require buffering since a small change in the pH will result in little to no change in the stability.

16.4.4 Buffers

As stated previously, buffers are commonly used in stability studies to maintain the pH of the solution over the course of the experiment, since the k_{obs} is dependent on the concentration of the H^+ and OH^-. However, the buffer can also catalyze (k_{cat}) the reaction through general acid or general base catalysis; although different ionized or un-ionized states of the buffer may have more or less catalytic activity. If this occurs then more terms must be added to the k_{obs} equation (Eq. 16.12) to account for this catalysis:

$$k'_{obs} = k_{obs} + k_{cat}[\text{Buffer}]_{total} \tag{16.17}$$

$$k_{cat} = k_{BH^+}f_{BH^+} + k_B f_B \tag{16.18}$$

In this example the buffer is a weak base with a single ionized state. When the pH is changed to study the stability at another pH, the k_{cat} will also change because the fractions of the ionized (f_{BH^+}) and neutral (f_B) buffer components will change.

Therefore, it is necessary to determine the k_{obs} so that the buffer catalysis does not impact the pH–rate profile. By increasing the concentration of the buffer while maintaining the pH, it is possible to extrapolate the k_{obs} from a plot of k'_{obs} versus the total concentration of the buffer (Fig. 16.5), where the *y*-intercept is k_{obs} and the slope is k_{cat}. The k_{obs} of studies at different pH values can then be combined to create a pH–rate profile. The k_{cat} can also be plotted versus the fraction of one of the ionized forms of the buffer (e.g., f_B) to determine the k_{BH^+} and k_B (Fig. 16.6); this may help in identifying which buffer to use in a formulation by helping to determine

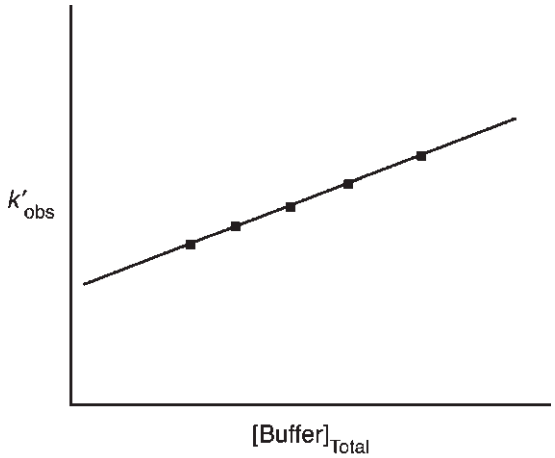


FIGURE 16.5 Theoretical plot of k'_{obs} versus total buffer concentration showing the effects of general acid/base catalysis on k'_{obs} when buffer concentration is changed.

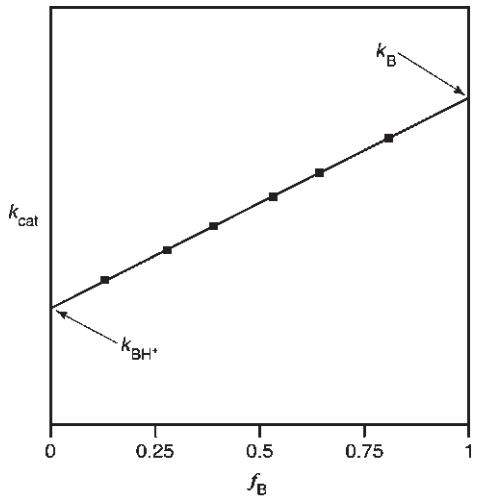


FIGURE 16.6 Theoretical plot of k_{cat} versus the fraction of the buffer that is in the neutral form, showing that the ionized form of the buffer is less catalytic than the neutral form.

which buffers do not catalyze the degradation or which do so only slightly. Note that ionic strength can also affect the rate of degradation; therefore, it is also important that the ionic strength be kept constant as the pH and the buffer concentration are changed.

16.4.5 Relative Humidity

In the solution state a drug is already dissolved in a solvent and thus water vapor has little to no interaction with the drug. However, solids can interact with water vapor in multiple ways. The interaction can be a physical one in which the solid takes up the water. If the solid is amorphous the water could increase the mobility of the matrix since water acts as a plasticizer to decrease the T_g of amorphous materials, or if the mobility increases sufficiently the drug may be able to crystallize [20]. On the other hand, a crystalline solid, either anhydrous or hydrated, could take up water stoichiometrically to form a hydrate or a higher order hydrate, respectively. Conversely, a hydrated form could lose water to the atmosphere if the humidity drops below a particular level. Water can also be involved in chemical reactions in the solid state. In the case of a hydrolysis reaction, the water from the atmosphere could diffuse into the solid and then react with the drug. The water may also produce a physical change, such as an increase in mobility, which may subsequently lead to the increased rate of a chemical reaction due to the increased mobility of the drug molecule. Additionally, there is a possibility that the water may form a thin layer on the surface of the particles in which the drug could dissolve and then degrade [42–44].

Since water can play such a vital role in the degradation of drugs in the solid state it is vital to characterize how water vapor interacts with solid materials. This is typically performed by water vapor sorption analysis in which the material is stored at multiple relative humidities while the temperature is held constant until the material reaches equilibrium. The water content of the material stored at each relative humidity is then measured and plotted against the corresponding relative humidity to form a water vapor sorption isotherm. The isotherm provides important information concerning the stability of different polymorphs or amorphous material to relative humidity. For example, if a drug has two anhydrate forms (A and B) by comparing their isotherms at 25 °C it can be seen that at 20% relative humidity Form A takes up 1 mole of water for every mole of drug and thus forms a monohydrate, and subsequently at 75% relative humidity this monohydrate produced from Form A takes up another mole of water for every mole of drug to produce a dihydrate. Form B does not take up any significant amount of water and remains anhydrous at all relative humidities. This information can be very important when preparing to develop a formulation. If Form B could not be used in the formulation, the consequences of the formation of the mono- and dihydrates must be considered (i.e., differences in the stability, solubility, or dissolution rate of Form A and the hydrates).

Deliquescence occurs when a solid, typically amorphous, adsorbs enough water vapor to liquefy. Conversely, if water evaporates to leave behind a solid this process is called efflorescence. When these processes occur the material is not simply labeled deliquescent or efflorescent; instead the temperature and relative humidity at which the water gain or loss occurs should be reported [21].

16.5 ACCELERATED STABILITY TESTING

The degradation of drugs means that eventually they will no longer be effective because too much of the drug will have degraded and the delivered dose will be too small. Therefore, drugs are given a shelf life that indicates how long they can be stored and still be guaranteed effective. For some drugs, this lifetime is defined as the amount of time that it takes for 10% of the drug to degrade or the t_{90}. However, occasionally the degradation of the drug produces a toxic compound, and the amount of this toxic substance that is produced will determine the shelf life of the formulation [40, 45]. The t_{90} for a first-order degradation reaction can be determined from the rate constant using

$$t_{90} = \frac{-\ln(0.9)}{k} \tag{16.19}$$

However, when determining if a potential formulation will be stable enough it is undesirable to wait for a year or longer to see if more than 10% of the drug has degraded. Thus, accelerated stability studies are used to stress the formulation (increased temperature and relative humidity) in an effort to predict the shelf life of the formulation under typical storage conditions. By stressing the formulation the study can be completed in several months and then, once an appropriate formulation is found, an extended stability study (~2 years) under typical storage conditions can be performed to verify the predictions.

While the principles are the same for accelerated stability testing in the solution and solid states, it is important to note that additional complications can arise in solids. For example, the drug may have polymorphs that are related enantiotropically and either some or all of the temperatures used may be above the crossover temperature; therefore, the accelerated stability testing may be done on one polymorph while the study is meant to predict the stability of the other polymorph. Also, the polymorphs could be related monotropically and some or all of the temperatures may be above the temperature at which the metastable form converts to the more stable form. However, it is not common to develop a formulation that contains a metastable polymorph, but it could be formed unintentionally in the processing steps used to produce the formulation. Additionally, it is possible that, when performing an accelerated stability study on an amorphous material, the material may be in its rubbery state and under normal storage conditions it would be in the glassy state, depending on the T_g of the material. In all of these cases, the accelerated stability study would give erroneous results and would not correctly predict the stability under normal storage conditions. It would not be possible to detect these errors unless the stability is studied under normal storage conditions, which may take a year or longer. Therefore, it is vital to thoroughly characterize solid-state systems before performing accelerated stability studies [46].

16.5.1 Isothermal Kinetics

Isothermal kinetics studies rely on the relationships between temperature and rate constant (as shown previously in Eq. 16.8 and 16.10) to predict the shelf life of a formulation. These studies are termed isothermal because the temperature is held

constant to measure the rate constant of the degradation. The rate constant is measured at multiple temperatures and subsequently fit to the Arrhenius equation (Eq. 16.9) to obtain the E_a of the reaction. The E_a of the reaction can subsequently be used to predict the rate of degradation and thus the t_{90} at lower temperatures using Eq. 16.8. This can be used to determine if the formulation is sufficiently stable or if a change must be made to increase the stability.

16.5.2 Nonisothermal Kinetics

Nonisothermal kinetics studies were first proposed by Rogers [47] as a way to simplify stability studies. In a nonisothermal kinetics study the temperature is increased throughout the course of the study. This allows the E_a to be determined from only one experiment and eliminates the need for measuring the degradation rate at multiple temperatures. The degradation of a drug that follows first-order kinetics can be described by Eq. 16.20 [47], which is achieved by substituting Eq. 16.8 into Eq. 16.5:

$$[\mathrm{D}]_t = [\mathrm{D}]_0 \exp[-tA\exp(-E_a/RT)] \tag{16.20}$$

$[\mathrm{D}]_t$ and $[\mathrm{D}]_0$ are the concentrations of the drug at the time t and zero, respectively; t is the time; A is the frequency term from the Arrhenius equation; R is the gas constant; and T is the temperature. However, Eq. 16.20 describes the isothermal degradation kinetics; King et al. [48] derived Eq. 16.21, which can be fit via nonlinear regression to directly estimate both E_a and t_{90}.

$$[\mathrm{D}]_t = [\mathrm{D}]_0 \exp\left\{-t\left(\frac{0.1054}{t_{90}}\right)\exp\left[\left(\frac{E_a}{R}\right)\left(\frac{1}{298}-\frac{1}{T(t)}\right)\right]\right\} \tag{16.21}$$

In this equation $T(t)$ is the temperature at time t. One major drawback to this method is that the quality of the prediction depends on the experimental design; thus, significant care must be taken to choose the correct experiment length, sampling interval, and temperature program (rate of temperature change) in order to get accurate predictions [49]. Unfortunately, isothermal studies are needed to obtain the correct variables for accurate nonisothermal studies. In practice, there is very little, if any, gain from performing nonisothermal studies. For this reason, these studies are not routinely performed. They do occasionally experience a resurgence in popularity due to their potential to reduce the number of experiments that are required. Additionally, nonisothermal kinetics also require very accurate temperature control but have the added requirement that the oven also be able to precisely increase the temperature according to a predefined program.

16.6 IMPROVING STABILITY

There are several ways to produce a stable formulation when the drug that it contains is inherently unstable. In general, this is accomplished by either adding excipients or making chemical modifications to the drug. If the drug is moderately stable and only a slight increase in stability is needed, then excipients will probably be

sufficient, although they will only slow the degradation. However, if the molecule is extremely unstable, it will probably require changes to its chemical structure to obtain sufficient stability.

Aside from the previously mentioned excipients (e.g., buffers to control pH, desiccants to control humidity), there are many excipients that can be used to increase stability. The drug may undergo photodegradation. If so, the formulation can be protected from light by being placed in an opaque container, a photoprotective coat can be applied to the outside of a tablet, or a colorant can be added to the formulation to absorb the light. Exposure to light can also cause the formation of compounds that can oxidize drug molecules. If the drug is sensitive to oxidation, an antioxidant (e.g., α-tocopherol, butylated hydroxyanisole, ascorbic acid, thioglycolic acid) could be added to the formulation to reduce the oxidation rate of the drug. Additionally, oxygen adsorbent can be used to reduce the oxygen concentration and thereby reduce the concentration of the reactive oxygen species [1].

Some drugs can be stabilized either by forming a complex or partitioning into another environment. There are several ways that a ligand can protect a drug molecule when forming a complex: the ligand can act as a barrier that blocks a reactant from reaching the drug molecule or it can restrict the freedom of the drug molecule and hold it in a more stable conformation. Cyclodextrins, in particular, have proved to be very useful in their ability to complex with and stabilize drugs; however, cyclodextrins can be toxic and therefore cyclodextrin derivatives are typically used instead [50]. In some cases liposomes or micelles can be used in a formulation to increase the stability of a drug, either through interactions at the "oil–water" interface or by the partitioning of the drug into the lipid phase [1].

In some cases excipients are not able to adequately stabilize a drug. In these cases chemical modification of the drug will likely be required, thereby creating a new chemical entity and requiring that the entire stability testing process be repeated. One potential solution is to develop a prodrug that will be converted to the active pharmaceutical after it is administered to the patient. Additionally, it may be necessary to remove the unstable functional group in the drug. This may create problems if that functional group is also needed for the pharmacological activity; if this is the case, it may be necessary to identify a similar functional group that will still result in the necessary pharmacological activity but is less reactive. Even if the unstable functional group is removed, the drug will almost certainly still degrade, but the degradation would likely be much slower, and occur through another mechanism [1].

16.7 METHODS OF ANALYSIS

When performing stability studies it is necessary to use an analytical method that is capable of determining the extent of the reaction by following either the disappearance of the drug or appearance of its degradation products, or ideally both. This requires a method that can identify and quantify either the drug or its products in a mixture containing the drug, all of its degradation products, excipients, and any impurities [51].

Chemical stability is commonly measured using a chromatography technique, such as HPLC, to separate the reactants and products and is coupled with a detec-

tion method for identification and quantification, including electromagnetic spectroscopy (e.g., UV or visible light absorption), mass spectrometry, and electrochemical detection [51]. The only difference between studying the solution and solid states is that the solid must be dissolved before analysis. Since the studies typically can go for long periods of time (days to months), it is not uncommon to store the samples until the end of the experiment and then perform the analysis of all of the samples at once to help reduce the day-to-day variability of the instruments. The samples are typically stored by freezing; however, it is imperative that a control be performed to verify that no changes occur in the samples when they are frozen [52].

Physical stability requires a much broader set of techniques. For peptides and proteins there are several spectroscopic techniques that can be used to follow changes in the secondary and tertiary structure. Circular dichroism and infrared spectroscopy can be used to follow changes in the secondary structure, while intrinsic fluorescence can be used to follow changes in the tertiary structure. UV-Vis spectroscopy and DSC can be used to monitor denaturation [10]. Light scattering techniques and size exclusion chromatography are available to analyze aggregation.

When it comes to solids there are several techniques available to identify and track physical changes. X-ray diffractometry can be used to determine the crystal structure of a crystalline material, and to identify and quantitate polymorphs, solvates, and amorphous materials [21, 53]. Hot-stage microscopy can be used to follow changes in solids during heating by changes in birefringence [21, 54]. Thermogravimetric analysis (TGA) can be used to determine water or other volatile content (identify solvates and determine their stochiometry) and to follow stability to thermal decomposition to form gaseous products [21]. Additionally, Karl Fischer titration can be used to determine water content. DSC can provide a variety of information including melting points of polymorphs, T_g of amorphous material [20], and identification of monotropic versus enantiotropic systems [21]. Infrared and Raman spectroscopy can be utilized to identify and quantitate polymorphs and solvates [21]. Water vapor sorption can be used to characterize how different solid forms (polymorphs, solvates, and amorphous materials) interact with atmospheric water. Solid-state NMR spectroscopy can provide mobility information (such as T_g) and identify polymorphs, solvates, and amorphous materials [55]. Solid-state NMR spectroscopy is gaining popularity for several reasons, including that it is nondestructive, that it can often differentiate the drug from the excipients in a formulation when other techniques cannot, and that it can be used to study many dosage forms *in situ* [21]. Solid-state NMR spectroscopy can also provide crystal structure information, such as the number of crystallographically inequivalent sites [56].

16.8 REGULATORY GUIDELINES

In the pharmaceutical industry, stability testing is typically performed according to guidelines established by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The ICH groups "stability" among its "Quality Topics" and thus identifies each individual "Quality" guideline with a Q-code. Some of the relevant guidances are shown in Table 16.3 and are available online at http://www.ich.org.

TABLE 16.3 ICH Stability Guidelines

Q-Code	Topic
Q1A(R2)	Stability Testing of New Drug Substances and Products
Q1B	Stability Testing: Photostability Testing of New Drug Substances and Products
Q1C	Stability Testing for New Dosage Forms
Q1D	Bracketing and Matrixing Designs for Stability Testing of New Drug Substances and Products
Q1E	Evaluation of Stability Data
Q1F	Stability Data Package for Registration Applications in Climatic Zones III and IV

There are also national and regional guidelines on stability testing. In the United States, the U.S. Food and Drug Administration (FDA) is the regulatory body and the FDA's Center for Drug Evaluation and Research (CDER) issues stability guidances. These documents can be found online at http://www.fda.gov/cder/guidance/index.htm and http://www.fda.gov/cber/guidelines.htm.

16.9 FURTHER READING

Stability is a very broad topic that has been widely studied for many years due to the need to understand the degradation of every potential pharmaceutical before being approved by the FDA, and every new compound offers its own new and unique challenges. There have been countless articles and numerous books written on the subject; thus, it is impossible to condense all of this information into one chapter. The following articles and books are good resources for further information about specific topics that have been discussed in this chapter.

Yoshioka and Stella [1] have written a book that provides extensive information regarding the stability of drugs (both small molecules and biopharmaceuticals) primarily in the solution state. They provide many examples for each of the possible degradation mechanisms and present a thorough discussion of kinetics and the factors that affect the chemical stability of drugs. Additionally, they include the ICH guidelines on stability and photostability testing of new drug substances and products (Q1A and Q1B) and the major concerns that have been raised by the European Union, the United States, and Japan regarding these guidelines. However, note that the ICH guidelines are revised occasionally. Therefore, it is best to refer to the most up-to-date copies, which are available at the ICH website (www.ich.org).

Byrn, Pfeiffer, and Stowell [21] wrote a useful book concerning drugs in the solid state. In the book they provide a good introduction to solid-state chemistry along with some of the more common methods of analysis that are used to study solids: X-ray diffractometry, microscopy, DSC, TGA, solubility, dissolution, particle-size analysis, infrared spectroscopy, and solid-state NMR spectroscopy. They include extensive discussions and examples of both crystalline (polymorphs, hydrates, and solvates) and amorphous materials and the physical and chemical changes that can occur within them, complete with a discussion about solid-state kinetics.

In addition to the two books discussed above there are also many books and review articles that have been written about stability but are more focused on specific issues. The photodegradation of drugs and formulations is described in a book

edited by Tønnesen [4], including information on how photostability testing is performed, how to treat and interpret data from photostability tests, and many other issues concerning the exposure of drugs to light. The book series entitled Pharmaceutical Biotechnology [11] contains several volumes concerning the stability and characterization of proteins and peptides. Additionally, Chi et al. [17] wrote a review paper discussing the physical stability of proteins in solution and the mechanism and driving forces for aggregation. There is another book series, Drugs and the Pharmaceutical Sciences, which also contains several volumes with very helpful information regarding the stability of drugs [12, 14, 18]. Connors, Amidon, and Kennon [57] wrote a book in which they discuss the kinetics of degradation and provide stability monographs of 30 drugs, including acetaminophen, aspirin, and vitamin A. There are also several review articles that discuss amorphous materials [19, 20], crystalline materials [53], polymorphism [58], the effects of water on drugs in the solid state [42, 44], and accelerated aging studies [40]. Finally, there are countless articles that have been published about the stability of specific compounds that may help to understand the degradation of a new drug. Therefore, a good stability study always begins with a thorough literature search.

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17

DOSAGE FORMULATION

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17.1 INTRODUCTION

17.1.1 Background

Advances in biotechnology have allowed the economical and large scale production of therapeutically important complex polymers of amino acids (peptides and proteins), nucleosides (antisense molecules), and other biological drugs to be used to combat poorly controlled diseases [1]. This rapid progress in molecular biology required substantial progress in the formulation and development of delivery systems for the next generation of drugs. Rational drug design does not necessarily mean rational drug delivery, which strives to incorporate into a molecule the properties necessary for optimal transfer between the site of administration and the pharmacological target site in the body [2]. In addition, the advent of high throughput screening has affected contemporary drug pipelines based in large part on the nature of the assay, which includes the evaluation of organic solutions (e.g., DMSO) of drug candidates in immobilized receptor matrices. As a result, new drug leads tend to be more lipophilic. On top of this, the hit to lead process reduces still further aqueous solubility, meaning that the formulator is often faced with significant challenges with regard to generating appropriately orally bioavailable dosage forms. Various new vehicles and delivery systems, such as liposomes, biodegradable microspheres, biopolymers, hydrogels, and bioavailability enhancers, have been introduced during the last decades to improve delivery and bioavailability of new chemical and biological drugs. Safety evaluation of new vehicles and excipients as well as their role on efficacy and toxicity of new drugs is an important part of new formulations assessment.

Drug formulation is a rapidly evolving field. The new formulations, their function and characterization, absorption mechanisms, and novel routes and modes of delivery are described in many journals including (but obviously not limited to) the following journals: *International Journal of Pharmaceutics* (http://www.sciencedirect.com/science/journal/03785173); *AAPS PharmSciTech; Journal of Control Release* (http://www.sciencedirect.com/science/journal/01683659); *AAPS PharmSciTech* (http://www.aapspharmscitech.org), an online-only journal of the American Association of Pharmaceutical scientists; *European Journal of Pharmaceutical Sciences* (http://sciencedirect.com/science/journal/092809870); *European Journal of Pharmaceutics and Biopharmaceutics* (http://www.sciencedirect.com/science/journal/09396411); and *Advanced Drug Delivery Reviews* (http://www.sciencedirect.com/science/journal/0169409X).

17.1.2 Objectives/Definitions

Formulation is a preparation of pharmaceutical product in various dosage forms containing a new active ingredient(s) (AI) [3]. The purpose of drug formulation is to determine experimentally all the variables necessary to develop an optimal formula and working directions for making the pharmaceutical product. Pharmaceutical technology applied to developing a drug formulation comprises selection of materials and procedures that are adaptable to various processes that lend themselves to inclusion in specific dosage forms (tablets, injections, capsules, ointments, etc.). Dosage formulations can include sterile products, tablets, capsules, topical

preparations, and nonconventional products such as semisolid or sustained release preparations. Formulations can be developed to enhance drug solubility, improve bioavailability, enhance drug stability, target tissue delivery, and treat specific patient populations. Stability and release properties of formulations can be evaluated *in vitro* prior to their use in animals.

Clinical drug formulation is a very complex multistep process that is significantly simplified at the stage of preclinical dosage formulations. A dosage form for preclinical study should optimize chances for a compound to exert its pharmacological action, determine the ways of solubilizing the compound, and determine photosensitivity and other aspects of stability. In a preclinical toxicity study, it is important that the formulation does not change the toxic effect of the active ingredient (either mask or enhance it). It is very important because in many cases the final clinical formulations may be quite different in composition from the dosage forms used for safety assessment in animals.

Stability is one of the most important factors that determine whether a compound or mixture of compounds can be developed in a therapeutically useful product. The stability of a dosage formulation can be defined as its degree of resistance to chemical and physical changes. The efficacy of the preparation must remain constant (or changed only within the specified limits) until the date of expiration. The formulation must be chemically, physically, microbiologically, therapeutically, and toxicologically stable.

Drug stability may be influenced by internal and external factors.

Internal Factors. Potential drug–drug (when mixtures are used) or drug–excipient interactions.

External Factors. The test articles (drugs) and dosage formulations come into contact with containers or packaging materials. For example, alkali from the glass containers, heavy metals from the metal containers, components of cork or rubber stopper, and plastics can penetrate and react with preparations. Various storage conditions may indicate three types of changes: (1) physical and colloidal–physical changes, (2) chemical changes, and (3) microbiological changes. Proper storage conditions should be determined and followed to minimize these changes.

There are numerous types of potential undesired interactions between the drug and excipients, which can occur during the process of formulation preparation, storage, and administration. For example, solubility, ionization pH effects, formation of complexes, and degradation reactions are possible. These changes may be *intentional* (e.g., prolonged release or enhanced delivery) or *unintentional* (e.g., discoloration, precipitation, liquefaction). The second group is usually referred to as incompatibilities. Incompatibilities are undesired physical, chemical, colloidal, or biopharmaceutical processes that take place during preparation, storage, or administration of the drug formulation. Incompatibilities may take place in visible or hidden manners. Visible incompatibilities include the following processes: (1) changes in solubility or degree of dispersion, turbidity, precipitation, coagulation, aggregation, crystallization, or crystal growth; (2) changes involving viscosity, physical state, crystal form, liquefaction, solidification, separation of phases, and uptake or loss of moisture; and (3) discoloration or change in odor or taste

due to chemical transformation such as oxidation, hydrolysis, or enzyme degradation [3].

Hidden incompatibilities may be the result of interactions between active ingredients and excipients or packing materials and environmental conditions and include chemical, physical, physicochemical, colloidal, technological, and biopharmaceutical interactions. Some typical chemical interactions include spatial rearrangements, hydrolysis, the formation of esters, oxidation and reduction, and polymerization and depolymerization. Incompatibilities may also be related to the electric charge of molecules (e.g., formation of new salts, double salts, and intermolecular associations and complexes as a result of the interaction of AI and excipients, which have opposite charge). Physical interactions include adhesion, absorption, molecular adhesion, and ionic absorption.

To assess stability of the dosage formulation in a preclinical safety study, usually purity of active ingredient in formulation is determined over time. A formulation is considered unstable when the purity of the active ingredient (AI) decreases more than 10%. This may depend on accuracy of the analytical method and type of formulation (e.g., diet formulations may be considered stable up to a 15–20% decrease of AI). When chromatographic methods are used for purity determination, the appearance of additional peaks may be another sign of AI decomposition over time. Stability of formulation under the storage and application conditions should be determined prior to any preclinical safety assessment. It is not acceptable to simply assume that the drug will be stable in the diet or ointment even if it is known to be stable for a long period of time at room temperature in a powder form. Mixing with the diet (for oral studies) or with "inert" excipient (e.g., Vaseline for dermal administration) or even with water can significantly shorten stability of very stable drugs. Stability at storage conditions of formulations determined prior to toxicological experiment will dictate how often formulations need to be prepared in a repeated dosing study. Stability at room temperature is important to know even in a single dose experiment to assure formulation stability throughout the time of administration, which may be long when a significant number of small animals per dose group are used (especially important for dermal applications and lengthy infusions).

17.2 GENERAL PRINCIPLES OF DOSAGE FORMULATION IN PRECLINICAL STUDIES

17.2.1 Dosage Formulation Preparation

The test article is rarely administered as a neat compound and in most cases a dosage formulation is prepared for dosing the animals. It may be as simple as a gelatin capsule filled with the drug for oral administration in dogs or a solution in water for gavage dosing of rodents. It can also be a complicated mixture of the drug, vehicle(s), surfactants, and so on. The desirable characteristics of the "ideal" formulation and its preparation are presented in Table 17.1. It is important to test solubility of dosage solutions or homogeneity/suspendability of suspensions prior to the dosing of animals. We suggest that the solubility/suspendability test be run with the proposed formulations several days prior to dosing. This will allow for adjustments of a formulation, if necessary. An example of the form to be completed to record the result of these tests is presented in Table 17.2.

TABLE 17.1 Desirable Characteristics of a Dosing Formulation and Its Preparation

1. Preparation of the formulation should not involve heating of the test material anywhere near to the point where its chemical or physical characteristics are altered.
2. If the material is a solid and it is to be assessed for dermal effects, its shape and particle size should be preserved.
3. Multicomponent test materials (mixtures) should be formulated so that the administered form accurately represents the original mixture (i.e., components should not be selectively suspended or taken into solution).
4. Formulation should preserve the chemical stability and identity of the test material.
5. Formulation should be such as to minimize total test volume. Use just enough solvent or vehicle.
6. Formulation should be easy to administer accurately.
7. The pH of dosing formulations should be between 5 and 9, if possible.
8. Acids or bases should not be used to divide the test material (for both humane reasons and to avoid pH partitioning in either the gut or the renal tubule).
9. If a parenteral route is to be employed, final solutions should be as nearly isotonic as possible.

Source: From Ref. 4, with permission.

The test substance can be administered to animals at a constant concentration across all dose levels (i.e., varying the dose volume) or at a constant dose volume (i.e., varying the dose concentration). The toxicity observed by administration of a constant concentration may be different from that observed for a constant dose volume. For instance, when a large volume of oil is given orally, gastrointestinal motility is increased, causing diarrhea and decreasing the time available for absorption of the test substance in the GI tract.

The volume of water or solvent–water mixture used to dissolve the chemical should be kept low, since excess quantities may distend the stomach and cause rapid gastric emptying. In addition, large volumes of water may carry the chemical through membrane pores and increase the rate of absorption. Thus, if dose-dependent absorption is suspected, it is important that the different doses are given in the same volume of solution [4].

Local irritation by a test substance generally decreases when the material is diluted. If the objective of the study is to establish systemic toxicity, the test substance should be administered in a constant volume to minimize gastrointestinal irritation that may, in turn, affect its absorption. If, however, the objective is to assess the irritation potential of the test substance, then it should be administered undiluted.

17.2.2 Role of the Route of Administration

The intended route of administration in humans dictates a route of administration in animals during preclinical studies. However, the drug formulations in animals are often different from the clinical formulations. The specific route of administration is a significant determinant of the commonly used formulations in preclinical testing. Specific types of formulations by route of administration are described in Section 17.3.

Generally, pharmaceutical companies prefer to develop an oral route of administration for a new chemical entity. Dosage forms associated with this route are

TABLE 17.2 Form for Preclinical Formulation Testing for Solubility/Suspensibility

SOLUBILITY/SUSPENSION TESTING

Chemical: _____

Performed By: _____

Date Performed: _____

The test article was mixed with the following vehicle(s):

Vehicle	Volume of Vehicle	Quantity of Test Article	Results
Water			
Corn Oil			
Peanut Oil			

Since the test article was not soluble in the above vehicles, it was suspended in the following vehicle(s):

Vehicle	Volume of Vehicle	Quantity of Test Article	Results

A = Test article was completely soluble.

B = Test article appeared partially soluble.

C = Test article appeared insoluble.

D = Test article was adequately suspended.

E = Test article was not adequately suspended.

— = Vehicle not utilized.

Comments: _____

_____

_____

Read and Reviewed by: _____ Date: _____

relatively cheap to manufacture, are simpler for patients to handle, and are widely accepted by the public. Because this is the preferred route of administration, it becomes crucial to evaluate the oral bioavailability of lead compounds in the earlier stage of the development process. Oral bioavailabilty of a compound can be a major selection criterion for the development candidate. Drawbacks of the low bioavailable compound from a pharmaceutical development standpoint are inherent variability in the oral bioavailablity, a requirement of large quantities of drug substance to establish a safety margin in preclinical safety studies, and, depending on the pharmacology of the molecule, a high dose to elicit the desired effect.

In contrast, with only a few exceptions, most new anticancer compounds are initially developed for intravenous (IV) use, despite some drawbacks such as the morbidity associated with gaining IV access; risk of IV catheter-related infection, thrombosis, and extravasation; and patients' preference for oral therapy when equally effective [5]. Important reasons for choosing IV use for initial drug development are the fact that usually less gastrointestinal toxicity occurs, there is immediate 100% bioavailability and instantaneous pharmacodynamic effects, and there is a possibility to modify the dosing rate or even halt the infusion if necessary. Solubility of the compound is a specific demand for IV administration, even for the newer chemotherapeutic agents, which are known to be poorly water soluble. Classical solubility approaches, which are discussed subsequently, include the use of colloidal systems, prodrug development, or solubilization techniques.

Colloidal systems such as liposomes, microcapsules, microspheres, nanoparticles, or macromolecule complexes may protect the anticancer drug from premature degradation or (chemical) inactivation within the systemic circulation. Prodrugs are inactive derivatives that release the active drug following spontaneous degradation or enzymatic reactions. Solubilization is the process of uptake of drugs through complex formation into, for example, oligomers of dextrose and fatty acids, through cosolvent systems (such as ethanol, polyethylene glycol, and glycerol), or through surfactant systems. The surfactant systems consist of amphoteric compounds (e.g., lecithin or gelatin), ionic surfactants (e.g., sodium palmitate), or nonionic surfactants (e.g., Tween 80 and Cremophor EL (CrEL)). Some of these solubilization approaches are discussed later in this chapter.

17.2.3 Role of Absorption/Enhancement of Oral Absorption

In the early stages of development, it is important to determine whether the dosage form targeted for clinical use has an acceptable level of bioavailability. To evaluate the dosage form in a clinical study is time consuming and costly. Large animals (e.g., dogs and monkeys) are used for assessment of bioavailability of different dosage formulations. Examples of the usefulness of such studies are presented in this volume in Chapter 32, Bioavailability and Bioequivalence Studies.

Oral administration is the most convenient mode of drug delivery and is associated with superior patient compliance as compared to other modes of drug intake. However, oral administration has only limited use for important drugs, from various pharmacological categories, that have poor oral bioavailability due to incomplete absorption and/or degradation in the gastrointestinal (GI) tract [6]. Some of these drugs are characterized by a narrow absorption window (NAW) at the upper part of the GI tract. This is because the proximal part of the small intestine exhibits

extended absorption properties (including larger gaps between the tight junctions, and dense active transporters). Despite the extensive absorption properties of the duodenum and jejunum, the extent of absorption at these sites is limited because the passage through this region is rapid. Enhancing the gastric residence time (GRT) of a NAW drug may significantly improve the net extent of its absorption. To further increase the GRT of drugs, a gastroretentive dosage form (GRDF) can be developed. It is quite complex to achieve extensive retention of the GRDF since the natural activity of the stomach is to evacuate its contents into the intestine. The development of the GRDF has generated enormous interest and the pharmaceutical aspects of these developments are reviewed elsewhere [7, 8]. Controlled release (CR) dosage forms have been extensively used to improve therapy of many important medications. However, in the case of NAW drugs this pharmaceutical approach cannot be utilized since it requires sufficient colonic absorption of the drug (which is, by definition, not the case for NAW agents). On the other hand, incorporation of the drug in a controlled release gastroretentive dosage form (CR-GRDF) can yield significant therapeutic advantages due to a variety of pharmacokinetic (PK) and pharmacodynamic (PD) factors. Hoffman et al. [9] reviewed these aspects in rat and dog animal models in order to suggest rational selection of drugs for which CR-GRDF would be a beneficial strategy. These authors found that a CR-GRDF formulation was superior compared to other modes of administration for levodopa and riboflavin, but not for metformin.

Incomplete oral bioavailability has various causes. These include poor dissolution or low aqueous solubility, degradation of the drug in gastric or intestinal fluids, poor intestinal membrane permeation, and presystemic intestinal or hepatic metabolism. Absorption of peptides, peptide analogues, or other polar, high molecular weight drugs may be significantly enhanced by using excipients that increase intestinal permeability. For some compounds, permeation through the intestinal epithelium is hindered by their active transport from the enterocyte back into the intestinal lumen. The secretory transporters involved may include P-glycoprotein (P-gp), the family of multidrug resistance-associated proteins (MRPs), and possibly others. For substrates of these secretory transporters, inhibiting secretory transport can increase permeation in the absorptive direction. This can be accomplished with pharmacologically inactive excipients.

Before permeation-enhancing excipients are evaluated for a particular drug of interest, it is important to understand the cause of its low oral bioavailability. Ideally, first-pass metabolism and solubility are not limiting oral bioavailability. Furthermore, the role of secretory transport should be assessed, because excipients that increase absorption by inhibiting secretory transport are different from those that increase absorption by other mechanisms.

Several reviews address intestinal permeation-enhancing excipients and evaluate how far some of the more advanced technologies have come toward fulfilling these criteria [6, 9–13].

17.2.4 Role of Animal Species

The key issue of an assessment of new dosage formulations in animal studies is to extrapolate the results to humans. The bioavailability of drug formulation in animals and the ability to predict its bioavailability in humans is one of the most important

factors. An important issue in determining the choice of animal species for bioavailability testing is its size. In most circumstances, it is not possible to administer intact, solid pharmaceutical formulations—tablets and capsules, for example—in small rodents such as mice, rats, or hamsters. If such dosage forms are to be tested, larger species, such as rabbits, dogs, and monkeys, are more appropriate.

Another problem associated with the use of smaller species is repetitive blood sampling. Urine measurements of drug excretion can be used for all animal species to determine bioavailability, overcoming many of the blood sampling difficulties. A reasonable proportion of the drug, however, must be excreted unchanged via kidney to make the results meaningful. In addition, extensive first-pass metabolism would preclude this approach if the total metabolite excretion is being used.

None of the animal models are ideal, and each has its advantages and disadvantages. Table 17.3 compares different animal species as a model for human bioavailability testing [14]. Nevertheless, many investigators have used animals for bioavailability testing and have obtained valuable information that has helped in their understanding of their product. The rabbit appears to be the least suitable animal model for bioavailability testing, despite its relatively large size (large enough to test solid formulations) and low cost. This is because it has a long gastric emptying time, and both its gastrointestinal pH and microflora bear little resemblance to those in humans. Table 17.4 presents gastric and intestinal pH values of common laboratory animals. The rat has the major disadvantage of being too small to test

TABLE 17.3 Advatages (+) and Disadvantages (–) of Different Animal Species as a Model for Humans in Bioavailability Testing

Parameters	Rat	Rabbit	Dog	Monkey
Cost	+	+	–	–
Ease of urine collection	+	+	+	–
Ease of blood sampling	–	+	+	+
Ease of handling	+	+	+	–
Intact solid formulation	–	+	+	+
Intestinal pH	+	–	+	+
Gastrointestinal transit time	–	–	–	–
Gastrointestinal microflora	–	–	+	+
Drug metabolism	–	(– +)	(– +)	+
Biliary excretion	–	+	–	+

Source: From Ref. 14, with permission.

TABLE 17.4 Gastric and Intestinal pH Values for Different Animal Species

	pH Values					
Gastrointestinal section	Rat	Guinea Pig	Rabbit	Dog	Monkey	Human
Stomach	3.3–5.5	4.1–4.5	1.9	3.5–5.5	2.8–4.8	1.5–3.5
Duodenum	6.5–6.7	7.6–7.7	6.0–6.8	6.2	5.8	5.0–7.0
Jejunum	6.7–6.8	7.7–8.1	6.8–7.5	6.2–6.6	5.8–6.0	6.0–7.0
Ilium	6.8–7.1	8.1	7.5–8.0	6.6–7.5	6.1	7.0
Colon	6.6	6.7	7.2	6.5	5.1	5.5–7.0

Source: From Ref. 14, with permission.

most solid formulations, as well as being difficult to obtain multiple blood samples from repetitive sampling. In addition, the gastrointestinal microflora is different from that of humans. This species does have the advantage of having a gastrointestinal pH similar to that in humans and, if urinary data are used for bioavailability testing, it is easy to obtain total urine collections using metabolism cages.

In most respects, the monkey offers the closest physiological and metabolic model for humans and would be the first choice if not for its extremely high cost and handling requirements. Additionally, the monkey is capable of transmitting several human pathogens, and care must be taken when handling analytical samples. Obviously, if primate facilities are available, the use of this species becomes more attractive. Dogs offer a reasonable compromise since (1) they are large enough to test solid dosage forms, (2) their gastrointestinal pH is similar to that in humans, (3) sampling is convenient, (4) they are easy to handle, and (5) special precautions are not needed when handling the biological samples after collection.

Throughout drug development, toxicology/safety studies are conducted in two species. As most drugs are intended for oral administration, the rat and dog are the primary species used for reasons of availability, costs, and long history of use. However, not all drugs are targeted for oral delivery and other routes (intravenous, dermal, etc.) and species (rabbit for reproductive toxicology, monkey) may be used.

The rat is the species most commonly used with oral administration—the route of choice—and is the primary rodent utilized in toxicology studies. Oral administration by gavage as a solution or suspension is the most frequently employed dosage form in rodents and is used in studies of up to 6–9 months duration. The primary consideration is the choice of vehicle. Solutions in water are chosen if the solubility of the drug is sufficient to deliver the highest dose proposed. For drugs of low or limited solubility, a dispersing agent for use in an aqueous milieu is selected. A vehicle of 0.5% aqueous Tween 80 (i.e., polysorbate 80) has a long history of use for a wide range of drugs. Cellulose and its derivatives (e.g., methyl-, carboxy-), starch, and different natural oils (e.g., corn, sesame) are often used as a vehicle for oral gavage formulations. For studies of greater duration than 9 months (i.e., lifetime carcinogenicity studies), oral drugs are usually administered as admixture in the diet of the rats. Dogs are often dosed with capsules.

Literature review of species differences in pharmacokinetics and bioavailabilty of different dosage formulations are presented in Section 32.2.5 of Chapter 32, Bioavailability and Bioequivalence Studies.

17.2.5 Protein/Peptide, DNA, Antisense Oligonucleotides (AS-ONs), siRNA, and Other Biological Formulations

Protein/Peptide Formulations Protein pharmaceuticals have high specificity and activity at relatively low concentrations, in comparison with small chemical drugs. These features have made protein pharmaceuticals indispensable in combating human diseases. Currently, the U.S. Food and Drug Administration (FDA) has approved over 30 different recombinant DNA-derived proteins (e.g., erythropoietin, interferon α-2a/b, somatropin and follitropin β), and many more are in an advanced stage of development [15].

The most challenging task in the development of protein pharmaceuticals is to deal with physical and chemical instabilities of proteins. Protein instability is one of the major reasons why protein pharmaceuticals are administered traditionally

through injection rather than taken orally like most small chemical drugs [16]. Problems such as acid catalyzed degradation in the stomach, proteolytic breakdown in the GI tract, poor permeability across the gastrointestinal mucosa, and first-pass metabolism during transfer across the absorption barrier and in the liver must be overcome for the efficient delivery of drugs into the bloodstream [17]. In order to achieve successful oral delivery of protein drugs, they need to be protected from the harsh environment in the stomach. For designing oral dosage forms, the formulator must consider that the natural pH environment of the GI tract varies from acidic (pH ~ 1.2) in the stomach to slightly alkaline in the intestine (pH ~ 7.4) [18]. The review by George and Abraham [19] describes the sources and physical and chemical properties of alginate and chitosan (naturally occurring biopolymers) that enable them to become suitable for protein delivery, the mechanisms of hydrogel formation, modifications that increase their protein encapsulation efficiency, and the recent trends in their application.

There are considerable hurdles to be overcome before practical use can be made of therapeutic peptides and proteins due to chemical and enzymatic instability, poor absorption through biological membranes, rapid plasma clearance, peculiar dose–response curves, and immunogenicity [1]. Many attempts have addressed these problems by chemical modifications or by coadministration of adjuvants to eliminate undesirable properties of peptides and proteins [20]. Cell permeant peptides and their delivery enhancement are reviewed by Ülo Langel [21]. Different types of protein/peptide formulations need to be developed based on clinical needs, patient compliance; delivery methods; drug stability, storage, and distribution; and market competitiveness. Having a clear vision of the type of formulation that is desired will allow one to design a better formulation. Liquid formulations have generally been preferred due to the convenience of manufacturing and use. However, protein drugs may not be stable enough to be handled as a liquid formulation. Dried formulations (e.g., lyophilized formulations) or suspension formulations have been successfully used to overcome stability problems. In addition, specific applications and/or delivery routes may demand an appropriate type of formulation such as a spray-dried formulation for pulmonary delivery.

Maximizing the stability of protein/peptide is one of the most important objectives of formulation optimization. Once the key degradation products, responsible stresses, and analytical methods are identified, the protein or peptide can be stabilized by optimizing various formulation variables. Often, well established scientific principles can be used to solve some stability problems. In other instances, solving stability issues can best be achieved through utilizing empirical approaches. When an optimized formulation does not provide sufficient stability, then alternative approaches like lyophilization are indicated. Table 17.5 provides some typical solutions for routine stability problems.

Gene Therapies Gene delivery has been regarded as a powerful tool for curing disease by replacing defective genes, substituting missing genes, or silencing unwanted gene expression. Gene therapy holds great promise for treating various forms of diseases with a genetic origin including cystic fibrosis, different forms of cancer, and cardiovascular disorders. Basically, there are two types of gene carriers that deliver foreign DNA into the diseased target cell population. DNA delivery systems are viral and nonviral vectors [22]. Viral vectors are the most effective because of their evolutionary optimization for this purpose. However, recently

TABLE 17.5 Problems and Solutions of Protein and Peptide Drug Development

Problems	Solutions
Deamidation, cyclic imide formation	pH optimization
Aggregation, precipitation	pH optimization; addition of sugars, salts, amino acids, and/or surfactants
Truncation	pH optimization, protease removal
Oxidation	Excipient purity analysis, addition of free-radical scavengers (mannitol, sorbitol), use of a competitive inhibitor (methionine)
Surface denaturation, adsorption	Addition of surfactants or excipient proteins
Dehydration	Addition of sugars or amino acids

reported safety issues such as random recombination, oncogenic potential, and immunogenicity [23, 24] have set back the rapid development of viral vectors. As compared to viral vectors, nonviral systems have a low or absent immunogenicity, can relatively easily be scaled-up, and have great flexibility with regard to vector modification and DNA incorporation [25, 26]. Nonviral vectors are safe to use but less efficient. In light of safety concerns, nonviral delivery systems have been developed for gene therapy experiments. Among those, cationic liposomes are widely used for almost all animal cells because they have nonspecific ionic interaction and low toxicity properties [27–29]. However, there are some limitations for cationic liposomes. When they are used for *in vivo* transfection, they are unstable. Therefore, many polymeric cationic systems such as gelatin, polyethylenimine (PEI), poly(L-lysines), tetraaminofullerene, poly(L-histidine)-graft-poly(L-lysines), DEAE-dextrans, cationic dendrimers, and chitosan have been studied for *in vitro* as well as *in vivo* application [30, 31].

The clinical use of gene therapy treatments, however, is restricted, mainly because of the absence of safe and efficient gene delivery technologies [32–34]. In the past decade, nonviral approaches, particularly cationic lipid or polymer-based systems, have received rapidly growing interest, because they offer several advantages over the viral counterparts. Nevertheless, currently investigated gene delivery polymers like polyethylenimine (PEI), poly(L-lysine) (PLL), polyamidoamine dendrimers (PAMAM), and poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA) have not yet advanced to clinical evaluation, mainly due to their acute cytotoxicities and low *in vivo* transfection activity [24, 35–37].

Recently, biodegradable cationic polymers such as poly(4-hydroxy-L-proline ester) [38, 39], poly[α-(4-aminobutyl)-L-glycolic acid][40], poly(L-lysine) conjugates [41], linear poly(ester amine)s [42–45], poly(ester amine) networks [46], cationic polyphosphazenes [47], and degradable polyethylenimine [48, 49] have been investigated as nonviral carriers for gene delivery. Generally, polyplexes based on these polymers showed lower cytotoxicity and comparable *in vitro* transfection activities as compared to the nondegradable counterparts. Furthermore, the degradation properties of these polymeric vectors can be employed as a valuable tool to regulate the unpacking and release of the DNA inside the cells. Linear poly(ester amine)s as recently reported by Langer and co-workers are of particular interest because the applied synthesis is straightforward and polymers with a great variety of different structures and properties can readily be prepared [42, 43, 45]. Chitosan oligosac-

charides (COSs) chemically modified with deoxycholic acid (DOCA) have low toxicity and possess a good transfection activity [50].

Zhong et al. [51] report a versatile family of degradable hyperbranched poly(ester amine)s that contain primary, secondary, and tertiary amino groups in their structures for nonviral gene delivery. These hyperbranched poly(ester amine)s could easily be synthesized in high yields by a Michael-type conjugate addition of trifunctional amines, containing both a primary and a secondary amino group to diacrylates [52]. As vectors for gene delivery, these hyperbranched polymers may have several advantages: (1) in general they exhibit much better solubility in water than their linear counterparts [53]; (2) primary amines present in the periphery of these hyperbranched polymers can interact more efficiently with the phosphate groups of DNA, resulting in the formation of nanosized polymer/DNA complexes, analogous to the behavior of hyperbranched PEI that was shown to condense DNA into a smaller size particle than linear PEI [54]; (3) the high density of secondary and tertiary amines will give rise to high buffering capacity over a wide pH range, facilitating endosomal escape of polymer/DNA complexes ("proton sponge hypothesis") [55]; and (4) due to their high functionality, the structure of hyperbranched polymers can easily be modified to further improve transfection efficacy and/or achieve targeted gene delivery *in vivo*. For example, hyperbranched PEI, consisting of 25%, 50%, and 25% of primary, secondary, and tertiary amines, is one of the most efficient polymeric gene carriers studied *in vitro* and *in vivo* [55–58].

A significant limitation in using nonviral gene therapy approaches for the treatment of disease has been poor delivery systems leading to poor cell transfection and protein expression [59]. Interest remains high in developing a robust nonviral system due to toxicity concerns associated with the alternative viral based approaches. The use of mechanical/physical delivery systems in combination with plasmid based vectors has produced encouraging results with regard to the high protein expression levels achieved [60–62]. These methodologies such as electroporation or hydrodynamic pressure are fairly complex in design and require special equipment and/or training; however, they are beginning to be clinically evaluated for various diseases and may ultimately prove to have therapeutic applicability [63]. Alternative nonviral approaches are directed at combining plasmid DNA with lipid or polymer based molecules that act to facilitate DNA uptake and expression by promoting interaction with cellular membranes, protecting DNA against endogenous nucleases and trafficking plasmid to the nucleus [64–66].

Polymeric gene carrier systems have been widely used for gene therapy applications and are emerging as a viable alternative to the current systems [35]. These systems are attractive because of ease of use and low cost. Furthermore, excellent molecular flexibility allows for sophisticated modifications and incorporation of novel chemistries with little difficulty [67, 68]. Use of polymers for gene therapy applications also offers the possibility of repeated injections in order to maximize and/or optimize the therapeutic applicability.

Polyethylenimine (PEI) as a gene delivery polymer has a distinct advantage over other nonviral gene delivery systems due to its inherent endosomolytic activity [69]. However, despite its use in animal studies, the clinical development of polyethylenimines has been sluggish due to molecular heterogeneity and acute toxicity [70, 71]. The transfection activity and cytotoxicity of PEI is directly related to its molecular size. Specifically, the larger the molecular size the higher the transfection

activity and cytotoxicity. Different strategies have been described to improve polymer activity without compromising safety including incorporating degradable linkages into high molecular weight polymers to improve clearance [49] or incorporating biocompatible membrane-permeating moieties into low molecular weight polymers to improve activity [36, 72].

Fewell et al. [59] describe the chemical synthesis and the physical and chemical properties of a novel condensing lipopolymer based on a low molecular weight polyethylenimine covalently linked to cholesterol (CHOL) and methoxypolyethylene glycol (PEG) molecules forming PEG–PEI–CHOL (PPC). The polymer offers a high degree of protection against DNases and high *in vitro* and *in vivo* transfection activity. Lv et al. [73] discuss the toxicity of main cationic gene vectors (cationic lipids and cationic polymers); emphasis is placed on the relationship between toxicity and structure of these compounds. The authors evaluate the structural features of cationic compounds and discuss which groups may increase the toxicity, what kind of linkages have relatively short half-life, and how proper modifications will decrease the toxicity of cationic lipids and cationic polymers for gene delivery.

Antisense oligonucleotides (AS-ONs) are specific inhibitors of gene expression. As single strands of DNA, these molecules hybridize to complementary RNA and induce RNase H-mediated degradation of the target RNA [74, 75]. Initially introduced by Zamecnik and Stephenson [76] and Stephenson and Zamecnik [77], AS-ONs represent a promising tool in fighting viral, malignant, and inflammatory diseases. Since unmodified phosphodiester oligonucleotides are susceptible to degradation by exo- and endonucleases, the more stable phosphorothioate oligonucleotides (S-ONs) have become the most extensively used antisense agents. In these molecules, one of the nonbridging oxygens of the phosphate backbone is substituted by a sulfur atom [78]. Several clinical trials with S-ONs are in progress for treatment of chronic lymphocytic leukemia, multiple myeloma, malignant melanoma, pancreatic carcinoma, and non-small-cell lung carcinoma [79, 80]. However, a major problem for the development of antisense therapy is the ineffective cellular uptake of antisense molecules. Oligonucleotides are relatively large hydrophilic molecules with a molecular weight ranging from 5000 to 10,000 Da and do not passively diffuse across cell membranes. Conflicting theories for oligonucleotide transport into the cell have been reported: (1) receptor-dependent [81], (2) independent [82], (3) via pinocytosis [83], or (4) via unknown mechanism (e.g., caveolar or potocytotic) [84]. However, in order to be active, AS-ONs must preferentially be present either in the nucleus or in the cytoplasm. The intracellular localization of ONs is confined to the endocytotic compartments. Only a small portion of ONs are somehow released into the cytoplasm [85].

Improvement of cellular uptake is a major topic of ongoing antisense research. Numerous potential delivery systems such as viral capsid structures, nanoparticles, and liposomes have been explored for efficient antisense delivery [86–88]. Currently, most research focuses on liposomes. Anionic liposomes can be loaded with ONs and then be linked to specific antibodies for tissue targeting [89]. Unfortunately, for these liposomes encapsulation of ONs is quite inefficient. In contrast, incubation of ONs with cationic lipids results in the high efficient formation of liposome–ON complexes by electrostatic interaction. The cellular uptake of ONs with cationic liposomes is strongly enhanced by a factor of 15–25 over ON only, resulting in a 1000-fold overall increase in ON activity [90–92]. This tremendous increase in

activity may be explained by the specific nuclear localization of ONs. In contrast to the cytoplasmic localization of noncomplexed ONs, ONs delivered by cationic liposomes accumulate predominantly in the cell nucleus [93].

Antisense oligonucleotides are being widely investigated for the downregulation of genes [94, 95]. To inhibit protein production, the ONs have to reach the cytoplasm or nucleus of the cells, where they act by specific binding to the target mRNA or DNA. In spite of this simple action mechanism, different barriers still limit the antisense activity. Actually, before the ONs can reach their target site, they first have to cross the cellular membrane, escape from the endosomal compartment, leave their pharmaceutical carriers (i.e., the delivery system), and hybridize to the target sequence [96].

Rapid degradation of phosphodiester ONs is known to be one of the factors limiting their success as therapeutics. One approach to improve antisense activity is to develop ONs with increased nuclease stability so that intracellular degradation should not be an issue [97]. However, modification of the ON backbone often induces undesirable features. The first generation of chemically modified ONs, namely, phosphothioate ONs, are known to frequently exhibit nonspecific, nonantisense effects, generally attributed to their increased nonspecific protein binding when compared to their phosphodiester analogues [98]. The second and third generation antisense compounds all work to some extent but have one or more problems such as solubility, delivery issues, lack of RNase H activation, or simply the cost of synthesis [99].

Another approach to improve the antisense activity of ONs is the development of suitable delivery systems [96, 100]. The delivery system, often composed of cationic lipids or cationic polymers, is designed to help the ONs enter the cell and escape from the endosomes. Clearly, in cases where (degradable) phosphodiester ONs (PO-ONs) are used, the protection of the ONs against nucleases, given by their carrier, becomes an important aspect. Ideally, the delivery system should protect the complexed ONs during the different steps of the trafficking pathway. In general, cationic carriers are believed to protect the complexed ONs against degradation. However, some carriers can establish an antisense effect with degradable phosphodiester ONs, while other carriers fail [101, 102]. The lack of antisense activity has been attributed to insufficient ON protection against nucleases with the carriers used, although no direct proof of this hypothesis was given.

RNAi is a recently developed technique to silence proteins in a sequence-specific manner by inhibiting mRNA and consequently reducing protein expression [103]. The functional mediator of RNA interference is a short dsRNA oligonucleotide called small interfering RNA (siRNA). A growing number of investigations are being reported on efforts to use siRNA as a candidate therapeutic agent [104]. To achieve therapeutic success, however, several hurdles must be overcome including rapid clearance, nuclease degradation, and inefficient intracellular localization. Most efforts reported to date are based on studies in rodents and rodent models of disease. With the exception of local administration into eye tissues, the results show low activity due to a strong dependence on methods for intracellular delivery, as found for antisense and gene therapy approaches. One surprising observation is that siRNA composed of unmodified RNA is rapidly cleared by the kidneys [105], indicating low binding to proteins or cells in the blood and a surprising lack of nuclease degradation as the major barrier. A delivery strategy for use of siRNA as a thera-

peutic modality should therefore firstly reduce glomerular filtration, and secondly optimize intracellular delivery to target cells, while also minimizing exposure to nuclease and distribution to nontarget tissues. Nanoparticle delivery systems developed with a ligand–polymer–polymer conjugate appear attractive to meet these needs and indeed neovasculature targeted forms, using a "cyclic" RGD peptide as the ligand, have produced successful results [106, 107].

Peptide nucleic acids (PNAs) are rapidly emerging as powerful tools in DNA hybridization and antisense techniques. PNAs are synthetic oligonucleotide analogues in which the natural nucleic acid backbone is replaced by an uncharged pseudopeptide backbone consisting of *N*-(2-aminoethyl)-glycine units linked to the natural purine and pyrimidine bases [108]. They form very stable complexes with complementary DNA or RNA strands with very high affinity, partially as a result of the neutrality and flexibility of the artificial backbone.

PNAs possess a strong resistance to enzymatic degradation in cells, fluids, and tissues, showing an exceptional stability in biological environments [109]. In addition, the unique chemical, physical, and biological properties of PNAs have been exploited to produce powerful biomolecular tools, antisense and antigene agents, molecular probes, and biosensors. Important new applications that could not be performed using natural oligonucleotides have emerged. Duplexes between PNA and DNA or RNA are in general thermally more stable than the corresponding DNA–DNA or DNA–RNA duplexes [109, 110]. The sequence dependence of the stability, however, is more complex than that found for DNA–DNA complexes because of the inherent asymmetry of the duplex. In fact, PNA–DNA duplexes show significantly increased stability when the purines are in the PNA strand. Thus, on top of the dependence on G–C content, the stability of PNA–DNA duplexes also depends on the purine fraction of the PNA strand [111]. In general, it is also observed that the thermal stability of PNA–PNA duplexes exceeds that of PNA–RNA duplexes, which again are more stable than PNA–DNA duplexes.

Very importantly, but not surprisingly, the stability of PNA–DNA duplexes is almost unaffected by the ionic strength of the medium (actually the stability decreases slightly with increasing Na^+ concentration due to counterion release upon duplex formation). This is in sharp contrast, of course, to the behavior of DNA–DNA (or RNA) duplexes, whose stability decreases dramatically at low ionic strength because of the required counterion shielding of the phosphate backbone [112].

PNA hybridization kinetics have also been studied by surface plasmon resonance (SPR) technology and the results, although they cannot be considered conclusive, indicate no major difference in PNA and DNA hybridization duplex formation [110]. Although it is not the intention to give an elaborate account of the ongoing development of gene therapeutic drugs based on PNA oligomers, it is fair to say that PNA does possess many of the properties desired for an antisense reagent. It binds strongly and with excellent sequence specificity to complementary mRNA, it has very high biological stability, and targeting of specific mRNAs with PNA has been shown to strongly inhibit its translation. This has been demonstrated in several biological systems *in vitro*, and the introduction of various novel methods for improved delivery of PNA to eukaryotic cells has recently also allowed good efficacy in cell culture *ex vivo* and, sometimes, *in vivo* [113–116]. In the last two years, PNA antisense and antigene technology has entered the realm of biological and preclinical studies. This is primarily due to the development of a number of methods able to improve PNA permeability across cell membranes [117–119] and to allow

efficient delivery of PNA oligomers to eukaryotic cells. Two novel delivery systems based on polymeric core–shell microspheres and autologous loaded opsonized RBC systems were able to specifically inhibit the expression of COX-2 or iNOS proteins in murine macrophages at nanomolar concentration, that is, with 3 log lower concentration with respect to those required by PNA linked to hydrophobic peptide [120]. Authors concluded that core–shell polymeric microspheres and loaded RBC represent promising delivery strategies for *in vivo* targeting of antisense PNAs to macrophages during inflammatory processes.

17.2.6 Enhanced Delivery and Semisolid or Sustained Release Preparations (Micronization and Nanoparticles)

In order to improve the specific delivery and bioavailability of drugs with low therapeutic index, several drug carriers such as liposomes, microparticles, nanoassociates, nanoparticles, drug polymer conjugates, and polymeric micelles have been developed. In recent years, polymeric micelles have been the object of growing scientific attention [121].

Micelles One of the widely used techniques for augmenting the water solubility of drug candidates is through modification of the solubilization medium by using cosolvents, complexing agents (i.e., cyclodextrins), or surfactants [122, 123]. In an aqueous medium, above a given concentration termed the critical micellar concentration (CMC), surfactants self-assemble into a colloidal dispersion of molecular aggregates called micelles (<50–100 nm) [124] and have been shown to solubilize hydrophobic drugs inside the core formed by the lipophilic part of the surfactant. The hydrophilic moieties form the shell or corona of these nanocarriers [124–128]. In addition to the increase in drug solubility, micelles can protect the incorporated drugs by insulating them from the aqueous environment.

Besides classic surfactants, micelles can also be formed from amphiphilic polymers. These polymers often provide better kinetic and thermodynamic stability than conventional surfactants [124–128]. However, they exhibit a number of disadvantages including a complex manufacturing process, which requires organic solvents followed by a dialysis step or an evaporation process depending on polymer polarity [125–128]. These limitations were addressed by the introduction of self-assembling polymeric micelles such as mmePEG$_{750}$P(CL-*co*-TMC) (monomethyletherpoly(oxyethylene glycol$_{750}$)-poly(caprolactone-*co*-trimethylene carbonate)). This amphiphilic copolymer aggregates spontaneously into 20 nm micelles upon contact with water [129] and is intended for oral delivery of poorly soluble drugs.

Micelles as drug carriers are able to provide a set of unbeatable advantages—they can solubilize poorly soluble drugs and thus increase their bioavailability, they can stay in the body (in the blood) long enough to provide gradual accumulation in the required area, their size permits them to accumulate in body regions with leaky vasculature, they can be targeted by attachment of a specific ligand to the outer surface, and they can be prepared in large quantities easily and reproducibly [125]. Being in a micellar form, the drug (poorly soluble drug, first of all) is well protected from possible inactivation under the effect of biological surroundings, it does not provoke undesirable side effects, and its bioavailability is usually increased.

The micelle is structured in such a way that the outer surface of the micelle exposed to the aqueous surrounding consists of components that are hardly reactive

toward blood or tissue components. This structural peculiarity allows micelles to stay in the blood (tissues) rather long without being recognized by certain proteins and/or phagocytic cells. This longevity is an extremely important feature of micelles as drug carriers. Long-circulating pharmaceuticals and pharmaceutical carriers currently represent a fast growing area of biomedical research; for example, see Refs. 130–134.

Micronization Developing intravenous formulations of hydrophobic drugs is particularly challenging. Possessing intravenous formulations of drugs can open new markets for drugs like antibiotics that could often be initially prescribed in a hospital setting. In addition, intravenous formulations can expand the market for the oral dosage formulation because physicians typically prefer to discharge patients on the oral formulation of the same drug administered intravenously in the hospital.

Many hydrophobic drugs are comprised of particles that are relatively large and therefore have a limited surface area available for interaction with water. These hydrophobic drugs are often formulated in less than ideal ways in order to make them dissolve. It is possible to increase the dissolution rate of hydrophobic drugs by increasing their aggregate surface area. To accomplish this, many pharmaceutical companies use a process, called micronization, which entails grinding hydrophobic drugs into smaller microparticles. However, the drug particles produced by micronization are often still not small enough to adequately improve dissolution, or to be administered intravenously. New technologies have been developed that convert drugs that do not dissolve well in water—hydrophobic drugs—into microparticles or nanoparticles of the drugs embedded in small microparticles. Alternatively, oils like Cremophor are used to dissolve the drugs. However, these oils are often not well tolerated and can require prolonged infusion rather than rapid injections. In addition, some hydrophobic drugs can be formulated into soft gelatin capsules, but these are only suitable for oral administration and encapsulate only a small volume of drug, requiring the administration of many capsules. Sometimes development of these drugs must be terminated because no suitable formulation can be found.

A continuous basal insulin supply may help to improve the condition of diabetics. A body weight gain in diabetic rats was observed as fast as in normal rats after a single injection of insulin-loaded microspheres in 28 days [135]. *In vitro* release of insulin from poly(lactide-*co*-glycolide) (PLGA) microspheres was improved by physically mixing microspheres prepared by water-in-oil-in-water double emulsion and solid-in-oil-in-water emulsion methods [136]. In this study, insulin-loaded microspheres maintained basal insulin level in diabetic rabbits for 40 days. Histological evaluations suggested that the insulin-loaded microspheres were biocompatible.

Macromolecular drugs such as peptides and proteins are unable to overcome the mucosal barriers and/or are degraded before reaching the bloodstream. Among the approaches explored so far in order to optimize the transport of these macromolecules across mucosal barriers, the use of nanoparticulate carriers represents a challenging but promising strategy. Nanostructures based on the mucoadhesive polysaccharide chitosan-coated systems showed an important capacity to enhance the intestinal absorption of the model peptide, salmon calcitonin, as shown by the important and long-lasting decrease in the calcemia levels observed in rats [137].

Alonso [138] reviews recent advances in the design of polymeric nanosystems intended to be used as carriers for nasal vaccine delivery. The information accumu-

lated regarding the *in vivo* behavior of these nanocarriers indicates that they are able to facilitate the transport of the associated antigen across the nasal epithelium, thus leading to efficient antigen presentation to the immune system. Furthermore, the results suggest that not only the size and surface properties but also the polymer composition and the structural architecture of the nanosystems are critical for the optimization of these antigen carriers.

Dendrimers Dendrimers are one of the emerging delivery systems with the capability to present such hydrophobic agents in a formulation with better prospective [139]. Dendrimers are synthetic, highly branched, monodisperse macromolecules of nanometer dimensions. These dendritic macromolecules with a large number of surface terminal groups and interior cavities offer a better opportunity for delivery by becoming charged and acting as static covalent micelles [140]. Started in the mid-1980s, the research investigations into the synthetic methodology and physical and chemical properties of these macromolecules are increasing exponentially with growing interest in this field. Although dendrimer drug delivery is in its infancy and toxicity is still a problem, it offers several attractive features. It provides a uniform platform for drug attachment that has the ability to bind and release drugs through several mechanisms. Properties associated with these dendrimers such as uniform size, water solubility, modifiable surface functionality, and available internal cavities make them attractive for biological and drug delivery applications. These are biocompatible, nonimmunogenic, and water soluble and possess terminal functional groups for binding various targeted or guest molecules [141]. The host–guest properties of dendrimers based on hydrophobic and ionic interactions apart from physical entrapment have been thoroughly studied [142]. The involvement of dendrimer in enhancing solubility of acidic anti-inflammatory drug through ionic interaction has been explored [143]. Dendrimers have also been explored for intracellular delivery of the anti-inflammatory drug ibuprofen [144]. As the dendrimer has many end functional groups, these would determine their solubility and physical and chemical interaction in the immediate surrounding environment [145].

Liposomes Liposomes are widely used as nanosized drug delivery vehicles for active and passive targeting [146]. Liposomes may also "plug" and "seal" the damaged myocyte membranes and protect cells against ischemic and reperfusion injury *in vitro* [147] and the delivery of the ATP into the ischemic myocytes at the same time [148, 149]. As a drug delivery system, liposomes can significantly alter the pharmacokinetics and pharmacodynamics of entrapped drugs, for example, by enhancing drug uptake, delaying rapid drug clearance, and reducing drug toxicity [150–152].

For example, pegylated liposomal formulation of 2′-deoxyinosine (a 5-fluorouracil modulator), through an optimized pharmacokinetic profile (near seven times clearance reduction as compared with the free form), displays a potent tumor reduction effect in xenografted mice [153].

17.2.7 Vehicles and Selection of Excipients

FDA guidance [154] means by *new excipients* any inactive ingredients that are intentionally added to therapeutic and diagnostic products, but that (1) we believe

are not intended to exert therapeutic effects at the intended dosage, although they may act to improve product delivery (e.g., enhance absorption or control release of the drug substance); and (2) are not fully qualified by existing safety data with respect to the currently proposed level of exposure, duration of exposure, or route of administration. Examples of excipients include fillers, extenders, diluents, wetting agents, solvents, emulsifiers, preservatives, flavors, absorption enhancers, sustained release matrices, and coloring agents.

In addition to their intended use, excipient candidates should also be qualified as appropriate pharmaceutical ingredients. Generally, it is preferable to select excipients that have been used in marketed products with a relevant route of delivery. Furthermore, excipients that have been used with a similar dosing frequency, history of chronic use, and patient population are also preferable. Otherwise, the approval and safety of the excipients need to be carefully examined. If the excipient is known to be safe on the basis of solid science or has a proven clinical safety record, it can be considered equivalent to approved excipients. The list of excipients used for parenteral pharmaceuticals is available in the literature [155–157]. The current information from the Inactive Ingredient Database can be found at the FDA website at http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm.

When it is necessary to introduce other excipients with minimal safety records, a significant risk associated with the excipients will be added to the product development process and additional preclinical and clinical studies may be needed. Not all excipients are inert substances; some have been shown to be potential toxicants. The Federal Food, Drug, and Cosmetic Act of 1938 (the Act) was enacted after the tragedy of the elixir of sulfanilamide in 1937 in which an untested excipient was responsible for the death of many children who consumed the pharmaceutical. The Act required manufacturers to perform safety testing of pharmaceuticals and submit new drug applications (NDAs) demonstrating safety before marketing. Since that time, the FDA has become aware that certain other excipients used in commerce can cause serious toxicities in consumers of prescription and over-the-counter (OTC) drug products in the United States and other countries.

The FDA's *Guidance for Industry: Nonclinical Studies for the Safety Evaluation of Pharmaceutical Excipients* [154] describes the types of toxicity data that the Agency uses in determining whether a potential new excipient is safe for use in human pharmaceuticals. It discusses recommended safety evaluations for excipients proposed for use in OTC and generic drug products, and describes testing strategies for pharmaceuticals proposed for short-term, intermediate, and long-term use. It also describes recommended excipient toxicity testing for pulmonary, injectable, and topical pharmaceuticals.

Another important requirement in qualifying an excipient is the purity of the raw material. Depending on its historical use as a pharmaceutical ingredient, excipients are available in several different pharmaceutical grades, for example, USP (U.S. Pharmacopoeia), Ph. Eur. (European Pharmacopoeia), and JP (Japan Pharmacopoeia). These pharmaceutical grade materials should be considered as a primary resource, but the quality provided may not be good enough for specific product development. For example, significant stability problems can be found with some impurities even at concentrations below their specification (e.g., metal ions, peroxides, protease, and reducing sugars). These problems are more prominent in low protein concentration products due to a high impurity/protein ratio, although problems like visible precipitation may be independent of protein concentration. If an

adjustment to the existing specification is necessary, it is critical to look into the availability of GMP quality raw materials with modified specifications as early as possible once the potential problem is identified. The use of excipients derived from animals or humans should be avoided if possible due to the risks associated with transmissible diseases like bovine spongiform encephalopathy, Creutzfeldt–Jakob disease, hepatitis virus, and HIV. Numerous regulatory guidelines have been issued to discourage the use of animal/human derived excipients (Note for Guidance on Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents Via Medicinal Products—EMEA (4/99); Note for Guidance on Plasma-Derived Medicinal Products—EMEA (7/98); Development Pharmaceutics for Biotechnological and Biological Products. Annex to Note for Guidance on Development). When animal derived excipients have to be included in a product, the manufacturer will need to fulfill its responsibilities to justify their selection and implement adequate safety measures.

A review of commercially available oral and injectable solution formulations presented by Robert Strickley [158] reveals that the solubilizing excipients include *water-soluble organic solvents* (polyethylene glycol 300, polyethylene glycol 400, ethanol, propylene glycol, glycerin, *N*-methyl-2-pyrrolidone, dimethylacetamide, and dimethylsulfoxide), *nonionic surfactants* (Cremophor EL, Cremophor RH 40, Cremophor RH 60, *d*-α-tocopherol polyethylene glycol 1000 succinate, polysorbate 20, polysorbate 80, Solutol HS 15, sorbitan monooleate, poloxamer 407, Labrafil M-1944CS, Labrafil M-2125CS, Labrasol, Gellucire 44/14, Softigen 767, and mono- and di-fatty acid esters of PEG 300, 400, or 1750), *water-insoluble lipids* (castor oil, corn oil, cottonseed oil, olive oil, peanut oil, peppermint oil, safflower oil, sesame oil, soybean oil, hydrogenated vegetable oils, hydrogenated soybean oil, and medium-chain triglycerides of coconut oil and palm seed oil), *organic liquids/semisolids* (beeswax, *d*-α-tocopherol, oleic acid, medium-chain mono- and diglycerides), various *cyclodextrins* (α-cyclodextrin, β-cyclodextrin, hydroxypropyl-β-cyclodextrin, and sulfobutylether-β-cyclodextrin), and *phospholipids* (hydrogenated soy phosphatidylcholine, distearoylphosphatidylglycerol, l-α-dimyristoylphosphatidylcholine, l-α-dimyristoylphosphatidylglycerol). The chemical techniques to solubilize water-insoluble drugs for oral and injection administration include pH adjustment, cosolvents, complexation, microemulsions, self-emulsifying drug delivery systems, micelles, liposomes, and emulsions.

An excellent reference for excipient information is the APA's *Handbook of Pharmaceutical Excipients* [159]. A very extensive and detailed list of vehicles and excipients commonly used in preclinical animal studies is presented by S. Gad in his book *Drug Safety Evaluation* [4, pp. 494–500]. In addition to this, we present below in more detail three other commonly or more recently used vehicles—Cremophor, chitosan, and cyclodextrin.

Cremophor EL (Polyoxyethyleneglycerol Triricinoleate) The heterogeneous nonionic surfactant CrEL is a white to off-white viscous liquid with an approximate molecular weight of 3 kDa and a specific gravity (25 °C/25 °C) of 1.05–1.06, and it is produced by the reaction of castor oil with ethylene oxide at a molar ratio of 1:35 [160]. Castor oil is a colorless or pale yellow fixed oil obtained from the seeds of *Ricinus communis*, with an extremely high viscosity, and consists mainly of the glycerides of ricinoleic, isoricinoleic, stearic, and dihydroxystearic acids. CrEL is usually of highly variable composition, with the major component identified as

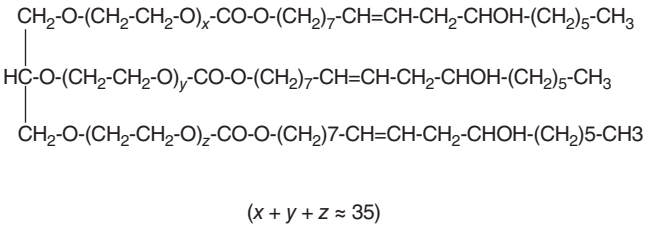


FIGURE 17.1 Chemical structures of the main component of CrEL (polyoxyethyleneglycerol triricinoleate 35).

oxylated triglycerides of ricinoleic acid (i.e., polyoxyethyleneglycerol triricinoleate 35) (Fig. 17.1). Polyvinyl chloride (PVC)-free equipment for CrEL administration is obligatory, since CrEL is known to leach plasticizers from PVC infusion bags and polyethylene-lined tubing sets, which can cause severe hepatic toxicity [161]. CrEL is being used as a vehicle for the solubilization of a wide variety of hydrophobic drugs, including anesthetics, photosensitizers, sedatives, immunosuppressive agents, and (experimental) anticancer drugs. The amount of CrEL administered with these drugs averages 5 mL (range 1.5–10.3 mL), although paclitaxel is an exception as the amount of CrEL is much higher per administration, approximately 26 mL.

Recent investigations have revealed that CrEL, a widely used formulation vehicle, is a biologically and pharmacologically active ingredient of various commercially available drugs. For example, when used in paclitaxel administrations, an exceptionally large amount of CrEL is inevitably coadministered with the IV infusions, causing important biological events that can lead to serious acute hypersensitivity reactions and neurological toxicity, depending on the dose and duration of infusion [5, 162–164 for a review]. In addition, the substantial effects of CrEL on the disposition of other coadministered drugs (e.g., anthracyclines) with a narrow therapeutic window in polychemotherapeutic regimens also can be potentially hazardous to the patient [165]. In view of the inherent problems associated with the use of CrEL, it can be anticipated that there may be a therapeutic advantage from using paclitaxel formulations in which CrEL is absent [5]. Currently, a large variety of new (CrEL-free) formulation vehicles for paclitaxel are in (pre)clinical development, including cosolvent systems (Tween 80/ethanol/Pluronic L64), water-soluble polymers (e.g., polyethylene glycols), emulsions (e.g., triacetin), liposomes, albumin, cyclodextrins, nanocapsules, and microspheres (reviewed in Ref. 166).

In contrast, the *in vitro* and *in vivo* observations of myeloprotective effects related to the use of CrEL in combination with some agents, such as cisplatin, might be used to reformulate such agents with CrEL in order to achieve an optimization of their therapeutic window [167–169]. Iwase et al. [170, 171] have shown that Cremophor EL augments the cytotoxicity of hydrogen peroxide in rat thymocytes.

Based on our laboratory experience (data not published), we have seen some hypersensitivity reactions to CrEL in dogs and would recommend replacement of this vehicle with less toxic vehicles in preclinical dosage formulation, especially in dog studies.

Chitosan Chitin is a copolymer of *N*-acetyl-glucosamine and *N*-glucosamine units randomly or block distributed throughout the biopolymer chain depending on the

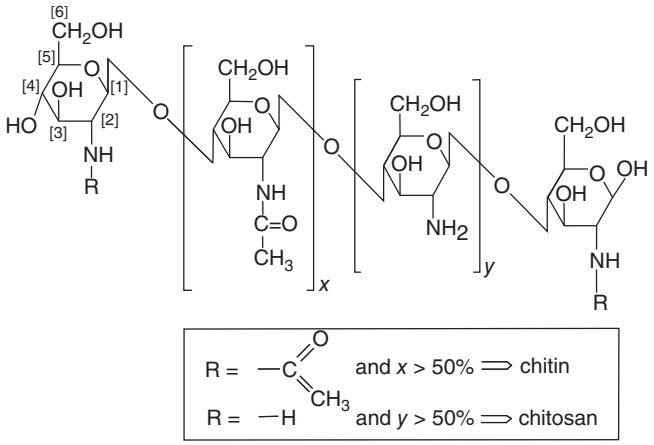


FIGURE 17.2 Schematic representation of the chitin and chitosan depicting the copolymer character of the biopolymers.

processing method used to derive the biopolymer (Fig. 17.2). When the number of *N*-acetyl-glucosamine units is higher than 50%, the biopolymer is termed chitin. Conversely, when the number of *N*-glucosamine units is higher, the term chitosan is used. Chitosan has been the better researched version of the biopolymer because of its ready solubility in dilute acids, rendering chitosan more accessible for utilization and chemical reactions.

Today, the production, chemistry, and applications of chitin and chitosan are well known. Commercially, chitin and chitosan are obtained from shellfish sources such as crabs and shrimps. It is likely that future sources of chitin and chitosan will come from biotechnology innovations, especially when medical applications are the focus.

Chitosan, a cationic polysaccharide obtained by alkaline *N*-deacetylation of chitin, is one of the most widely utilized polysaccharides. The sugar backbone of chitosan consists of β-1,4-linked *d*-glucosamine. In its structure, chitosan is very similar to cellulose, except for the amino group replacing the hydroxyl group on the C-2 position [172]. Recently, chitosan has been widely employed in pharmaceutical and biomedical fields owing to its unique properties such as nontoxicity, biocompatibility, and biodegradability [173, 174]. These characteristics make chitosan an excellent candidate for various biomedical applications such as drug delivery, tissue engineering, and gene delivery [173–178].

Chitosan has been investigated as an excipient in the pharmaceutical industry, to be used in direct tablet compression, as a tablet disintegrant, for the production of controlled release solid dosage forms, or for the improvement of drug dissolution [174]. Compared to traditional excipients, chitosan has been shown to have superior characteristics and especially flexibility in its use. Furthermore, chitosan has been used for production of controlled release implant systems for delivery of hormones over extended periods of time. Lately, the transmucosal absorption promoting characteristics of chitosan have been exploited, especially for nasal and oral delivery of polar drugs to include peptides and proteins and for vaccine delivery. These

properties, together with the very safe toxicity profile, make chitosan an exciting and promising excipient for the pharmaceutical industry for present and future applications.

The transfection efficiency of low molecular weight chitosan (LMWC, molecular weight of 22 kDa) was significantly higher than naked DNA and higher than poly-L-lysine (PLL); it was also less cytotoxic than PLL [176]. For the further development of efficient gene carriers, highly purified chitosan oligosaccharides (COSs) were chemically modified with deoxycholic acid (DOCA). Owing to the amphiphilic characters, the DOCA-conjugated COSs (COSDs) formed self-aggregated nanoparticles in aqueous milieu. COSDs showed great potential as gene carriers with a high level of gene transfection efficiencies, even in the presence of serum. Considered with the negligible cytotoxic effects, DOCA-modified chitosan oligosaccharides can be considered as potential candidates for efficient nonviral gene carriers [50]. A vehicle containing 1.5% chitosan at pH 6.7 increased the bioavailability of intraduodenally administered buserelin in rats from 0.1% to 5.1% [179].

Cyclodextrins Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six or more glucose units linked by α-(1,4)-glycosidic bonds. α-, β-, and γ-Cyclodextrins (α-CD, β-CD, and γ-CD) are composed of six, seven, and eight unsubstituted glucose units, respectively (Fig. 17.3). In nature, cyclodextrins are produced as a storage form of carbohydrate (reserve substrate) by different microorganisms [180]. On an industrial scale, cyclodextrins are obtained by enzymatic hydrolysis of starch with cyclodextrin-glucosyltransferases. These amylolytic enzymes are produced by different strains of bacilli (e.g., *Bacillus macerans, Bacillus circulans*, and *Bacillus firmus*) and other organisms of other species (e.g., *Klebsiella oxytoca, Micrococcus* sp., and *Thermoanaerobacter* sp.). Cyclodextrin-glucosyltransferase (CGTase) cleaves the helical amylose molecule at regular intervals of six, seven, or eight glucose units, forming at the same time a ring by an intramolecular glucosyltransferase reaction [181].

Cyclodextrins have attracted the interest of the pharmaceutical, cosmetic, and—more recently—the food industry because they can entrap lipophilic compounds in the cavity of their torus-like structure [182–184]. Because of their larger ring, γ-CD and β-CD can host a wider range of molecules than α-CD. The usefulness of α-CD as a carrier and protectant of lipophilic molecules is therefore limited. However,

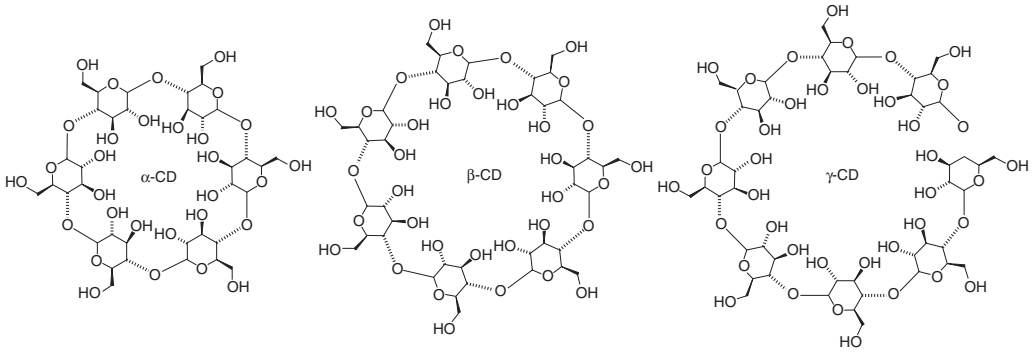


FIGURE 17.3 Chemical structure of the three main types of cyclodextrins.

α-CD merits interest as a potential food ingredient for a completely different reason. It has been found that α-CD is not digested by pancreatic amylase and therefore has the physiological properties of a water-soluble, dietary fiber. Consisting of α-(1,4)-linked glucose molecules, it structurally and metabolically resembles retrograded or crystalline nongranular starch ("resistant starch" of the RS3 type according to Englyst's classification) [185]. However, unlike resistant starch, α-CD is freely soluble in water (about 13 g/100 mL at 25 °C), yielding clear solutions with a low viscosity. Because of these properties, α-CD is very well suited for use as a dietary fiber not only in solid but also in semiliquid foods (e.g., yogurt) and all types of beverages.

The structural features of CDs are evaluated for their effect on complexation performance in pharmaceuticals [186]. Optimal specifications, quality production, and safety of each CD are presented. The current and future regulatory process facing excipients is summarized, and the current regulatory status of CDs in Japan, the United States, and Europe is presented.

Cyclodextrin complexation seems to be an attractive delivery system for proteins and peptides helping to overcome chemical and enzymatic instability, poor absorption through biological membranes, and rapid plasma clearance of these macromolecules [187]. The results from the study by Oda et al. [188] suggest that β-CD is considered as one of the most suitable solubilizing agents for evaluating poorly water-soluble drugs using *in situ* loop and perfusion techniques. The capacity to dissolve phenytoin was great in β-CD and hydroxypropyl β-cyclodextrin, followed by Tween 80. Those of methanol, dimethyl sulfoxide, dimethyl acetoamide, and polyethylene glycol 400 were much lower than expected.

However, CDs have been reported to interact with cell membrane constituents such as cholesterol, phospholipids, and phosphatidylinositols, resulting in the induction of hemolysis of RBCs [189–191]. Additionally, randomly methylated β-cyclodextrin (M-β-CD) disrupted the structures of lipid rafts and caveolae [192, 193], which are lipid microdomains formed by lateral assemblies of cholesterol and sphingolipids in the cell membrane, through extraction of cholesterol from the microdomains [194]. The magnitude of the hemolytic activity of the parent CDs is reported to increase in the order of γ-CD < α-CD < β-CD [189, 195]. Recently, various hydrophilic CD derivatives have been developed to improve aqueous solubility and complexation ability of parent CDs. Of the various hydrophilic CD derivatives, hydroxypropylated CDs, sulfobutyl ether CDs, and branched CDs are able to lower the hemolytic activity of each parent CD [196]. Conversely, methylation of the hydroxyl group at the 2 and 6 positions of a glucopyranose unit of β-CD is known to severely augment the hemolytic activity in high concentrations [197]. Arima et al. [198] and Motoyama et al. [199] reported that 2,6-di-*O*-methyl-α-CyD (DM-α-CyD) is predominantly likely to release CD14, a glycosylphosphatidylinositol (GPI)-anchored protein, from lipid rafts through the extractions of phospholipids from murine macrophages without cytotoxicity compared to α-CyD and 2-hydroxypropyl-α-cyclodextrin (HP-α-CyD). Recently, Motoyama et al. [200] showed that DM-α-CyD has higher hemolytic and morphological change activity than α-CyD and HP-α-CyD through more extraction of phospholipids including sphingomyelin and proteins, not cholesterol, from RBC membranes than α-CyD and HP-α-CyD. These toxicity reports for different types of cyclodextrins should be taken into account for their selection in dosage formulations.

17.3 TYPES AND CHARACTERISTICS OF DOSAGE FORMULATIONS BY ROUTE OF ADMINISTRATION

17.3.1 Oral Formulations

Pharmaceutical preparations for peroral administration form the majority of drug products most frequently administered. Within this group, availability for absorption usually decreases as solutions > suspensions > capsules > compressed tablets > coated tablets. Changes in this order may occur but are exceptions [201].

Solutions This dosage form presents the drug component in a form most suitable for absorption. Hence, if there is no complexation or micellization with components of the formula, and if there is no degradation in the stomach, this dosage form will give maximal bioavailability and a rapid onset of action. The other advantage is that since solutions are homogeneous mixtures, the medication is uniformly distributed throughout the preparation. A number of drugs irritate the gastric mucosa when given in a concentrated form, such as a capsule or a tablet. This irritancy may be reduced by an adequate dilution factor if the drug is administered as a solution.

The disadvantage is that the solution form is bulky and hence requires increased costs for containers, handling, and storage. A drug in solution is usually the form most susceptible to degradation. Because solutions are, in many instances, fair to good microbiological growth media, adequate care must be taken to ensure a low initial bacterial count and prevention of growth during storage. The formulations are usually kept under refrigerated conditions to increase stability and decrease microbiological growth.

Emulsions An emulsion is a thermodynamically unstable heterogeneous system consisting of at least one immiscible liquid intimately dispersed in another in the form of droplets. Oil emulsions (o/w) are usually manufactured to make the active oil phase easier to handle and sometimes more bioavailable. For example, griseofulvin, a poorly water-soluble drug, shows an increase in the rate and extent of absorption when dispersed in corn oil and then emulsified (o/w) [202]. The smaller amounts of drug present in the upper region of the small intestine at any particular time after oral administration of the corn oil emulsion, coupled with the inhibitory effect of lipid on proximal small intestine motility, allows the drug more time to dissolve in, and be absorbed from, the region of the intestinal tract where absorption is optimal. Also, the presence of emulsified corn oil in the intestinal tract stimulates bile excretion.

Emulsions, however, present problems with stability, not only of the emulsion itself, but also of the active ingredient. Changes in the solubility of emulsifiers and their hydrophilic–lipophilic balance, the viscosity of the emulsion, and partitioning of ingredients should always be considered.

Suspensions A suspension is a two-phase system composed of a solid material dispersed in a liquid. A suspension may be considered the most desirable dosage form for a particular drug for a number of reasons. Certain drugs are chemically unstable when in solution but stable when a suitable salt or derivative is suspended. The suspension presents most of the advantages of the solution dose form: ease in

swallowing, greater ease in administration of unusually large doses, and infinite variability of dosage. The disadvantages of suspensions are that they are heterogeneous systems: homogeneity of dose throughout the entire use period is difficult to maintain, or components may settle before the dose is measured. To overcome this problem, a suspending agent is usually added to increase viscosity and hence slow settling in the system.

Powders and Granules Powders and granules, in general, provide a better environment for maintaining stability of the active drug than solutions, emulsions, and suspensions. They may be used to advantage in a number of different ways as follows:

1. The powder volume is usually less than an equivalent liquid volume.
2. The powder or granule could be administered by being mixed with the animal's food. Less common, the powder may be formulated as a soluble powder, to be added to drinking water.
3. There is good stability (may be difficult with some premix components) and easy tailoring of dose.
4. Specialized dosing equipment or unnecessary handling of the animal is not required.

A disadvantage would be that the dose actually consumed may vary widely, depending on the adequacy of the mix and the animal's eating/drinking habits. A medication that changes the odor and taste characteristics of the usual diet (or water) may cause problems with palatability.

Capsules Capsules are the most common formulations used in dogs. Unlike other dosage forms, the capsule is a tasteless, easily administered and digested container for different materials such as powders, granules, pellets, suspensions, emulsions, or oils. There are two basic types of capsules. The hard gelatin capsule [203] is usually used for solid fill formulations and the soft gelatin capsule [204] for liquid or semisolid fill formulations. Soft gelatin capsules should be used in preference to hard gelatin capsules probably only if the fill is fluid or if bioavailability of the hard gelatin capsule formulation does not meet with requirements. The capsule offers an advantage in that the particle size and distribution of the original starting compound are rarely altered by the final filling process. Capsules are efficient in hiding the taste and odor of an unpleasant drug. Filling of the capsules takes time and labor costs are therefore high. The gelatin capsule does not offer protection from oxygen or moisture, although if it has an opaque shell, it will protect the contents from light. Hence, information on the stability and moisture sensitivity of the formulation must be known before the gelatin shell dosage form is selected.

Paste Use of an oral paste formulation should be considered to overcome some of the dosing problems associated with other conventional dose delivery systems [205, 206]. A well formulated paste can provide accurate dose delivery, the dose being easily individually tailored to the animal's body weight. A paste affords a high degree of safety to both the administrator and animal.

TABLE 17.6 Advantages and Disadvantages of a Prolonged Release Delivery System

Advantages	Disadvantages
1. The maintenance of a relatively constant drug concentration in blood can reduce fluctuations of drug concentrations in tissue and at biological target sites for a more uniform pharmacological response.	1. Controlled release formulations are comparatively more costly to manufacture.
2. The incidence and intensity of side effects that might be caused by excessively high peak plasma concentrations resulting from the administration of conventional dosage forms may be reduced.	2. They do not permit termination.
3. The number and frequency of doses is decreased; hence, there is a reduction in labor costs and trauma to the animal.	3. Drugs with a low margin of safety may cause concern.
4. Slow accumulation (so-called enhanced permeability and retention effect, EPR) in pathological sites with affected and leaky vasculature (such as tumors, inflammations, and infarcted areas) and improved or enhanced drug delivery in those areas can be achieved.	4. A type of first-pass effect could be experienced.
5. Gradual release of a drug from a dosage form may reduce or prevent irritation to the gastrointestinal mucosa by drugs that are irritating to the tissue in high concentration.	

Tablets or Boluses A tablet or bolus offers many advantages over other dose forms. The tablet offers relative ease of administration of an "accurate" dose and is readily adaptable to various dose sizes of medicinal substances. Tablets are portable and compact. They usually present the fewest problems with stability. However, the tablet form is usually not available in the early stages of preclinical safety drug assessment. Tablets also pose a problem because an animal can easily eject the tablet when out of sight of the doser, if the tablet has not been properly administered. To achieve optimal drug delivery to the intended site of absorption or action, the active ingredient should be protected from stomach function. Encapsulation or ptotective coating to achieve this should be considered. Gastric irritants, gastric degradable drugs, glandular products, and gastric nauseants, as well as drugs intended for action in the intestine, should be enteric coated to attain maximum effectiveness with least drug loss or side effects [205].

Controlled Release Pharmaceuticals Long-circulating pharmaceuticals and new pharmaceutical carriers are rapidly developing. These dosage forms allow maintenance of a required level of a pharmaceutical agent in the blood for extended time intervals for better drug availability. The main advantages and disadvantages of prolonged release delivery are presented in Table 17.6.

17.3.2 Intravenous and Other Parenteral Formulations

The intravenous (IV) dosage form must be a solution and thus the physicochemical properties of the drug are taken into consideration. Once the formulation is established, its compatibility with blood must be determined to establish venous and perivenous tolerance. In addition, the duration of injection (or infusion) is

TABLE 17.7 Advantages and Disadvantages of Parenteral Therapy and Drug Formulations

Advantages	Disadvantages
1. The rate of onset of action can easily be controlled by the type of formulation and by the site of injection.	1. IV infusion warrants special apparatus (i.e., pumps) and sometimes animal surgery prior to dose (e.g., surgically implanted jugular catheter or VAP).
2. Nausea and vomiting are overcome.	2. The dosage form has to be administered by trained personnel (especially for IV) and requires strict adherence to aseptic technique.
3. Degradation of some drugs in the GI tract is avoided.	3. Once a drug is injected into the veins, tissue, or other body fluids, its removal is impossible.
4. Onset of action is faster and blood levels are more predictable.	4. Subcutaneous and intramuscular injections can produce severe local irritation and tissue necrosis.
5. Recommended for drugs that are poorly absorbed from the gastrointestinal tract.	5. Parenteral administrations (especially IV infusion) are time consuming.
6. Rapid onset of pharmacological effect.	6. Special care has to be taken in packaging parenterals.
7. When an animal is uncooperative or unconscious, parenteral administration is warranted.	
8. Parenteral administration can be used for local effects when desired (e.g., in anesthesiology).	

evaluated to establish the safety margin; however, it should be stated that this factor may not be applicable to human use because of anatomical and physiological differences.

Parenteral dose forms include aqueous, aqueous organic, and oily solutions, emulsions, suspensions, and solid forms for implantation. The parenterals should be sterile and pyrogen free; they are, if possible, buffered close to normal physiological pH and are preferably isotonic with body fluids [201]. The advantages and disadvantages of parenteral therapy should be considered and are presented in Table 17.7.

Severe local irritation and even tissue necrosis at the site of injection may be due to the solvent used [207, 208], or in many cases, the active drug results in a reaction. Rasmussen [209] has studied the tissue-damaging effect at the site of intramuscular injection of various preparations of antibiotics, other chemotherapeutic agents (sulfonamides, trimethoprim), certain drugs (lidocaine, diazepam, digoxin), and some vehicles. After intramuscular injection of physiological saline or sterile water, little or no tissue reaction was observed, while vehicles containing glycerol or propylene glycol caused severe damage at the injection site in swine, hens, and rabbits [210–212]. Pain is also likely to occur after intramuscular injection. Formulation techniques can often be used to overcome irritation and pain at the injection site.

17.3.3 Alternatives to Parenteral Administration (Nasal, Buccal, Transdermal, Rectal, and Vaginal)

Nasal Recent progress in biotechnology led to an enormous increase in the number of peptide and protein drugs that usually require parenteral administration to become therapeutically useful [213, 214]. Nonparenteral administration across mucosal surfaces (e.g., rectal, buccal, vaginal, nasal, and pulmonary epithelia) has been considered as an alternative route of application to replace parenteral injections [215, 216]. Among them, nasal delivery of peptides attracted strong interest because of its favorable biopharmaceutical properties. An overview is given by Gizurarson [217] on the relevance of the intranasal administration of drugs, peptides, and vaccines, and some physiological factors that present a barrier to the use of this route. The structure of the nasal epithelium, its high blood perfusion, and lower detrimental enzyme concentrations may result in higher bioavailabilities as compared to oral administration [218]. Nasal drug administration facilitates self-medication, thereby improving patient compliance. However, protein and peptide drugs show frequently low and variable bioavailabilities after nasal administration, for example, for insulin <1% [219]. Therefore, nasal insulin therapy necessitates coadministration of absorption enhancers such as surfactants, bile salts, fusidic acid derivatives, medium-chain fatty acids, chelators, or enzyme inhibitors to increase absorption and hence bioavailability [220, 221]. Although these absorption enhancers significantly increase transmucosal peptide transport, local tolerability, and safety concerns related to membrane damage, ciliary toxicity, mucus discharge, and epithelial disruption need to be overcome [222].

Consequently, the design of appropriate peptide carrier systems avoiding harmful additives has become a topic of intensive research. Nanoscale colloidal carriers from hydrophilic and biocompatible polymers, such as chitosan [223], were found to be useful for nasal vaccine [224] and insulin delivery [225] without employing additional penetration enhancers. Not only size and surface properties but also the polymer composition and architecture affected the functional properties of nanocarriers, such as transport of macromolecules across biological surfaces. In general, nanoscale dimensions favor transport of particles across mucosal epithelia [226, 227].

Moreover, nanocarriers with bioadhesive properties (e.g., nanoparticles (NP) coated with Carbopol) prevented rapid nasal clearance [228]. Increasing residence time in the nasal cavity was thought to improve drug absorption [229, 230]. Through the design of polymer properties, nanoparticle characteristics such as hydrophilicity and surface charge can be manipulated. In particular, polycations such as poly(L-lysine), protamine, polyethylene imine, chitosan, and dextran derivatives seem to increase mucosal permeability [231–233]. The combination of enhancing properties, mediated through cationic surface charges, with the formation of nanoparticulate carriers was achieved by encapsulating insulin into chitosan NP by ionotropic gelation, which yielded a significant blood glucose reduction in rabbits after nasal administration [225]. Recent study demonstrates that the nanocomplexes significantly enhanced insulin absorption, suggesting that amphiphilic biodegradable comb-polymers offer a promising approach for nasal peptide delivery [234].

In the development of a nasal drug delivery system (NDDS), formulation characteristics and device capabilities must be harmonized in order for consistent delivery into the nasal cavity. The approach to improve nasal bioavailability is the use of

polymeric gel vehicles to increase nasal residence times and to control the rate of drug absorption. The aerosol droplet size distribution (DSD) is an important variable in defining the efficiency of aerosolized drugs. Low viscosity or shear-thinning vehicle systems were effectively atomized into small droplets using different nasal pump sprays, as previously reported [235]. There have been many reports that solutions of mixtures of certain polymers, surfactants, and excipients can exhibit molecular interactions that affect the rheological and physicochemical properties of the solutions [236]. The nature of these interactions can affect the ability of the solution to be aerosolized into small droplets and may alter the stability and liberation of the active components. The characteristic of nasal aerosol generation is dependent on a combination of actuation force, viscosity, rheological properties (e.g., changes in viscosity and appearance of the thixotropic system), surface tension, and pump design [235].

In the pharmaceutical industry, polymers are routinely used in the formulation of gels and in the stabilization of emulsions. In a recent publication, the insulin gel was formulated for intranasal administration using the combination of carbopol and hydroxypropyl methylcellulose as gelling agent [237]. The use of bioadhesive nasal gel containing insulin not only promoted the prolonged contact between the drug and the absorptive sites in the nasal cavity but also facilitated direct absorption of medicament through the nasal mucosa in rat and human studies. This study further demonstrates that administration of insulin intranasally in gel form is a pleasant and painless alternative to injectable insulin.

Buccal Among the various transmucosal routes, buccal mucosa has excellent accessibility, an expanse of smooth muscle, and relatively immobile mucosa, hence suitable for administration of retentive dosage forms. Direct access to the systemic circulation through the internal jugular vein bypasses drugs from the hepatic first-pass metabolism, leading to high bioavailability. Other advantages, such as low enzymatic activity, suitability for drugs or excipients that mildly and reversibly damage or irritate the mucosa, painless administration, easy drug withdrawal, facility to include permeation enhancer/enzyme inhibitor or pH modifier in the formulation, and versatility in designing multidirectional or unidirectional release systems for local or systemic actions, project buccal adhesive drug delivery systems as promising options for continued research [238, 239]. Morishita et al. [240] demonstrate in a rat model that 20% Pluronic PF-127 gels containing unsaturated fatty acids are potential formulations for the buccal delivery of insulin. Sudhakar et al. [239] discuss the implication of various approaches for buccal adhesive delivery strategies applied for the systemic delivery of orally less inefficient drugs, in addition to the widely used local drug delivery. The growth rate for transmucosal drug delivery systems is expected to increase 11% annually through 2007. Worldwide market revenues are at $3B with the United States at 55%, Europe at 30%, and Japan at 10%. However, the effect of salivary scavenging and accidental swallowing of the delivery system and the barrier property of buccal mucosa stand as major limitations in the development of buccal adhesive drug delivery systems.

An ideal buccal adhesive system must have the following properties:

1. Should adhere to the site of attachment for several hours.
2. Should release the drug in a controlled fashion.

3. Should provide drug release in a unidirectional way toward the mucosa.
4. Should facilitate the rate and extent of drug absorption.
5. Should not cause any irritation or inconvenience to the patient.
7. Should not interfere with normal functions, such as talking or drinking.

Buccal adhesive drug delivery systems using matrix tablets, films, layered systems, disks, microspheres, ointments, and hydrogels have been studied and reported by several research groups. However, limited studies exist on novel devices that are superior to those of conventional buccal adhesive systems for the delivery of therapeutic agents through buccal mucosa. A number of formulation and processing factors can influence properties of the buccal adhesive system. There are numerous important factors, including biocompatibility (both the drug–device and device–environment interfaces), reliability, durability, environmental stability, accuracy, delivery scalability, and permeability, to be considered while developing such formulations. Traditionally, pharmaceutically acceptable polymers were used to enhance the viscosity of products to aid their retention in the oral cavity. Dry mouth is treated with artificial saliva solutions that are retained on mucosal surfaces to provide lubrication. These solutions contain sodium CMC as bioadhesive polymer.

Several buccal adhesive delivery devices were developed at the laboratory scale by many researchers either for local or systemic actions. They are broadly classified into three groups presented in Table 17.8.

Selection of Animal Species for Buccal Studies Apart from the specific methodology used to study buccal drug permeation characteristics, special attention is warranted for the selection of experimental animal species for such experiments. Many researchers have used small animals including rats and hamsters for permeability studies [241, 242]. But unlike humans, most laboratory animals have a totally keratinized oral lining and hence are not suitable. The rat has a buccal mucosa with a very thick, keratinized surface layer. The rabbit is the only laboratory rodent that has nonkeratinized mucosal lining similar to human tissue, but the sudden transition to keratinized tissue at the mucosal margins makes it hard to isolate the desired nonkeratinized region [243].

Among the larger experimental animals, monkeys are not practical models because of the difficulties associated with their maintenance. Dogs [244, 245] are easy to maintain and less expensive than monkeys [246] and their buccal mucosa is nonkeratinized and has a close similarity to that of the human buccal mucosa. However, we found in our laboratory that it is difficult to precisely deliver tablets intrabuccally to dogs as they tend to swallow tablets. Pigs also have nonkeratinized buccal mucosa similar to that of humans and their inexpensive handling and main-

TABLE 17.8 Classification of Buccal Adhesive Delivery Devices

Solid Buccal Adhesive Dosage Forms	Semisolid Buccal Adhesive Dosage Forms	Liquid Buccal Adhesive Dosage Forms
Tablets	Gels	Solutions
Microparticles	Patches/films	Suspensions
Wafers		
Lozenges		

tenance costs make them a highly suitable animal model for buccal drug delivery studies. In fact, the oral mucosa of pigs resembles that of humans more closely than any other animal in terms of structure and composition [247].

Adhesion of buccal adhesive drug delivery devices to mucosal membranes leads to an increased drug concentration gradient at the absorption site and therefore improved bioavailability of systemically delivered drugs. In addition, buccal adhesive dosage forms have been used to target local disorders at the mucosal surface (e.g., mouth ulcers) to reduce the overall dosage required and minimize side effects that may be caused by systemic administration of drugs. Researchers are now looking beyond traditional polymer networks to find other innovative drug transport systems. Much of the development of novel materials in controlled release buccal adhesive drug delivery is focusing on the preparation and use of a responsive polymeric system using a copolymer with desirable hydrophilic/hydrophobic interaction, block or graft copolymers, complexation networks responding via hydrogen or ionic bonding, and new biodegradable polymers especially from natural edible sources. At the current global scenario, scientists are finding ways to develop buccal adhesive systems through various approaches to improve the bioavailability of orally less inefficient drugs by manipulating the formulation strategies like inclusion of pH modifiers, enzyme inhibitors, and permeation enhancers. Novel buccal adhesive delivery systems, where drug delivery is directed toward buccal mucosa by protecting the local environment, is also gaining interest. Currently, solid dosage forms and liquids and gels applied to the oral cavity are commercially successful. The future direction of buccal adhesive drug delivery lies in vaccine formulations and delivery of small proteins/peptides. Microparticulate bioadhesive systems are particularly interesting as they offer protection to therapeutic entities as well as the enhanced absorption that results from increased contact time provided by the bioadhesive component. Exciting challenges remain to influence the bioavailability of drugs across the buccal mucosa. Many issues are yet to be resolved before safe and effective delivery through buccal mucosa. Successfully developing these novel formulations requires assimilation of a great deal of emerging information about the chemical nature and physical structure of these new materials.

Transdermal In recent years, the development of transdermal dosage forms has been attracting increasing attention, owing to the several advantages that this administration route offers [248]. Transdermal delivery systems, when compared with conventional formulations, generally show a better control of blood levels, a reduced incidence of systemic toxicity, no hepatic first-pass metabolism, and higher patient compliance [249, 250]. In addition, the transdermal route provides sustained and controlled delivery [250]. It also allows continuous input of drugs with short biological half-lives and can eliminate pulsed entry into systemic circulation, which often causes undesirable side effects [251–254]. Release of drug from transdermal patches is controlled by the chemical properties of drug and delivery form as well as the physiological and physicochemical properties of the biological membrane [255]. Technological discoveries, over the last decade, have proved the feasibility of using several methodologies for enhancing transdermal drug delivery [256]. With a diverse set of tools to enhance skin permeability, the future of transdermal drug delivery looks brighter. The challenge now lies in converting these discoveries into useful products using newer excipients and technologies [257].

However, drug delivery via the skin is not an easy task because of the formidable barrier properties of the stratum corneum (SC). The majority of drugs do not appear to penetrate the skin at a sufficiently high rate to have therapeutic effectiveness. The feasibility of the transdermal route is thus limited to powerful actives presenting the appropriate features such as appropriate low molecular weight and high lipophilicity. Different approaches have been studied to overcome these limitations in order to develop efficient transdermal delivery systems [258–264].

The implication of skin permeation of drug on release rate profiles of the experimental formulations should not be ignored because the skin is known to have a substantial role in variation of release kinetics [265]. At an early stage and in a steady-state of skin permeation, diffusion of drug through appendages is considered to be significant. Selection of receptor fluid is also important for *in vitro* studies. A biphasic characteristic of the study fluid is desirable as the diffusion for the drug molecules through skin is routed through both aqueous and nonaqueous heterogeneous media. PEG 400 and water or normal saline are commonly selected to provide biphasic characteristics of the liquid [266].

The enhancement of skin flux with increase of drug concentrations may be due to accumulation of a greater amount of drug on the skin surface. The improvement in skin flux with the increase of PVP content may be owing to its antinucleating effect that converts the crystalline drug into an amorphous state, which generally possesses a high energy state with high solubility. The enhancement in drug solubility provides increased thermodynamic activity, which facilitates the skin permeation of drug [267].

One longstanding approach for improving transdermal drug delivery uses penetration enhancers (also called sorption promoters or accelerants), which penetrate into skin to reversibly decrease the barrier resistance [256]. Numerous compounds have been evaluated for penetration-enhancing activity, including sulfoxides (such as dimethylsulfoxide, DMSO), Azones (e.g., laurocapram), pyrrolidones (e.g., 2-pyrrolidone, 2P), alcohols and alkanols (ethanol or decanol), glycols (e.g., propylene glycol, PG, a common excipient in topically applied dosage forms), surfactants (also common in dosage forms), and terpenes. Different aspects of enhanced transdermal delivery are reviewed in Refs. 256 and 268.

Another way of enhancing transdermal delivery is iontophoresis. Iontophoresis involves application of small amounts of current to push charged drug molecules through skin, resulting in higher fluxes of the drug molecules for which permeation through skin is otherwise negligible. It provides the advantages of improved patient compliance, avoidance of first-pass metabolism, control rate of drug release from the patch, and the possibility of programmed delivery [269, 270]. The self-contained iontophoretic patches successfully delivered granisetron hydrochloride by iontophoresis and depot formation was observed in the dermal and subcutaneous structures in the skin [271]. In addition, the prodrug approach and, most recently, the use of colloidal carriers represent very promising strategies to improve transdermal delivery [260, 261].

Rectal This route is used for local action on the rectum and lower colon. Glucose, digested proteins, and anesthetics are occasionally administered by high colonic irrigation to obtain systemic effect.

Vaginal Vaginal tablets are ovoid or pear-shaped and prepared by granulation and compression. They can be formulated to exhibit two types of release mechanisms: (1) a slow release dissolution that retains the tablet's original shape; this tablet is ideal for drugs requiring low concentrations in the cavity for long periods; and (2) effervescent and disintegrating tablets that release quickly and ensure rapid distribution of the active drug for total local effect throughout the cavity. Both forms may often contain a buffer to maintain or change the vaginal pH to that required for normal physiologic vaginal flora.

Vaginal pessaries require specialized manufacturing equipment. The mass is usually a glycogelatin, theobroma oil, or synthetic base solid at room temperature but that dissolves or is fluid at body temperature. Pessaries pose special stability problems and should only be considered if the vaginal tablet does not give satifactory drug efficacy. Intravaginal and intrauterine drug delivery devices are often used.

17.3.4 Topical Formulations for Local Disorders (Dermal, Ocular, Ear)

Topical preparations may be used for local protective or therapeutic reasons (dusting powders, solutions, suspensions, lotions, liniments, creams, ointments, aerosols).

Dermal Formulations Delivery of drugs to the skin is an effective and targeted therapy for local dermatological disorders. This route of drug delivery has gained popularity because it avoids first-pass effects, gastrointestinal irritation, and metabolic degradation associated with oral administration. Topical gel formulations provide a suitable delivery system for drugs because they are less greasy and can easily be removed from the skin. Percutaneous absorption of drugs from topical formulations involves the release of the drug from the formulation and permeation through skin to reach the target tissue. The release of the drug from topical preparations depends on the physicochemical properties of the vehicle and the drug employed. In order to enhance drug release and skin permeation, methods such as the selection of a suitable vehicle [271], coadministration of a chemical enhancer [268], and iontophoresis [272] have been studied.

The important considerations in the formulation of a dermatological preparation are, however, whether it is to be applied to a broken wound or an abrasion, whether it is to be rubbed into the affected area, whether it has to exhibit adhesiveness upon application to the skin, and whether it will deliver the active ingredient to the site required. Dusting powders, lotions, and aerosols are recommended formulations for application to abraded sites: lotions, liniments, creams, and ointments are best for unabraded sites.

For local therapeutic and systemic effects, the following should be considered: (1) the rate of dissolution of the drug in the vehicle, (2) the rate of diffusion of solubilized drug through the vehicle to the skin, and (3) the rate of permeation of the drug through the stratum corneum.

Formulation overcomes the problem of drug dissolution in the vehicle. Either the vehicle can be changed or the chemical structure of the active drug can be suitably altered (e.g., salt formation). The vehicle also governs the rate of diffusion and release of the drug to the stratum corneum. Thus, the vehicle is exceptionally

important in determining topical bioavailability [273–278]. Nevertheless, vehicle design is often ignored in the development of a suitable delivery system.

In the design of a suitable delivery system, the following should be considered [201]:

1. Permeability of the stratum corneum may be increased if it is hydrated by a suitable vehicle (dimethyl sulfoxide can result in superhydration [279, 280]) or by occlusive dressings or vehicles. Transport in some species, however, may be via skin appendages; hence, agents promoting increased stratum corneum permeability may have no or even a negative effect [281].
2. The thermodynamic activity of the drug in the vehicle should be higher than in the stratum corneum [282].
3. The formulation should be buffered to a pH such that the active ingredient is in the un-ionized state.
4. Keratolytics, lipid and polar solvents (acetone and alcohol), surfactants after protracted use, and some vehicles may cause damage to the stratum corneum, thus increasing penetrability.
5. Viscosity of the medium is inversely related to flux.

There are various topical gel formulations on the market for both local and systemic delivery of drugs and several others are in clinical trials [283]. Various delivery systems, such as liposomes [284], biodegradable microspheres [285, 286], and hydrogels [287], have been investigated in order to circumvent the susceptibility of peptides to degradation during formulation, storage, and administration. Proteins and peptides are compatible with hydrogels, such as nonionic cellulose polymers and poloxamer gels, which are nontoxic and exhibit reversible thermal characteristics. Poloxamers have been reported as suitable gel systems for various proteins such as insulin [240], α-chymotrypsin, and lactate dehydrogenase [288] and peptides such as deslorelin and gonadotropin-releasing hormone (GnRH) [289]. Pluronic F127 (PF127), a type of poloxamer, has been shown to enhance the stability of proteins, such as urease, and interleukin-2 [290]. These formulations are targeted to deliver both small molecules and macromolecules. Spantide II, a neurokinin-1 receptor (NK-1R) antagonist, for the treatment of inflammatory skin disorders (e.g., dermatitis and psoriasis) showed significant retention in epidermis and dermis from lotion and gel formulations when the lotion and gel formulations contained *N*-methyl-2-pyrrolidone (NMP) as a penetration enhancer [291].

Recently, patch devices were introduced for local dermal (as well as systemic) drug delivery [292, 293]. The optimized bioadhesive patch device offered a more patient-compliant and convenient alternative to tetracaine percutaneous anesthetic gel, particularly where large areas of skin are to be treated [292].

Ocular Formulations Ophthalmic preparations are sterile aqueous or oily solutions, suspensions, emulsions, or ointments for topical administration by instillation. Ophthalmic solutions are usually isotonic and buffered to minimize irritation to the eye. All multiple-dose eye preparations must contain a bacteriostatic agent. These, however, cannot be used in the injured eye or during surgical intervention in the anterior eye chamber, because of possible irritation.

Solutions exhibit a fast drug pulse delivery: they produce an initial high dosage that rapidly lapses to very low concentrations. Ophthalmic suspensions should preferably have an aqueous vehicle containing the drug of low solubility. The suspension duration of action is more prolonged than in aqueous solution. Their disadvantage is the possibility of irritation due to suspended crystals or particles. Ointments remain in the eye longer than do solutions, both in the precorneal tear film and the conjunctival fornices, thereby increasing absorption of active ingredients [294]. The commonly used ointment bases may be toxic to the interior of the eye and should not be used when the cornea has been penetrated. Invasion of the ointment base into the internal chambers of the eye causes toxic endothelial damage, corneal edema, vascularization, and scarring [294].

Topical therapy with corticosteroids is quite common in the treatment of ocular inflammatory disorders. Many nonsteroidal anti-inflammatory drugs (NSAIDs) have been tested as ocular anti-inflammatory agents [295–297] so as to diminish the well documented ocular side effects caused by corticosteroids. Ketorolac tromethamine (KT), an aryl-acetic acid NSAID, is nonirritating to the eye at 0.5% wt/vol concentration [298]. Aqueous ocular drop of KT is an effective and safe anti-inflammatory agent for topical use following cataract surgery and intra ocular lens implantation [299–301].

Compared with aqueous drop, sesame and soybean oil drops of ketorolac provided higher ocular availability followed by ophthalmic ointment in rabbit ocular studies. The ointment formulation provided maximum sustained effect. Ketorolac aqueous drop with BAC and EDTA improved the rate of ocular absorption though not the extent of absorption [302]. Sesame and soybean oil drops containing 0.2% (wt/vol) ketorolac free acid and benzyl alcohol (0.5% vol/vol) and ophthalmic ointment containing 0.5% (wt/wt) KT (in dissolved state) showed higher *in vitro* transcorneal permeation with minimum corneal damage [303].

Iontophoresis as a noninvasive technique for ocular drug delivery has been investigated for many years. The technology of ocular iontophoresis has reached maturity in the aspect of device development. Therapeutic levels of drugs can be reached, in the anterior and posterior segments of the eye, after applying a convenient and noninvasive iontophoretic treatment [304]. It is clearly seen that ocular iontophoresis has clinical potential and importance as a local delivery system for many drugs. The iontophoretic treatment is already a promising tool for delivering anti-inflammatory and antibiotic drugs to the eye. Iontophoresis will soon be used in delivering chemotherapeutic agents for retinoblastoma and ocular melanomas or as home-use device for treating glaucoma. It is a matter of time until iontophoresis will routinely be used in the ophthalmic field [304].

Transscleral iontophoresis is a good potential alternative for multiple intravitreal injections or systemic therapy used for posterior ocular disorders, such as endophthalmitis, uveitis, retinitis, optic nerve atrophy, pediatric retinoblastoma, and age-related macular degeneration (AMD). The search for an alternative drug delivery system derives from the serious intraocular complications occurring after recurrent intravitreal injections including retinal detachment, vitreous hemorrhage, endophthalmitis, and cataract. One recent study [305] showed that compared to IV administration, transscleral Coulomb Control iontophoresis (CCI) achieved higher and more sustained tissue concentrations of methylprednisolone with negligible systemic absorption. These data demonstrate that high levels of methylprednisolone

can safely be achieved in intraocular tissues and fluids of the rabbit eye, using CCI. Results from another study [306] suggest that focal delivery of carboplatin using subconjunctival injection or iontophoretic delivery transmits drug more effectively than intravenous delivery into the target tissues of the vitreous, choroid, retina, and optic nerve in the rabbit eye. Both focal applications resulted in significantly higher peak concentrations of carboplatin in the choroid, retina, optic nerve, and vitreous than those obtained after intravenous delivery. Focal chemotherapeutic delivery resulted in dramatically decreased carboplatin levels in the blood plasma compared with intravenous delivery.

Ear Formulations Otic dosage forms are intended for administration either on the outer ear or into the auditory canal. They include a number of dosage forms: solutions, suspensions, ointments, otic cones, and powders. Their primary use is either to remove wax or supply local drug treatment. Direct delivery of new therapies for treatment of sensorineural hearing loss to the fluids of the inner ear is necessary because of the presence of a blood–labyrinth drug barrier, which is anatomically and functionally similar to the blood–brain barrier [307, 308]. Direct delivery also has significant potential advantages for therapeutic application. Drugs are largely unaltered by metabolic changes that inevitably occur with other routes of administration, and have ready access to the sensory cells of the inner ear (the hair cells) and the synaptic regions of hair cells [309]. Delivery directly to the inner ear can avoid undesirable systemic side effects that some drugs may produce. The performance of an extracorporeal reciprocating perfusion system in guinea pigs was recently described by Chen et al. [310].

17.3.5 Inhalation Formulations (Vapors and Aerosols)

Many biologically active peptides have been discovered recently and have attracted attention as new drugs. Because of transport and enzymatic barriers, clinical dosage forms of these peptides have been primarily parenteral forms [311]. Development of sustained release forms of these peptide drugs is also being actively researched [312–314] and the pulmonary route would seem to be a promising alternative for delivering them, because many drugs that are poorly absorbed from other mucosal sites are well absorbed from the lungs [315]. This route of administration offers several advantages over the conventional gastrointestinal pathway, including large surface area, extensive vasculature, easily permeable membrane, and low intracellular and extracellular enzymatic activity [316–319]. Recent clinical and preclinical reports reveal that delivery of peptide drugs such as leuprolide acetate and insulin is feasible through the pulmonary route [320–323]. However, the bioavailability of the drugs having relatively high molecular weight is still poor through the pulmonary route compared with the parenteral route.

Recent research is focused on the delivery of systemically acting drugs via the pulmonary route [324]. Dry powder inhalations are a promising application form for peptides and proteins for systemic delivery because they overcome the drawbacks of oral and invasive delivery forms [315].

The administration of liposome-encapsulated drugs by aerosols seems to be a feasible way of targeting these delivery systems to the lung. The tolerability and safety of liposome aerosols have previously been tested in animals as well as in

human volunteers; no untoward effects have been recognized [325, 326]. Liposomes are also known to sustain the release of the entrapped drug(s) and to decrease the mucociliary clearance of the drug(s) because of their surface viscosity. Therefore, more effective and sustained systemic absorption of a drug would be attained by administering the drug containing liposomes in the respiratory tract. The developed liposomal dry powder inhaler of Leucinostatin demonstrated approximately 50% bioavailability compared with the SC route in rats [311]. This study justifies the role of the pulmonary route as a promising alternative to the presently available SC route. Green et al. [327] review the formulation process of inhalable drugs. They showed that high pressure media milling (HPMM) yields stable, fine-particle dispersion of medicament in hydrofluorocarbone propellant (HCP), which enhances a metered dose inhaler. HCP is currently mostly used for inhalation dose formulation as a more environmentally friendly product than previously used chlorofluorocarbons. However, eliminating chlorine from modern propellants introduced a solubility challenge for which the HPMM process helps to compensate.

17.4 CONCLUSION

Rapid developments in the field of molecular biology and gene technology resulted in generation of many macromolecular drugs including peptides, proteins, polysaccharides, and nucleic acids in great numbers and possessing superior pharmacological efficacy with site specificity and devoid of untoward and toxic effects. However, the main impediment for the oral delivery of these drugs as potential therapeutic agents is their extensive presystemic metabolism and instability in an acidic environment, resulting in inadequate and erratic oral absorption. This requires development of new drug delivery systems and increased usage of other routes of administration compared to the traditional oral and parenteral routes. These new therapies and new delivery systems require new formulations, which should be evaluated for toxicity and efficacy in preclinical studies using the proposed routes of administration.

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18

CYTOCHROME P450 ENZYMES

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18.1 INTRODUCTION

The cytochrome P450 (CYP) enzymes, also known as the microsomal mixed function oxidase system, are the predominant biotransformation pathway in the body for lipid-soluble endogenous and xenobiotic compounds. CYP enzymes play a key role in the biotransformation of pharmaceutical agents and thus are major determinants of duration of action and clearance of drugs. CYP enzymes are also involved in complications arising from drug therapy, such as drug interactions, which may be caused by induction or inhibition of CYP enzymes. Moreover, CYP enzymes may contribute to disease states. For example, CYP enzymes are implicated in the etiology of some cancers because chemical procarcinogens require enzymatic conversion to carcinogens, a process that is mediated by CYP enzymes. This chapter presents aspects of the characterization, function, and regulation of CYP enzymes, in mammals generally and in humans specifically, that are relevant to our understanding of how CYP enzymes can metabolize a large variety of drugs.

CYP enzymes are membrane-bound proteins localized predominantly in the smooth endoplasmic reticulum of liver and other tissues and are quantitatively and qualitatively among the most important enzymes of the phase I pathway for xenobiotic biotransformation [1, 2]. CYP enzymes are responsible for oxidizing hydrophobic compounds to more polar metabolites for further biotransformation or subsequent excretion. CYP enzymes function as terminal oxidases of an electron transport chain in which one atom of oxygen from molecular oxygen is incorporated into the substrate and the other atom is reduced to water. The multicomponent electron transport chain also includes a flavoprotein, NADPH-dependent cytochrome P450 reductase, which serves to transfer reducing equivalents (electrons) supplied by NADPH (and sometimes NADH) to the CYP enzymes. CYP, as the terminal oxidase of the chain, is the enzyme active site, the oxygen- and substrate-binding site, and the determinant of substrate specificity (Fig. 18.1).

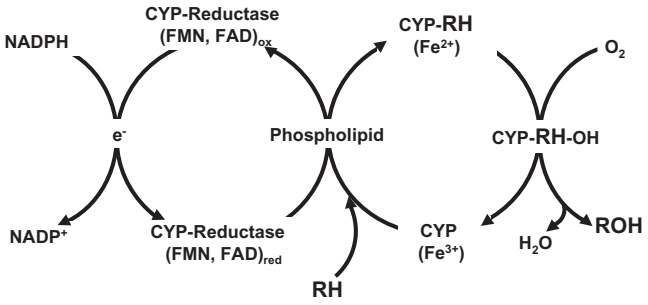


FIGURE 18.1 Electron transfer and metabolism of substrates by the microsomal CYP system. Electrons, transferred through a series of oxidation–reduction reactions from NADPH to CYP by NADPH-dependent CYP reductase, are used to reduce molecular oxygen with the concomitant oxidation of the substrate. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; RH, substrate; ROH, oxidized product.

18.2 IDENTIFICATION OF CYP ENZYMES

18.2.1 Characterization of CYP Enzymes

CYP enzymes contain a heme prosthetic group, which gives the enzymes a reddish brown color. The heme group is iron protoporphyrin IX and is the same heme moiety found in hemoglobin. As is the case for hemoglobin, the iron protoporphyrin IX group in CYP enzymes has a strong affinity for molecular oxygen but binds carbon monoxide with higher affinity. The iron atom of the heme prosthetic group can exist in two oxidation states, Fe^{2+} (reduced) and Fe^{3+} (oxidized). The heme moiety bound to CYP enzymes is essential for activity, and loss of the heme group or "poisoning" by carbon monoxide is accompanied by loss of enzymatic activity.

In all CYP enzymes, the heme prosthetic group (iron protoporphyrin IX) is bound to a single polypeptide chain composed of approximately 500 amino acids. Considerable evidence indicates that the amino terminus of the apoprotein anchors CYP in the lipid membrane bilayer, with most of the enzyme including the active site exposed on the cytoplasmic side of the endoplasmic reticulum [3, 4]. The central iron atom of the heme group is bound noncovalently to the sulfur atom of a cysteine residue situated close to the C terminus of the apoprotein chain.

Interactions between the heme group and apoprotein produce unique spectral and magnetic properties. Normally, the iron in the heme prosthetic group of CYP is in the oxidized state, which exhibits low affinity for carbon monoxide and absorbs visible light with a spectral maximum at a wavelength of approximately 418 nm. However, if the iron is in the reduced state, it can then bind carbon monoxide with high affinity, and the resulting reduced carbon monoxide-bound CYP complex exhibits a large spectral maximum at 450 nm. This characteristic spectral property is a signature of CYP enzymes from which the name, cytochrome P450, was derived. Other heme proteins, such as hemoglobin, do not produce an absorbance at 450 nm when complexed with carbon monoxide.

Another essential component for CYP activity is NADPH-dependent CYP reductase, which is also located primarily on the surface of the phospholipid bilayer of the smooth endoplasmic reticulum in close proximity to the CYP enzymes. NADPH-dependent CYP reductase forms a temporary complex with CYP, permitting the flow of electrons to the heme prosthetic group and the resulting oxidation of the substrate. NADPH-dependent CYP reductase has two domains, one containing flavin adenine dinucleotide and the second containing flavin mononucleotide. Two electrons are acquired from NADPH and migrate from FAD to FMN, then to the heme group of CYP. In some cases, CYP enzymes can also accept electrons from cytochrome b_5, a small membrane-bound heme protein that receives electrons from NADH.

18.2.2 Detection and Isolation

Studies by Axelrod [5] and by Brodie and co-workers [6] in the mid-1950s revealed the existence of an enzyme system in liver that converted drugs and aromatic compounds into more polar metabolites and that required NADPH for the reaction. This enzyme system was subsequently identified as a carbon monoxide-binding pigment in liver microsomes by Klingenberg [7] and Garfinkel [8].

Microsomes is the name given to the endoplasmic reticulum membrane fraction, which can be isolated by breaking open and grinding cells to prepare a whole cell homogenate. If the homogenate is subjected to centrifugation at approximately 9000 *g*, cell debris, nuclei, and mitochondria spin down in the pellet. The resulting supernatant suspension, called the S9 fraction, contains both cytosolic and microsomal proteins. When the supernatant is spun at higher speed (e.g., 100,000 *g*), the resulting supernatant fraction contains the cytosolic fraction and the pellet contains the microsomal fraction. The pellet can be resuspended with 0.25 M sucrose or with 0.1 M potassium phosphate buffer containing 10% glycerol to give the microsomes, which can be stored at −80 °C for substantial periods of time. As long as the microsomal fraction is sufficiently concentrated (i.e., has a protein concentration >10 mg/mL), the CYP activity of the frozen microsomal fraction can be maintained for up to five years. The total CYP concentration of tissue preparations such as the S9 or microsomal fractions can be determined in a scanning spectrophotometer by measuring the height of the reduced carbon monoxide-bound CYP complex at 450 nm as reported by Omura and Sato [9].

The identity of CYP enzymes involved in the metabolism of a specific drug can be determined using a combination of approaches. Well characterized, specific inhibitory antibodies (either polyclonal or monoclonal) against various CYP enzymes are a valuable tool for identification of CYP enzymes in S9 and microsomal fractions. Chemical inhibitors (discussed later), especially highly selective inhibitors, can also be used. Finally, purified or recombinant enzymes can be used to determine the intrinsic ability of an individual or panel of CYP enzymes to catalyze a particular reaction or metabolize a specific drug. Once the CYP enzymes involved in the metabolism of a specific drug have been identified, it may be necessary to measure levels of the CYP enzymes in the liver or other relevant tissue. Immunological assays with specific antibodies, such as immunoblot analysis, are the preferred method for quantifying tissue levels of individual CYP enzymes.

18.2.3 Occurrence and Distribution

CYP enzymes are present in most organisms including mammals, birds, fish, reptiles, amphibians, insects, plants, fungi, and bacteria [10, 11]. The finding that CYP enzymes are found in every class of biota suggests that the diverse CYP enzymes identified to date have evolved from a single ancestral gene over a period of 1.36 billion years [12].

In humans and other mammals, CYP enzymes are found in almost all tissues including liver, intestine, lung, kidney, stomach, brain, adrenal gland, gonads, heart, nasal and tracheal mucosa, and skin [13, 14]. Concentrations and expression of individual CYP enzymes differ between tissues. Liver contains the largest number and the highest levels of CYP enzymes involved in drug biotransformation [2].

The endoplasmic reticular membrane is a structure with an extensive surface area in most cells. In rat liver, for example, the endoplasmic reticular membrane comprises 7–11 m^2/g of liver weight [15], and total microsomal protein represents 1.8–2% of liver wet weight. CYP enzymes comprise from 4% to 6% of total microsomal protein. This translates to a typical specific content of 0.8–1.1 nmol of total CYP per milligram of microsomal protein or approximately 20 nmol of total CYP per gram rat liver. In human liver, the total CYP content is lower, with CYP enzymes typically

TABLE 18.1 Total CYP Content in Various Human and Rat Organs

Organ	Human (nmol/mg microsomal protein)	Rat (nmol/mg microsomal protein)
Liver	0.30–0.60	0.8–1.1
Adrenal	0.23–0.54	0.5
Small intestine	0.03–0.21	0.02–0.13
Kidney	0.03	0.05–0.2
Lung	0.01	0.035–0.05
Brain	0.10	0.025–0.05
Testis	0.005	0.07–0.12
Skin	Not determined	0.05
Mammary	<0.001	0.001–0.003

Source: Compiled from Refs. 15–20.

comprising 1.5–3% of total microsomal protein, giving a specific content of 0.3–0.6 nmol of total CYP per milligram of microsomal protein (Table 18.1), or approximately 5 nmol of total CYP per gram human liver. For tissues other than liver, the CYP content, expressed in terms of microsomal protein or tissue weight, is lower (Table 18.1).

18.2.4 Nomenclature of CYP Enzymes

Multiple CYP enzymes have been identified in diverse species. Each CYP enzyme is encoded by a separate gene. To date, nearly 4000 CYP genes have been identified [21]. CYP enzymes are named and categorized according to their amino acid sequences. A systematic nomenclature has been developed to classify CYP enzymes into families and subfamilies based on the degree of similarity of their primary protein structure [22]. According to this system, CYP enzymes within the same family should exhibit at least 40% amino acid sequence identity, and CYP enzymes that share less than 40% identity in amino acid sequences are assigned to different families, which are designated using CYP as an abbreviation for cytochrome P450, followed by an Arabic numeral (e.g., CYP1, CYP2, CYP3, CYP4) [12]. Within a family, members that share more than 55% identity are assigned to the same subfamily, which is designated by a capital letter following the Arabic numeral (e.g., CYP2A, CYP2B, CYP2C) [12]. Individual enzymes are identified using a final number to specify the individual protein (e.g., CYP2A1, CYP2A2, CYP2B1).

To date, over 300 different CYP enzymes representing 50 families, 82 subfamilies, and 67 different species have been identified [12, 15]. In humans, 57 CYP genes belonging to 18 families and 42 subfamilies have been identified thus far [11, 23] (Table 18.2). In addition, more than 50 pseudogenes have been found. Pseudogenes are defective genes that do not produce functional proteins. They are thought to be relics of gene duplications in which one of the copies has degenerated and lost its function.

Although all members of the CYP superfamily possess highly conserved regions of amino acid sequences, there is variation in the primary sequences among individual CYP enzymes and among related enzymes across species. Small changes in amino acid sequences are reflected in profound differences in substrate specificity.

TABLE 18.2 Human CYP Enzymes

Family	Subfamily	Individual CYP Enzymes
CYP1	CYP1A	CYP1A1, CYP1A2
	CYP1B	CYP1B1
CYP2	CYP2A	CYP2A6, CYP2A7, CYP2A13
	CYP2B	CYP2B6
	CYP2C	CYP2C8, CYP2C9, CYP18, CYP19
	CYP2D	CYP2D6
	CYP2E	CYP2E1
	CYP2F	CYP2F1
	CYP2J	CYP2J2
	CYP2R	CYP2R1
	CYP2S	CYP2S1
	CYP2U	CYP2U1
	CYP2W	CYP2W1
CYP3	CYP3A	CYP3A4, CYP3A5, CYP3A7, CYP3A43
CYP4	CYP4A	CYP4A9, CYP4A11
	CYP4B	CYP4B1
	CYP4F	CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12
	CYP4V	CYP4V2
	CYP4X	CYP4X1
	CYP4Z	CYP4Z1
CYP5	CYP5A	CYP5A1
CYP7	CYP7A	CYP7A1
	CYP7B	CYP7B1
CYP8	CYP8A	CYP8A1
	CYP8B	CYP8B1
CYP11	CYP11A	CYP11A1
	CYP11B	CYP11B1, CYP11B2
CYP17	CYP17	CYP17
CYP19	CYP19	CYP19
CYP20	CYP20	CYP20
CYP21	CYP21A	CYP21A1
CYP24	CYP24	CYP24
CYP26	CYP26A	CYP26A1
	CYP26B	CYP26B1
	CYP26C	CYP26C1
CYP27	CYP27A	CYP27A1
	CYP27B	CYP27B1
	CYP27C	CYP27C1
CYP39	CYP39	CYP39
CYP46	CYP46	CYP46
CYP51	CYP51	CYP51

Source: Adapted from D. Nelson website [396].

In addition, CYP enzymes differ in spectral, electrophoretic, and immunologic properties, modes of regulation, and inducibility.

Of the 18 CYP gene families identified in mammals thus far [12, 15], the major hepatic drug-metabolizing enzymes belong to families CYP1, CYP2, and CYP3. CYP enzymes belonging to families such as CYP5, CYP7, CYP8, CYP24, CYP27,

and CYP51 are involved in the biosynthesis and catabolism of endogenous compounds.

The human CYP1 family, for example, consists of three enzymes, CYP1A1 and CYP1A2 in the CYP1A subfamily, and CYP1B1 in the CYP1B subfamily, although all three enzymes are not expressed in all tissues. Enzymes belonging to the CYP1 family are normally present in liver at very low levels but can be highly induced by treatment with planar halogenated and nonhalogenated aromatic compounds such as 3-methylcholanthrene, β-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [15, 24–26].

The CYP2 family is represented by five major subfamilies, CYP2A, CYP2B, CYP2C, CYP2D, and CYP2E, in liver. CYP2E1, which is the only member of the CYP2E subfamily in various species and is involved in the metabolism of ethanol and other low molecular weight organic solvents, is induced by ethanol, acetone, and the antitubercular drug, isoniazid [24, 27]. The CYP2C enzymes, a quantitatively important group of enzymes in human and rodent liver, appear to be relatively refractory to induction by most drugs and other xenobiotics [27].

The CYP3 family is represented in liver by only one subfamily, CYP3A. CYP3A enzymes are inducible by glucocorticoids, phenobarbital, and macrolide antibiotics [15, 24, 28]. CYP3A enzymes are the most predominant CYP enzymes in human liver and intestine and are involved in the metabolism of a large percentage of therapeutic agents [1].

The CYP4 family, which is represented in liver by CYP4A and CYP4F subfamilies, is less important in xenobiotic metabolism. CYP4A and CYP4F enzymes catalyze the metabolism of fatty acids, leukotrienes, eicosanoic acids, and prostaglandins and are inducible by hypolipidemic agents, such as clofibrate, and by phthalates and other peroxisomal proliferators [15, 29].

In contrast to the CYP enzymes, NADPH-dependent CYP reductase is encoded by one gene. Thus the single form of NADPH-dependent CYP reductase, which exists in human liver, must be capable of interacting with the multiple CYP enzymes found in each cell. Moreover, NADPH-dependent CYP reductase is expressed at a lower level than CYP, with a reductase to CYP ratio of approximately 1:10 or less. NADPH-dependent CYP reductase is slightly inducible (a maximal two- to three-fold increase) by some of the same compounds (e.g., 3-methylcholanthrene, phenobarbital, TCDD) that induce CYP enzymes.

A list of CYP enzymes that have been detected in human tissues is presented in Table 18.3.

18.2.5 Genetic Polymorphism

Genetic polymorphism is a common phenomenon in the CYP superfamily. A polymorphism arises from a single base change or multiple nucleotide base changes in a gene and leads to a variant form of the coded protein. The mutation can occur in both coding and noncoding regions of the gene. The variant form of the polymorphic CYP enzyme may have enzymatic activity that is identical to that of the native enzyme, or it may exhibit greater or lesser activity, or it may not be expressed. For example, more than 60 polymorphisms are known for CYP2D6 [44]. The different polymorphic variants are designated by the addition of an asterisk followed by a number to the CYP enzyme name. For example, CYP2D6*1 is a commonly

TABLE 18.3 CYP Enzymes Detected in Various Human Organs

Organ	CYP Genes Expressed (mRNA or Protein)
Liver[a]	CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP3A7, CYP4A11
Small intestine[a,b]	CYP1A1 (5.6), CYP2C9 (8.4), CYP2C19 (1), CYP2D6 (0.5), CYP2J2 (0.9), CYP3A4 (43), CYP3A5 (16)
Nasal mucosa	CYP2A6, CYP2A13, CYP2B6, CYP2C, CYP2J2, CYP3A
Trachea	CYP2A6, CYP2A13, CYP2B6, CYP2C, CYP2J2, CYP2S1, CYP3A, CYP4X1
Lung	CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C18, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP3A4, CYP3A5, CYP4B1
Stomach	CYP1A1, CYP1A2, CYP2C, CYP2J2, CYP2S1, CYP3A4
Colon	CYP1A1, CYP1A2, CYP1B1, CYP2J2, CYP2S1, CYP3A4, CYP3A5, CYP4F12
Kidney	CYP1B1, CYP2A6, CYP2B6, CYP2E1, CYP2R1, CYP2S1, CYP3A5, CYP4A11, CYP4F2, CYP4F12
Skin	CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2S1, CYP3A4, CYP3A5, CYP4A11
Brain	CYP1A1, CYP2B6, CYP2D6, CYP2E1, CYP2U1
Mammary	CYP1A1, CYP1B1, CYP2C, CYP2D6, CYP3A4, CYP3A5, CYP4Z1
Placenta	CYP1A1, CYP2E1, CYP2F1, CYP2S1, CYP3A4, CYP3A5, CYP4B1

[a]CYP enzyme levels in liver and small intestine were detected by protein expression, whereas CYP enzymes in other tissues were detected mainly by mRNA expression.

[b]CYP enzyme levels in the small intestine were determined by immunoblot analysis using intestinal microsomes from 31 individuals. The numbers shown in parentheses in the table are mean CYP enzyme levels, expressed as picomoles per milligram microsomal protein, in the small intestine [14]. Note: CYP1A1 was detected in 3, and CYP3A5 was detected in 11, of the 31 samples, and CYP1A2, CYP2A6, CYP2B6, CYP2C8, and CYP2E1 were below the limit of detection [14].

Source: Compiled from Refs. 2, 13, 14, and 30–43.

occurring variant of CYP2D6 that codes for an enzyme with high activity toward a number of substrates including debrisoquine and sparteine, whereas CYP2D6*9 codes for an enzyme with low activity. CYP2D6*4 and CYP2D6*5 are nonfunctional alleles, meaning that the CYP2D6 protein is not expressed. Although allelic variation can occur with any CYP gene, polymorphisms of CYP2A6, CYP2C9, CYP2C19, and CYP2D6 are the best characterized (Table 18.4) [45].

Enzyme polymorphism is inherited and is a major cause of interindividual and ethnic differences in the rates at which individuals metabolize drugs. The activities of the different polymorphic enzymes may vary by more than 100-fold when tested with the same drug substrate. Thus individuals bearing a nonfunctional allele such as CYP2D6*5 or an allele that codes for a CYP enzyme with low activity, such as CYP2D6*9, will metabolize debrisoquine, a drug that is hydroxylated at the 4-position by CYP2D6, more slowly than individuals bearing an allele that codes for a CYP enzyme with high activity, such as CYP2D6*1. People in the former group are classified as poor/slow metabolizers and people in the latter group are classified as extensive/fast metabolizers. Moreover, other people may have multiple copies of a variant allele, such as CYP2D6*2, and are classified as ultrarapid metabolizers [46].

TABLE 18.4 Human CYP Polymorphisms with Relatively High Population Frequency

CYP Enzyme	Allele[a]	Function
CYP2A6	*2	Inactive
	*4	Inactive
CYP2D6	*2	Decreased activity
	*3	Inactive
	*4	Inactive
	*5	Inactive
	*6	Inactive
	*7	Inactive
	*9	Decreased activity
	*10	Decreased activity
	*17	Decreased activity
	*2xN	Ultrarapid activity
CYP2C9	*2	Decreased activity
	*3	Decreased activity
CYP2C19	*2	Inactive
	*3	Inactive
	*4	Inactive
	*5	Inactive

[a]The *1 allele, which is not presented in the table, codes for CYP enzymes with high activity (i.e., represents the rapid/extensive metabolizer phenotype). The allelic variants listed in the table represent polymorphisms that occur with relatively high frequency and have functional relevance. Many more CYP allelic variants have been detected but are not listed in the table.

Source: Adapted from Ref. 45.

18.3 CYP ENZYME REACTIONS

18.3.1 CYP Substrates

The CYP enzyme system is capable of catalyzing the oxidative biotransformation of an almost limitless number of xenobiotics including drugs, solvents, anesthetics, dyes, petroleum products, pesticides, halogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs), and plant products. An exhaustive list of substrates for the mammalian CYP enzymes is presented in Table 18.5. These substrates share a common feature in that most are lipid soluble and are converted by CYP into more water-soluble metabolites. This facilitates their eventual excretion from the body. Xenobiotics are metabolized by a relatively small number of CYP enzymes belonging to families CYP1, CYP2, and CYP3. Many lipid-soluble physiological compounds including fatty acids, steroids, eicosanoids, retinoids, and amino acids also serve as substrates of CYP1, CYP2, and CYP3 enzymes. A characteristic of the CYP1, CYP2, and CYP3 enzymes that contributes to their ability to metabolize such a diverse array of different substrates is that they possess broad and overlapping substrate selectivity.

By contrast, a large subset of CYP enzymes, especially those found in steroidogenic organs such as the adrenal gland and gonads, but also including enzymes present in the liver, are not involved in xenobiotic metabolism. These CYP enzymes

TABLE 18.5 Substrates for Mammalian CYP Enzymes[a]

Category/Subcategory of Substrates	Examples of Substrates[b]
	Animal/Fungal/Plant Natural Products
Alkaloids	Californine, protopine
Components of garlic	Diallyl disulfide, diallyl sulfide
Coumarins, furanocoumarins, psoralens	Bergamottin, BFC, coumarin, 7-ethoxycoumarin, methoxsalen
Drugs	Caffeine, cocaine, ipomeanol, morphine, nicotine, quinidine, quinine
Flavonoids	Aminoflavone, apigenin, 7,8-benzoflavone, biochanin A, daidzein, eriodictyol, flavanone, galangin, genistein, hesperetin, kaempferide, methoxyflavanone, naringenin, prunetin, tangeretin, tamarixetin
Flavorants, herbal constituents, spices	Aristolochic acids, capsaicin, glabridin
Indoles	Ibogaine, indole, 3-methylindole
Mycotoxins	Territrems A, B, C
Phytoalexins	Resveratrol
Retinoids	All-*trans*-retinoic acid, retinal, 9-*cis*-retinoic acid, 13-*cis*-retinoic acid
Safroles	Elemicin, myristicin
Terpenes and derivatives	Betulinic acid, eucalyptol, germander, limonenes, (R)-(+)-pulegone, verbenone
Vitamins	1α,25-Dihydroxyvitamin D_3, 1α-hydroxyvitamin D_2, 1α-hydroxyvitamin D_3, vitamin A, vitamin B_2, vitamin D_2, vitamin D_3, vitamin E
Various natural products	Benzylisothiocyanate, odorants, oleuropein, pheromones, pyrrolizidine, tannins, tentoxin, yohimbine
	Endogenous Compounds
Amino acids, amines	Arginine, *N*-hydroxyarginine, octopamine, synephrine, tryptamine, tyramine
β-Carbolines	Methoxydimethyltryptamine, pinoline
Heme compounds	Bilirubin
Pineal hormones	Melatonin
Hydrocarbons	Acetone, acetol, H_2O_2
Lipids	Eicosanoic acids, fatty acids, leukotrienes, lipid peroxides, lipoxins, prostaglandins, thromboxanes
Steroid hormones and various steroids	Androgens, bile acids, cholesterol, estrogens, glucocorticoids, lanosterol, mineralocorticoids, progestins, steroid hydroperoxides
	Xenobiotics
Alcohols, aldehydes, alkanes, and related compounds	Acetal, acetoacetate, butanol, butanone, 3,4-dimethylhexane, ethanal, ethane, ethanol, glycerol, hexane, 2-hexanone, methanol, methyl-*t*-butyl ketone, *p*-nitrobenzaldehyde, pentane, pentanol, propanol, nonane, *p*-tolualdehyde

Alkenes, alkynes	Biphenyl acetylene, divinyl ether, ethene, fluroxene, propene
Amines and related compounds	Acetanilide, acetylhydrazine, aniline
Azo compounds, azoles	Azoxymethane, methylazoxymethanol, pyrazole
Benzene and derivatives	Benzene, bromobenzene, 2,6-di-*t*-butyl-4-methylphenol, nitrobenzene, phenol
Carcinogens[c]	
Alkaloids, alkenes, allylarenes, spices	β-Asarone, butadiene, estragole, isosafrole, methyleugenol, safrole, senecionine, styrene
Amines and derivatives	Acetylaminofluorene, aminoazotoluene, aminobiphenyl, benzidine, dimethylaminoazobenzene, Glu-P-1, Glu-P-2, HMP, IQ, MeIQ, MeIQx, 2-naphthylamine, PhIP, Trp-P-1, Trp-P-2
Carbamates, ethers	Diethylether, ethyl carbamate, methyl-*t*-butyl ether, vinyl carbamate
Estrogens	Diethylstilbestrol, 17β-estradiol
Halogenated hydrocarbons	Benzyl chloride, CCl_4, chloroform, bis(chloromethyl)ether, dibromochloropropane, 1,4-dichlorobenzene, dichloromethane, dichloropropane, dimethylcarbamoyl chloride, epichlorohydrin, ethylene dibromide, ethylene dichloride, methyl chloride, methylene chloride, MOCA, trichloroethane, trichloroethylene, Tris-BP, vinyl bromide, vinyl chloride, vinylidene chloride
Hydrazines, nitriles, triazenes	Acrylonitrile, agaritine, dimethylhydrazine, dimethylphenyltriazene, procarbazine
Mycotoxins	Aflatoxin B_1, aflatoxin G_1, aflatoxin M_1, ochratoxin A, sterigmatocystin
Nitro compounds	DEN, DMN, *p*-nitroanisole, 4-nitrobiphenyl, 2-nitrofluorene, nitrosodimethylmorpholine, nitrosomethylbenzylamine, NNAL, NNK, NNN
Polycyclic aromatics	Aminoanthracene, 6-aminochrysene, benzo[*a*]pyrene, chrysene, benzophenanthrene, dibenzanthracene, DMBA, MC, naphthalene, nitrobenzo[*a*]pyrene, 6-nitrochrysene, nitropyrene, phenanthrene
Catechols, hydroquinones	α-Methyldopa, *m*-AMSA
Fungicides	Furametpyr, *o*-phenylphenol
Halogenated hydrocarbons	Bromobenzene, chlorobenzene, 1,2-dichlorobenzene, enflurane, halothane, methoxyflurane, tetrachloroethane, tetrachloroethylene, trichlorobenzene
Heteroaromatics, nitroaromatics	Isaxonine, lasciocarpaine, *p*-nitrophenol, *N*-nitrosopyrrolidine, suprofen, thiophene, ticrynafen
Hydroperoxides	BOOH, *t*-butylhydroperoxide, cumene hydroperoxide, DBOOH, EBOOH, TMOOH
Insect repellents	*N*,*N*-Diethyl-*m*-toluamide
Oral contraceptives	Desogestrel, dienogest, ethinylestradiol, gestodene, medroxyprogesterone, mestranol, norethisterone
Organic solvents, petroleum products	CCl_4, chloroform, methylene chloride, pyridine, toluene, vinyl chloride, xylene
Pesticides	Aldrin, azobenzene, carbaryl, carbofuran, chlordanes, chlorpyrifos, chlortoluron, DCBN, DDT, diazinon, dieldrin, endosulfan, endrin, heptachlor, kepone, methiocarb, methoxychlor, parathion, phorate, pyrethrins, rotenone, strychnine
Plasticizers	Di-(2-ethylhexyl)phthalate

TABLE 18.5 ***Continued***

Category/Subcategory of Substrates	Examples of Substrates[b]
Polyhalogenated aromatics	PBBs, PBDEs, PCBs, TCDD, TCDF
Sulfur compounds	Carbon disulfide, thioacetamide, thiobenzamide
	Therapeutic/Pharmacologic Drugs[c,d]
Abortifacients	Mifepristone
Alcohol deterrents	Diethyldithiocarbamate, disulfiram
Analgesics	
Antimigraine drugs	Almotriptan, butalbital, divalproex, eletriptan, dihydroergotamine, ergotamine, flunarizine, lisuride, naratriptan, propranolol, valproic acid, zolmitriptan
Antineuralgia drugs	Capsaicin, carbamazepine, duloxetine
COX-3 inhibitors	Acetaminophen, aminopyrine, antipyrine
Opioid analgesics	Alfentanil, buprenorphine, codeine, dihydrocodeine, ethylmorphine, fentanyl, hydrocodone, hydromorphone, ketobemidone, meperidine, methadone, morphine, noralfentanil, oxycodone, pentazocine, phenazocine, propoxyphene, sufentanil, tramadol
Various analgesics	Acetaminophen, acetanilide, carisoprodol, phenacetin
Anesthetics, general	
Opioid anesthetics	Alfentanil, fentanyl, sufentanil
Fluorinated hydrocarbons	Desflurane, enflurane, fluroxene, halothane, isoflurane, methoxyflurane, sevoflurane
Various general anesthetics	Ketamine, phencyclidine, propofol, thiamylal, thiopental
Anesthetics, local	Bupivacaine, levobupivacaine, lidocaine, ropivacaine
Anorectic/antiobesity drugs	Dexfenfluramine, fenfluramine, phenmetrazine, phentermine, sibutramine
Antiacne drugs	Retinoic acid, erythromycin, metronidazole, salicylic acid, tazarotenic acid
Antiandrogenics	Bicalutamide, flutamide, finasteride, valproate
Anti-alopecia drugs	Finasteride, minoxidil
Anti-Alzheimer drugs	DMP543, donepezil, galantamine, milameline, tacrine
Anti-amyotrophic lateral sclerosics	Riluzole
Antiasthmatics/bronchodilators/nasal decongestants	Flunisolide, fluticasone, methoxyphenamine, montelukast, paraxanthine, pseudoephedrine, salmeterol, theobromine, theophylline, tiaramide, zafirlukast, zileuton
Anti-ADHD drugs	d-Amphetamine, imipramine, methamphetamine, methylphenidate, nortriptyline, tamoxetine
Antibacterials	
Antileprotics	Dapsone, rifabutin
Anti-MAC drugs	Rifabutin
Antirickettsials	Chloramphenicol, rifampicin

Antituberculars	Isoniazid, rifabutin, rifalazil, rifampicin
Fluoroquinolones	Ciprofloxacin, grepafloxacin, pefloxacin
Macrolides	Azithromycin, clarithromycin, erythromycin, miocamycin, roxithromycin, troleandomycin
Various antibacterials	Chloramphenicol, clindamycin, rifabutin, metronidazole, sulfamethoxazole, sulfamidine, trimethoprim
Antibacterials, topical	Sulfadiazine silver
Anti-benign prostatic hyperplasia drugs	Alfuzosin, finasteride, prazosin, tamsulosin
Anticancer/antineoplastic drugs	All-*trans*-retinoic acid, aminoflavone, anastrozole, AQ4N, artemisinin, O^6-benzylguanine, berberine, bexarotene, bicalutamide, bropirimine, busulfan, *t*-butylhydroxyanisole, carmustine, coumarin, cyclophosphamide, dacarbazine, daidzein, DF203, docetaxel, doxorubicin, dutasteride, ellipticine, etoposide, exemestane, fadrozole, finasteride, flutamide, ftorafur, gefitinib, genistein, hexamethylmelamine, ifosfamide, imatinib, imiquimod, irinotecan, karenitecin, ketoconazole, letrozole, lonafarnib, mitotane, mitoxantrone, MMDX, oracin, paclitaxel, procarbazine, sirolimus, tamoxifen, targretin, tauromustine, tegafur, teniposide, thioguanine, 6-thiopurine, thioTEPA, toremifene, trofosfamide, urethane, valproic acid, vinblastine, vincristine, vindesine, vinorelbine
Antidepressants	
Monamine oxidase inhibitors	Amiflamine, brofaromine, iproniazid, moclobemide, phenelzine, tranylcypromine
NMRIs, tricyclic antidepressants	Amitriptyline, clomipramine, desipramine, doxepin, imipramine, medifoxamine, norclomipramine, nortriptyline, opipramol, trimipramine
SNRIs, S&NRIs, SSRIs	Citalopram, duloxetine, escitalopram, fluoxetine, fluvoxamine, maprotiline, milnacipran, norfluoxetine, paroxetine, reboxetine, sertraline, triazoledione, tomoxetine, venlafaxine
Various antidepressants	Adinazolam, bupropion, clonazepam, cotinine, gepirone, iprindole, iproclozide, mianserin, minaprine, mirtazapine, nefazodone, trazodone, triazoledione
Anti-dermatitis herpetiformis drugs	Dapsone
Antidiabetics	Acetohexamide, chlorpropamide, CPO, glibenclamide, gliclazide, glimepiride, glipizide, nateglinide, phenformin, pioglitazone, repaglinide, rosiglitazone, tolbutamide, troglitazone
Antiepileptics	Carbamazepine, clobazam, clomethiazole, clonazepam, diazepam, ethosuximide, felbamate, ganaxolone, losigamone, mephenytoin, mephobarbital, nimetazepam, phenobarbital, phenytoin, primidone, tiagabine, topiramate, trimethadione, valproic acid, zonisamide
Antierectile dysfunction drugs	Sildenafil, vardenafil, yohimbine
Antiestrogenics	Tamoxifen, toremifene
Antifungals	Clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole, sirolimus, terbinafine, voriconazole
Antigout, uricosuric drugs	Benzbromarone, colchicine, dexamethasone, hydrocortisone, indomethacin, methylprednisolone, phenylbutazone, sulfinpyrazone, tienilic acid, zoxazolamine

TABLE 18.5 ***Continued***

Category/Subcategory of Substrates	Examples of Substrates[b]
Antihistamines	Azelastine, brompheniramine, chlorpheniramine, cinnarizine, clemastine, cyproheptadine, diphenhydramine, doxylamine, ebastine, flunarizine, hydroxyzine, loratadine, medrylamine, mequitazine, orphenadrine, pheniramine, promethazine, rupatadine, tripelennamine
Antihyperlipoproteinemics	
Fibrates	Bezafibrate, ciprofibrate, clofibrate, fenofibrate, gemfibrozil
HMGRIs	Atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, simvastatin
Antihyperprolactinemics	Lisuride, terguride
Anti-inflammatory drugs	
COSAIDs	Budesonide, dexamethasone, fluticasone, mometasone, prednisolone
NSAIDs	
Anti-FMF drugs	Colchicine
COX-2 selective inhibitors	Celecoxib, etoricoxib, lumiracoxib, rofecoxib, valdecoxib
Various NSAIDs	Aceclofenac, acetylsalicylic acid, alclofenac, berberine, diclofenac, etodolac, flurbiprofen, ibufenac, ibuprofen, indomethacin, ketorolac, lisofylline, lornoxicam, mefenamic acid, meloxicam, naproxen, oxyphenbutazone, phenylbutazone, piroxicam, salicylate, suprofen, tazofelone, tenoxicam, tiaramide, tolmetin, zomepirac
Anti-malignant hyperthermia drugs	Dantrolene
Antimanics	Carbamazepine
Antinarcolepsy drugs	d-Amphetamine, methylphenidate, modafinil
Anti-OCD drugs	Clomipramine, fluoxetine, fluvoxamine, paroxetine, sertraline
Anti-opioid addiction drugs	Buprenorphine, methoxycoronaridine, vanoxerine
Anti-osteoporosis drugs	Raloxifene
Anti-parkinsonian drugs	Amantadine, benztropine, bromocriptine, lisuride, orphenadrine, procyclidine, rasagiline, ropinirole, terguride, tolcapone
Antiprogestins	Valproate
Antipruritics	Camphor, cyproheptadine, doxepin
Antipsoriatics	Bergapten, retinoic acid, methoxsalen, phenol, salicylic acid, tazarotenic acid
Antipyretics	Aceclofenac, acetaminophen, acetanilide, acetylsalicylic acid, alclofenac, aminopyrine, ibuprofen, indomethacin, naproxen, phenacetin, tiaramide
Antirheumatics	
Corticosteroids	Cortisone, dexamethasone, methylprednisolone, prednisolone
Various antirheumatics	Azathioprine, capsaicin, chloroquine, hydroxychloroquine sulfate, leflunomide
Antiseptics	Naphthalene, phenol, terpineol
Antithyroid drugs	Methimazole, methylthiouracil, propylthiouracil
Antitussives	Codeine, dextromethorphan, dihydrocodeine, dimemorfan, dropropizine, ethylmorphine, hydrocodone
Anti-urinary incontinence drugs	Oxybutynin, scopolamine, tolterodine

Antivirals	
Anti-influenza drugs	Amantadine, chloroquine
Anti-hepatitis B drugs	Pradefovir
Anti-herpetics	Valacyclovir
Anti-HIV drugs	Amprenavir, atazanavir, capravirine, delavirdine, efavirenz, indinavir, lopinavir, methoxycoronaridine, nelfinavir, nevirapine, ritonavir, saquinavir, tipranavir, zidovudine
Anti-papillomavirus drugs	Imiquimod, resiquimod
Anxiolytics/hypnotics/sedatives	Adinazolam, alpidem, alprazolam, brofaromine, brotizolam, buspirone, chlordiazepoxide, citalopram, clomethiazole, clomipramine, clonazepam, diazepam, escitalopram, fenobam, flunitrazepam, fluvoxamine, gespirone, imipramine, meprobamate, mexazolam, midazolam, nimetazepam, nordiazepam, opipramol, oxazepam, paroxetine, pimozide, sertraline, temazepam, triazolam, zaleplon, zolpidem, zopiclone
Anti-insomnials	Brotizolam, eszopiclone, zaleplon, zolpidem, zopiclone
Cardiovascular drugs	
ADR receptor agonists	Octopamine, synephrine
Antianginals	Amiodarone, amlodipine, bunitrolol, diltiazem, isosorbide dinitrate, metoprolol, nicardipine, nifedipine, oxprenolol, perhexiline, pronethalol, propranolol, ranolazine, timolol
Antiarrhythmics	Ajmaline, amiodarone, aprindine, azimilide, bepridil, bunitrolol, cibenzoline, digoxin, diltiazem, diprafenone, disopyramide, lidocaine, mexiletine, oxprenolol, phenytoin, procainamide, pronethalol, propafenone, propranolol, quinidine, sparteine, timolol, verapamil
Anticoagulants	Acenocoumarol, dicoumarol, phenprocoumon, warfarin
Antiglaucomics	Betaxolol, brinzolamide, dorzolamide, pilocarpine, pindolol, timolol
Antihypotensives	Desglymidodrine
Antihypertensives	
α-ADR receptor antagonists	Alfuzosin, indoramin, labetolol, prazosin, tamsulosin, yohimbine
β-ADR receptor antagonists	Betaxolol, bisoprolol, bufuralol, bunitrolol, bupranolol, carteolol, carvedilol, labetolol, metoprolol, nebivolol, oxprenolol, pindolol, pronethalol, propranolol, timolol
ACE inhibitors	Captopril, enalapril
AII receptor antagonists	Candesartan, irbesartan, losartan, tasosartan, valsartan

TABLE 18.5 ***Continued***

Category/Subcategory of Substrates	Examples of Substrates[b]
Calcium channel blockers	Amlodipine, barnidipine, bepridil, diltiazem, felodipine, gallopamil, isradipine, lomerizine, mibefradil, nicardipine, nifedipine, niludipine, nilvaldipine, nimodipine, nisoldipine, nitrendipine, norverapamil, oxodipine, pranidipine, verapamil
Various antihypertensives	Ajmaline, debrisoquine, eplerenone, guanabenz, guanoxan, pargyline, pheniprazine
Antiplatelets	Acetylsalicylic acid, cilostazol, clopidogrel, seratrodast, sulfinpyrazone, ticlopidine
Cardiac inotropics	Digitoxin, digoxin, pimobendan, vesnarinone
Lipid peroxidase inhibitors	Tirilazad
Vasodilators	Bosentan, buflomedil, cinnarizine, dihydralazine, flosequinan, flunarizine, glyceryltrinitrate, hydralazine, isosorbide dinitrate, minoxidil, nimodipine, perhexiline
Diuretics	Caffeine, furosemide, perhexiline, tienilic acid, torasemide, triamterene
Estrogenics	17α-Ethinylestradiol
Gastrointestinal drugs	
Antidiarrheals	Berberine, loperamide
Antiemetics	Aprepitant, diphenidol, dolasetron, dronabinol, ezlopitant, granisetron, meclozine, metoclopramide, ondansetron, scopolamine, tropisetron, zatosetron
Antigastroenteritis drugs	Berberine
Antiulceratives	Cimetidine, esomeprazole, lansoprazole, omeprazole, pantoprazole, rabeprazole, ranitidine
Prokinetic drugs	Ecabapide, metoclopramide, mosapride
Immunosuppressants/ immunomodulators	Azathioprine, busulfan, cyclosporin A, imiquimod, laquinimod, lisofylline, mycophenolic acid, resiquimod, roquinimex, sirolimus, tacrolimus
Mucolytics	Ambroxol
Multidrug resistance modulators	Valspodar
Muscle relaxants	Carisoprodol, chlordiazepoxide, chlorzoxazone, cyclobenzaprine, dantrolene, diazepam, meprobamate, nimetazepam, orphenadrine, oxybutynin, tizanidine, tolterodine, zoxazolamine
Mydriatics	Atropine, scopolamine, yohimbine
Neuroleptics	Aripiprazole, bromoperidol, chlorpromazine, clozapine, etoperidone, fluperlapine, fluphenazine, haloperidol, harmaline, harmine, iloperidone, mazapertine, methoxyamphetamine, methoxyphenamine, olanzapine, perazine, perospirone, perphenazine, pimozide, protriptyline, quetiapine, remoxipride, risperidone, sertindole, thioridazine, tiospirone, trazodone, trifluperidol, ziprasidone, zotepine, zuclopenthixol
Nootropics	Nefiracetam, tacrine
Oxytocics	Sparteine

Parasiticides	
Anthelmintics	Albendazole, diethylcarbamazine, ivermectin, naphthalene, praziquantel, pyrantel, thiabendazole
Antimalarials	Amodiaquine, arteether, artelinic acid, artemether, artemisinin, artesunate, berberine, chloroquine, chlorproguanil, halofantrine, lumefantrine, mefloquine, primaquine, proguanil, pyrimethamine, quinidine, quinine, tafenoquine
Ectoparasiticides	Carbaryl, deltamethrin, lindane, malathion, methoxychlor, permethrin
Various parasiticides	Metronidazole, pentamidine, sulfamethoxazole, tinidazole
Photosensitizers	Methoxsalen, trioxsalen
Signal transduction inhibitors	Imatinib
Smoking cessation aids	Bupropion
Stimulants	
Hallucinogenics	Ibogaine, LSD, MBDB, MDA, MDE, MDMA, mescaline, methoxyamphetamine, phencyclidine, THC
Various stimulants	d-Amphetamine, benzphetamine, caffeine, methamphetamine, methylphenidate, modafinil
Topical keratolytics	Salicylic acid
Ultraviolet screens	*p*-Aminobenzoic acid

[a]Substrates presented in this Table are metabolized by CYP enzymes derived primarily from human and rodent species.

[b]Abbreviations: AII, angiotensin II; ACE, angiotensin converting enzyme; ADHD, attention-deficit hyperactivity disorder; ADR, adrenergic; AQ4N, 1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; BOOH, 2-*t*-butyl-4-hydroperoxy-4-methyl-2,5-cyclohexadienone; CNS, central nervous system; COSAIDs, corticosteroidal anti-inflammatory drugs; COX, cyclooxygenase; CPO, 5-{4-[3-(4-cyclohexyl-2-propylphenoxy)propoxy]phenyl}-1,3-oxazolidine-2,4-dione; DBOOH, 2,6-di-*t*-butyl-4-methyl-4-hydroperoxy-2,5-cyclohexadienone; DCBN, 2,6-dichlorobenzonitrile; DDT, dichlorodiphenyltrichloroethane; DEN, diethylnitrosamine; DF203, 2-(4-amino-3-methylphenyl)benzothiazole; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMN, dimethylnitrosamine; DMP543, 10,10-bis(2-fluoro-4-pyridinylmethyl)-9(10*H*)anthracenone; EBOOH, 2,6-di-*t*-butyl-4-ethyl-4-hydroperoxy-2,5-cyclohexadienone; FMF, familial Mediterranean fever; Glu-P-1, 2-amino-6-ethyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; Glu-P-2, 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole; HIV, human immunodeficiency virus; HMGRIs, 3-hydroxy-3-methylglutarylcoenzyme A reductase inhibitors; HMP, hexamethylphosphoramide; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; LSD, lysergic acid diethylamide; MAC, *Mycobacterium avium* complex; MBDB, *N*-methylbenzodioxazolylbutanamine; MC, 3-methylcholanthrene; MDA, 3,4-methylenedioxyamphetamine; MDE, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MeIQ, 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline; MMDX, methoxymorpholinodoxorubicin; MOCA, 4,4′-methylene-bis(2-chloroaniline); NMRIs, nonselective monamine reuptake inhibitors; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, nornitrosonicotine; NSAIDs, nonsteroidal anti-inflammatory drugs; OCD, obsessive–compulsive disorder; PBBs, polybrominated biphenyls; PBDEs, polybrominated diphenyl ethers; PCBs, polychlorinated biphenyls; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PPAR, peroxisome proliferator-activated receptor; SNRIs, selective noradrenaline reuptake inhibitors; S&NRIs, serotonin and noradrenaline reuptake inhibitors; SSRIs, selective serotonin reuptake inhibitors; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; THC, Δ^9-tetrahydrocannabinol; thioTEPA, triethylenethiophosphoramide; TOOH, 4-hydroperoxy-2,4,6-trimethyl-2,5-cyclohexadienone; Tris-BP, tris(2,3-dibromopropyl)phosphate; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole.

[c]Some of the substrates listed as carcinogens or drugs are considered to be procarcinogens or prodrugs, respectively, and need to be bioactivated by CYP enzymes [15, 47–59].

[d]Therapeutic properties of the drugs were determined using human subjects. Drugs are listed under several alphabetically arranged categories/subcategories and are further classified under specific pharmacologic/chemical subheadings within a category. Certain drugs that are used for more than one indication are classified under more than one section. Drugs have been selected on the basis of past and present importance. Certain drugs are no longer marketed as therapeutic agents.

Source: Compiled from Refs. 10 and 60–108. A comprehensive listing of substrates for CYP enzymes can be found in book chapters [2, 15, 50, 53, 55], a review article [89], and websites [397, 398].

have much narrower substrate specificity and function exclusively in the biotransformation of physiological compounds. For example, CYP5 and CYP8A are involved in the production of thromboxane and prostacyclin. CYP11, CYP17, CYP19, and CYP21 play important roles in steroid biosynthesis. CYP7, CYP8B, and CYP27A are involved in the synthesis of bile acids, and CYP51 is involved in cholesterol biosynthesis. CYP24 and CYP27B metabolize vitamin D, while enzymes in the CYP26 family metabolize retinoic acid [23].

18.3.2 Types of Reactions Catalyzed by CYP Enzymes

CYP reactions are typically oxidative reactions that expose or add a functional group on to the substrate molecule. In addition to catalyzing the biotransformation of xenobiotics, CYP enzymes are also involved in the metabolism of endogenous compounds and the biosynthesis of signaling molecules used to control homeostasis and development [21].

Depending on the structure of the substrate, CYP enzymes catalyze a wide range of monooxygenase reactions (i.e., insertion of one atom of oxygen) including aromatic and aliphatic hydroxylations, *N*-, *O*-, and *S*-dealkylations, deaminations, *N*-, *P*-, and *S*-oxidations, and dehalogenations. Less commonly, CYP enzymes can catalyze reduction reactions under conditions of low oxygen tension (Table 18.6). CYP-catalyzed biotransformation often results in inactivation or a decrease in the biological activity of the substrate, although it can also lead to formation of chemically reactive metabolites that can bind to macromolecules such as proteins and DNA and initiate toxic, teratogenic, and carcinogenic events [47, 48]. Other products may be stable molecules possessing intrinsic biological activity that differs from that of the substrate.

It must be emphasized that one CYP enzyme may catalyze several pathways of drug metabolism, and one pathway may be catalyzed by several CYP enzymes. For example, human CYP3A4 can catalyze the hydroxylation of testosterone, *N*-demethylation of alfentanil, and *N*-oxidation of nifedipine, to name just a few reactions, and a drug such as acetaminophen can be metabolized by CYP1A2, CYP2E1, and CYP3A4. Furthermore, CYP enzymes often exhibit regio- and stereoselectivity toward a particular substrate. A good example is testosterone, which undergoes selective ring hydroxylation by various CYP enzymes at either the alpha or beta positions at carbons 2, 6, 7, and 16. Unfortunately, there is no relationship between various CYP enzymes and their abilities to catalyze specific types of reactions so that it is not possible, for example, to characterize an individual CYP enzyme as strictly an *N*-demethylase or an aromatic hydroxylase.

18.3.3 CYP Enzyme Marker Activities

An ideal marker enzyme activity should be catalyzed by a single CYP enzyme. However, in view of the versatile and nonspecific nature of CYP enzymes, it is difficult to find completely specific marker activities for individual CYP enzymes under all conditions.

Human and rodent liver contains more than 25 or 30 different CYP enzymes that can, under *in vitro* conditions, participate to varying degrees in the biotransformation of a given xenobiotic. However, at the concentration of substrates (e.g., drugs)

TABLE 18.6 Reactions Catalyzed by CYP Enzymes

Types of Reactions[a]	Examples of Substrates[a,b]	Product(s) Formed
Oxidative reactions		
Alicyclic carbon hydroxylation	Acetohexamide	*trans*-4-Hydroxyacetohexamide
	Cyclophosphamide	4-Hydroxycyclophosphamide
	Digitoxin	Digoxin
	Minoxidil	4′-Hydroxyminoxidil
Aliphatic amine hydroxylation	Amantadine, benzylamphetamine, chlorphentermine, nicotine, phenmetrazine, phentermine	*N*-Hydroxy derivatives
Aliphatic carbon hydroxylation	*n*-Alkanes, barbiturates, bile acids, cyclohexane, fatty acids, prostaglandins, steroids, drugs (e.g., capsaicin, chlorpropamide, flutamide, glutethimide, ibuprofen, meprobamate, phenylbutazone, valproic acid)	ω- and (ω-1)-Hydroxylation; hydroxylation at other sites
Alkene epoxidation	Aflatoxin B_1	Aflatoxin B_1 2,3-oxide
	Carbamazepine	Carbamazepine 10,11-oxide
	Diethylstilbestrol	Diethylstilbestrol oxide
	Styrene	Styrene oxide
Aromatic amide hydroxylation	Acetaminophen, 2-acetylaminofluorene	*N*-Hydroxy derivatives
Aromatic carbon epoxidation	Benzene, benzo[*a*]pyrene, bromobenzene, DMBA, 4-ipomeanol, naphthalene, phenytoin	Epoxide derivatives
Aromatic carbon hydroxylation	Amphetamine, chlorpromazine, coumarins, diazepam, imipramine, lidocaine, lovastatin, mephenytoin, methadone, phenobarbital, polycyclic aromatics, promethazine, propranolol, salicylic acid, warfarin	Hydroxylation at different sites (certain reactions may proceed via an epoxide intermediate)
Aromatization	Felodipine, nicardipine, nifedipine	Aromatization derivatives
Arylamine hydroxylation	Aniline	Phenylhydroxylamine
	Dapsone	*N*-Hydroxydapsone
Cyclization	Proguanil	Cycloguanil

TABLE 18.6 ***Continued***

Types of Reactions[a]	Examples of Substrates[a,b]	Product(s) Formed
N-Dealkylation	Aminopyrine	Monomethyl-4-aminoantipyrine
	Caffeine	1,7-Dimethylxanthine
	Diazepam	Nordiazepam
	Halofantrine	Desbutylhalofantrine
	Iproniazid	Isoniazid
	Lidocaine	Monoethylglycylxylidine
	(*S*)-Mephenytoin	Nirvanol
	Mephobarbital	Phenobarbital
	Methamphetamine	Amphetamine
	Promethazine	10-(2-Aminopropyl)phenothiazine
	Propranolol	Desisopropylpropranolol
	Sibutramine	Norsibutramine, dinorsibutramine
	Sildenafil	Norsildenafil
O-Dealkylation	Artelinic acid	Dihydroquinghaosu
	Astemizole	Desmethylastemizole
	Codeine	Morphine
	7-Ethoxycoumarin	7-Hydroxycoumarin
	Ethylmorphine	Morphine
	Methoxyamphetamine	Hydroxyamphetamine
	Phenacetin	Acetaminophen
	Reboxetine	Desethylreboxetine
S-Dealkylation	2-Benzylthio-5-trifluoromethylbenzoic acid	2-Thio-5-trifluoromethylbenzoic acid
	Methitural	Desmethylmethitural
	Methylmercaptan	Mercaptan
	6-Methylthiopurine	6-Thiopurine
Deamination	Amphetamine	Phenylacetone
	Bisdesmethylbrompheniramine	3-(*p*-Bromophenyl)-3-pyridyl-propionate
	(*S*)-(+)-α-Methyldopamine	3,4-Dihydroxyphenylacetone
	Propranolol	Desisopropylpropranolol
Dehalogenation	Chloramphenicol	Oxamic acid derivative
	Chloroform	Phosgene
	Dibromomethane	Hydrogen bromide
	Halothane	Trifluoroacetic acid

Dehydrogenation	*N*-Methylformamide, valproic acid, zafirlukast	Dehydrogenated derivatives
Denitrification	2-Nitropropane	Nitrite, acetone
Denitrosation	*N*-Dimethylnitrosamine	Nitrite, methylamine
Descarbethoxyation	Loratadine	Descarboethoxyloratadine
Desulfuration	Chlorpyrifos, diazinon, parathion	Oxon derivatives
	Thiopental	Pentobarbital
Heterocyclic ring oxidation	Phenmetrazine	3-Oxophenmetrazine
N-Hydroxylation	Acetaminophen, acetylhydrazine, 6-aminochrysene, aminoglutethimide, amphetamine, isoniazid, phenacetin, phenmetrazine, phenytoin, riluzole	*N*-Hydroxy derivatives
Hydroxylation at allylic carbon atoms	Hexobarbital	3′-Hydroxyhexobarbital
	Pentazocine	*trans*- and *cis*-Hydroxypentazocine
	Quinidine	3-Hydroxyquinidine
	THC	7-Hydroxy-THC
Hydroxylation at benzylic carbon atoms	Amphetamine	α-Hydroxyamphetamine
	Methaqualon	2′-Hydroxymethylmethaqualon
	3-Methylcholanthrene	3-Hydroxymethylcholanthrene
	Metoprolol	α-Hydroxymetoprolol
	Tolbutamide	4-Hydroxytolbutamide
Hydroxylation of carbon atoms adjacent to a carbonyl or imine group	Diazepam	3-Hydroxydiazepam
	Flurazepam	3-Hydroxyflurazepam
	Glutethimide	4-Hydroxyglutethimide
	Nimetazepam	3-Hydroxynimetazepam
Hydroxylation of indole ring	Dolasetron, indole, ondansetron, tropisetron	Hydroxy derivatives
Isoxazole ring cleavage	Leflunomide	α-Cyanoenol derivative
Methyl hydroxylation	Dacarbazine, etoricoxib, ibuprofen, 3-methylindole, midazolam, montelukast, naproxen, omeprazole, terfenadine, tolterodine, triazolam, zolpidem	Hydroxymethyl derivatives
N-Oxidation	Dapsone, dolasetron, mephentermine, nifedipine, quinidine, senecionine, sparteine, trimethylamine	*N*-Oxidation derivatives
P-Oxidation	Diethylphenylphosphine	*P*-Oxidation derivative
S-Oxidation	Chlorpromazine, cimetidine, lansoprazole, metiamide, montelukast, promethazine, thioacetamide, thiobenzamide, thioguanine, thioridazine, zotepine	Sulfoxide derivatives

TABLE 18.6 *Continued*

Types of Reactions[a]	Examples of Substrates[a,b]	Product(s) Formed
Pyridine ester deesterification	Dehydrogenated pranidipine	OPC-13463
Ring formation	Pulegone	Menthofuran
Transannular dioxygenation	9,10-Dimethyl-1,2-benz[*a*]anthracene	9,10-Dimethyl-1,2-benz[*a*]anthracene -9,10-epidioxide
Reductive reactions		
t-Amine *N*-oxide reduction	AQ4N	AQ4
	N,*N*-Dimethylaniline *N*-oxide	*N*,*N*-Dimethylaniline
	Imipramine *N*-oxide	Imipramine
Arene oxide reduction	Benzo[*a*]pyrene-4,5-oxide	Benzo[*a*]pyrene
Azido reduction	Zidovudine	3′-Amino-3′-deoxythymidine
Azo reduction	Azobenzene	Aniline
	Azsulfidine	Sulfapyridine, *p*-aminosalicylic acid
	Prontosil	2,4-Diaminoaniline, sulfanilamide
Chromate reduction	Chromate (VI)	Chromate (III)
Hydroperoxide reduction	*t*-Butyl hydroperoxide, cumene hydroperoxide, H_2O_2, linoleic acid hydroperoxide, steroid hydroperoxides	Hydroxy derivatives, other products
Nitro reduction	Chloramphenicol	Dichloro-*N*-[2-hydroxy-1-(hydroxymethyl)-2-(4-aminophenyl) ethyl]acetamide
	Nitrobenzene	Aniline
	p-Nitrobenzoic acid	*p*-Aminobenzoic acid
Oxygen reduction	Oxygen	H_2O, H_2O_2, superoxide
Reductive dehalogenation	Carbon tetrachloride	Chloroform
	Halothane	Chlorodifluoroethylene
Reductive dehydrogenation	Hexachlorocyclohexane	Hexachlorocyclohexene
Reductive ring cleavage	Zonisamide	2-Sulfamoylacetylphenol

[a]The examples listed are not meant to be comprehensive but are meant to illustrate the diversity of types of reactions and substrates.
[b]Abbreviations: AQ4, 1,4-bis-{[2-dimethylamino)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; AQ4N, 1,4-bis-{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; DMBA, 7,12-dimethylbenz[*a*]anthracene; OPC-13463, methyl-2,6-dimethyl-4-(3-nitrophenyl)-3-carboxy-5-pyridinecarboxylate; THC, Δ^9-tetrahydrocannabinol.

Source: Compiled from Refs. 15, 50–55, 60, 61, 89, and 109–122.

typically found in tissue after exposure, enzyme kinetics favors a single form of CYP as being the primary catalyst of metabolism for that substrate [27, 49]. Representative examples of CYP enzymes in human liver and their characteristic activities are presented in Table 18.7. The number of competing CYP enzymes, the amounts of individual enzyme present, and the catalytic efficiencies of each enzyme determine the rate and predominant pathway of metabolism of a compound.

TABLE 18.7 Marker Activities and Inhibitors of the Major Human Hepatic CYP Enzymes

CYP	Characteristic Marker Activities	Inhibitors[a]
CYP1A1	7-Ethoxyresorufin *O*-deethylation	9-Hydroxyellipticine
CYP1A2	7-Methoxyresorufin *O*-demethylation	7,8-Benzoflavone, fluvoxamine, furafylline
	Phenacetin *O*-deethylation	7,8-Benzoflavone, fluvoxamine, furafylline
CYP1B1	17β-Estradiol 4-hydroxylation	Propofol
CYP2A6	Coumarin 7-hydroxylation	Diethyldithiocarbamate, methoxsalen
CYP2B6	Bupropion hydroxylation	ThioTEPA, ticlopidine
	Efavirenz 8-hydroxylation	ThioTEPA
	7-Ethoxytrifluoromethylcoumarin *O*-deethylation	9-Ethynylphenanthrene
	(*S*)-Mephenytoin *N*-demethylation	ThioTEPA
CYP2C8	Amadiaquine *N*-deethylation	Quercetin
	Paclitaxel 6α-hydroxylation	Quercetin
CYP2C9	Celecoxib methyl hydroxylation	Sulfaphenazole
	Diclofenac 4′-hydroxylation	Sulfaphenazole, tienilic acid
	(*S*,*R*)-Flurbiprofen 4′-hydroxylation	Sulfaphenazole
	Naproxen *O*-demethylation	Sulfaphenazole, tienilic acid
	Tolbutamide *p*-methyl hydroxylation	Sulfaphenazole, tienilic acid
	Torasemide tolylmethyl hydroxylation	Sulfaphenazole
CYP2C19	(*S*)-Mephenytoin 4′-hydroxylation	Fluconazole, tranylcypromine
CYP2D6	Bufuralol 1′-hydroxylation	Quinidine
	Debrisoquine 4-hydroxylation	Ajmalicine, quinidine
CYP2E1	Chlorzoxazone 6-hydroxylation	Chlormethiazole, 4-methylpyrazole
	Lauric acid ω1-hydroxylation	Diethyldithiocarbamate
CYP3A4	Alfentanil *N*-dealkylation	Ketoconazole, troleandomycin
	Alprazolam 4-hydroxylation	Ketoconazole
	Midazolam 1′-hydroxylation	Ketoconazole
	Nifedipine *N*-oxidation	Gestodene, troleandomycin
	(*S*)-Quinidine 3-hydroxylation	Ketoconazole
	Testosterone 6β-hydroxylation	Troleandomycin
CYP4A11	Lauric acid ω-hydroxylation	10-(Imidazolyl)decanoic acid, TS-011

[a]Abbreviations: thioTEPA, triethylenethiophosphoramide; TS-011, [*N*-(3-chloro-4-morpholin-4-yl) phenyl-*N*-hydroxyimidoformamide].

Source: Compiled from Refs. 2, 15, 50, 51, 53, 55, 89, 123–149, and 398.

18.3.4 CYP Reaction Cycle

The fact that CYP enzymes have been conserved throughout evolution suggests that they serve a vital role in the preservation of life. Many believe the endogenous substrates of CYP enzymes are steroids or some as yet unidentified endogenous compounds [49, 150]. Alternatively, CYP enzymes may serve a critical protective role against the possible harmful effects of exogenous compounds ingested in the diet to which all organisms are exposed continually during their lifetimes. It has also been suggested that, from an evolutionary standpoint, the primary biological role of CYP can be considered to be the detoxification of molecular oxygen (O_2) in tissue to water [15]. This is accomplished by first activating oxygen with reducing equivalents supplied by NADPH, but the activation of oxygen is dangerous if not tightly regulated. The regulation is achieved by a mechanism that prevents CYP from activating oxygen unless a substrate molecule is bound to the enzyme to act as a recipient for the reactive oxygen insertion [15].

The basic aspects of the catalytic mechanism and the interactions of the components of the CYP system are generally agreed upon. The overall reaction for a typical CYP-mediated monooxygenation can be represented as follows:

$$RH + NAD(P)H + O_2 \rightarrow ROH + NAD(P) + H_2O$$

where RH is the substrate and ROH is the oxidized product (Fig. 18.1).

The catalytic cycle can be divided into several discrete steps but basically involves oxygen activation and oxygen insertion (Fig. 18.2). Binding of substrate to the CYP enzyme occurs in the first step. The substrate binds to a site on the enzyme in close proximity to the heme moiety, which results in displacement of the water molecule that normally occupies the sixth coordination site of the heme iron. Substrate binding also facilitates a change in spin state and a reduction of the heme iron, from the low spin ferric (Fe^{3+}) to the high spin ferrous (Fe^{2+}) state, by the addition of an electron from NADPH. Molecular oxygen is then able to bind to the high spin ferrous heme iron to form a dioxygen (Fe^{2+}—O_2) complex. The addition of a second electron results in conversion to a peroxoiron ($Fe^{2+}OOH$) complex. Protonation and cleavage of the O—O bond produces water and a reactive iron–oxo (FeO^{3+}) complex, which transfers its oxygen to the substrate [15, 21, 151–153]. The oxidized substrate is then released, permitting CYP to return to its initial state and repeat the cycle. Oxidation (oxygen insertion) of the substrate typically occurs at the most reactive carbon–hydrogen bond.

18.3.5 CYP Inhibitors

Many clinically important drug interactions involve the effect of one drug on the metabolism of another. Enzyme inhibition, specifically inhibition of CYP enzymes, is a very common mechanism behind drug interactions. Some CYP inhibitors and the CYP enzymes affected are listed in Table 18.7.

The effect of enzyme inhibition is to reduce the rate of metabolism of drugs, taken concurrently with the inhibitor, that are also substrates of the inhibited CYP enzyme. The affected drugs begin to accumulate in the body and their therapeutic activity and side effects are increased. For example, if erythromycin, an inhibitor of

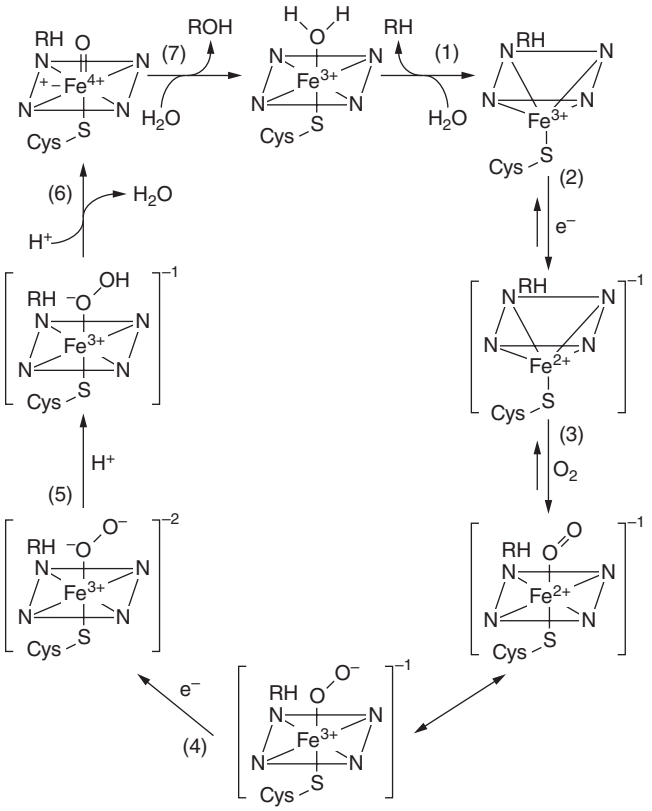


FIGURE 18.2 CYP catalytic cycle. Enzymatic steps by which CYP enzymes catalyze the hydroxylation of a substrate (RH) to a product (ROH). Adapted from Ref. 153.

CYP3A4, is taken by a patient who is also being treated with carbamazepine, which is extensively metabolized by CYP3A4, this may lead to toxicity due to higher concentrations of carbamazepine.

Inhibition is dose related, so that inhibition will not appear until a sufficient concentration of inhibitor is present in the liver. The effects are often maximal when steady-state plasma concentrations of the inhibitor are reached. Moreover, at low concentrations, the inhibitor may be relatively selective for a single CYP enzyme, whereas at high concentrations, the inhibitor may be relatively nonselective and several CYP enzymes will be inhibited.

There are several mechanisms by which drugs and other compounds inhibit CYP enzymes. The commonest mechanism is simple competition by two drugs for metabolism by the same CYP enzyme. The two drugs compete at the substrate binding site to produce reversible and rapid inhibition of the metabolism of one of the drugs, usually the drug with the lower affinity for the enzyme or the drug that is present at substantially lower concentration. Simple competitive inhibition is usually short-acting and is possible with any two drugs. A second more selective type of reversible inhibition is produced by drugs containing an azole group, such as the antifungal agents, ketoconazole, miconazole, and fluconazole, or by drugs containing an

imidazole group, such as cimetidine. In this type of inhibition, a basic nitrogen atom of the azole or imidazole group interacts, via its free electron pair, with the iron in the heme group of CYP and forms a relatively stable but reversible substrate–enzyme complex. A more slowly reversible form of inhibition is produced when a drug is metabolized to a reactive metabolite that binds in a noncovalent fashion with the iron in the CYP heme group. Examples of drugs that produce this type of inhibitory metabolite (i.e., metabolism-dependent inhibitors) include nortriptyline, amiodarone, and the macrolide antibiotics such as erythromycin and clarithromycin. This type of inhibition often increases with repeated dosing and the effects may continue for several weeks after stopping the drug if the drug has a long half-life. Irreversible inhibition is relatively rare but the effects are long-lasting. Irreversible inhibitors such as gestodene, ethinyl estradiol, levonorgestrel, and spironolactone are metabolized to reactive metabolites that form covalent bonds with either the heme group or the apoprotein of CYP, a process that inactivates the enzyme and is only overcome when the inhibited CYP enzyme turns over (i.e., is destroyed and new enzyme is synthesized).

18.4 STRUCTURE AND REGULATION

18.4.1 Overview of Individual CYP Enzymes

The total CYP level in liver may vary two- to threefold, at most, among humans. However, there is considerably greater variation in the enzyme activities and protein levels of individual CYP enzymes within a population. Table 18.8 illustrates the variability and range of CYP enzyme levels that have been measured for 14 different enzymes in human liver microsomes. The interindividual variation in human

TABLE 18.8 CYP Enzyme Levels[a] in Human Liver Microsomes

CYP Enzyme	Content (pmol CYP/mg microsomal protein)	Percentage of Total CYP
CYP1A1	Not detected	0
CYP1A2	<1–45	<0.5–15
CYP1B1	Not detected	0
CYP2A6	<1–68	4–17
CYP2B6	1–39	<1–7
CYP2C8	1–60	12–15
CYP2C9	9–100	14–18
CYP2C18	<1	<0.5
CYP2C19	0.5–19	1–17
CYP2D6	1.5–10	1–4
CYP2E1	7–49	3–9
CYP3A4	40–140	18–29
CYP3A5	<1	<0.5
CYP4A11	Not determined	10

[a]CYP enzyme levels were compiled from Refs. 55, and 155–158. CYP enzyme levels were measured in individual human liver samples by immunoquantification. Values represent the approximate range of CYP levels reported.

hepatic CYP levels and activities is related to several factors including genetic polymorphism, enzyme inhibition, and enzyme induction.

18.4.2 CYP1 Family

Two CYP enzymes are present in the 1A subfamily, CYP1A1 and CYP1A2. These enzymes are highly conserved among species [15]. Human CYP1A1 and CYP1A2 share approximately 70% amino acid sequence identity.

CYP1A1 and CYP1A2 preferentially metabolize planar aromatic molecules but exhibit distinct substrate selectivity (Table 18.9). CYP1A1 and CYP1A2 both catalyze the *O*-dealkylation of 7-methoxyresorufin and 7-ethoxyresorufin and are involved in the bioactivation of chemical carcinogens [2, 159]. CYP1A1 preferentially catalyzes the hydroxylation of benzo[*a*]pyrene leading to the 7,8-epoxide intermediate, whereas CYP1A2 preferentially catalyzes the *N*-hydroxylation of aromatic amines including 4-aminobiphenyl, 2-naphthylamine, and 2-acetylaminofluorene [26, 51]. With respect to drug metabolism, CYP1A2 catalyzes the *N*-hydroxylation of acetaminophen, leading to formation of the reactive benzoquinoneimine metabolite, *N*3-demethylation of caffeine to paraxanthine, and *O*-dealkylation of phenacetin. Ellipticine is an inhibitor of human CYP1A1 and furafylline is a potent metabolism-dependent inhibitor of human CYP1A2 [2, 13].

CYP1A1 is not normally expressed in the liver of humans and most mammals but is detected in extrahepatic tissues such as lung and placenta in humans. CYP1A2 is expressed constitutively and almost exclusively in the liver [26, 158, 160]. Hepatic levels of CYP1A2 vary widely, exhibiting as much as a 40- to 100-fold difference, among individuals [155, 158].

CYP1B1 is the only member of the CYP1B subfamily. Recombinant CYP1B1 catalyzes the oxidation of 7,12-dimethylbenz[*a*]anthracene and the 4-hydroxylation of 17β-estradiol (Table 18.9). CYP1B1 protein has not been detected in liver [158] and is found primarily in extrahepatic tissues including thymus and the adrenal cortex [55]. CYP1B1 is linked to eye development as mutations in the human CYP1B1 gene are associated with congenital glaucoma [180].

CYP1A1, CYP1A2, and CYP1B1 are inducible enzymes, which are upregulated by the activation of the aryl hydrocarbon receptor (AhR) [26, 181]. In humans, CYP1A enzymes can be induced by polycyclic aromatic hydrocarbons found in charbroiled meat, cigarette smoke, and cruciferous vegetables. Omeprazole, a proton pump inhibitor, has also been shown to induce CYP1A enzymes [2]. In rodents, CYP1A1 levels increase from undetectable to approximately 40% of the total CYP content in the liver, following treatment with xenobiotic inducers such as 3-methylcholanthrene [160]. CYP1A1 levels in the lung, small intestine, and kidneys are also induced. CYP1A2 levels in the liver are increased but CYP1A2 remains undetectable in extrahepatic tissues following administration of inducers such as 3-methylcholanthrene [160].

18.4.3 CYP2 Family

The CYP2 family is the largest CYP family present in humans in terms of number of different CYP enzymes identified. CYP2 enzymes are responsible for steroid

TABLE 18.9 Substrates for Human CYP1 Enzymes[a]

CYP	Category of Substrates	Examples of Substrates[b]
CYP1A1	Benzene derivatives	*o*-Aminoazotoluene, toluene
	Carcinogens	
	Amino compounds	2-Acetylaminofluorene, 2-aminoanthracene, 4-aminobiphenyl, 6-aminochrysene, 2-aminofluorene, IQ, MeIQ, MeIQx, methoxyaminoazobenzene, NNK, PhIP, Trp-P-1, Trp-P-2
	Polycyclic aromatics	Benz[*a*]anthracene-3,4-diol, benzo[*g*]chrysene-11,12-diol, benzo[*b*]fluoranthene-9,10-diol, benzo[*a*]perylene, benzo[*a*]pyrene-7,8-diol, chrysene-1,2-diol, dibenz[*a*,*h*]anthracene, dibenzo[*a*,*l*]pyrene-11,12-diol, DMBA, DMBA-3,4-diol, 5,6-dimethylchrysene-1,2-diol, 1-ethylpyrene, 5-methylchrysene-1,2-diol, 6-nitrochrysene, 2-naphthylamine, 2-nitronaphthalene, nitropyrene, phenanthrene
	Various carcinogens	Aflatoxin B_1, diethylstilbestrol, 17β-estradiol, ochratoxin A
	Chloroaromatics	1,2,4-Trichlorobenzene, 1,2,3,5-tetrachlorobenzene
	Coumarins, resorufins	Coumarin, 7-ethoxycoumarin, ethoxyresorufin
	Endogenous compounds	Tryptamine, uroporphyrinogen
	Flavonoids	7,8-Benzoflavone, eriodictyol, galangin, genistein, hesperetin, kaempferide, methoxyflavanone
	Fungicides	Furametpyr
	Retinoids	All-*trans*-retinoic acid, retinal, retinol
	Steroids	Androstenedione, estrone, pregnenolone, progesterone, testosterone
	Drugs	Abecarnil, albendazole, aminopyrine, amiodarone, amodiaquine, benzydamine, bufuralol, caffeine, capsaicin, carvedilol, chloroquine, chlorzoxazone, cinnarizine, clomethiazole, dacarbazine, diclofenac, ellipticine, flunarizine, flutamide, fluvastatin, lisofylline, lisuride, MDE, nicardipine, nicotine, nifedipine, pentamidine, phenacetin, phencyclidine, pranidipine, propranolol, pyrantel, quinine, riluzole, ropivacaine, tegafur, terguride, theophylline, thiabendazole, tolbutamide, toremifene, troglitazone, warfarin, zotepine
CYP1A2	Benzene derivatives	*o*-Aminoazotoluene, toluene
	β-Carbolines	Harmaline, harmine
	Carcinogens	
	Amines and derivatives	2-Acetylaminofluorene, 2-aminoanthracene, aminoazotoluene, 4-aminobiphenyl, 6-aminochrysene, 2-aminofluorene, benzidine, dimethylaminoazobenzene, furylfuramide, Glu-P-1, Glu-P-2, IQ, MeIQ, MeIQx, methoxyaminoazobenzene, NNK, PhIP, Trp-P-1, Trp-P-2, 2-naphthylamine
	Polycyclic aromatics	Benz[*a*]anthracene-3,4-diol, benzo[*a*]pyrene-7,8-diol, 1,3-dinitropyrene, DMBA, DMBA-3,4-diol, 5-methylchrysene-1,2-diol, 1-methylpyrene, nitropyrene, naphthalene, 6-nitrochrysene, phenanthrene
	Various carcinogens	Aflatoxin B_1, butadiene, 1,4-dichlorobenzene, 17β-estradiol, methyleugenol, ochratoxin A

TABLE 18.9 ***Continued***

CYP	Category of Substrates	Examples of Substrates[b]
	Chloroaromatics	Bromodichloromethane, 1,2-dichlorobenzene, 1,2,4-trichlorobenzene
	Coumarins, resorufins	BFC, coumarin, 7-ethoxycoumarin, methoxyresorufin
	Endogenous compounds	Arachidonic acid, linoleic acid, PGH_2, phosphatidylcholine, uroporphyrinogen
	Flavonoids	Aminoflavone, apigenin, biochanin A, daidzein, formononetin, galangin, genistein, hesperetin, kaempferide, kaempferol, methoxyflavanone, naringenin, prunetin, tangeretin, tamarixetin
	Fungicides	Furametpyr, *o*-phenylphenol
	Hormones	Estrone, melatonin, progesterone, testosterone
	Insect repellents	*N*,*N*-Diethyl-*m*-toluamide
	Pesticides	Ametryne, atrazine, chlorpyrifos, diazinon, methoxychlor, parathion
	Phytoalexins	Resveratrol
	Pneumotoxins, other toxins	3-Methylindole, myristicin
	Retinoids	Retinal, 13-*cis*-retinoic acid, retinol
	Xanthines	1,7-Dimethylxanthine, 8-ethylfurafylline, furafylline, paraxanthine
	Drugs	Acenocoumarol, acetaminophen, acetanilide, albendazole, aminopyrine, amiodarone, amitriptyline, amodiaquine, antipyrine, benzydamine, O^6-benzylguanine, bropirimine, bufuralol, bupivacaine, bupropion, caffeine, capsaicin, carbamazepine, carvedilol, chlordiazepoxide, chlorpromazine, chlorzoxazone, cilostazol, cinnarizine, ciprofloxacin, cisapride, clomipramine, clozapine, cyclobenzaprine, dacarbazine, dapsone, desipramine, diazepam, diclofenac, diethylcarbamazine, dihydralazine, doxepin, ellipticine, enoxacin, etoposide, fenfluramine, flunarizine, flunitrazepam, fluoxetine, flutamide, fluvoxamine, ftorafur, grepafloxacin, guanabenz, haloperidol, hydromorphone, iloperidone, imipramine, imiquimod, ipomeanol, leflunomide, lidocaine, lisofylline, maprotiline, MBDB, MDE, MDMA, (*S*)-mephenytoin, methadone, metoclopramide, mexiletine, mianserin, mirtazapine, naproxen, nicardipine, nicotine, nifedipine, nordiazepam, nortriptyline, olanzapine, ondansetron, pefloxacin, perazine, perphenazine, phenacetin, pimobendan, pimozide, pranidipine, praziquantel, primaquine, proguanil, propafenone, propofol, propranolol, ranitidine, rasagiline, resiquimod, riluzole, ritonavir, rofecoxib, ropinirole, ropivacaine, tacrine, tamoxifen, tauromustine, tegafur, terbinafine, theobromine, theophylline, thiabendazole, tizanidine, tolbutamide, tolcapone, toremifene, triamterene, verapamil, warfarin, zileuton, zolmitriptan, zolpidem, zotepine, zoxazolamine

TABLE 18.9 ***Continued***

CYP	Category of Substrates	Examples of Substrates[b]
CYP1B1	Carcinogens	
	Amino compounds	2-Aminofluorene, IQ, MeIQ, MeIQx, methoxyaminoazobenzene, NNK, PhIP, Trp-P-1, Trp-P-2
	Polycyclic aromatics	2-Aminoanthracene, 6-aminochrysene, benz[*a*]anthracene-1,2-diol, benzo[*g*]chrysene-11,12-diol, benzo[*b*]fluoranthene-9,10-diol, benzo[*c*]phenanthrene-3,4-diol, benzo[*a*]pyrene, benzo[*a*]pyrene-7,8-diol, chrysene-1,2-diol, dibenzo[*a*,*l*]pyrene-11,12-diol, DMBA, DMBA-3,4-diol, 5,6-dimethylchrysene-11,12-diol, dinitropyrene, 9-hydroxybenzo[*a*]pyrene, 1-ethylpyrene, 5-methylchrysene, 5-methylchrysene-1,2-diol, 1-methylpyrene, 6-nitrochrysene, nitrofluoranthene, nitropyrene
	Various carcinogens	Aflatoxin B_1, 17β-estradiol
	Coumarins, resorufins	Coumarin, 7-ethoxycoumarin, ethoxyresorufin
	Flavonoids	Eriodictyol, genistein, hesperetin
	Steroid hormones	Estrone, progesterone, testosterone
	Fatty acids	Arachidonic acid
	Retinoids	Retinal, retinol
	Drugs	Albendazole, amodiaquine, artelinic acid, artemisinin, artesunate, bufuralol, caffeine, DF203, docetaxel, ellipticine, flutamide, nifedipine, pyrimethamine, theophylline, thiabendazole, tolbutamide

[a]Involvement of specific human CYP enzymes in the metabolism of substrates listed in Tables 18.9–18.12 was determined using *in vivo* and *in vitro* procedures. *In vivo* techniques included administering drugs and other substrates together with selective CYP inhibitors to human subjects and measuring metabolites in plasma and urine. *In vitro* studies included incubating human hepatic microsomes with substrate along with selective CYP inhibitors or antibodies and measuring inhibition of enzyme activity and product formation. Other *in vitro* studies included incubating purified human hepatic CYP enzymes, or a panel of cDNA expressed human CYP enzymes, with substrate and measuring product formation. Liver is the primary site of expression for the majority of CYP enzymes listed in Tables 18.9–18.12.

[b]Abbreviations: BFC, 7-benzyloxy-4-trifluoromethylcoumarin; DF203, 2-(4-amino-3-methylphenyl)benzothiazole; DMBA, 7,12-dimethylbenz[*a*]anthracene; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; Glu-P-2, 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MBDB, *N*-methylbenzodioxazolylbutanamine; MDE, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MeIQ, 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PGH_2, prostaglandin H_2; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole.

Source: Compiled from Refs. 10, 51–56, 130, and 161–179. A comprehensive listing of substrates for CYP1 enzymes can be found in book chapters [2, 15, 50, 53, 55] and a review article [89].

hydroxylation and the biotransformation of a wide variety of drugs and xenobiotics, as well as many endogenous compounds (Table 18.10).

The CYP2A subfamily consists of three genes in humans, namely, CYP2A6, CYP2A7, and CYP2A13. CYP2A6, CYP2A7, and CYP2A13 exhibit >90% identity

TABLE 18.10 Substrates for Human CYP2 Enzymes

CYP	Category of Substrates	Examples of Substrates[a]
CYP2A6	Carcinogens	
	Amino compounds	6-Aminochrysene, PhIP
	Nitro compounds	*p*-Nitroanisole, DEN, DMN, NNAL, NNK, NNN
	Various carcinogens	Aflatoxin B_1, butadiene, chloroform, 17β-estradiol, MOCA, phenanthrene, safrole
	Components of garlic	Diallyl sulfide
	Coumarins	Coumarin, 7-ethoxycoumarin, psoralen
	Ethers, phenols	Ethyl *t*-butyl ether, methyl *t*-butyl ether, *p*-nitrophenol
	Fluorinated hydrocarbons	Halothane, methoxyflurane, sevoflurane
	Indoles, xanthines	1,7-Dimethylxanthine, indole, 3-methylindole, paraxanthine
	Insect repellants	*N*,*N*-Diethyl-*m*-toluamide
	Retinoids	All-*trans*-retinoic acid
	Steroid hormones	Progesterone, testosterone
	Terpenes	Verbenone
	Drugs	Acetaminophen, aminopyrine, amodiaquine, artelinic acid, artesunate, bupropion, chlorzoxazone, cinnarizine, cisapride, clomethiazole, clozapine, cotinine, cyclobenzaprine, cyclophosphamide, fadrozole, flunarizine, flunitrazepam, ftorafur, ifosfamide, letrozole, lidocaine, lisofylline, losigamone, medifoxamine, methoxsalen, mexiletine, naproxen, nicotine, nifedipine, omeprazole, phenacetin, primaquine, propofol, quinoline, ritonavir, tacrine, tamoxifen, tegafur, valproic acid, zidovudine
CYP2B6	Alkanes	Hexane
	Aromatic hydrocarbons	*o*-Phenylphenol, toluene
	Nitrosoamines	*N*-Nitrosomethylbutylamine, *N*-nitrosomethylethylamine, nitrosomorpholine
	Carcinogens	
	Polycyclic aromatics	6-Aminochrysene, benzo[*a*]pyrene-7,8-diol, dibenzo[*a*]pyrene, DMBA, naphthalene, phenanthrene
	Various carcinogens	Aflatoxin B_1, butadiene, chloroform, 17β-estradiol, isoprene, DMN, NNK, styrene
	Coumarins, resorufins	Coumarin, 7-ethoxycoumarin, ethoxyresorufin, benzyloxyresorufin, pentoxyresorufin
	Fatty acids	Arachidonic acid, lauric acid
	Flavonoids	Flavanone, methoxyflavanone
	Fluorinated hydrocarbons	Chlorodifluoroethane, halothane, methoxyflurane, sevoflurane
	Insect repellents	*N*,*N*-Diethyl-*m*-toluamide

TABLE 18.10 ***Continued***

CYP	Category of Substrates	Examples of Substrates[a]
	Pesticides	Acetochlor, ametryne, butachlor, chlorpyrifos, diazinon, endosulfan, methoxychlor, parathion, terbutryne
	Polychlorinated aromatics	2,4,5,2′,4′,5′-Hexachlorobiphenyl
	Retinoids	All-*trans*-retinoic acid
	Steroid hormones, sterols	5β-Cholestane-3α,7α,12α-triol, estrone, testosterone
	Terpenes	Verbenone
	Drugs	Alfentanil, aminopyrine, amitriptyline, antipyrine, arteether, artelinic acid, artemisinin, artesunate, benzphetamine, O^6-benzylguanine, bergamottin, bupropion, capsaicin, carteolol, cinnarizine, clobazam, clomethiazole, cyclophosphamide, diazepam, diclofenac, diethylcarbamazine, efavirenz, erythromycin, ethylmorphine, flunitrazepam, ifosfamide, ketamine, lidocaine, MDA, MDE, MDMA, (*S*)-mephenytoin, mephobarbital, mianserin, midazolam, nevirapine, nicotine, pentobarbital, primaquine, procainamide, promethazine, propofol, quinoline, ropivacaine, selegiline, seratrodast, sertraline, tamoxifen, tazofelone, temazepam, thioTEPA, tinidazole, trofosfamide, valproic acid, verapamil, zotepine
CYP2C8	Carcinogens	Benzo[*a*]pyrene, benzo[*a*]pyrene-7,8-diol, naphthalene, styrene
	Fatty acids	Arachidonic acid, 9,10-epoxystearic acid, leukotoxin
	Fluorescent probes	Dibenzylfluorescein
	Pesticides	Chlorpyrifos, diazinon, methoxychlor, parathion
	Retinoids	All-*trans*-retinoic acid, 13-*cis*-retinoic acid, retinol, tazarotenic acid
	Steroids	Androstenedione, testosterone
	Drugs	Albendazole, aminopyrine, amiodarone, amodiaquine, antipyrine, artelinic acid, benzphetamine, bufuralol, capsaicin, carbamazepine, cerivastatin, chloroquine, cisapride, clozapine, CPO, dapsone, diazepam, diclofenac, fluvastatin, ftorafur, gallopamil, halofantrine, karenitecin, lidocaine, lonafarnib, (*R*)-mephenytoin, mephobarbital, mirtazapine, mycophenolic acid, naproxen, omeprazole, paclitaxel, perospirone, phenprocoumon, phenytoin, pioglitazone, pyrantel, repaglinide, rosiglitazone, sulfadiazine, tegafur, temazepam, terbinafine, tiaramide, tienilic acid, tolbutamide, torasemide, trimethoprim, troglitazone, verapamil, warfarin, zidovudine, zopiclone

CYP2C9	Carcinogens	
	Polycyclic aromatics	Benzo[*a*]pyrene, benzo[*a*]pyrene-7,8-dihydrodiol, dibenzo[*a*]pyrene, naphthalene, phenanthrene
	Various carcinogens	Butadiene, 17β-estradiol, MeIQ, methyleugenol, safrole
	Components of garlic	Diallyl disulfide
	Coumarins	7-Ethoxycoumarin
	Fatty acids	Arachidonic acid, 9,10-epoxystearic acid, isoleukotoxin, leukotoxin, linoleic acid
	Flavonoids	Biochanin A, formononetin, tamarixetin
	Fluorescent probes	Dibenzylfluorescein
	Oral contraceptives	Desogestrel
	Retinoids	Retinoic acid, retinol
	Steroids	5α-Androstane-3α,17β-diol, testosterone
	Terpenes	Limonene
	Drugs	Aceclofenac, acenocoumarol, acetylsalicylic acid, albendazole, amitriptyline, antipyrine, artelinic acid, benzbromarone, bosentan, bufuralol, bupropion, candesartan, capsaicin, carvedilol, celecoxib, chloramphenicol, chlorpheniramine, cinnarizine, clozapine, cyclophosphamide, dapsone, dextromethorphan, diazepam, diclofenac, dicoumarol, dolasetron, dorzolamide, dronabinol, eletriptan, ellipticine, etodolac, etoricoxib, flunarizine, flunitrazepam, fluoxetine, flurbiprofen, fluvastatin, glimepiride, glipizide, hexobarbital, hydromorphone, ibuprofen, ifosfamide, imipramine, indomethacin, irbesartan, ketamine, ketobemidone, lornoxicam, losartan, lovastatin, lumiracoxib, mefenamic acid, meloxicam, (*R*)-mephenytoin, mirtazapine, moclobemide, montelukast, naproxen, nateglinide, nelfinavir, nevirapine, nicotine, ondansetron, perazine, perphenazine, phenobarbital, phenprocoumon, phenylbutazone, phenytoin, piroxicam, pravastatin, proguanil, propofol, pyrantel, ritonavir, rosiglitazone, selegiline, seratrodast, sertraline, sildenafil, simvastatin, sulfadiazine, sulfamethoxazole, sulfinpyrazone, suprofen, tafenoquine, tamoxifen, targretin, tauromustine, tegafur, temazepam, tenoxicam, terbinafine, THC, thiamylal, tiaramide, tienilic acid, tolbutamide, torasemide, trimethadione, trimethoprim, tropisetron, valproic acid, valsartan, venlafaxine, verapamil, voriconazole, warfarin, zafirlukast, zidovudine, zileuton, zopiclone
CYP2C18	Pesticides	Methoxychlor
	Retinoids	All-*trans*-retinoic acid
	Steroids	Progesterone
	Drugs	Aminopyrine, antipyrine, clobazam, diazepam, imipramine, lansoprazole, lidocaine, mephenytoin, naproxen, perphenazine, piroxicam, propofol, tienilic acid, tolbutamide, verapamil, warfarin

TABLE 18.10 ***Continued***

CYP	Category of Substrates	Examples of Substrates[a]
CYP2C19	Carcinogens	Benzo[*a*]pyrene-7,8-dihydrodiol, benzo[*a*]pyrene-7,8-diol, chloroform, 17β-estradiol, methyleugenol
	Components of garlic	Diallyl disulfide
	Fatty acids	Arachidonic acid
	Hormones	Melatonin
	Indoles	Indole
	Insect repellents	*N*,*N*-Diethyl-*m*-toluamide
	Pesticides	Chlorpyrifos, diazinon, furametpyr, methoxychlor, parathion
	Prostaglandins	9,11-Epoxymethano-PGH_2, 11,9-epoxymethano-PGH_2, 9,11-diazo-15-deoxy-PGH_2
	Retinoids	Retinal, retinoic acid
	Steroids	Desogestrel, progesterone, testosterone
	Terpenes	Limonene
	Drugs	Acenocoumarol, adinazolam, albendazole, aminopyrine, amitriptyline, azelastine, benzbromarone, bufuralol, capsaicin, carisoprodol, chlorproguanil, citalopram, clobazam, clomethiazole, clomipramine, clozapine, CPO, dapsone, dextromethorphan, diazepam, diphenylhydantoin, divalproex, eletriptan, esomeprazole, etoricoxib, flunitrazepam, glibenclamide, hexobarbital, imipramine, lansoprazole, loratadine, (*R*)- and (*S*)-mephenytoin, mephobarbital, methadone, methoxycoronaridine, metoprolol, mianserin, moclobemide, naproxen, nelfinavir, nirvanol, nordiazepam, nortriptyline, omeprazole, pantoprazole, pentamidine, perazine, perphenazine, phenobarbital, phenytoin, piroxicam, praziquantel, proguanil, propranolol, pyrimethamine, quinine, rabeprazole, ranitidine, ritonavir, rofecoxib, selegiline, sertraline, tauromustine, temazepam, terbinafine, thalidomide, THC, tolbutamide, topiramate, valproic acid, venlafaxine, verapamil, voriconazole, warfarin, zidovudine, zotepine
CYP2D6	Amines	Octopamine, synephrine, tryptamine, tyramine
	β-Carbolines	Harmaline, harmine, methoxydimethyltryptamine, pinoline
	Carcinogens	Butadiene, 17β-estradiol, NNK, safrole
	Components of garlic	Diallyl disulfide
	Fungicides	*o*-Phenylphenol
	Insect repellents	*N*,*N*-Diethyl-*m*-toluamide
	Pesticides	Chlorpyrifos, diazinon, methoxychlor, parathion
	Steroids	Progesterone

	Drugs	Ajmaline, albendazole, alprenolol, amiflamine, aminopyrine, amitriptyline, amodiaquine, aprindine, aripiprazole, artemisinin, azelastine, barnidipine, benztropine, betaxolol, bisoprolol, brofaromine, bromoperidol, buflomedil, bufuralol, bunitrolol, bupranolol, capsaicin, captopril, carteolol, carvedilol, chloroquine, chlorpheniramine, chlorpromazine, cibenzoline, cinnarizine, citalopram, clemastine, clomipramine, clozapine, codeine, cyclobenzaprine, dapsone, debrisoquine, delavirdine, desglymidodrine, desipramine, desmethylcitalopram, dexfenfluramine, dextromethorphan, diazepam, diclofenac, diltiazem, diphenhydramine, diprafenone, dolasetron, donepezil, doxepin, doxylamine, duloxetine, efavirenz, eletriptan, encainide, ethylmorphine, etoricoxib, ezlopitant, fenfluramine, fentanyl, flecainide, flunarizine, fluoxetine, fluperlapine, fluphenazine, fluvastatin, fluvoxamine, galantamine, gallopamil, gepirone, guanoxan, haloperidol, hydrocodone, hydromorphone, hydroxyzine, ibogaine, imipramine, indinavir, indoramin, karenitecin, labetalol, lidocaine, lisuride, lopinavir, loratadine, lovastatin, maprotiline, MBDB, MDA, MDE, MDMA, meperidine, mequitazine, methadone, methamphetamine, methoxyamphetamine, methoxyphenamine, metoclopramide, metoprolol, mexiletine, mianserin, milameline, minaprine, mirtazapine, moclobemide, morphine, nebivolol, nefazodone, nelfinavir, nevirapine, norcodeine, norfluoxetine, nortriptyline, olanzapine, ondansetron, opipramol, oxycodone, oxyprenolol, paroxetine, pentazocine, perhexiline, perospirone, perphenazine, phenformin, pilocarpine, pimozide, pindolol, praziquantel, primaquine, procainamide, promethazine, propafenone, propoxyphene, propranolol, *N*-propylajmaline, pyrantel, quetiapine, quinidine, ranitidine, remoxipride, risperidone, ritonavir, rofecoxib, ropivacaine, selegiline, sertindole, sertraline, simvastatin, sparteine, sumatriptan, tamoxifen, tamsulosin, tauromustine, terguride, thebaine, thioridazine, timolol, tolterodine, tomoxetine, tramadol, trazodone, trifluperidol, trimipramine, tripelennamine, tropisetron, venlafaxine, yohimbine, zolpidem, zotepine, zuclopenthixol
CYP2E1	Alkanes and related compounds	Acetaldehyde, acetonitrile, diethylether, ethanol, glycerol
	Amides	Dimethylformamide, methylformamide
	Aromatic hydrocarbons	Aniline, naphthylhydrazine, *p*-nitrophenol, phenol, pyridine, toluene, xylene
	Carcinogens	
	Amine derivatives	DEN, DMN, NNAL, NNK, NNN
	Halogenated hydrocarbons	CCl_4, chloroform, 1,4-dichlorobenzene, dichloromethane, 1,2-dichloropropane, ethylene dibromide, ethylene dichloride, methyl chloride, methylene chloride, tetrachloroethane, trichloroethane, trichloroethylene, vinyl bromide, vinyl chloride
	Polycyclic aromatics	Benzo[*a*]pyrene-7,8-diol, 1-methylpyrene
	Various carcinogens	Acrylonitrile, benzene, butadiene, ethyl carbamate, isoprene, methyleugenol, *p*-nitroanisole, safrole, styrene, vinyl carbamate

TABLE 18.10 ***Continued***

CYP	Category of Substrates	Examples of Substrates[a]
	Chloroaromatics	1,2-Dichlorobenzene, 1,2,4-trichlorobenzene, 1,2,3,5-tetrachlorobenzene, pentachlorobenzene
	Components of garlic	Diallyl disulfide, diallyl sulfide
	Coumarins	7-Ethoxycoumarin
	Endogenous compounds	Acetone, PGH_2, phosphatidylcholine
	Fatty acids	Lauric acid, linoleic acid, oleic acid
	Flavonoids	Genistein
	Fluorinated hydrocarbons	Desflurane, enflurane, halothane, isoflurane, methoxyflurane, sevoflurane
	Indoles	Indole, 3-methylindole
	Insect repellents	*N*,*N*-Diethyl-*m*-toluamide
	Pesticides	Methoxychlor, parathion
	Drugs	Acetaminophen, albendazole, artesunate, bupropion, caffeine, capsaicin, chloroquine, chlorzoxazone, dacarbazine, dapsone, disulfiram, dorzolamide, eszopiclone, felbamate, fluoxetine, isoniazid, ondansetron, phenobarbital, primaquine, quinoline, ritonavir, salicylate, sulfadiazine, tamoxifen, tegafur, theobromine, theophylline, thiamylal, tolcapone, trimethadione, vesnarinone, zidovudine
CYP2J2	Fatty acids	Arachidonic acid, linoleic acid
	Steroids	Testosterone
	Vitamins	1α-Hydroxyvitamin D_3, vitamin D_2, vitamin D_3
	Drugs	Astemizole, bufuralol, diclofenac, ebastine
CYP2R1	Vitamins	1α-Hydroxyvitamin D_2, 1α-hydroxyvitamin D_3, vitamin D_2, vitamin D_3

[a]Abbreviations: CPO, 5-{4-[3-(4-cyclohexyl-2-propylphenoxy)propoxy]phenyl}-1,3-oxazolidine-2,4-dione; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; MBDB, *N*-methylbenzodioxazolylbutanamine; MDA, 3,4-methylenedioxyamphetamine; MDE, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MeIQ, 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline; MOCA, 4,4 -methylene-bis(2-chloroaniline); NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, nornitrosonicotine; PGH_2, prostaglandin H_2; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; THC, Δ^9-tetrahydrocannabinol; thioTEPA, triethylenethiophosphoramide.

Source: Compiled from Refs. 40, 112, and 182–252. A detailed listing of substrates for CYP2 enzymes can be found in book chapters [2, 15, 50, 53, 55] and a review article [89].

at the amino acid level [253]. CYP2A6 is expressed primarily in liver. CYP2A7, which was originally named CYP2A4, is considered nonfunctional. CYP2A13 is expressed mainly in the nasal mucosa, followed by the trachea and lung [13]. Expression of CYP2A6 is subject to genetic polymorphism (see Table 18.4), with four allelic variants having been described. CYP2A6 is involved in the metabolism of several compounds found in tobacco. For example, CYP2A6 catalyzes the *C*-oxidation of nicotine, hydroxylation of cotinine at the 3′-position, hydroxylation of *N*-nitrosonornicotine at the 5′-position, and oxidation of 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone and *N*-nitrosodiethylamine [253]. In terms of drug substrates, CYP2A6 preferentially catalyzes the hydroxylation of coumarin at the 7-position, which serves as a marker activity for this enzyme. Methoxypsoralen (methoxsalen), a structural analogue of coumarin, is a potent inhibitor of CYP2A6 [2].

There is only one CYP2B enzyme in humans, CYP2B6. A second CYP2B gene, CYP2B7, was identified as a splice variant of CYP2B6 but is not transcribed into protein [254]. CYP2B6 is expressed mostly in the liver [15, 254]. In a panel of 19 human liver microsome samples, CYP2B6 protein expression showed a 100-fold interindividual variability [255]. CYP2B6 shows sex and ethnic differences in expression and activity [256]. CYP2B6 is involved in the metabolism of relatively few drugs. It has been shown to catalyze the 4-hydroxylation of cyclophosphamide, and the *N*-demethylation of benzphetamine, (*S*)-mephenytoin, and diazepam [2]. Moreover, CYP2B6 catalyzes the *O*-dealkylation of 7-benzyloxyresorufin, methoxychlor, and 7-ethoxycoumarin [253]. However, the activities are not specific to CYP2B6 as other CYP enzymes such as CYP2C9 and CYP3A4 also contribute to these reactions. In rats, members of the CYP2B subfamily include CYP2B1, CYP2B2, and CYP2B3 [396]. Hepatic CYP2B1 and CYP2B2 are highly inducible in the rat following exposure to phenobarbital [15, 257]. Unlike the rat, little is known about induction of CYP2B6 in humans. In cultured human hepatocytes, CYP2B6 expression was increased following treatment with rifampicin, dexamethasone, and phenobarbital [255].

The CYP2C subfamily consists of four genes in humans, namely, CYP2C8, CYP2C9, CYP2C18, and CYP2C19. The four CYP2C enzymes are >80% identical in terms of amino acid sequence [15]. All four CYP2C enzymes are expressed primarily in liver (see Table 18.8), with CYP2C9 typically present at a higher level than CYP2C8, CYP2C18, or CYP2C19. CYP2C9 and CYP2C19 exhibit genetic polymorphism (see Table 18.4), with two allelic variants described for CYP2C9 and five allelic variants described for CYP2C19. CYP2C enzymes are involved in the metabolism of several important drugs. For example, CYP2C8 catalyzes the 6α-hydroxylation of taxol and the 4-hydroxylation of retinol and all-*trans* retinoic acid. CYP2C9 catalyzes tolbutamide hydroxylation, tienilic acid hydroxylation, phenytoin 4′-hydroxylation, diclofenac 4′-hydroxylation, and (*S*)-warfarin 7-hydroxylation [2]. CYP2C19 preferentially catalyzes the hydroxylation of (*S*)-mephenytoin at the 4′-position, which serves as a marker activity for this enzyme. No specific reactions have been identified for CYP2C18, which is expressed in trace amounts in liver. Sulfaphenazole is a potent selective inhibitor of CYP2C9 [2]. Inhibitors of CYP2C8 and CYP2C19 are known but they are not very specific.

There is a single functional member of the CYP2D subfamily in humans. CYP2D6 is expressed primarily in liver. Low levels have also been detected in extrahepatic tissues. CYP2D6 is a highly polymorphic enzyme. More than 60 allelic variants,

which affect the amount of expression of the enzyme and its catalytic activity, have been described (see Table 18.4). The polymorphisms affecting CYP2D6 have clinical relevance because this enzyme is involved in the metabolism of at least 100 different drugs (see Table 18.10). For example, CYP2D6 catalyzes the 4-hydroxylation of propranolol and debrisoquine, *O*-demethylation of dextromethorphan, 1′-hydroxylation of bufuralol, 5′-hydroxylation of propafenone, and hydroxylation of sparteine, all of which can be used as marker activities for this enzyme. Quinidine is a relatively selective inhibitor of CYP2D6 [2].

CYP2E1 is the only member of the CYP2E subfamily in humans. It is expressed constitutively in liver, lung, and kidney [2]. Relatively few drugs are metabolized by CYP2E1, but this enzyme catalyzes ethanol oxidation, *N*1- and *N*7-demethylation of caffeine, *N*-oxidation of acetaminophen, and chlorzoxazone 6-hydroxylation, which serves as a marker activity for CYP2E1. CYP2E1 can be induced by ethanol and isoniazid. 4-Methylpyrazole is a relatively selective inhibitor for CYP2E1 [2].

CYP2F and CYP2J are two minor subfamilies with only one functional gene in each. CYP2F1 is expressed primarily in human lung. It has been shown to catalyze the oxidation of ethoxycoumarin and propoxycoumarin, and of 4-ipomeanol and 3-methylindole, two lung toxicants [13]. CYP2J2 is expressed in liver and intestine. It has been shown to catalyze the hydroxylation of ebastine, a second-generation antihistamine [357].

Additional enzymes in the CYP2 family include CYP2S1, CYP2U1, and CYP2W1, but their function remains unknown, as substrates for these enzymes have not been identified [11].

18.4.4 CYP3 Family

CYP3A enzymes are the predominant CYP enzymes expressed in human liver and intestine [14, 154]. The human CYP3A subfamily consists of CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A enzymes catalyze the biotransformation of a wide variety of xenobiotic compounds, as well as bile acids and steroids (Table 18.11). Indeed, CYP3A substrates represent diverse chemical structures including cyclosporin A, which is the largest CYP substrate known. The prominent role played by CYP3A enzymes in drug metabolism is highlighted by an estimate that CYP3A enzymes are responsible for the biotransformation of greater than 50% of all prescription medications [154].

CYP3A enzymes catalyze the *N*-dealkylation of alfentanil, benzphetamine, codeine, dextromethorphan, erythromycin, imipramine, and tamoxifen, hydroxylation of midazolam at the 1′-position, hydroxylation of alprazolam at the 4-position, and *N*-oxidation of dihydropyridines such as nifedipine [50]. A catalytic activity that is common to CYP3A enzymes in many mammalian species, and is often used as a marker activity, is the stereoselective hydroxylation of testosterone and cortisol at the 6β-position [15] (see Table 18.7). Inhibitors of CYP3A include the macrolide antibiotics, antifungal agents such as clotrimazole and ketoconazole, HIV protease inhibitors, particularly ritonavir, and gestodene [2].

CYP3A4 is the single most abundant CYP enzyme in human liver and small intestine [14, 154]. All human liver and small intestine samples appear to contain CYP3A4, but levels vary by 10-fold or more among individuals [14, 155]. In contrast, CYP3A5 is expressed at a lower level (approximately one-third or less) than

TABLE 18.11 Substrates for Human CYP3A4

	Category of Substrates	Examples of Substrates[a]
CYP3A4	Azole antifungals	Clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole, voriconazole
	Bile acids	Chenodeoxycholic acid, cholic acid, deoxycholic acid, lithocholic acid, taurochenodeoxycholic acid
	Carcinogens	
	Mycotoxins	Aflatoxin B_1, aflatoxin G_1, ochratoxin A, sterigmatocystin
	Polycyclic aromatics	6-Aminochrysene, benzo[*a*]pyrene, benzo[*a*]pyrene-7,8-diol, DDB[*a*]P, DDB[*b*]F, DDMBA, 1,6-dinitropyrene, 1-ethylpyrene, 1-methylpyrene, naphthalene, nitropyrene, phenanthrene
	Various carcinogens	17β-Estradiol, MeIQ, methoxyaminoazobenzene, MOCA, NNK, NNN, senecionine, Tris-BP
	Chloroaromatics	1,2-Dichlorobenzene, 1,2,4-trichlorobenzene, 1,2,3,5-tetrachlorobenzene, pentachlorobenzene
	Coumarins	BFC
	Endogenous compounds	9,10-Epoxystearic acid, *N*-hydroxyarginine, leukotoxin, PGH_2
	Ergot alkaloids	Bromocriptine, dihydroergotamine
	Flavonoids	Apigenin, 7,8-benzoflavone, genistein, kaempferol, naringenin, tamarixetin
	Fluorescent probes	Dibenzylfluorescein
	Fungicides	Furametpyr, *o*-phenylphenol
	Hormones	1α,25-Dihydroxyvitamin D_3
	Insect repellents	*N*,*N*-Diethyl-*m*-toluamide
	Macrolides	Clarithromycin, erythromycin, miocamycin, roxithromycin, sirolimus, tacrolimus, troleandomycin
	Mycotoxins, other toxins	Myristicin, territrems B and C
	Pesticides	Acetochlor, alachlor, aldrin, ametryne, atrazine, butachlor, carbofuran, chlorpyrifos, chlortoluron, diazinon, endosulfan, methoxychlor, parathion, terbutryne
	Retinoids	Retinal, all-*trans*-retinoic acid, 9-*cis*-retinoic acid
	Rifamycins and derivatives	Benzoxazinorifamycins, rifalazil, rifampicin
	Steroid hormones, steroids	Androstenedione, budesonide, corticosterone, cortisol, dehydroepiandrosterone, dexamethasone, DHEA, dutasteride, estrone, 17α-ethinylestradiol, finasteride, fluticasone, gestodene, levonorgestrel, lilopristone, medroxyprogesterone, mestranol, methandienone, methylprednisolone, mifepristone, norethisterone, onapristone, prednisolone, prednisone, pregnenolone, progesterone, testosterone
	Terpenes	Eucalyptol, limonene

TABLE 18.11 ***Continued***

Category of Substrates	Examples of Substrates[a]
Drugs	Acetaminophen, adinazolam, albendazole, alfentanil, alfuzosin, alpidem, alprazolam, ambroxol, aminopyrine, amiodarone, amitriptyline, amlodipine, amprenavir, anastrozole, antipyrine, aprepitant, AQ4N, aripiprazole, arteether, artelinic acid, artemether, artemisinin, astemizole, atazanavir, atorvastatin, azelastine, azimilide, barnidipine, benzphetamine, bepridil, bezafibrate, bicalutamide, bisoprolol, bosentan, brinzolamide, bromoperidol, brotizolam, bupivacaine, buprenorphine, bupropion, buspirone, busulfan, capravirine, capsaicin, carbamazepine, carvedilol, cerivastatin, chloroquine, chlorpromazine, cibenzoline, cimetidine, ciprofibrate, cisapride, citalopram, clindamycin, clobazam, clofibrate, clomethiazole, clomipramine, clonazepam, clopidogrel, clozapine, cocaine, codeine, colchicine, cyclobenzaprine, cyclophosphamide, cyclosporine, dantrolene, dapsone, delavirdine, desipramine, desmethyladinazolam, dextromethorphan, diazepam, diclofenac, digitoxin, dihydralazine, dihydrocodeine, diltiazem, disopyramide, docetaxel, dolasetron, donepezil, dorzolamide, doxepin, doxorubicin, ebastine, ecabapide, efavirenz, eletriptan, ellipticine, enalapril, eplerenone, esomeprazole, eszopiclone, ethosuximide, ethylmorphine, etizolam, etoperidone, etoposide, etoricoxib, exemestane, ezlopitant, felbamate, felodipine, fenofibrate, fentanyl, flosequinan, flunitrazepam, flutamide, fluvastatin, galantamine, gallopamil, ganaxolone, gefitinib, gemfibrozil, gepirone, germander, glibenclamide, glyceryltrinitrate, granisetron, halofantrine, haloperidol, hydrocodone, hydromorphone, ifosfamide, imatinib, imipramine, imiquimod, indinavir, ipomeanol, irinotecan, isoniazid, isosorbide dinitrate, isradipine, ivermectin, karenitecin, ketamine, ketobemidone, lansoprazole, laquinimod, letrozole, lidocaine, lisofylline, lisuride, lonafarnib, loperamide, lopinavir, loratadine, losartan, lovastatin, lumefantrine, MBDB, MDA, MDE, MDMA, medifoxamine, mefloquine, meloxicam, methadone, mexazolam, mianserin, mibefradil, midazolam, mifepristone, mirtazapine, MMDX, modafinil, montelukast, mosapride, mycophenolic acid, nateglinide, navelbine, nefazodone, nefiracetam, nelfinavir, nevirapine, nicardipine, nifedipine, niludipine, nilvaldipine, nimodipine, nisoldipine, nitrendipine, noralfentanil,

nordiazepam, nortriptyline, norverapamil, omeprazole, ondansetron, orphenadrine, oxodipine, oxybutynin, paclitaxel, pantoprazole, perazine, perospirone, perphenazine, phencyclidine, phenprocoumon, pimozide, pioglitazone, piroxicam, pradefovir, pranidipine, pravastatin, praziquantel, primaquine, propafenone, proguanil, pyrilamine, quetiapine, quinidine, quinine, rabeprazole, raloxifene, ranolazine, reboxetine, repaglinide, resiquimod, risperidone, ritonavir, rofecoxib, ropinirole, ropivacaine, roquinimex, rupatadine, salmeterol, saquinavir, selegiline, seratrodast, sertindole, sertraline, sibutramine, sildenafil, simvastatin, sterinodole, sufentanil, sulfadiazine, sulfamethoxazole, sulfamidine, tafenoquine, tamoxifen, tamsulosin, targretin, tasosartan, tauromustine, tazofelone, temazepam, teniposide, tentoxin, terbinafine, terfenadine, terguride, THC, theophylline, thiamylal, thioTEPA, tiagabine, ticlopidine, tinidazole, tipranavir, tirilazad, tolmetin, tolterodine, toremifene, trazodone, triazolam, triazoledione, trimethadione, trimethoprim, trofosfamide, troglitazone, tropisetron, valdecoxib, valspodar, vanoxerine, vardenafil, venlafaxine, verapamil, vesnarinone, vinblastine, vincristine, vindesine, vinorelbine, warfarin, yohimbine, zafirlukast, zaleplon, zatosetron, zidovudine, zileuton, ziprasidone, zopiclone, zolpidem, zomepirac, zonisamide, zotepine

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[a]Abbreviations: AQ4N, 1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; DDB[*a*]P, 7,8-dihydroxy-7,9-dihydrobenzo[*a*]pyrene; DDB[*b*]F, 9,10-dihydroxy-9,10-dihydrobenzo[*b*]fluoranthene; DDMBA, 3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenz[*a*]anthracene; DHEA, dehydroepiandrosterone; MBDB, *N*-methylbenzodioxazolylbutanamine; MDA, 3,4-methylenedioxyamphetamine; MDE, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MeIQ, 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline; MMDX, methoxymorpholinodoxorubicin; MOCA, 4,4′-methylene-bis(2-chloroaniline); NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, nornitrosonicotine; PGH_2, prostaglandin H_2; THC, Δ^9-tetrahydrocannabinol; thioTEPA, triethylenethiophosphoramide; Tris-BP, tris-(2,3-dibromopropyl)phosphate.

Source: Compiled from Refs. 59, 118, 199, 212, 221, 238, 244, 247, and 258–344. A comprehensive listing of substrates for CYP3A enzymes can be found in book chapters [2, 15, 50, 53, 55], a review article [89], and websites [397, 398].

CYP3A4 and in only 10–30% of human liver and intestine samples [14, 155]. CYP3A5 has also been detected in human kidney [2]. The lack of CYP3A5 expression in most humans is thought to be due to a splice site mutation [345]. CYP3A7 is considered to be a fetal enzyme and accounts for 30–50% of total CYP content in fetal liver [345]. CYP3A7 is now recognized to be expressed in some adult livers [345]. CYP3A43 mRNA is expressed at low levels in the liver and at relatively high levels in the testis and prostate. In earlier studies, a fifth CYP3A enzyme, CYP3A3, was identified. This enzyme showed strong homology to CYP3A4. It is now believed that CYP3A3 is in fact an allelic variant of CYP3A4 [345]. Both CYP3A5 and CYP3A7 have similar substrate specificities as CYP3A4. The substrate specificity of CYP3A43 is unknown since only low levels of expression have been achieved in recombinant systems [345]. The human CYP3A enzymes can be induced by treatment with drugs such as phenobarbital, rifampicin, phenytoin, and troglitazone [50], and this induction can result in clinically relevant drug interactions.

In rats, the CYP3A subfamily includes CYP3A1, CYP3A2, CYP3A9, CYP3A18, and CYP3A62 [346, 396]. A sixth rat CYP3A enzyme, CYP3A23, has also been reported in the literature, but this is now believed to be an allelic variant of CYP3A1 [346, 347]. CYP3A2, CYP3A9, CYP3A18, and CYP3A62 display age- and sex-specific regulation and induction [348, 349]. For example, CYP3A2 is constitutively expressed in hepatic microsomes from immature rats of both sexes and mature male rats but not in adult female rats [348]. CYP3A18 is expressed predominantly in adult male rats, whereas CYP3A9 is expressed at higher levels in adult female rats than in male rats [350]. Dexamethasone treatment increases hepatic microsomal levels of CYP3A2 in immature rats and adult male but not adult female rats [348]. CYP3A1 is undetectable in hepatic microsomes from untreated rats of either sex but following administration of dexamethasone, hepatic levels of CYP3A1 are increased dramatically [348]. CYP3A1 can account for 30–37% of total CYP content in hepatic microsomes prepared from dexamethasone-treated immature and mature rats of both sexes. Expression of hepatic CYP3A9, CYP3A18, and CYP3A62 enzymes is also increased by administration of dexamethasone [346, 348]. Other inducers of rat CYP3A enzymes include phenobarbital, rifampicin, isosafrole, and pregnenolone 16α-carbonitrile [348].

18.4.5 CYP4 Family

Members of the CYP4 family generally metabolize endogenous fatty acid substrates such as lauric, palmitic, and arachidonic acids and their derivatives including prostaglandins, thromboxane, prostacyclin, and leukotrienes (Table 18.12). CYP4 enzymes play a minor role in the metabolism of drugs and other xenobiotics.

The CYP4A subfamily consists of CYP4A9 and CYP4A11. CYP4A11 is expressed in human liver and kidney. The expression and function of CYP4A9 is not known at present. CYP4A11 preferentially catalyzes the ω (terminal carbon) hydroxylation of fatty acids, although it can also catalyze hydroxylation at the ω-1 (penultimate carbon) position [2]. Hence the characteristic activity of CYP4A11 is hydroxylation of lauric acid at the 12-(ω) position (see Table 18.7). CYP4A enzymes in rats are inducible by peroxisomal proliferators such as clofibrate. Induction of CYP4A11 by clofibrate has also been reported in primary cultures of human hepatocytes [360].

TABLE 18.12 Substrates for Human CYP4 Enzymes

CYP	Category of Substrates	Examples of Substrates[a]
CYP4A11	Fatty acids, leukotrienes	Arachidonic acid, lauric acid, LTB_4, linoleic acid, palmitic acid, 9,10-epoxystearic acid, isoleukotoxin, leukotoxin
	Eicosanoic acids	15-HETE
	Prostaglandins	9,11-Epoxymethano-PGH_2, 11,9-epoxymethano-PGH_2, 9,11-diazo-15-deoxy-PGH_2
	Drugs	Artesunate, pentamidine
CYP4F2	Aromatic hydrocarbons	7-Ethoxycoumarin, *p*-nitroanisole
	Fatty acids, leukotrienes, lipoxins	Arachidonic acid, hydroxystearic acids, LTB_4, 6-*trans*-LTB_4, oleic acid, lipoxin A_4, 9,10-epoxystearic acid, isoleukotoxin, leukotoxin
	Eicosanoic acids	5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 15-HETE
CYP4F3B	Fatty acids, leukotrienes	Arachidonic acid, 9,10-dihydroxyoctadec-12-enoic acid, 12,13-dihydroxyoctadec-9-enoic acid, 9,10-dihydroxystearic acid, LTB_4, 9,10-epoxystearic acid, isoleukotoxin, leukotoxin
	Eicosanoic acids	5,6-DHET, 8,9-DHET, 11,12- DHET, 14,15-DHET, 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET
CYP4F11	Fatty acids, leukotrienes	Arachidonic acid, LTB_4
	Eicosanoic acids	8-HETE
	Drugs	Benzphetamine, chlorpromazine, erythromycin, ethylmorphine, imipramine, theophylline, verapamil
CYP4F12	Fatty acids, leukotrienes	Arachidonic acid, LTB_4
	Prostaglandins	11,9-Epoxymethano-PGH_2, 9,11-diazo-15-deoxy-PGH_2
	Drugs	Ebastine

[a]Abbreviations: DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; LTB_4, leukotriene B_4; PGH_2, prostaglandin H_2.

Source: Compiled from Refs. 15, 29, 55, 89, and 351–361.

The human CYP4F subfamily consists of five genes, CYP4F2, CYP4F3, CYP4F8, CYP4F11, and CYP4F12 [358, 359]. CYP4F enzymes have been detected at the level of mRNA in numerous tissues in humans including leukocytes, liver, lung, kidney, intestine, heart, brain, and skin [358, 359]. CYP4F enzymes are involved in the oxidation of fatty acids, leukotrienes, and prostaglandins (Table 18.12).

18.4.6 Regulation of CYP Enzyme Expression

The expression of CYP genes in many species is highly regulated by age, hormonal factors including sex steroids, growth hormone, and thyroid hormones, diet and nutritional status, and disease states. Indeed, regulation of hepatic CYP enzymes is complex. Although the mechanisms and consequences of induction of CYP enzymes have been studied intensively, relatively little is known about the mechanisms by which CYP enzymes are regulated by physiological factors. In humans, CYP enzyme

expression appears to be regulated mainly via induction by xenobiotics (i.e., drugs, environmental compounds including those present in cigarette smoke, dietary components, and ethanol) and possibly by hormones. CYP enzyme induction has consequences in terms of drug pharmacokinetics, accumulation, and toxicity.

Induction of CYP enzymes usually occurs at the level of transcription and results in *de novo* protein synthesis. There are several mechanisms that involve interactions with regulatory elements in the relevant CYP gene, by which CYP genes can be induced. Most commonly, inducers bind to a receptor protein, which then binds to regulatory elements in the DNA, and activates gene transcription and translation. Alternatively, the inducer can interact, either directly or indirectly, with a repressor protein that would normally be bound to the operator region of the CYP gene and repress its expression. Binding of the inducer would release the repressor and result in increased transcription of the structural region of the gene [15]. Specific xenobiotic receptor proteins have now been identified as being involved in gene induction by four distinct mechanisms [369, 370]. Inducing agents can bind to four different xenobiotic receptors, namely, the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the peroxisomal proliferator-activated receptor (PPAR). Typical inducers of human CYP enzymes are presented in Table 18.13.

The Ah receptor is a ligand-activated nuclear transcription factor that controls the expression of a battery of genes including CYP1A1, CYP1A2, and CYP1B1 [371, 372]. The AhR is ubiquitously expressed in most organs and is conserved across many species including mammals and invertebrates [181]. Once activated, the AhR translocates to the nucleus, where it forms a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT) [373]. This heterodimer complex then binds to a

TABLE 18.13 Inducers of Human CYP Enzymes

CYP	Inducers[a]
CYP1A1	Chargrilled meat, omeprazole, PCBs, polycyclic aromatics (e.g., DMBA, MC), TCDD
CYP1A2	Chargrilled meat, cruciferous vegetables (e.g., broccoli, Brussels sprouts), hyperforin, omeprazole, PCBs, polycyclic aromatics, TCDD
CYP1B1	PCBs, polycyclic aromatics, TCDD
CYP2A6	Phenobarbital
CYP2B6	Nevirapine, phenobarbital, dexamethasone, troglitazone
CYP2C8	Rifampicin
CYP2C9	Hyperforin, phenobarbital, rifampicin
CYP2C19	Rifampicin, ritonavir
CYP2D6	Unknown
CYP2E1	Ethanol, isoniazid
CYP3A4	Bosentan, carbamazepine, cortisol, dexamethasone, efavirenz, felbamate, hyperforin, nevirapine, phenobarbital, phenytoin, primidone, rifampicin, rifapentin, sulfadimidine, topiramate, troglitazone
CYP4A11	Clofibrate

[a]Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; MC, 3-methylcholanthrene; PCBs, polychlorinated biphenyls; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Source: Compiled from Refs. 19, 26, 158, and 360–370. A comprehensive listing of CYP inducers can be found in book chapters [2, 15, 50, 55], a review article [89], and a website [398].

xenobiotic response element in the promoter region upstream of the CYP1A1 and CYP1A2 genes. Binding of the heterodimer complex to the xenobiotic response element results in recruitment of coregulatory proteins and relaxation of chromatin structure, which promotes transcription [181]. One of the most potent inducers of CYP1A expression is the highly toxic compound, TCDD. Other xenobiotic AhR ligands include 3-methylcholanthrene, indolo[2,3-*b*]carbazole, and coplanar PCBs [181] (Table 18.14). Endogenous AhR ligands include tryptamine, bilirubin, and indirubin [181].

Phenobarbital is a prototype agent for a variety of structurally unrelated inducers that elicit induction of CYP2B enzymes in a number of mammalian species. Induction of the CYP2B enzymes occurs mainly through the activation of CAR, a novel nuclear receptor. Following exposure to phenobarbital-type inducers, CAR translocates from the cytoplasm to the nucleus, where it binds and activates a phenobarbital-responsive element upstream of the CYP2B genes [374]. CAR, which is highly expressed in liver, is known to dimerize with the retinoid X receptor (RXR) and can constitutively activate its target genes even in the absence of inducers. Normally, endogenous androstane-like steroids bind to CAR and maintain it in an inactive state. The binding of phenobarbital-type inducers interferes with the binding of the inhibitory steroids so that receptor activity is derepressed and gene transcrip-

TABLE 18.14 Receptors and Xenobiotic Activators/Ligands of Human CYP Genes

Receptors[a]	Xenobiotic Activators/Ligands[a]	Regulated CYP Genes
AhR	Guanabenz, omeprazole, PCBs, polycyclic aromatics (e.g., benzo[*a*]pyrene, MC), TCDD, TCDF, thiabendazole	CYP1A1, CYP1A2, CYP1B1
CAR	CITCO, hyperforin, methoxychlor, phenobarbital, phenytoin, rifampicin	CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4
PPARα	Bezafibrate, clofibrate, fenofibrate, fenoprofen, flufenamic acid, gemfibrozil, herbicides, ibuprofen, indomethacin, phthalates, Wy-14,643	CYP4A11
PXR	Amprenavir, avasimibe, bosentan, carbamazepine, chlorpyrifos, CITCO, clotrimazole, cypermethrin, dexamethasone, DDT, dieldrin, efavirenz, endosulfan, endrin, etoposide, glibenclamide, hyperforin, lovastatin, mifepristone, methoxychlor, nelfinavir, nifedipine, nonylphenol, paclitaxel, phenobarbital, phenytoin, rifabutin, rifampicin, ritonavir, simvastatin, tamoxifen, TCPOBOP, topiramate, triclosan, troglitazone	CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4

[a]Abbreviations: AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime; DDT, dichlorodiphenyltrichloroethane; MC, 3-methylcholanthrene; PCBs, polychlorinated biphenyls; PPARα, peroxisome proliferator-activated receptor α; PXR, pregnane X receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; Wy-14,643, [4-chloro-6-(2,3-xylidino)pyrimidynylthio]acetic acid.

Source: Compiled from Refs. 23, 26, 94, 154, 181, 366, and 369–395.

tion can proceed [369, 370]. CYP2B enzymes can be induced by a variety of antimicrobials, barbiturates, and pesticides. Some examples include clotrimazole, rifampicin, phenytoin, methoxychlor, and dichlorodiphenyltrichloroethane (DDT) [374, 375].

Another novel nuclear receptor, PXR (also known as PAR and SXR) [376], mediates induction of CYP3A genes in response to glucocorticoids such as dexamethasone and drugs such as rifampicin [28] (Table 18.14). CYP3A4 and CYP3A5 enzymes are inducible following administration of rifampicin although CYP3A5 is induced to a lesser extent than CYP3A4 [345]. Induction of the CYP3A subfamily occurs mainly through activation of PXR. PXR binds as a heterodimer with RXR to xenobiotic response elements in the proximal promoter regions of the CYP3A genes [154]. Induction of the CYP3A subfamily shows distinct species-specific differences. For example, rifampicin is a potent inducer of human CYP3A4 but is a weak inducer of rat CYP3A2 [154]. Studies have suggested that CAR may also be involved in transcriptional activation of the CYP3A genes since CAR can bind to xenobiotic response elements in the promoter region of the CYP3A genes [154]. Experimental evidence indicates that the glucocorticoid receptor may participate in the induction of CYP3A4, but the molecular mechanism is not clear [377].

The peroxisomal proliferator-activated receptor (PPAR) is highly expressed in liver and mediates peroxisome proliferation and induction of CYP4A enzymes [369] (Table 18.14).

18.5 CONCLUSION

Insight into the biotransformation of drugs by CYP and other drug-metabolizing enzymes is a prerequisite for the evaluation of drug safety and risk assessment. Moreover, comprehensive knowledge of the regulation and activity of CYP enzymes and an awareness of possible drug interactions is needed to avoid possible adverse effects associated with the common practice of prescribing several drugs simultaneously.

There are still many gaps in our knowledge of CYP enzymes. There is a practical need for better and more specific reagents to measure CYP enzyme activity and CYP enzyme levels in various tissues. For example, the ready availability of highly specific inhibitory antibodies and potent specific chemical inhibitors for each CYP enzyme would be highly desirable. This would allow investigators to identify the individual CYP enzymes involved in the metabolism of a particular drug and to quantify the enzymes in both hepatic and extrahepatic tissues. Also needed are highly selective and safe *in vivo* probes of CYP activity to allow investigators to estimate CYP levels in patients without the need to take liver biopsy samples. The large interindividual variability in CYP enzyme levels complicates drug therapy and efficacy. Expression profiling studies with larger banks of human tissues and human subjects are needed to discover the extent of variation of individual CYP protein levels, to delineate the causes of the variation, and to map distribution patterns of CYP enzymes within a population.

Recent advances are expanding our knowledge of the CYP enzymes and providing therapeutic benefits. For example, the development of CYP knockout mouse models is enabling investigators to explore the role and importance of CYP enzymes

in normal physiology. Pharmacogenomic testing is also currently receiving much attention because of the increasing awareness of the benefits of incorporating pharmacogenomics into the selection of drug regimens. The selection of proper drug regimen and dose based on the patient's individual genetic makeup could eliminate the unpredictable response of drug treatment, which may arise because of genetic polymorphisms that affect drug metabolism, clearance, and tolerance. Lastly, computational models can be used to predict the metabolism of specific drugs by CYP enzymes, to "design out" unfavorable drug interactions, and to help guide the drug design process.

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