7

Protein Binding

Upon entering into plasma, most drugs bind rapidly to blood constituents. When the phenomenon of protein binding of a drug is considered, it is usually the protein binding of drug molecules to blood components, including blood cells, albumin, and α_1-acid glycoprotein. The extent of binding can vary with both drug and protein concentrations. Binding of a drug to plasma and tissue proteins is a saturable process, and is generally considered reversible with rapid equilibrium within milliseconds. For most drugs, protein binding at physiologically relevant concentrations seems to be concentration-independent.

It is generally assumed that only unbound drug is able to transport across membranes and become subject to absorption, distribution, metabolism, and excretion processes. Characterization of protein binding of a drug and the effects of various pathophysiological conditions, such as disease states and concomitant medications, on protein binding are important for an understanding of the pharmacokinetic behavior of a drug. Furthermore, as only unbound drug is considered to be able to interact with pharmacological receptors, the proper integration of drug pharmacokinetics and pharmacodynamics should be based on a thorough understanding of the nature and extent of drug protein binding in both plasma and tissues.

7.1. DEFINITION

Protein binding indicates how much of the total amount of a drug in plasma or tissue is bound to plasma or tissue proteins.

Plasma proteins: Important physiological functions of plasma proteins include maintenance of the osmotic pressure of the blood and transport capacity for numerous endogenous and exogenous substrates through specific and/or nonspecific binding. Albumin and α_1-acid glycoprotein are the two major proteins in plasma, with albumin being by far the most abundant [approximately 4% (w/v) of plasma] (Table 7.1). Albumin in plasma is approximately 40% of the total albumin in the body with the rest being found mainly in interstitial fluid (Fig. 7.1). The distribution pattern of albumin in the human body is summarized in Table 7.2. In general, albumin appears to have higher binding affinity for acidic compounds. The content of α_1-acid glycoprotein in plasma is less than 0.1% (w/v), and basic compounds tend

Table 7.1. Plasma Proteins in Humans

Plasma protein	Molecular weight (kDa)[a]	Concentration in plasma (g/dl)	Drugs binding to the protein[b]
Albumin	69	3.5–5.0	Acidic
α_1-acid glycoprotein	44	0.04–0.1	Basic
Lipoproteins	200–3400	Variable	Basic
Globulins	140	2.5	—
Steroid binding globulin (transcortin)	53	0.003–0.007	Steroids (cortisol)
Fibrinogen	400	0.3	—

[a]kDa = 1000.
[b]Preferable characteristics of drugs binding to the corresponding protein.

to bind more to α_1-acid glycoprotein, although they do bind to albumin to a significant extent as well. In general, the binding affinity for α_1-acid glycoprotein, which is referred as the acute phase reactant protein, is much higher than for albumin. The primary physiological role of lipoproteins is the synthesis and transport of endogenous fatty acids such as triglycerides, phospholipids, and cholesterol. It has also been found that lipoproteins can be important in the binding of very lipophilic and/or basic compounds. Various specific proteins are responsible for the plasma binding and transport of certain endogenous compounds, including hormones. In addition, the red and white blood cells as well as the platelets can bind drugs, especially basic drugs, although such binding is usually minor (Wilkinson,1983).

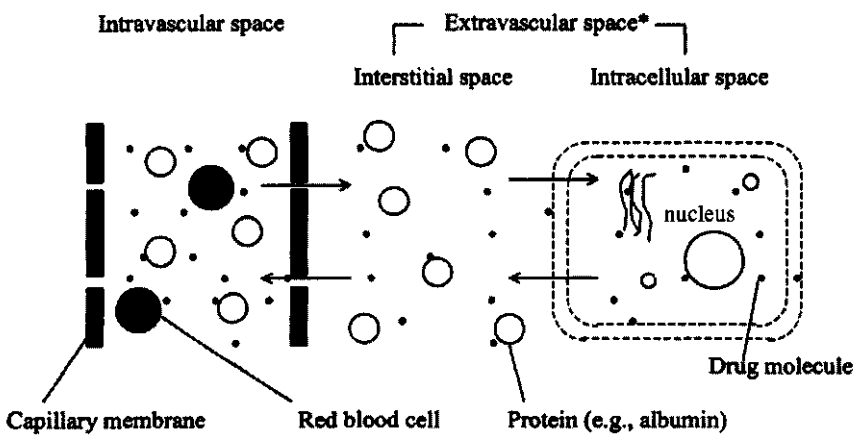

Figure 7.1. Schematic description of drug distribution. Note that only drug molecules not bound (•) to proteins (O) can distribute from intravascular space into interstitial and intracellular spaces throughout the rest of the total body water. *Approximately, 55–60% of the total extracellular (intravascular and interstitial) albumin is found in interstitial space (after Φie and Tozer, 1979).

Table 7.2. Distribution of Albumin in Human Plasma and Tissue[a]

Organ	Concentration (mg/g organ)	Amount (g/70 kg man)	Total albumin in the body (ca. %)
Intravascular			
Plasma[b]	43	140	40
Extravascular[c]			
Skin[d]	12	60	18
Muscle[e]	1.7	50	15
Intestine	4	8	2
Liver	1.4	2	1
Heart, kidney, lungs, spleen	—	11	3
Other tissues	3	79	21
Total	—	210	60
Total in the body		350	100

[a]Data taken from Φie and Tozer (1979) and Rothschild *et al.* (1955).
[b]The plasma volume is about 5% of body weight.
[c]Extravascular space consists of interstitial and intracellular space, and interstitial fluid volume is about 17% of body weight in a normal 70-kg man. Albumin is located primarily in the interstitial fluid.
[d]Skin mass is about 6–7%of the body weight and about 60% of the skin weight is interstitial fluid.
[e]Muscle constitutes about 40–45%of the body weight and about 10–16% of muscle is interstitial fluid.

7.2. ESTIMATING THE EXTENT OF PROTEIN BINDING

The binding of a drug to proteins can be viewed as a reversible and rapid equilibrium process. In the simplest case, assuming only one reversible-binding site in protein for a drug molecule, the binding equilibrium between a drug and proteins can be described in the following scheme:

$$[D] + [P] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [DP] \tag{7.1}$$

where [D] is the unbound-(free)-drug concentration and [P] is the concentration of protein that is free of drug; [DP] is the concentration of the drug–protein complex, ie., the concentration of drug bound to protein; k_1 and k_{-1} are the association and dissociation rate constants, respectively. At equilibrium,

$$k_1 \cdot [D] \cdot [P] = k_{-1} \cdot [DP] \tag{7.2}$$

Rearranging Eq. (7.2) yields the association constant (K_a):

$$K_a = \frac{k_1}{k_{-1}} = \frac{[DP]}{[D] \cdot [P]} \tag{7.3}$$

The ratio of unbound and total plasma drug concentrations (f_u) is

$$f_u = \frac{[D]}{[D] + [DP]} \tag{7.4}$$

Table 7.3. Advantages and Disadvantages of Equilibrium Dialysis and Ultrafiltration for Measuring the Extent of Protein Binding of Drug in Plasma[a]

	Equilibrium dialysis	Ultrafiltration
Advantages	Considered as standard method	Needs small amount of sample (< 1 ml)
	Temperature controlled	Fast (takes ~30 min)
	Thermodynamically sound	No buffer needed
		Commercially available kits
		Disposable device (easy cleanup)
		Small changes in drug concentration during filtration
Disadvantages	Long time to reach equilibrium[b]	Nonspecific binding of drug to plastic tube or ultrafiltration membrane
	Need of buffer	Volume of ultrafiltrate may not be sufficient for drug assay
	Degradation of unstable compounds	Usually not temperature controlled
	Dilution of drug[c]	Constriction of membrane pores during ultrafiltration
	Donnan ion effect	
	Volume shift[d]	Donnan ion effect
	pH changes	
	Nonspecific binding to dialysis device and membrane	
Applications	More suitable for highly (> 98%) protein bound drugs	Suitable for fast screening when nonspecific binding is less than 10%.
		More applicable for highly concentrated protein solutions or tissue homogenates

[a]Information taken from Bower *et al.* (1984) and Pacifici and Viani (1992).
[b]Using a commercial dialysis membrane, a dialysis of 4 hr at 37°C appears to be optimal for most drugs. Owing to the long incubation time, the possible degradation of proteins and the chemical or enzymatic stability of the drug should be taken into consideration.
[c]The initial concentration of drug in plasma decreases during incubcation as the plasma and the buffer equilibrate. Equilibrium dialysis may be inappropriate when there are significant changes in the extent of protein binding of a drug resulting from its dilution in plasma with buffer during equilibrium.
[d]Owing to the osmotic pressure difference between plasma (high) and buffer (low), water molecules from the buffer side are continuously moving into the plasma side during incubation, causing an increase in plasma volume and a decrease in buffer volume as compared to the original values.

There are several *in vitro* methods for measuring the unbound drug concentration in plasma, including equilibrium dialysis, ultrafiltration, ultracentifugation, gel filtration, and albumin column (Oravcova *et al.,* 1996). Among them, equilibrium dialysis and ultrafiltration are the two most commonly used for determining the unbound drug concentrations in plasma, serum, or diluted tissue homogenate. In general, equilibrium dialysis is considered to be the standard method for protein binding measurements; however, ultrafiltration can be adopted as the initial method for conducting protein binding studies, because it is less time-consuming and involves simpler sample preparation. Neither of these methods is free of biological, chemical, and physical artifacts, and the advantages and disadvantages of each are summarized in Table 7.3.

7.2.1. Equilibrium Dialysis

Equilibrium dialysis is based on the establishment of an equilibrium state between plasma containing a drug and a buffer after a period of incubation, usually longer than 2 hr, at a fixed temperature (e.g., 37°C). The equilibrium dialysis chambers for plasma containing a drug and a buffer free of drug are separated by a semipermeable membrane, which allows only low-molecular-weight ligands, such as drug molecules, to transport between the two chambers (Fig. 7.2). Sodium or potassium phosphate buffers at pH 7.4 are the ones most commonly used, although for some compounds others are required owing to the formation of insoluble salts or interactions with drug binding sites in protein molecules.

As illustrated in Fig. 7.2, water molecules from the buffer side are continuously moving into the plasma side during incubation because of the difference in osmotic pressure between the plasma and the buffer and/or the Donnan ion effect. This phenomenon is called the "volume shift," i.e., an increase in plasma volume and a decrease in buffer volume compared to their initial values. The ratio of unbound and total drug concentrations in plasma (f_u) can be estimated after equilibrium dialysis using the following equation with a correction factor for the volume shift, which is usually about 15–20%:

$$f_u = \frac{C_{be}}{(C_{pe} - C_{be}) \cdot (V_{pe}/V_{pb}) + C_{be}} \tag{7.5}$$

C_{pe} and C_{be} are the concentrations of the drug in the plasma and buffer sides of the

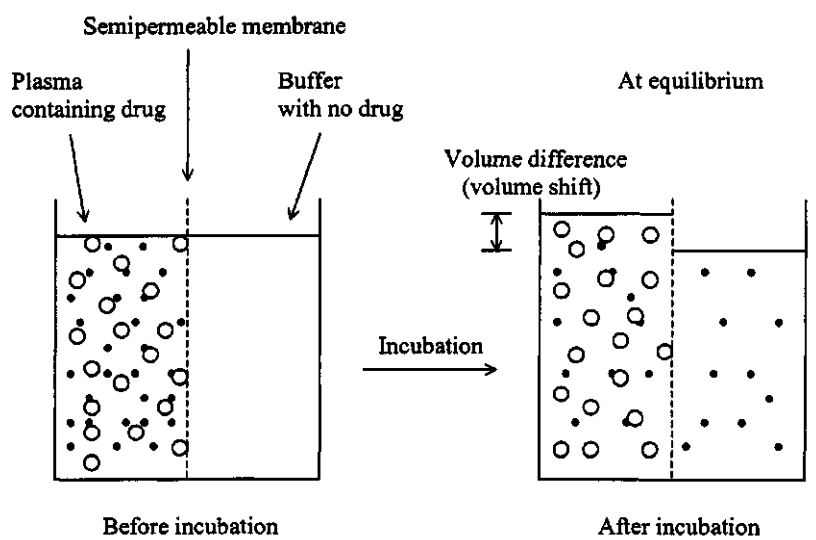

Figure 7.2. Schematic description of the equilibrium dialysis process between drug molecules not (•) bound to proteins (O) in plasma and a buffer with no drug molecules via a semipermeable membrane. There is a volume shift from the buffer side to the plasma side, while reaching an equilibrium of free drug molecules between the two sides owing to the higher osmotic pressure of the plasma containing the drug.

equilibrium chambers at equilibrium after incubation, respectively, and V_{pb} and V_{pe} are the original volume of the plasma before incubation and the volume of the plasma at equilibrium after incubation, respectively. It should be stressed that maintenance of physiological temperature and pH during the experiment is important for accurate assessment of protein binding of drugs (Boudinot and Jusko, 1980; McNamara and Bogardus, 1982; Tozer et *al.,* 1983).

7.2.2. Ultrafiltration

Ultrafiltration is based on physical separation of free drug molecules in plasma water (plasma without proteins) from drug bound to plasma proteins by filtering plasma samples through a semipermeable membrane under a positive pressure generated by centrifugation (Judd and Pesce, 1982). Concentration of a drug in an ultrafiltrate is an unbound drug concentration at the particular plasma drug concentration examined. There are several advantages to the ultrafiltration method over equilibrium dialysis. Since ultrafiltrate is free of proteins, small fraction sample preparation for drug assay is relatively simple. Ultrafiltration takes about 30 min, which is significantly faster than equilibrium dialysis, and as the ultrafiltration device is disposable, cleanup after experiments is easy. A major drawback of this method is the potential nonspecific binding of a drug to the plastic tube and ultrafiltration membrane. Although the nonspecific drug binding to the ultrafiltration apparatus can be corrected by performing separate studies with plasma water spiked with the drug, equilibrium dialysis is considered to be more reliable for protein binding measurements, if nonspecific binding to ultrafiltration apparatus is greater than 20%.

In order to correct nonspecific binding of a drug to the centrifuge device, plasma water (PW), can be spiked with a known amount of the drug and ultrafiltrated. A correction factor for nonspecific binding, ie., the ratio between drug concentration in the original PW (C_{pw}) and concentration of the drug in PW ($C_{pw,f}$) after

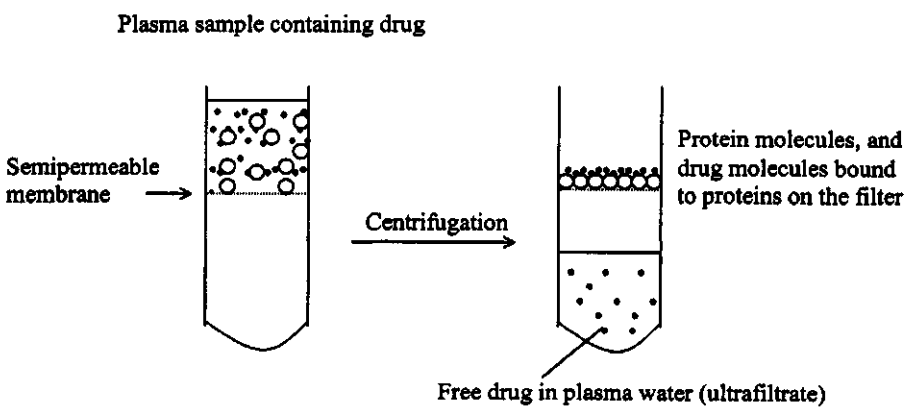

Figure 7.3. Schematic description of ultrafiltration. Drug molecules (•) not bound to protein (O) and plasma water can pass through a semipermeable filter with a molecular-weight cutoff against plasma protein, whereas drug molecules bound to proteins remain on the top of the filter.

ultrafiltration, can be incorporated into the estimate of f_u, using Eq. (7.6):

Correction factor for nonspecific binding measured using PW spiked with drug

$$f_u = \frac{C_f \cdot (C_{pw}/C_{pw,f})}{C_p} \tag{7.6}$$

C_f is the drug concentration to be measured in the ultrafiltrate *after* centrifugation of the plasma containing the drug and C_p is the concentration of the drug in the plasma *before* centrifugation.

NOTE: EFFECTS OF pH CHANGES IN PLASMA ON PROTEIN BINDING. The physiological pH of blood (or plasma) is tightly controlled *in vivo* within a range between 7.2 and 7.6. Immediately upon withdrawal from animals, the pH of blood starts to rise, up to 8.2 owing to the escape of blood CO_2 into the atmosphere, which may affect the nature and extent of protein binding of a drug. To avoid this problem, plasma has to be treated with a small volume of concentrated phosphoric acid prior to protein binding experiments to adjust the pH to 7.4.

7.2.3. Microdialysis

Microdialysis has been used to estimate concentrations of unbound drug present in the extracellular fluids in various tissues or organs or in blood *in vivo* (Fig. 7.4). For microdialysis sampling, a dialysis capillary with a typical outer diameter of 500 μm is implanted in tissues or within blood vessels. and the capillary is then

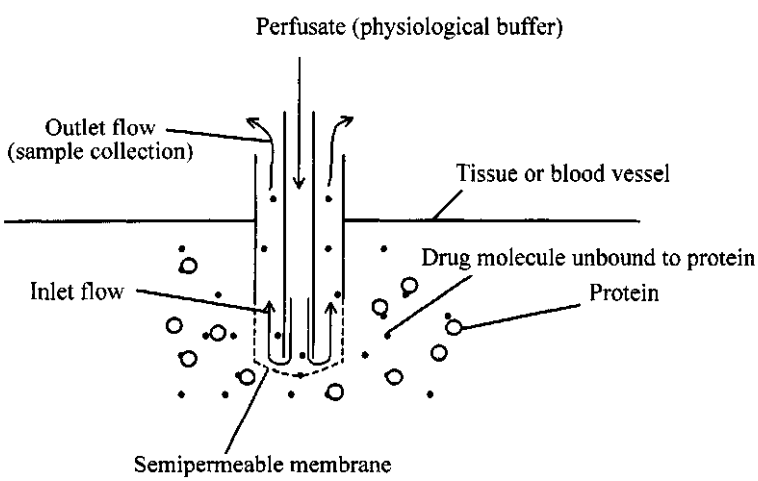

Figure 7.4. Schematic diagram describing a microdialysis probe in tissues or blood vessels.

perfused with a physiological buffer such as Ringer's solution at a low flow rate (usually < 2 μl/min). Owing to the semipermeable membrane of microdialysis with a typical molecular weight cutoff of 20 kDa, only low-molecular-weight compounds transport across the membrane into the perfusate. Concentrations of a drug in perfusate collected from microdialysis, therefore, are concentrations of drug that is not bound to blood or tissue components (Elmquist and Sauchuk, 1997). Microdialysis allows continuous sampling from awake, freely moving animals, along with simple sample preparation for assay (Telting-Diaz *et al.,* 1992). Owing to the relatively small volume of samples (typically 0.1–10 μl/min for 1–5min), the major limitation of microdialysis is the sensitivity of the assay.

Assessment of microdialysis probe recovery: Since microdialysis operates under nonequilibrium conditions, the concentration of analyte in the dialysate tends to be lower than that in the extracellular fluid surrounding the dialysis probe. The ratio between these concentrations is defined as a relative recovery of analyte. Relative recovery decreases as the flow rate of the perfusate increases. It is important to assess recovery efficiency *in vivo* rather than *in vitro,* since diffusion coefficients determined *in vitro* can be substantially different from those measured *invivo.*

7.3. PHARMACOKINETIC AND PHARMACODYNAMIC IMPLICATIONS OF PROTEIN BINDING

It is assumed that only unbound drug is available for uptake into tissue, subject to the intrinsic elimination activities, and able to bind to pharmacological target sites or tissue proteins governing the onset, duration, and intensity of its pharmacological efficacy [free hormone hypothesis (Mendel, 1989)]. Bound drug must dissociate from the drug–protein complex before it becomes available for these processes. The extent of protein binding in plasma is, therefore, considered one of the important physiological factors affecting disposition profiles as well as the pharmacological efficacy of a drug.

7.3.1. Effects on Clearance

Clearance of a drug can be significantly affected by the extent of its protein binding. In general, a high-protein-bound drug is considered to have lower systemic clearance than a low-protein-binding drug, when other pharmacokinetic conditions are similar.

7.3.1.1. Hepatic Clearance

To be eliminated via hepatic metabolism and/or renal excretion, the drug molecules in blood must dissociate from the plasma proteins, after partitioning out of the red blood cells in case they are bound to those cells. For a better understanding of the relationship between protein binding in plasma (in this case, *blood* to be exact) and hepatic clearance (Cl_h), let us consider the well-stirred hepatic

clearance model (see Chapter 6):

$$Cl_h = \frac{Q_h \cdot f_{u,b} \cdot Cl_{i,h}}{Q_h + f_{u,b} \cdot Cl_{i,h}} \tag{7.7}$$

$f_{u,b}$, $Cl_{i,h}$, and Q_h are the ratio of the unbound and total drug concentrations in blood, the intrinsic hepatic clearance, and the hepatic blood flow rate, respectively. For a drug with a low extraction ratio, i.e., $f_{u,b} \cdot Cl_{i,h} \ll Q_h$, Cl_h becomes proportionally related to $f_{u,b}$ and $cl_{i,h}$. For a drug with a high extraction ratio, i.e., $f_{u,b} \cdot Cl_{i,h} \gg Q_h$, Cl_h becomes similar to Q_h, and independent of $f_{u,b}$ and $Cl_{i,h}$ (Balant and Gex-Fabry, 1990). The relationship between $f_{u,b}$ and Cl_h can be better understood after Eq. (7.7) is rearranged in terms of $f_{u,b}$ and Cl_h:

$$\frac{1}{f_{u,b}} = Cl_{i,h} \cdot \left(\frac{1}{Cl_h}\right) - \frac{Cl_{i,h}}{Q_h} \tag{7.8}$$

As indicated in Eq. (7.8), the reciprocal of $f_{u,b}$ is positively related to the reciprocal of Cl_h. The extensive protein binding of a drug in blood can, therefore, result in a low hepatic clearance and vice versa. It is important to note that $f_{u,b}$ is the ratio of unbound and total drug concentrations estimated under *equilibrium* conditions between association and dissociation of drug molecules with *blood components.* An estimate of hepatic clearance of a drug based on the conventional hepatic clearance models such as the well-stirred model (see Chapter 6) is valid only when it can be assumed that the binding equilibrium between unbound and bound drug molecules exists within the sinusoids of the liver.

Basically, three different processes determine the fate of drug bound to proteins in the capillary bed (or blood) during perfusion through the eliminating organ: the rate of dissociation of drug from its protein complex, the permeability of unbound drug across the capillary membrane, and the mean transit time of drug molecules passing through the organ. Binding of a drug to plasma proteins is considered to be more rapid than the dissociation of the drug–protein complex. For some drugs, dissociation from the drug–protein complex may take longer than the transit time along the capillary bed during perfusion, and, thus, there may not be binding equilibrium between unbound and bound drugs in the capillary bed in the case of rapid cellular uptake of a drug from the blood. In this case, the rapid cellular uptake and subsequent elimination of unbound drug from the blood can result in the dissociation of the drug from proteins becoming the rate-limiting process in overall cellular uptake and elimination (Weisiger, 1985). On the other hand, rapid dissociation of a drug in addition to fast membrane permeation and subsequent elimination can make the extent of dissociation of the complex and cellular uptake of a supplementary amount of free drug *in vivo* greater than expected from *in vitro* $f_{u,b}$ measurements. This phenomenon becomes more apparent when the capillary transit time of a drug in the organ is long (Tillement *et al.,* 1988).

Relationship between unbound fractions of drug in blood and plasma: In general, the free drug concentration in red blood cells is considered to be the same as that

in plasma, unless there is an active transport system(s) in the membranes of red blood cells:

Unbound drug concentration in blood ↘ ↙ *Unbound drug concentration in plasma*

$$f_{u,b} \cdot C_b = f_u \cdot C_p \tag{7.9}$$

C_b and C_p are the (total) drug concentrations in blood and plasma, and $f_{u,b}$ and f_u are the ratios of unbound and total drug concentrations in the blood and plasma, respectively.

7.3.1. Renal Clearance

Protein binding is especially important in the urinary excretion of unchanged drug, as renal clearance (Cl_r) is closely related to $f_{u,b}$:

$$Cl_r = f_{u,b} \cdot \left(GFR + \cdot \frac{Q_r \cdot Cl_{i,s}}{Q_r + f_{u,b} \cdot Cl_{i,s}} \right) \cdot (1 - F_r) + Cl_{rm} \tag{7.10}$$

$Cl_{i,s}$ and Q_r are the intrinsic clearance for renal tubular secretion by active transporter(s) and renal blood flow rate, respectively; Cl_{rm} is the renal metabolism; F_r is the fraction of the drug reabsorbed back into the blood from the urine after excretion; and GFR is the glomerular filtration rate. As indicated in Eq. (7.10), protein binding of a drug can affect its glomerular filtration, active secretion, and renal metabolism. Clearance by glomerular filtration, in particular, is directly related to $f_{u,b}$, as this is a physical filtration process with a molecular-weight cutoff (Balant and Gex-Fabry, 1990). Like Cl_h, Cl_r of a high-protein-bound drug tends to be lower than that of a less extensive protein-binding drug, when other conditions are similar.

7.3.2. Effects on the Volume of Distribution

The volume of distribution of a drug at steady state (V_{ss}) referred to drug concentration in plasma is affected by the extent of binding to both plasma proteins and tissue components [Eq. (7.1 1)], because only the unbound drug is considered to be capable of transporting across the membranes:

$$V_{ss} = V_P + \frac{f_u}{f_{u,t}} \cdot V_t \tag{7.11}$$

f_u and $f_{u,t}$ are the ratios of unbound and total drug concentrations in plasma and tissues, respectively; and V_p and V_t are the actual physiological plasma and extravascular volumes into which the drug distributes. As indicated in Eq. (7.1 1), the degree of protein binding in both plasma and tissue can significantly affect the extent of V_{ss}. For instance, a drug with extensive binding to plasma proteins (small f_u) generally exhibits a small V_{ss}. On the other hand, when a drug has a high affinity for tissue components (small $f_{u,t}$), V_{ss} can be much greater than the actual volume of

the body. It is, however, difficult to reliably assess the effects of tissue protein binding on V_{ss}, because unlike protein binding in plasma, it is difficult to obtain an accurate estimate of the extent of drug binding in tissues (Benet and Zia-Amirhusseini, 1995).

7.3.3. Effects on Half-Life

Owing to the fact that the terminal half-life of a drug ($t_{1/2}$) is dependent on both the volume of distribution in the pseudodistribution equilibrium phase (V_β) and the systemic clearance, it is difficult to reliably predict the effects of changes in protein binding on $t_{1/2}$:

(7.12) $$t_{1/2} = \frac{0.693 \cdot V_\beta}{Cl}$$

7.3.4. Effects on Pharmacological Efficacy

The blood protein binding of drugs targeting receptors inside cells can have a significant effect on their efficacy since only unbound drug is available for interaction with receptors (du Souich *et al.,* 1993). In fact, *in vitro* potency of a drug can be drastically reduced in the presence of plasma protein in an incubation buffer, compared to that in the absence of protein. In most clinical cases, however, the changes in the extent of protein binding of a drug alone may have only a limited effect on its efficacy because the changes in protein binding are usually small *in vivo,* and thus may not alter the unbound drug concentrations to any significant extent.

7.3.5. Effects on Drug–Drug Interaction

Displacement from plasma binding of one drug by another *in vivo* may not lead to an increase in the concentration of unbound drug in plasma, because the drug molecules released from the plasma proteins can further distribute into tissues and the transient increase in the unbound drug concentration will be buffered by tissue binding. As indicated in Table 7.4, displacement of a drug from plasma proteins by

Table 7.4. Potential Effects of a Decrease in Plasma Protein Binding of One Drug by Another on Systemic Clearance, Volume of Distribution at Steady State, Terminal Half-Life, and Unbound Concentrations in Plasma

	Effects of an increase in f_u of drug A by drug B[b]				
Clearance of drug A[a]	Cl	V_{ss}	$t_{1/2}$	C_p	C_u
High ($\approx Q_h$)	↔	↑	↑	↔[c], ↓[d]	↑[c], ↔[d]
Low ($\approx f_u \cdot Cl_{i,h}$)	↑	↑	↓	↓	↔

[a] Assuming systemic elimination of drug A occurs primarily via hepatic clearance.
[b] C_u: unbound concentration of A in plasma ($Cu = f_u C_p$); $Cl_{i,h}$: intrinsic hepatic clearance; f_u: ratio of unbound and total concentrations of drug A in plasma; and Q_h: hepatic blood flow rate. Note that C_u is not affected by an increase in f_u, except after parental administration of the drug.
[c] After parenteral administration of drug A.
[d] After oral administration of drug A. Complete oral absorption is assumed. C_p: total concentration of drug A in plasma.

other drugs *in vivo* does not significantly alter its unbound drug concentrations (C_u), so plasma protein binding interactions between different drugs are rarely of clinical significance. Exceptions can be found for a drug with a high systemic clearance after parenteral administration, in which case its C_u increases when it is displaced in proteins by other drug(s).

7.4. FACTORS AFFECTING PROTEIN BINDING

In general, basic compounds tend to bind more to x_1-acid glycoprotein, whereas albumin seems to have higher binding affinity for acidic compounds. Various endogenous ligands such as bilirubin, free fatty acids, heparin, pregnancy factors, uremic middle molecules, and uremic peptides can inhibit protein binding of drugs. Studies of drug binding to a particular protein(s) in plasma are useful for defining the major binding site(s) for a particular drug; however, physiological or clinical implications are rather limited. Concentrations of albumin and α_1-acid glycoprotein in plasma can be altered under various pathophysiological conditions (Table 7.5).

Table 7.5. Various Physiological or Pathological Conditions Altering Protein Concentrations in Plasma[a]

Changes	Albumin	α_1-acid glycoprotein	Lipoprotein
Increase	Benign tumor, exercise, hypothyroidism, gynecological disorder, myalgia, neurological diseases, psychosis, schizophrenia	Age (geriatric) acute illness, burns,[b] chronic pain syndrome, enzyme induction by phenobarbital (in dogs), infection, inflammations,[b] morbid, obesity,[b] myocardiac infarction, neoplastic diseases, renal failure, rheumatoid arthritis, smoking, stress, surgery,[b] trauma[b]	Diabetes, hypothyroidism, liver disease, nephrotic syndrome
Decrease	Injury, acute infection, age (neonates, geriatric), bone fractures, burns,[c] chronic bronchitis, cystic fibrosis, freezing, gastrointestinal diseases, leprosy, liver diseases,[c] malnutrition, neoplasms, pregnancy, renal failure,[c] surgery, trauma	Age (neonates), nephrotic syndrome, oral contraceptives, pregnancy (?)	Hyperthyroidism, trauma

[a]Data taken from Jusko and Gretch (1976), Notarianni (1990), Φie (1986), and Verbeeck *et al.* (1984).
[b]Factors causing more than 50% increase in x_1-acid glycoprotein, compared to the normal value.
[c]Factors causing a larger decrease in albumin concentration than the others.

7.5. NONLINEARITY OF PLASMA PROTEIN BINDING

The extent of protein binding of a drug can be drug- or protein-concentration-dependent, based on the affinity and capacity of the plasma protein. It is desirable to measure the extent of protein binding *in vitro* at more than two different concentrations, one close to the therapeutic concentration and another close to a toxic concentration level. For drugs with multiple protein binding sites, the fraction of a drug free in plasma can vary with drug concentrations such that linear protein binding can occur over multiple ranges of drug concentrations, as illustrated in Fig. 7.5 (Boudinot and Jusko, 1980).

7.6. PLASMA *VS.* SERUM AND *IN VITRO VS. EX VIVO* PROTEIN BINDING MEASUREMENTS

In general, the extent of protein binding of drugs to plasma proteins is based in "drug-spiked serum or plasma'' *in vitro* Between serum and plasma, serum is preferable for protein binding studies because of the possible interference of anticoagulants, such as heparin, EDTA, or citric acid, when plasma is used. The extent of plasma protein binding of a drug can be affected by qualitative and/or quantitative changes in the plasma proteins under various pathophysiological conditions (Table 7.4). Because of the possible interactions of metabolites of drugs copresent in plasma, for a highprotein-bound drug, it is more desirable to measure *ex vivo* protein binding in blood samples obtained from animals or individuals exposed to the drug than otherwise.

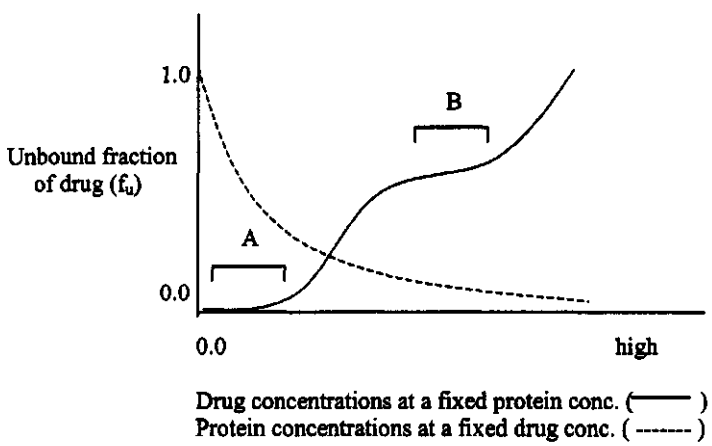

Figure 7.5. Drug- or protein-concentration-dependent changes in protein binding of a hypothetical drug with two discrete binding sites of protein molecules. A, B: Linear protein binding region over the ranges of drug concentrations, reflecting high-affinity/low-capacity and low-affinity/high-capacity binding sites of the protein, respectively.

7.7. PROTEIN BINDING IN TISSUES

Determining the "true" unbound and total drug concentrations in pharmacological target tissues at the sampling time is of utmost pharmacological importance in understanding the relationships among *in vitro* potency, *in vivo* efficacy, and toxicological responses of a drug. In addition, the degree of protein binding to the tissues can have a more pronounced effect on the extent of drug distribution because the total amount of protein in the tissues exceeds by several times the quantity of plasma protein. Not much is known about tissue binding of a drug compared with plasma protein binding, owing to the fact that the reliable *in vitro* estimation of drug binding to tissue components *in vivo* is experimentally much more difficult than in plasma. In addition to tissue proteins, there are also membrane lipids and other macromolecular constituents involved in drug binding, all of which make protein-binding study in tissues more complicated.

Several methods using isolated organ perfusion, tissue slices, tissue homogenate, or isolated subcellular organelles have been investigated to estimate the extent of drug binding to or within tissues. All the techniques, however, suffer from various methodological problems that confine the determination of tissue binding to relatively simple experimental approaches, such as ultrafiltration of diluted tissue homogenates.

7.7.1. General Trends in Drug Binding to (Muscle) Tissues

Not much is known concerning the characteristics of protein binding of drugs in tissues. The following is a summary of some experimental findings, but their general applicability to a large number of compounds has not been thoroughly examined (Fichtl *et al.,* 1991; Kurz and Fichtl, 1983).

- For many drugs, the extent of binding to intact muscle tissues *in vivo* can be extrapolated from *in vitro* data obtained using tissue homogenates. This is because the extent of protein binding in tissue homogenates is found to be almost linearly related to the protein concentrations within them.
- Ultrafiltration appears to be more suitable than equilibrium dialysis for measuring the extent of protein binding in tissue homogenates. In general, tissue homogenate diluted with saline or phosphate buffer [1:1–1:4 (w/v)] is used for ultrafiltration studies. Equilibrium dialysis of tissue homogenate tends to take much longer (sometimes up to 48 hr) than ultrafiltration.
- Unlike in plasma protein binding, there appear to be no species-dependent differences in the extent of binding of drugs to muscle.
- For acidic compounds, there tends to be more binding to plasma proteins than to muscle.
- For basic compounds, there tends to be more binding to tissue than to plasma proteins.
- There is a good positive correlation between plasma and muscle tissue binding of structurally related compounds.

- Muscle tissue binding seems to be linear over a wide range of drug concentrations of many drugs.
- In general, drug binding to different tissues decreases in the rank order of liver > kidney > lung > muscle.

7.7.2. Pharmacokinetic Implications of Tissue Binding

The following is the summary of important effects of changes in tissue binding on drug disposition profiles:

- *Clearance:* Clearance of a drug is not affected by the extent of the tissue binding [Eq. (7.7)].
- *Volume of distribution:* Drugs with extensive tissue binding [Eq. (7.11)] tend to have larger volumes of distribution at steady state compared to those with limited tissue binding.
- *Terminal half-life:* Drugs with extensive tissue binding tend to have a longer terminal half-life than those with limited tissue binding, when other parameters are similar.
- *Drug–drug interaction:* If a displacement of one drug by another takes place only at tissue binding sites and does not occur in plasma, the total concentration of the drug may decrease, but unbound or total drug concentrations in plasma and unbound drug concentration in tissues are not changed because drug clearance is not affected by changes in tissue binding.

7.8. SPECIES DIFFERENCES IN PROTEIN BINDING

The extent of drug binding to plasma proteins can vary considerably among different species. There are slight differences in the amino acid sequences in protein molecules such as albumin among different species, despite their structural and functional homologies (approximately 590 amino acids), and similar concentrations (e.g., 500–600 μM of albumin in plasma in rats and humans). This may cause the differences in binding affinity and/or number of binding sites of drugs in protein molecules among different species. In general, more extensive plasma protein binding is expected in larger animals, including humans, than in small laboratory animals. However, a similar extent of tissue binding of drugs has been found among different species with a few exceptions (Fichtl and Schulmann, 1986; Fichtl *et al.,* 1991; Sawada *et al.,* 1984).

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8

Metabolism

8.1. INTRODUCTION

Metabolism (biotransformation) is the major elimination pathway for most drugs. A thorough understanding of the metabolic pathways and profiles of a drug is important in improving its pharmacokinetic profiles and addressing potential metabolism-related issues such as toxic metabolites, metabolic interactions, and polymorphic metabolism. In general, enzymatic metabolism transforms lipophilic parent drugs to more hydrophilic metabolites, which can be readily excreted into bile or urine. Drug metabolism in the body can be divided into two different types of reactions: phase I and phase II metabolism. Phase I metabolism generally results in the introduction of a functional group into molecules or the exposure of new functional groups of molecules, whereas phase II metabolism involves conjugation of functional groups of molecules with hydrophilic endogenous substrates (Caldwell et *al.,* 1995; Parkinson, 1996a).

$$\text{Drugs (lipophilic and less polar)} \xrightarrow{\text{Metabolism}} \text{Metabolites (hydrophilic and more polar)}$$

Phase I: addition (or revealing) of hydrophilic moieties
Phase II: conjugation with hydrophilic endogenous substrates

Important characteristics of phase I and phase II metabolism and metabolizing enzymes are summarized below.

8.1.1. Phase I Metabolism

Phase I metabolism is sometimes called a "functionalization reaction," since it results in the introduction of new hydrophilic functional groups to compounds.

1. Function: introduction (or unveiling) of functional group(s) such as –OH, $–NH_2$, –SH, –COOH into the compounds.
2. Reaction types: oxidation, reduction, and hydrolysis
3. Enzymes:
   3.1. Oxygenases and oxidases: Cytochrome P450 (P450 or CYP), flavin-containing monooxygenase (FMO), peroxidase, monoamine oxidase

(MAO), alcohol dehydrogenase, aldehyde dehydrogenase, and xanthine oxidase.
3.2. Reductase: Aldo-keto reductase and quinone reductase.
3.3. Hydrolytic enzymes: esterase, amidase, aldehyde oxidase, and alkylhydrazine oxidase.
3.4. Enzymes that scavenge reduced oxygen: Superoxide dismutases, catalase, glutathione peroxidase, epoxide hydrolase, *y*-glutamyl transferase, dipeptidase, and cysteine conjugate β-lyase.

Examples of phase I metabolism:

1. Oxidation
   1.1. Oxidation by cytochrome P450 isozymes (microsomal mixed-function oxidases).
   1.2. Oxidation by enzymes other than cytochrome P450s—most of these enzymes are involved primarily in oxidation of endogenous substrates: (a) oxidation of alcohol by alcohol dehydrogenase, (b) oxidation of aldehyde by aldehyde dehydrogenase, and (c) N-dealkylation by monoamine oxidase.
2. Reduction
   Enzymes responsible for reduction of xenobiotics require NADPH as a cofactor. Substrates for reductive reactions include azo- or nitrocompounds, epoxides, heterocyclic compounds, and halogenated hydrocarbons: (a) azo- or nitroreduction by cytochrome P450; (b) carbonyl (aldehyde or ketone) reduction by aldehyde reductase, aldose reductase, carbonyl reductase, quinone reductase; and (c) other reductions including disulfide reduction, sulfoxide reduction, and reductive dehalogenation.
3. Hydrolysis
   Esters, amides, hydrazides, and carbamates can be hydrolyzed by various enzymes.

8.1.2. Phase II Metabolism

Phase II metabolism includes what are known as conjugation reactions. In general, the conjugation reaction with endogenous substrates occurs on the metabolite(s) of the parent compound after phase I metabolism; however, in some cases, the parent compound itself can be subject to phase II metabolism.

1. Function: conjugation (or derivatization) of functional groups of a compound or its metabolite(s) with endogenous substrates.
2. Reaction types: glucuronidation, sulfation, glutathione-conjugation, N-acetylation, methylation and conjugation with amino acids (e.g., glycine, taurine, glutamic acid).
3. Enzymes: Uridine diphosphate-glucuronosyltransferase (UDPGT): sulfotransferase (ST), N-acetyltransferase, glutathione S-transferase (GST), methyl transferase, and amino acid conjugating enzymes.

Examples of phase II metabolism:

1. Glucuronidation by uridine diphosphate-glucuronosyltransferase
2. Sulfation by sulfotransferase
3. Acetylation by N-acetyltransferase
4. Glutathione conjugation by glutathione S-transferase
5. Methylation by methyl transferase
6. Amino acid conjugation

8.1.3. Subcellular Locations of Metabolizing Enzymes

ENDOPLASMIC RETICULUM (microsomes): the primary location for the metabolizing enzymes. (a) Phase I: cytochrome P450, flavin-containing monooxygenase, aldehyde oxidase, carboxylesterase, epoxide hydrolase, prostaglandin synthase, esterase. (b) Phase 11: uridine diphosphate-glucuronosyltransferase, glutathione S-transferase, amino acid conjugating enzymes.

CYTOSOL (the soluble fraction of the cytoplasm): many water-soluble enzymes. (a) Phase I: alcohol dehydrogenase, aldehyde reductase, aldehyde dehydrogenase, epoxide hydrolase, esterase. (b) Phase 11: sulfotransferase, glutathione S-transferase, N-acetyl transferase, catechol 0-methyl transferase, amino acid conjugating enzymes.

MITOCHONDRIA. (a) Phase I: monoamine oxidase, aldehyde dehydrogenase, cytochrome P450. (b) Phase II: N-acetyl transferase, amino acid conjugating enzymes.

LYSOSOMES. Phase I: peptidase.

NUCLEUS. Phase II: uridine diphosphate-glucuronosyltransferase (nuclear membrane of enterocytes).

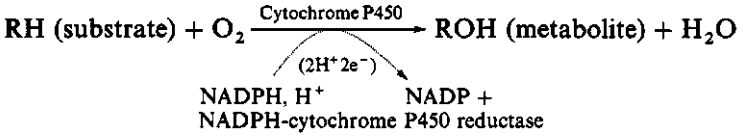
Brief descriptions of the metabolic implications and biological importance of several metabolizing enzymes in humans are described below.

8.2. PHASE I ENZYMES

8.2.1. Cytochrome P450 Monooxygenase (Cytochrome P450, P450, or CYP)

METABOLIC IMPLICATIONS. Cytochrome P450 monooxygenases (Cytochrome P450, P400, or CYP) play a major role in the biosynthesis and catabolism of various endogenous compounds such as steroid hormones, bile acids, fat-soluble vitamins, and fatty acids. P450 enzymes are also considered the most important metabolizing enzymes for xenobiotics >85% of the drugs in the market are metabolized by P450s) (Rendic and DiCarlo, 1997).

REACTION TYPE. Mainly oxidation in the presence of oxygen or reduction under low oxygen tension. A basic reaction scheme for the oxidation of a substrate by P450 is



1. Oxidation
   (a) Aromatic hydroxylation: $R-C_6H_5 \rightarrow R-C_6H_4OH$
   (b) Aliphatic hydroxylation: $R-CH_3 \rightarrow R-CH_2 \rightarrow OH$
   (c) N, O, S-dealkylation:
   $R-NH\ (O, S)-CH_3 \rightarrow R-NH_2\ (OH, SH) + HCHO$

   (d) N-oxidation: $R-C_6H_4-NH_2 \rightarrow R-C_6H_4-N(OH)(H)$

   (e) S-oxidation: $R-S-CH_3 \rightarrow R-SO-CH_3$
   (f) Epoxidation: $R_1-C=C-R_2 \rightarrow R_1-C(O)-C-R_2$

   (g) Dehalogenation: $R_1R_2CH-X \rightarrow R_1R_2C=O$ (X: Cl, Br)
2. Reduction
   (a) Azo- or nitroreduction: $R_1-N=N-R_2 \rightarrow R_1-NH_2 + R_2-NH_2$ (or $R-NO_2$) (or $R-NH_2$)
   (b) Dehalogenation: $CCl_4 \rightarrow CHCl_3$
3. Others
   (a) Hydrolysis of esters
   (b) Dehydrogenation

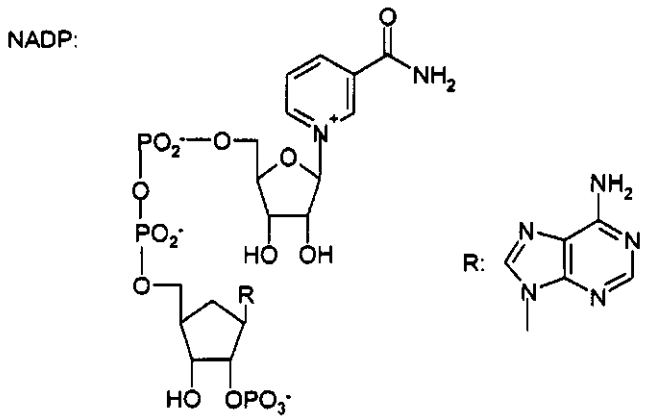
COFACTORS. Nicotinamide-adenine-dinucleotide-phosphate (NADPH) and 0_2.

TISSUE DISTRIBUTION. Ubiquitous, especially in liver, intestine, kidney, lung and skin.

SUBCELLULAR LOCALIZATION. Endoplasmic reticulum (microsomes), mitochondria (P450 enzymes in mitochondria are involved mainly in steroid biosynthesis and vitamin D metabolism).

ISOZYMES. There are at least eight mammalian P450 gene families. In humans, at least seventeen liver cytochrome P450 isoforms have been characterized.

POLYMORPHISM. Polymorphic metabolism by CYP2C18, CYP2C19, CYP2D6, and CYP3A5 in humans.



8.2.1. 1. Cytochrome P450 Enzyme System

The P450 system consists of three protein components embedded in the phospholipid environment of the endoplasmic reticulum, including: (a) Cytochrome P450, a membrane-bound hemoprotein, which binds directly to substrates and molecular oxygen. (b) NADPH-cytochrome P450 reductase, a membrane-bound flavoprotein, containing 1 mole each of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which transfers electrons from NADPH to the cytochrome-P45O–substrate complex; cytochrome P450 and NADPH–cytochrome-P450 reductase are embedded in the phospholipid bilayer of the endoplasmic reticulum, which facilitates their interaction. (c) Cytochrome b_5, a membrane-bound hemoprotein, which enhances the catalytic efficiency (rate) of some P450 isoforms by donating the second of the two electrons required for cytochrome P450 reactions.

(a) Relationship between P450 and NADPH-Cytochrome P450 Reductasel Cytochrome b_5. There are various cytochrome P450 isoforms, but only one form of NADPH-cytochrome P450 reductase and cytochrome b_5 in liver microsomes. Approximately 10–20 molecules of cytochrome P450s and 5–10 molecules of cytochrome b_5 surround each molecule of NADPH-cytochrome P450 reductase in livermicrosomes.

(b) Nomenclature.

(i) Cytochrome P450. When the heme iron [usually in ferric (Fe^{+3}) state] in cytochrome P450 is reduced to ferrous (Fe^{+2}) state, cytochrome P450 can bind ligands such as 0_2 and CO. The name cytochrome P450 was derived from the findings that the complex between ferrous cytochrome P450 and CO absorbs light maximally between 447 and 452 nm (an average of 450 nm).

(ii) Cytochrome P420. When the thiolate bond in the fifth ligand (cysteine–thiolate) to the heme moiety of cytochrome is disrupted, cytochrome P450 is

converted to a catalytically inactive form called cytochrome P420, which absorbs light maximally at 420 nm upon binding CO.

(iii) Cytochrome P450 isoforms. Classification of cytochrome P450 depends on the extent of amino acid sequence identity of different P450 enzymes, not on catalytic activities or substrate specificity (Nelson, 1996).

- Gene families (e.g., CYP1, CYP2, CYP3, etc). P450 enzymes with less than 40% amino acid sequence identity are assigned to different gene families. There are at least eight mammalian P450 gene families.

- Subfamilies (e.g., CYP2A, CYP2B, CYP2C, etc). P450 enzymes with 40–55% amino acid sequence identity are assigned to different subfamilies.

- Isoforms (e.g., CYP2C8, CYP2C9, etc). P450 enzymes with more than 55% amino acid sequence identity are classified as members of the same subfamily.

Most cytochrome P450s are named irrespective of species. CYP1A1, CYP1A2, and CYP2E1 are present in all mammalian species. Similar isoform names of cytochrome P450 imply similar structure, but not necessarily similar catalytic functions, since slight changes in enzyme structure can cause marked differences in metabolic activities.

(c) Human Cytochrome P450 Isoforms. Currently, four P450 gene families, eight subfamilies, and at least seventeen human liver cytochrome P450 isoforms involved in drug metabolism have been characterized to varying degrees. CYP1, CYP2, and CYP3 are the three main P450 gene families in the human liver. Metabolically important isoforms include CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. Table 8.1 lists the cytochrome P450 families, subfamilies, and isoforms known in humans, and Table 8.2 summarizes the average content of different P450 isoforms in the human liver. The relative amount of individual P450 isoforms present in the liver is 3A subfamily (mainly 3A4) > 2C subfamily > 1A2 > 2E1 > 2A6 > 2D6 > 2B6 (Fig. 8.1).

Table 8.1. Human Cytochrome P450 Isoforms

Category	Cytochrome P450 isoforms			
Family	CYP1	CYP2	CYP3	CYP4
	↓	↓	↓	↓
Subfamily	1A	2A, B, C, D, E	3A	4A
	↓	↓	↓	↓
Isoforms	1A1, 1A2	2A6 2B6 2C8, 2C9, 2C10, 2C18, 2C19 2D6 2E1	3A3, 3A4, 3A5, 3A7	4A9, 4A11

Table 8.2. The Average Content of Cytochrome P450 Enzymes in Human Liver Microsomes[a]

P450 enzymes	Average content	
	pmol/mg microsomal protein	nmol/g liver[b]
Total P450 (determined spectrally)	344	18.1
Total P450 (determined immunochemically)	240	12.6
CYP isoforms		
1A2	42	2.2
2A6	14	0.7
2B6	1	0.05
2C[c]	60	3.2
2D6	5	0.3
2E1	22	1.2
3A[d]	96	5.0

[a]Data taken from Shimada *et al* (1994)
[b]The values were converted to nmol/g liver, considering the microsomal protein content of 52.5 mg/g liver
[c]Sum of CYP2C8, 2C9, 2C10, 2C18, and 2C19
[d]Sum of CYP3A3, 3A4,3A5, and 3A7

(d) Important Human Cytochrome P450 Isoforms in Drug Metabolism. Most of the drugs on the market today are metabolized by CYP3A4 and 2D6, followed by CYP2C9, 2C19, 1A2, and 2E1 (Fig. 8.2). The discrepancy between the relative abundance of P450 isoforms in the liver and the extent of their contribution to the overall metabolism of xenobiotics is due to differences in the affinity (K_m values) of

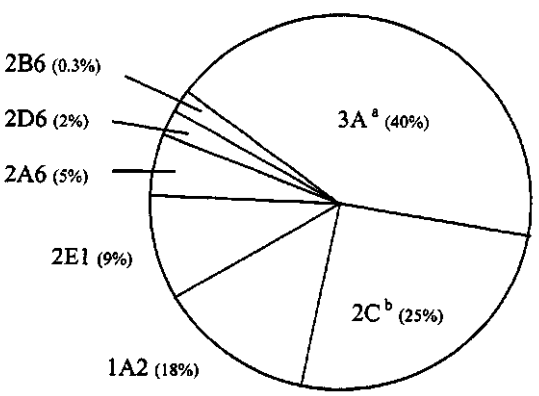


Figure 8.1. Percent amount of individual P450 isoforms in the total P450 determined by immunochemical methods in human liver microsomes (Shimada, 1994).

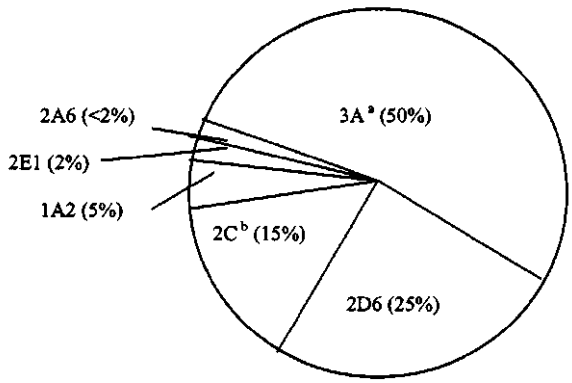


Figure 8.2. Percent of drugs on the market metabolized by various cytochrome P450 isoforms.

the enzymes to substrates. Since the rate of metabolism by an enzyme is determined by the amount (capacity) of the enzyme (V_{max}) as well as its affinity (K_m) to substrates, the enzyme with high affinity can exhibit a fast turnover rate despite its smaller quantity compared to other enzymes. For instance, CYP2D6 metabolizes more drugs than CYP2C9 owing to the higher affinity (lower K_m values) of CYP2D6, although the absolute amount of CYP2D6 present in the liver is substantially lower (smaller V_{max}) than the amount of CYP2C9. This becomes especially important for the metabolism of substrates at low concentrations. As a rule of thumb, $K_m > 100$ μM is usually considered "low affinity," whereas $K_m <$ 20 μM can be viewed as "high affinity" of a particular enzyme for a substrate of interest.

(e) Characteristics of Cytochrome P450 Reactions.

1. Cytochrome P450-mediated metabolism is responsible for the metabolism of more than 85% of the drugs on the market.
2. Cytochrome P450 metabolism often precedes phase II metabolism and is generally slower. As a result, in many cases, cytochrome P450–mediated metabolism is the rate-limiting step for the biotransformation of drugs.
3. The same compound can be a substrate for many different P450 isoforms with markedly different affinities, and also one P450 isoform can metabolize the same compound in multiple sites at different rates.
4. There is a large variability in P450 functions among different mammalian species.
5. Genetic and environmental factors have significant effects on P450 expression, which leads to substantial interindividual and species variability in P450 mediated–metabolism.

Typical substrates and inhibitors of human cytochrome P450 isoforms, and their tissue locations and inducibility are listed in Table 8.3.

NOTE: SUICIDE INHIBITOR (MECHANISM-BASED INHIBITOR). A suicide inhibitor (sometimes called a mechanism-based inhibitor) is a compound that inhibits the activity of a metabolizing enzyme such as cytochrome P450 by forming a covalent bond(s) with the enzyme as a result of its own metabolism by the enzyme. For instance, 1-aminobenzotriazole (ABT) is a suicide inhibitor of various cytochrome P450 enzymes. To be activated, ABT undergoes a P450-catalyzed oxidation to form benzyne, a reactive intermediate, which covalently binds to the prosthetic heme group of cytochrome P450 and thereby causes the irreversible loss of its enzymatic activity (Mugford *et al.,* 1992).

(f) Substrate Structure Specificity. Certain structural properties of substrates for several cytochrome P450 isozymes have been characterized, although there are some exceptions (DeGroot and Vermeuler, 1997; Smith and Jones, 1992; Smith, 1994 *a,b).*

CYP1A: Most substrates are planar and aromatic [so-called, polycyclic aromatic hydrocarbon (PAH)].

CYP2C9: Most substrates are lipophilic, and neutral or acidic with strong hydrogen-bonding and/or ion-pairing ability. (b) Oxidation of substrates usually occurs 5–20 Å from a proton-donor heteroatom in substrates.

CYP2D6 Most substrates are arylalkylamines, which are basic (cationic) at physiological pH. (b) Oxidation of substrates usually occurs 5 to 7 Å from a protonated nitrogen in the substrates, which interacts with an anionic residue ($G1u^{301}$) of the enzyme.

CYP3A4: No apparent selectivity in terms of overall structure of substrates. (b) Most substrates are lipophilic, and neutral or basic at physiological pH. (c) Oxidation often occurs on a basic nitrogen atom (N-dealkylation) or an allylic position in a substrate. (d) Metabolism can occur at multiple sites in a single molecule. (e) K_m values of substrates for CYP3A4 are usually higher than those for CYP2D6.

8.2.2. Flavin-Containing Monooxygenase (FMO)

METABOLIC IMPLICATIONS. In addition to the cytochrome P450, hepatic microsomes contain a second class of monooxygenase, the flavin-containing monooxygenase (FMO). Although the FMOs are considered to be important for metabolizing heteroatom (N, S, Se or P)-containing compounds rather than direct oxidation at a carbon atom, the quantitative role of FMOs in hepatic drug metabolism in humans is limited (Cashman, 1995).

Table 8.3. Typical Model Substrates and Inhibitors, Tissue Distribution, and Inducibility of Human Cytochrome P450s[a]

CYP isoforms	Model substrates	Inhibitors	Notes
1A1	Acetoaminophen Caffeine 7-Ethoxyresorufin[b] (polycyclic hydrocarbons)	α-Naphthoflavone	Lung, liver, placenta Inducible by smoking, charcoal-broiled meat, and cruciferous vegetables
1A2	Acetoaminophen Caffeine[c] Theophylline R-Warfarin (heterocyclic amines)	Furafylline α-Naphthoflavone	Liver only (large interindividual variability in levels) Inducible by smoking, charcoal-broiled meat, cruciferous vegetables, omeprazol, and lansoprazole
2A6	Coumarin[d]	Pilocarpine	Hepatic (a large interindividual variability in levels) Inducible by barbiturates, dexamethasone, and rifampicin
2B6	7-Ethoxy-4-trifluoromethyl-coumarin[e]		Hepatic Constitutive Neonatal form (?) Inducible by rifampicin
2C8, 9, 10	Phenytoin Taxol[f] Tolbutamide[g] S-Warfarin (many NSAIDs)	Sulfaphenazole (2C9, 10)	Hepatic, renal (2C8) Inducible by barbiturates and rifampicin Rare genetic variation [poor metabolizer ~0.2% (2C8)]
2C18, 19	Diazepam S-Mephenytoin[h]	Tranylcypromine	Hepatic Inducible by rifampicin, and omeprazole Polymorphic [poor metabolizer: 3% in Caucasians (2C18, 2C19) and 20% in Asians (2C19)]
2D6	Bufarolol Codein Debrisoquine[i] Dextromethorphan[j] Sparteine (amines)	Quinidine Yohimbine	Hepatic Constitutive Not inducible Polymorphic (poor metabolizer 5–10% in Caucasians and <2% in Asians)
2E1	Chlorzoxazone[k] p-nitrophenol Nitrosamine Ethanol Vinyls (small molecules)	Diethyldithiocarbamate Disulfiram 4-Methylpyrazole	Hepatic and extrahepatic Constitutive Inducible by acetone, ethanol and isoniazid Rare genetic variation (<0.3%)

Table 8.3. Continued

CYP isoforms	Model substrates	Inhibitors	Notes
3A3, 4	Caffeine[l] Cortisol[m] Cyclosporin Erythromycin[n] Lidocaine Midazolam[o] Nifedipine Tamoxifen Testosterone[p]	Gestodene Ketoconazole Naringenin (grapefruit juice) Quercetin Troleandomycin	Hepatic and extrahepatic (intestine) Constitutive Inducible by barbiturate, carbamazepine, dexamethason, phenytoin, rifampicin, and troleandomycin
3A5	Cyclosporin Midazolam[q] Nifedipine Testosterone	Gestodene Troleandomycin	Hepatic and extrahepatic Not inducible Polymorphism (expressed 10–30% and 80% in all human livers and kidneys, respectively)
3A7	Dehydroepiandrosteron (DHEA)		Hepatic Fetal enzyme (also found in adult liver)
4A9, 11	Lauric acid[r]		Hepatic Not inducible

[a]Data taken from Birkett *et al.* (1993), Bourrié *et al.* (1996), Gonzalez (1992), Halpert *et al.* (1994), Meyer (1996), Newton *et al.* (1995), Thummel (1994), Wrighton and Stevens (1992), and Wrighton *et al.* (1993). NSAID stands for nonsteroidal antiinflammatory drug. Footnotes b–r refer to the metabolic reactions of common marker substrates used for phenotyping the *in vivo* catalytic activities of P450 isozymes in human.

[b]7-Ethoxyresorufin 0-dealkylation.
[c]Caffeine-3-demethylation.
[d]Coumarin 7-hydroxylation.
[e]7-Ethoxy-4-trifluoromethyl coumarin0-dealkylation.
[f]Taxol 6α-hydroxylation.
[g]Tolbutamidemethyl-hydroxylation.
[h]S-Mephenytoin 4-hydroxylation.
[i]Debrisoquine-4-hydroxylation.
[j]Dextromethorphan *0*-demethylation
[k]Chlorzoxazone-6-hydroxylation.
[l]Caffeine-8-hydroxylation.
[m]Cortisol-6-hydroxylation.
[n]Erythromycin N-demethylation.
[o]Midazolaml-hydroxylation.
[p]Testosterone 6β-hydroxylation.
[q]Midazolam-4-hydroxylation.
[r]Lauric acid 12-hydroxylation.

REACTION TYPE. Oxidation

$$\mathrm{R{-}N(S,P) \rightarrow R{-}N(S,Se,P)} \quad \downarrow \quad \mathrm{O}$$

SUBSTRATES. Compounds containing a heteroatom (N, S, Se, or P).

COFACTORS. NADPH and O_2.

TISSUE DISTRIBUTION. Liver, kidney, lung, and skin.

SUBCELLULAR LOCALIZATION. Endoplasmic reticulum.

ISOZYMES. FMO^{I} (fetal liver FMO) and FMO^{II} (major form of FMO in adult liver).

POLYMORPHISM. Unknown.

NOTE: THERMAL INSTABILITY OF FMOs. FMOs are heat labile and can be inactivated in the absence of NADPH by warming microsomes at 50°C for 1 min.

8.2.3 Esterase

METABOLIC IMPLICATIONS. Esters, amides, hydrazides, and carbamates can be hydrolyzed by various esterases. Ester hydrolysis can occur in the plasma mainly by cholinesterase (nonspecific acetylcholine esterases, pseudocholine esterases, and other esterases) or in the liver by specific esterases for particular groups of compounds. Enzymatic hydrolysis of esters and amides can be important in determining the duration of action of certain drugs. Especially, the rate of enzymatic cleavage of ester or amide moiety from ester or amide prodrugs can play a critical role in the onset of pharmacological activity and its duration (Satoh and Hosokawa, 1998).

REACTION TYPE. Hydrolysis:
(a) Hydrolysis of esters: $R_1–CO–OR_2 \rightarrow R_1–COOH + R_2–OH$
(b) Hydrolysis of amides: $R_1–CO–NH-R_2 \rightarrow R_1–COOH + R_2–NH_2$

Hydrolysis of amides can occur by amidases in the liver and in general, enzymatic hydrolysis of amides is slower than that of esters. Amides can be also hydrolyzed by esterases with a much slower rate than the corresponding esters.

SUBSTRATES. Esters and amides.

SUBCELLULAR LOCALIZATION. Endoplasmic reticulum and cytosol.

TISSUE DISTRIBUTION. Ubiquitous, liver (centrilobular region), kidney (proximal tubules), testis, intestine, lung, plasma, and red blood cells.

ISOZYMES.

Main groups	Substrates
A-esterase (arylesterase)	aromatic esters
B-esterase (carboxylesterase)	aliphatic esters
C-esterase (acetylesterase)	acetyl esters
Cholinesterase	choline esters

POLYMORPHISM. Approximately 2% of Caucasians have defective serum cholinesterase activity (Daly *et al.,* 1993).

SPECIES DIFFERENCES. In general, esterase activity is higher in small laboratory animals such as the rat and mouse than in humans.

8.2.4. Alcohol Dehydrogenase (ADH)

METABOLIC IMPLICATIONS. Alcohol dehydrogenase (ADH) is a major enzyme responsible for oxidation of alcohol (ethanol) to aldehyde (acetaldehyde). Other quantitatively less important microsomal and peroxisomal enzymes for ethanol oxidation include CYP2El and catalase, respectively.

REACTION TYPE. Oxidation of alcohol to aldehyde:

$$R\text{–}CH_2\text{–}OH \rightarrow R\text{–}CHO$$

SUBSTRATES. Aliphatic or aromatic alcohols.

COFACTORS. NAD^+.

ENZYME STRUCTURE. Zinc-containing dimer of two 40 kDa subunits.

TISSUE DISTRIBUTION. Liver, kidney, lung and gastric mucosa.

SUBCELLULAR LOCATION. Cytosol.

ISOZYMES.

Classes	Substrates
Class I (ADH_1, ADH_3, and ADH_3)	Small alcohols such as ethanol
Class II (ADH_4)	Larger aliphatic and aromatic alcohols
Class III (ADH_5)	Long-chain aliphatic and aromatic alcohols

POLYMORPHISM. Approximately 85% of Asians express the class I isozymes (so-called atypical ADH responsible for rapid conversion of ethanol to acetaldehyde), whereas fewer than 20% of Caucasians express the atypical ADH (Agarwal and Goedde,1992).

8.2.5. Aldehyde Dehydrogenase (ALDH)

METABOLIC IMPLICATIONS. Aldehyde dehydrogenase (ALDH) is a major enzyme responsible for oxidation of xenobiotic aldehydes to acids. In particular, acetaldehyde formed from ethanol by alcohol dehydrogenase is oxidized to acetic acid by ALDH, which is further oxidized to carbon dioxide and water.

REACTION TYPE. Oxidation of aldehyde to acid:

$$R–CH_2–CHO \rightarrow R–CH_2–COOH$$

SUBSTRATES. Aliphatic or aromatic aldehydes.

COFACTORS. NAD^+ or $NADP^+$.

ENZYME STRUCTURE. Tetramer of 54 kDa subunits (ALDH1 and ALDH2) or dimer of 85 kDa subunits (ALDH,).

TISSUE DISTRIBUTION. Liver, kidney, lung, and gastric mucosa.

SUBCELLULAR LOCATION. Cytosol (ALDH1 and ALDH3), mitochondria (ALDH2).

ISOZYMES.

Classes	Substrates
ALDH1	Various xenobiotic aldehydes
ALDH2	Small aldehydes such as acetaldehyde
ALDH3	

POLYMORPHISM. Approximately 50% of Asians have a defective ALDH2 gene causing impaired ALDH2 activity (Goedde and Agarwal, 1992).

8.2.6. Monoamine Oxidase (MAO)

METABOLIC IMPLICATIONS. Monoamine oxidase (MAO) has been seen to be related to the metabolism of exogenous tyramine and the "cheese effect" produced as a result of the ingestion of large amounts of tyramine-containing foods under certain conditions. MAO catalyzes the oxidative deamination of biogenic amines (Benedetti and Tipton, 1998).

REACTION TYPE. Oxidative deamination of amines:

$$RCH_2–NR_1R_2 \rightarrow R–CHO + NHR_1R_2$$

SUBSTRATES. Primary, secondary, and tertiary amines.

COFACTORS. Oxygen from water, not from molecular oxygen. MAO converts amines into the corresponding imines, which are then further hydrolyzed to aldehydes with oxygen taken from water.

TISSUE DISTRIBUTION. Ubiquitous, liver, intestine, lung, blood platelets, and lymphocytes.

SUBCELLULAR LOCATION. Primarily in mitochondria, although some MAO activity has also been reported in the microsomal fraction.

ISOZYMES. MAO-A and MAO-B.

8.3. PHASE II ENZYMES

8.3.1. Uridine Diphosphate-Glucuronosyltransferase (UDPGT)

METABOLIC IMPLICATIONS. Glucuronidation is quantitatively the most important conjugation reaction of xenobiotics mediated by uridine diphosphate-glucuronosyltransferase (UDPGT) (Clarke and Burchell, 1994). It is generally considered a low-affinity and high-capacity reaction. More than 95% of the drugs in the market are metabolized by cytochrome P450s, UDPGT, and sulfotransferases.

REACTION TYPE. Glucuronidation (Fig. 8.3):
(a) O-glucuronidation:

R–OH → R–O–glucuronic acid (ether glucuronidation)
R–COOH → R–COO-glucuronic acid (acyl (or ester) glucuronidation)

(b) N, S-glucuronidation:

$R–NH_2$ (or SH) → R–NH (or S)-glucuronic acid

SUBSTRATES. Glucuronidation can occur on nucleophilic moieties of molecules such as alcohol, acid (O-glucuronidation), amine (N-glucuronidation), and thiol (S-glucuronidation).

ENZYME STRUCTURE. Oligomers of between 1 and 4 subunits with a molecular weight of between 50 and 60 kDa.

COFACTOR. Uridine-5′-diphospho-α-D-glucuronic acid (UDPGA).

TISSUE DISTRIBUTION. Liver, lung, kidney, stomach, intestine, skin, spleen, thymus, heart, and brain Most tissues have some glucuronidation activity.

SUBCELLULAR LOCATION. Mainly endoplasmic reticulum and some in the nuclear membrane.

ISOZYMES. More than 15 UDPGTs are known in humans, and they can be categorized into two major subfamilies, UDPGT1 and UDPGT2. The UDPGT protein sequences exhibit greater than 60% similarity within the subfamily.

SPECIES DIFFERENCES. Glucuronidation occurs in most mammalian species with the exception of the cat and related felines and the Gunn rat.

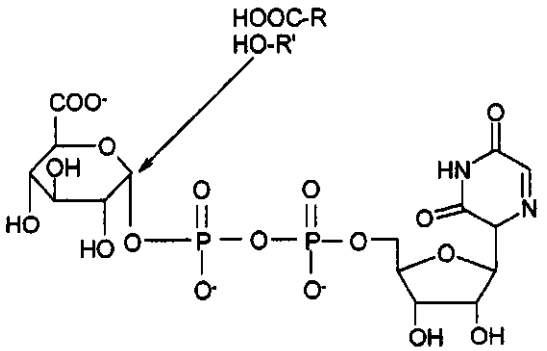


Figure 8.3. Substitution reaction of a nucleophilic substrate (R–OH, R–COOH) on the C_1 carbon atom of uridine diphosphate-glucuronic acid (UDPGA) by uridine diphosphate-glucuronosyltransferase (UDPGT).

INDUCIBILITY. Inducible by phenobarbital, 3-methylcholanthrene (MC), or pregnenolone-1 6α -carbonitrile (PCN) in rats. In humans, the induction of various UDPGT activities by phenobarbital, phenytoin, and oral contraceptives has been observed.

POLYMORPHISM. Approximately 2–5% of the population have a defective UDPGT1 gene complex causing hyperbilirubinemia (Gilbert's syndrome) (Burchell *et al.,* 1995).

IN VITRO EXPERIMENTAL CONDITIONS. There are important *in vitro* experimental considerations, which can affect the degree of activities and substrate specificity of UDPGTs as a result of the latency of enzyme activity and chemical instability of acylglucuronides.

8.3.1.1. Latency and Membrane Disruption by Detergents

The active site of the UDPGT lies on the lumenal side of the endoplasmic reticulum (ER), which restricts the access of substrates and UDPGA from cytosol. It has been suggested that UDPGA transport from cytosol onto the lumenal side of the ER across the ER membranes may be the rate-determining step for glucuronidation in the intact microsomes. Owing to this membrane barrier, UDPGTs are latent enzymes and their activities in freshly isolated microsomes cannot be fully revealed without disrupting the membranes to a certain degree. For instance, more than 95% of UDPGT activity can be latent in liver microsomes prepared in 0.25 M sucrose with 5 mM HEPES, pH 7.4. Disruption of microsomal membranes with detergents such as Lubrol PX can remove UDPGT latency and increase enzyme activity by up to 10- to 20-fold under the optimal conditions. Often, the ratio of protein and detergent (0.01–0.5 mg detergent/mg microsomal protein) has to be tested empirically for optimal activation of UDPGT, and preincubation of microsomes with detergent(s) is required before an experiment (Burchell and Coughtrie, 1989;Mulder,1992).

8.3.1.2. Chemical Instability of Acyl Glucuronides

In buffer or biological matrices at neutral or slightly alkaline pH, acyl glucuronides of many drugs with carboxylic acid moiety can undergo hydrolysis converting back to the parent drugs (futile cycling) and/or rearrangement (intramolecular rearrangement and intermolecular transacylation), whereas ether glucuronides are relatively stable. When acyl glucuronidation is anticipated, it is important to treat biological samples with acetic acid or HCl upon collection from animals, adjusting the pH to below 5, in order to minimize hydrolysis or rearrangement so that the amount of acyl glucuronides can be measured accurately (Kaspersen and Van Boeckel, 1987; Musson *et al.,* 1985; Watt *et al.,* 1991).

8.3.1.3. Acyl Migration

The rearrangement or isomerization reaction of acyl glucuronides involves the nonenzymatic migration of the drug moiety from the biosynthetic C_1 position of the glucuronic acid ring to the neighboring C_2, C_3, or C_4 positions (Fig. 8.4). In particular, intramolecular rearrangement (isomerization via acyl migration) of acyl glucuronide of drugs in plasma or albumin solutions can potentially lead to covalent binding of the drug moiety to proteins (intermolecular transacylation). These protein adducts with the (rearranged) acyl glucuronides of drugs have been proposed as possible causes of the *in vivo* toxicity seen in drugs with acid moieties (Hayball, 1995; Smith *et al.,* 1986).

8.3.2. Sulfotransferase (ST)

METABOLIC IMPLICATIONS. Sulfation is a predominant conjugation reaction of a compound at a low concentration mediated by sulfotransferase (ST), whereas glucuronidation becomes an important conjugation pathway at a higher substrate concentration. Sulfation is considered in general a high-affinity and low-capacity reaction (Weinshilboum and Otterness, 1994).

REACTION TYPE. Sulfation:

(a) O-sulfation:

$$R\text{–}OH \rightarrow R\text{–}OSO_3H$$

(b) N-sulfation:

$$R\text{–}NH\text{-}COR' \rightarrow R\text{–}\underset{\displaystyle SO_3H}{\overset{\displaystyle |}{N}}\text{–}COR',\ R\text{: aryl}$$

SUBSTRATES. Sulfation can occur on nucleophilic moieties of such molecules as phenol, alcohol, and arylamine.

ENZYME STRUCTURE. Homodimers *in vivo* with a molecular weight between 32 and 34 kDa.

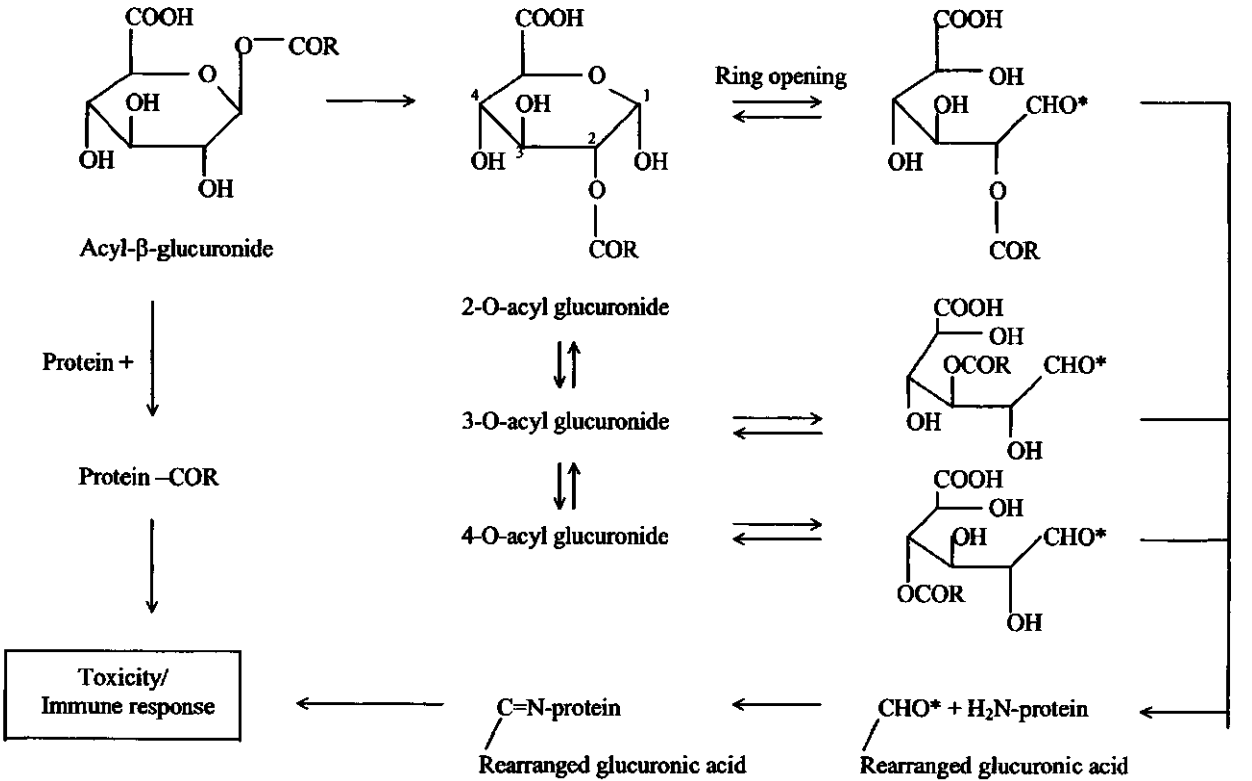
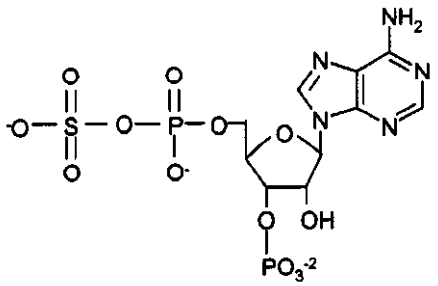


Figure 8.4. Acyl migration of acyl glucuronides. Diagrammatic representation of the migration of acyl group (RCO-) from C_1 to C_2, C_3, or C_4 positions of the glucuronic acid moiety of an acyl glucuronide, and formation of protein adducts potentially related to *in vivo* toxicity of acyl glucuronides.

COFACTOR. 3′-Phosphoadenosine-5′-phosphosulfate (PAPS).



PAPS

TISSUE DISTRIBUTION. Liver, kidney, adrenals, lung, brain, jejunum, and blood platelets, and, to a lesser extent, skin and muscle.

SUBCELLULAR LOCATION. Cytosol.

ISOZYMES. Six different phenol sulfotransferases (PST) and seven different steroid/bile acid sulfotransferases have been characterized in rats. In humans, four subfamilies have been identified: TS ST (thermostable ST or PST), TL ST (thermolabile ST, or monoamine ST), EST (estrogen ST), and DHEA ST (dehydroepiandrosterone ST).

POLYMORPHISM. A low-activity allele has been reported at a frequency of 0.2% for TS ST and 0.08% for TL ST. Bimodal frequency distribution of DHEA ST activity suggests that approximately 75% of the population are poor metabolizers (Weinshilboum and Aksoy, 1994).

SPECIES DIFFERENCES. The pig and opossum are defective in their capability regarding sulfate conjugation of phenolic compounds.

NOTE: RELATIONSHIPS BETWEEN UDPGT AND ST. Often, UDPGT and ST are considered to be complementary to each other for conjugation of the same substrates, except in connection with acyl glucuronidation, which cannot be replaced by sulfation. In general, glucuronidation is considered a low-affinity (K_m) and high-capacity (V_{max}) reaction, whereas sulfation is known as a high-affinity and low-capacity conjugation. Thus, at low substrate concentrations sulfation may be more predominant, but as concentration increases, glucuronidation becomes quantitatively more important. This is sometimes due in part to rapid depletion of the cofactor of ST, i.e., PAPS.

FUTILE CYCLING OF CONJUGATION AND DECONJUGATION: The glucuronide and sulfate conjugates of various compounds in tissues can undergo hydrolysis back to the parent compounds, which can be subsequently reconjugated. This futile cycling of conjugation and deconjugation is further facilitated because the enzymes involved in

these processes are localized in the same or adjacent subcellular compartments. For instance, both UDPGT [glucuronidation (conjugation)] and β-glucuronidase [hydrolysis of glucuronide conjugates (deconjugation)] are localized in the endoplasmic reticulum of hepatocytes. Aryl sulfatase (hydrolysis of sulfate conjugates) is located on the cytosolic surface of the endoplasmic reticulum and is readily accessible to sulfate conjugates formed by sulfotransferases in cytosol (Coughtrie *et al.,* 1998). In addition to enzyme activities, biliary excretion, transport of conjugates and free substrates across cellular membranes, and protein binding can also influence the extent and rate of futile cycling. Futile cycling of conjugation and deconjugation may be one of the important mechanisms for regulating the net production of conjugated metabolites of xenobiotics (Kauffman, 1994).

8.3.3. N-Acetyltransferase (NAT)

METABOLIC IMPLICATIONS. The first genetic polymorphism described for an enzyme involved in human drug metabolism was for N-acetyltransferase (NAT). Approximately half of the Caucasian population are poor acetylators.

REACTION TYPE. Acetylation on amine moiety.

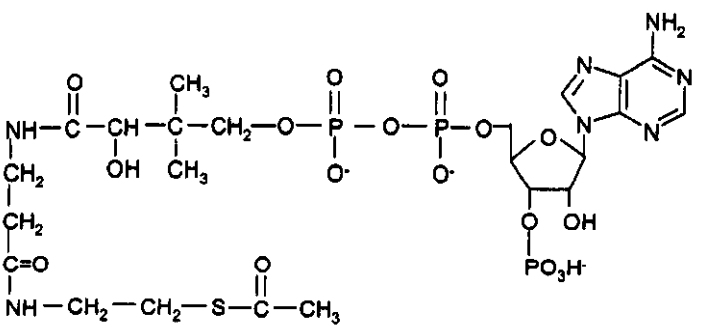
$$R\text{–}NH_2 \rightarrow R\text{–}NH\text{–}COCH_3$$

$$R\text{–}SO_2NH_2 \rightarrow R\text{–}SO_2NH\text{–}COCH_3$$

SUBSTRATES. N-acetylation reactions are common for aromatic amines (R–NH_2), sulfonamides (R–SO_2–NH_2), or hydrazine (R–NH–NH_2) derivatives.

ENZYME STRUCTURE. N-Acetyltransferase has a molecular weight of 26.5 kDa.

COFACTOR. Acetyl-coenzyme A(CoA).



CoA

TISSUE DISTRIBUTION. Liver (in the Kupffer cells, not in the hepatocytes), spleen, lung, and intestine.

SUBCELLULAR LOCATION. Cytosol.

ISOZYMES. NAT1 and NAT2 enzymes (Vatsis and Weber, 1994).

POLYMORPHISM. The slow acetylator has a genetic defect in the NAT2 gene. Approximately, 40–60% of Caucasians and 10–30% of Asians are slow acetylators.

SPECIES DIFFERENCES. Dogs and guinea pigs have a deficiency in N-acetylation.

NOTE: Acetylation of sulfonamides leads to a less water-soluble metabolite (acetylsulfonamides) than the parent compound. Precipitation of acetylsulfonamides of some sulfonamide drugs in the kidney is found to be related to renal toxicity.

8.3.4. Glutathione S-Transferase (GST)

METABOLIC IMPLICATIONS. Glutathione S-transferase (GST) represents an integral part of the phase II detoxification system. GST protects cells from oxidative- and chemical-induced toxicity and stress by catalyzing the glutathione conjugation reaction with an electrophilic moiety of lipophilic and often toxic xenobiotics (van der Aar *et al.,* 1998). In the liver, GST accounts for up to 5% of the total cytosolic proteins.

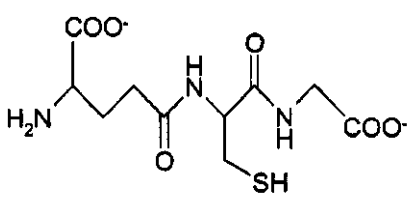
REACTION TYPE. Glutathione conjugation.

$$R\text{–}X \rightarrow R\text{–}S\text{–glutathione} + X^{-} \quad (X\text{: halide, sulfate, or phosphate})$$

$$R\text{–}C=C\text{–}COR \rightarrow R\text{–}\underset{\displaystyle S\text{–glutathione}}{\overset{\displaystyle |}{C}}\text{–}C\text{–}COR$$

SUBSTRATES. In general, substrates for GST are lipophilic and have an electrophilic moiety. Glutathione conjugates of xenobiotics formed in the liver are usually excreted in bile and urine, or further metabolized to mercapturic acids in the kidney and excreted in urine. Glutathione conjugation occurs preferably in substrates with reactive or good leaving groups such as epoxide, halide, sulfate, phosphate, or nitro moiety attached to an allylic or a benzylic carbon. The addition of glutathione may be facilitated by electron-withdrawing groups, such as –CHO, –COOR, –COR, or –CN, adjacent to the electrophilic moiety of the compounds.

COFACTOR. Glutathione, a tripeptide cofactor (GSH, L-*y*-glutamyl-L-cysteinylglycine (Gly-Cys-Glu)), is present in virtually all tissues, often in relatively high (0.1–10 mM) concentrations. Concentration of GSH in the liver is approximately 10 mM.



Glutathione

ENZYME STRUCTURE. Dimer *in vivo* with a molecular weight of 24–28 kDa.

TISSUE DISTRIBUTION. Liver, gut, kidney, testis, adrenal, and lung.

SUBCELLULAR LOCATION. Cytosol (major) and endoplasmic reticulum (minor).

ISOZYMES. The mammalian GSTs are divided into six classes, i.e., five classes of cytosolic enzymes, α, μ, π, θ, and σ and one class of microsomal enzyme.

POLYMORPHISM. (a) GSTM1 (the class μ enzyme): 40–50% of individuals from various ethnic groups have a deficiency. (b) GSTT1 (the class θ enzyme): 10–30% of Europeans have a deficiency.

8.3.5. Methyl Transferase

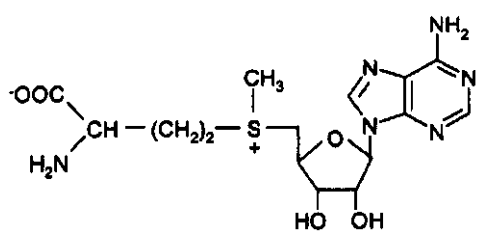
METABOLIC IMPLICATIONS. Methyl transferases are mainly involved in methylation of endogenous substrates such as histamine, catecholamines, and norepinephrine. Some drugs, however, can be methylated by nonspecific methyl transferases. Methyl transferases are important in the metabolism of chemotherapeutic agents.

REACTION TYPE. 0, N, S-methylation:

$$R_2{=}NH \rightarrow R_2{=}N{-}CH_3$$

$$R{-}SH \rightarrow R{-}S{-}CH_3$$

COFACTOR. S-adenosylmethionine(SAM).



SAM

TISSUE DISTRIBUTION. Liver, brain, lung, kidney, adrenals, skin, and erythrocytes.

SUBCELLULAR LOCATION. Cytosol.

ISOZYMES. At least four different enzymes can perform S-, N-, or 0-methylation reactions.

POLYMORPHISM. Approximately 0.3% of the European population have a deficiency in thiopurine S-methyltransferase activity.

NOTE: In general, methylation of a compound produces a less polar metabolite than the parent compound, and thus, unlike other conjugation reactions, tends to decrease the rate of its excretion.

8.3.6. Amino Acid Conjugation

METABOLIC IMPLICATIONS. Exogenous carboxylic acid, especially acetates, can be activated to coenzyme A derivatives (acyl CoA thioether) *in vivo* by acyl-CoA synthetase and further conjugated with endogenous amines such as amino acids by acyl-CoA:amino acid N-acyltransferase. Amino acid conjugates of xenobiotics are eliminated primarily in urine by tubular active secretion mechanisms.

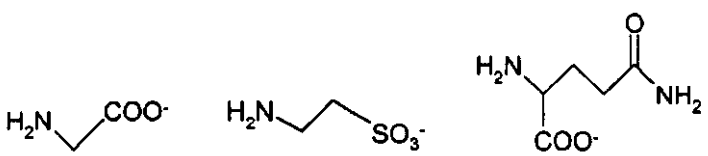
REACTION TYPE. Two-step amino acid conjugation:
(a) Acylation of substrate:

$$\text{R–COOH} + \text{CoA–SH} \xrightarrow{\text{acyl-CoA synthetase and ATP}} \text{RCO–S–CoA}$$

(b) Conjugation of amino acid:

$$\text{RCO–S–CoA} + \underset{\text{(amino acids)}}{\text{H}_2\text{N–R}'} \xrightarrow{\text{acyl-CoA:amino acid N-acyltransferase}} \text{RCO–NH–R}'$$

COFACTORS. Coenzyme A (CoA-SH) for acyl-CoA synthetase, and amino acids such as glycine, glutamine, ornithine, arginine, and taurine, for acyl-CoA:amino acid N–acyltransferase.



Glycine **Taurine** **Glutamine**

TISSUE DISTRIBUTION. Liver and kidney.

SUBCELLULAR LOCATION. Mitochondria and endoplasmic reticulum for acyl-CoA synthetase, and cytosol and mitochondria for acyl-CoA:amino acid N-acyltransferase.

SPECIES DIFFERENCES. The amino acid used for conjugation is both species- and compound-dependent. For instance, amino acid conjugation of bile acids occurs with both glycine and taurine in most species, whereas in cats and dogs, conjugation of bile acids occurs only with taurine.

Table 8.4. Summary of Characteristics of Important Metabolizing Enzymes in Humans

	Reaction	Cofactor	Tissue distribution	Subcellular location	Isozymes	Polymorphism	Route of elimination	Inducibility
Cytochrome P450 (P450 or CYP)	Mainly oxidation	NADPH	Most tissues (liver, intestine, etc)	Endoplasmic reticulum (ER)	~20	2C18, 2C19, 2D6, 3A5	Urine, bile	Yes
Flavin-mono-oxygenase	Oxidation	NADPH	Liver,kidney, lung, skin	ER	FMOI and FMOII	—	—	—
Esterase	Hydrolysis	—	Liver,blood, intestine	ER,cytosol	A, B,C-Esterase and choline esterase	Rare in cholinesterase	—	Yes
Uridine diphosphate-glucuronosyl-transferase (UDPGT)	Glucuronylation on –OH, –COOH,=NH, $-NH_2$, –SH	UDPGA	Most tissues (liver, intestine, etc)	ER	> 15 in two subfamilies (UDPGT1 and UDPGT2)	UDPGT1	Mainly bile (MW>350)	Yes
Sulfotransferase (ST)	Sulfation on –OH,$-NH_2$, –NHOH	PAPS	Liver, adrenals lung, brain, jejenum, blood platelets	Cytosol	TS ST, TL ST, EST, DHEA ST	Rare in TS ST, TL ST	Mainly urine	—
N-acetyltransferase (NAT)	Acetylation on $-NH_2$	Acetyl-coA	Liver,spleen, lung,intestine	Cytosol	NAT1,NAT2	NAT2	—	—
Glutathione S-transferase (GST)	Glutathione conjugation on electrophilic moieties with good leaving group	GSH	Liver,kidney, intestine	Cytosol, ER	6classes	GSTM1, GSTT1	Mainly bile	—
Amino acid conjugation	Amino acid conjugation on acyl-CoA of substrates	CoA, glycine, taurine, etc	Liver, kidney	Mitochondria,[a] ER,a cytosol,[b] mitochondria[b]	—	—	Mainly urine	—

[a]Subcellular locations for acyl-CoA synthetase.
[b]Subcellular locations for acyl-CoAamino acid N-acyltransferase.

NOTE: Amino acid conjugation of carboxylic acid–containing xenobiotics is an alternative metabolism pathway to glucuronidation. Acyl glucuronides of xenobiotics can be potentially toxic, whereas conjugation with amino acids is a detoxification reaction.

8.4. EXTRAHEPATIC METABOLISM

Most tissues have some metabolic activity; however, quantitatively the liver is by far the most important organ for drug metabolism. Important organs for extrahepatic metabolism include the intestine (enterocytes and intestinal microflora), kidney, lung, plasma, blood cells, placenta, skin, and brain. In general, the extent of metabolism in the major extrahepatic drug-metabolizing organs such as the small intestine, kidney, and lung is approximately 10–20% of the hepatic metabolism. Less than 5% of extrahepatic metabolism compared to hepatic metabolism can be considered low with negligible pharmacokinetic implications (Connelly and Bridges, 1980; deWaziers *et al.,* 1990; Krishna and Klotz, 1994; Ravindranath and Boyd, 1995).

8.4.1. Intestinal Metabolism

Because most drugs are administered orally, there has been much emphasis in presystemic metabolism on the effects of gastrointestinal metabolism on the bioavailability of drugs. Recent studies have indicated that P450 isoforms such as CYP2C19 and 3A4 in enterocytes might play an important role in the presystemic intestinal metabolism of drugs and the large interindividual variability in systemic exposure after oral administration (I1ett *et al.,* 1990; Kaminsky and Fasco, 1992; Schwenk, 1988; Zhang *et al.,* 1996). The cytochrome P450 content of the intestine is about 35% of the hepatic content in the rabbit, but accounts for only 4% of the hepatic content in the mouse. Cytochrome P450 levels and activities are highest in the duodenum near the pyrolus, and then decrease toward the colon. A similar trend in regional activity levels along the intestine has been observed for glucuronide, sulfate, and glutathione conjugating enzymes. The rate and extent of first-pass intestinal metabolism of a drug after oral administration are dependent on various physiological factors such as:

1. Site of absorption: If the absorption site in the intestine is different from the metabolic site, first-pass intestinal metabolism of a drug may not be significant.
2. Intracellular residence time of drug molecules in enterocytes: The longer the drug molecules stay in the enterocytes prior to entering the mesenteric vein, the more extensive the metabolism.
3. Diffusional barrier between splanchnic bed and enterocytes: The lower the diffusibility of a drug from the enterocytes to the mesenteric vein, the longer its residence time.
4. Mucosal blood flow: Blood in the splanchnic bed can act as a sink to carry drug molecules away from the enterocytes, which reduces intracellular residence time of drug in the enterocytes.

Treatment with certain drugs such as methylcholanthren (MC) and phenobarbital can increase metabolizing enzyme levels in the intestine. Enzyme induction requires 2–4 days and is more extensive when the inducer is administered orally as opposed to parenterally.

8.4.2. Renal Metabolism

In addition to physiological functions of homeostasis in water and electrolytes and the excretion of endogenous and exogenous compounds from the body, the kidneys are the site of significant biotransformation activities for both phase I and phase II metabolism. The renal cortex, outer medulla, and inner medulla exhibit different profiles of drug metabolism, which appears to be due to heterogeneous distribution of metabolizing enzymes along the nephron. Most metabolizing enzymes are localized mainly in the proximal tubules, although various enzymes are distributed in all segments of the nephron (Guder and Ross, 1984; Lohr *et al.,* 1998). The pattern of renal blood flow, pH of the urine, and the urinary concentrating mechanism can provide an environment that facilitates the precipitation of certain compounds, including metabolites formed within the kidneys. The high concentration or crystallization of xenobiotics and/or their metabolites can potentially cause significant renal impairment in specific regions of the kidneys.

8.4.3. Metabolism in Blood

Blood contains various proteins and enzymes. As metabolizing enzymes, esterases, including cholinesterase, arylesterase, and carboxylesterase, have the most significant effects on hydrolysis of compounds with ester, carbamate, or phosphate bonds in blood (Williams, 1987). Esterase activity can be found mainly in plasma, with less activity in red blood cells. Plasma albumin itself may also act as an esterase under certain conditions. For instance, albumin contributes about 20% of the total hydrolysis of aspirin to salicylic acid in human plasma. The esterase activity in blood seems to be more extensive in small animals such as rats than in large animals and humans. Limited, yet significant monoamine oxidase activities can be also found in blood.

8.5. VARIOUS EXPERIMENTS FOR DRUG METABOLISM

In this section, various *in vitro, in situ,* and *in vivo* experiments for drug metabolism and important considerations in regard to *in vitro* drug metabolism are discussed.

8.5.1. Examining Metabolic Profiles of Drugs

Suitability of a particular experimental system for the metabolism of a compound of interest can be significantly affected by experimental conditions such as compound availability and assay sensitivity (Rodrigues, 1994). The advantages and limitations of various *in vitro, in situ,* and *in vivo* experimental systems are summarized in Table 8.5. A schematic description of the preparation of liver S9 and microsomes is shown in Fig. 8.5.

Table 8.5. Advantages and Limitations of Various Experimental Methods for Investigating Drug Metabolism in the Liver[a]

Method	Advantages	Limitations
In vitro system		
S9 fraction[b]	Useful for both phase I and phase II metabolism	Difficult to assess the effects of membrane transport on metabolism
Microsomes[c]	Useful for cytochrome P450 metabolism Can be used for UDPGT activity Hight throughput screening for metabolic stability of compounds is possible Easy to maintain and stable for a long-term storage at −80°C Freezing and thawing (up to 10 cycles) with little loss of cytochrome P450 activity Simple sample preparation for assay	Difficult to asses the effects of membrane transport on metabolism In case of UDPGT activity measurement, proper extrapolation of UDPGT activity to *in vivo* conditions may be difficult owing to the usage of detergent to enhance UDPGT activity Production of metabolites potentially different from those under *in vivo* conditions owing to the closed experimental system of microsome studies (no further elimination of metabolites formed in microsomes) Limited integrated drug metabolism for phase I and II simultaneously
Purified enzumes	Useful for studying metabolism by specific metabolizing enzymes	Cross-contamination of enzymes during isolation
Recombinant enzymes	Useful for identifying specific isozyme(s) of the metabolizing enzyme responsible for metabolism of substrate of interest Potential replacement of enzymes obtained from tissues (large-scale preparation is possible) Easy to handle; unlike enzymes prepared from animal tissues, recombinant enzymes are not hazardous	Technical difficulties in preparing recombinant enzymes Difficult to fully characterize enzyme activities Apparent discrepancy in enzyme affinities (K_m or K_i) between some recombinant enzymes and those in other *in vitro* systems for certain compounds
Hepatocytes[d]	Useful for both phase I and phase II metabolism Can examine the effects of membrane transport in metabolism	Only freshly isolated hepatocytes are suitable for metabolism studies, since metabolic activities diminishes within a few hours, especially in rat hepatocytes, after isolated from the

8.5. Continued

Method	Advantages	Limitations
Hepatocytes	Primary cultures of hepatocytes can be used for studying the inducibility of metabolizing enzymes such as P450 under certain incubation conditions Cryopreservation of hepatocytes is possible for long-term storage	liver (this limitation has been significantly improved by recent progress in cryopreservation techniques for hepatocytes) Not suitable for high throughput screening Need to assay not only buffer but also hepatocytes for metabolite identification and extent of metabolism Difficult to prepare mixture of hepatocytes, which can reflect average population for metabolic profiles of compounds tested
Liver slices[a]	Integration of all cell types in the liver for metabolism study Tissue architecture and cell-to-cell communication maintained Useful for both phase I and phase II metabolism Can examine the effects of membrane transport on metabolism	Only freshly obtained liver slices are suitable for metabolism studies (limited applicability of cryopreservation to liver slices for metabolism studies) Release of cytosolic enzymes from damaged cells at the slice surface and poor oxygen supply to the center of the slice can cause impaired metabolic activities Not suitable for high throughput screening Need to assay both buffer and liver slices for metabolite identification and extent of metabolism Difficult to prepare mixture of liver slices representing population Lower estimate of intrinsic clearance as compared to that from studies with hepatocytes
In situ system		
Liver perfusion	Useful for both phase I and phase II metabolism Sequential metabolism can be studied Useful for examining biliary excretion Can study the effects of blood flow, protein binding, etc, on metabolism Closer system to *in vivo* metabolism and organ clearance than *in vitro* systems (maintaining the spatial heterogeneity and architecture of the liver)	Requires more sophisticated equipment than other *in vitro* methods Limited supply of the fresh liver (especially fresh human liver for perfusion study) Slow throughout Similar limitations to those of liver slices

Table 8.5. Continued

Method	Advantages	Limitations
In vivo system		
Metabolism study in bile-cannulated animals after intravenous administration of drug	The most comprehensive model for investigating drug metabolic profiles By measuring the amount of unchanged drug excreted in the urine and bile, the quantitative contributions of metabolism to the overall clearance can be estimated	Slow throughput Labor intensive

[a] Data taken from deKanter *et al.* (1998), Elkins (1996), Hawksworth (1994), Pearce *et al.* (1996), Price *et al.* (1998), Remmel and Burchell (1993). Rodrigues (1994). Silvo *et al.* (1998), Shett (1994), Thummel (1994), Vickers (1997). and Wrighton *et al.* (1993).

[b] S9 subcellular fraction of the liver can be obtained as a supernatant after centrifugation of liver homogenate at 9000 g, which removes nuclei and mitochondria pellets. S9 contains both cytosolic (soluble) and microsomal metabolizing enzymes and is capable of performing both phase I and phase II metabolism.

[c] A microsomal fraction of the liver can be prepared as a pellet after centrifuging S9 fraction at 100,000 g. Microsomes consist mainly of the endoplasmic reticulum, where important phase I metabolizing enzymes such as cytochrome P450s are located. Microsomes are not present in phase II metabolizing enzymes, except a few transferases including UDPGT.

[d] Primary hepatocyte suspension can be obtained by perfusing the liver with a collagenase-containing perfusate. In terms of drug metabolism, the one important fact that limits the use of isolated hepatocytes is the rapid loss of activities of metabolizing enzymes including cytochrome P450s. when the cells are kept for any extended period, sometimes, within a matter of several hours. Recent progress in cryopreservation techniques, however, greatly improved utilities of hepatocytes for metabolism studies. In general, cultured hepatocytes such as HepG2 cells are consdered to be not suitable for metabolism studies due to the lower levels of metabolizing enzymes including cytochrome P450s compared to primary hepatocytes.

[e] A commercially available tissue slicer can produce liver slices with a thickness of approximately 200 μm, containing five to six layers of hepatocytes from cylindrical cores of the liver. Liver slices are capable of both phase I and II metabolism. Owing to limitations of drug penetration through the multilayers of hepatocytes in liver slices, the metabolism of drugs is often limited to hepatocytes located in an outer layer of the slices, resulting in an underestimate of the extent of the metabolism.

8.5.2. Phenotyping of Cytochrome P450 Isoforms

Despite the fact that many of P450 isoforms exhibit partially overlapping substrate specificity, it has become apparent that in most cases a single P450 isoform may be exclusively or primarily responsible for metabolism of a particular drug at therapeutic concentrations *in vivo* (Parkinson, 1996b; Guengerich, 1996). Information on P450 isoform(s) responsible for the metabolism of the drug of interest is important for an understanding of two critical aspects of drug metabolism in humans. (a) drug–drug interaction in metabolism between coadministered drugs, and (b) metabolic polymorphism of a drug. Four *in vitro* methods have been used for phenotyping P450 isoforms, and a combination of at least two different approaches is generally necessary to identify the P450 isoform(s) responsible for the metabolism of the substrate of interest.

8.5.2.1. Correlation between Metabolism Rates and P450 Isoform Activities

The particular P450 isoform responsible for the metabolism of a particular compound can be identified by examining the relationship between the initial rate

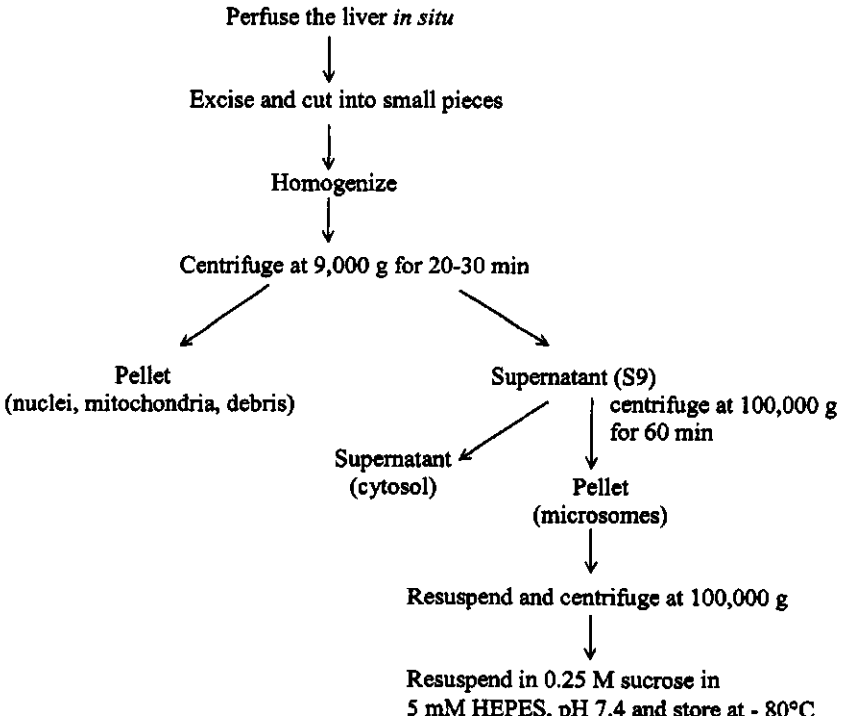


Figure 8.5. Schematic description of preparation processes for liver S9 and microsomes.

of metabolism of the compound in several different human microsomes and the level of activity of the individual P450 isoforms in the same microsomes. Information on activities of the individual P450 isoforms can be obtained by measuring the disappearance rates of known substrates for those P450 isoforms in the microsomes (Beaune *et al.,* 1986). If the rates of metabolism of the two different reactions show a linear correlation, both metabolic reactions can be considered to be mediated predominantly by the same P450 isoform. This approach is possible because the levels of the individual P450 isoforms in liver microsomes vary significantly among different subjects. Let us assume that the initial rates of disappearance of compound A and known substrates for different cytochrome P450 isoforms in a panel of human liver microsomes (Hl-H10) are as shown in Table 8.6. Disappearance rates of compound A exhibit a good linear correlation only with those of a known substrate for CYP2D6 among the three isoforms examined in the same human microsome samples, suggesting that a major cytochrome P450 responsible for metabolism of compound A is CYP2D6.

8.5.2.2. Competitive Inhibition

If metabolism (disappearance rate) of the compound of interest in human liver microsomes is markedly inhibited by a known inhibitor against a particular P450

Table 8.6. Initial Rates of Disappearance of Compound A and Known Substrates for Different Cytochrome P450 Isoforms in a Panel of Human Liver Microsomes

	Initial disappearance rates in microsomes			
		Known substrates for		
Human liver microsome ID	Compound A	CYP2C9	CYP2D6	CYP3A4
H1	1	3	2	57
H2	2	6	4	35
H3	3	5	6	27
H4	4	9	8	3
H5	5	10	10	48
H6	6	3	12	5
H7	7	6	14	23
H8	8	9	16	39
H9	9	14	18	42
H10	10	7	20	7

isoform, then that P450 isoform may be involved in the metabolism of the compound to a significant extent. Results from these studies must be interpreted with caution, because most of the known inhibitors for cytochrome P450s can act on more than one isoform. These studies can be done using either liver microsomes or recombinant P450 enzymes.

8.5.2.3. Antibody Specific for Particular P450 Isoforms

The inhibitory effects of specific antibodies against selected cytochrome P450 isoforms on the metabolism of a compound in human liver microsomes are evaluated. Owing to the ability of an antibody to selectively inhibit a specific cytochrome P450 isoform, information from these studies is sufficient to establish which P450 isoform is responsible for metabolism of the compound (Gelbonin, 1993).

8.5.2.4. Metabolism with Purified or Recombinant P450 Isozymes

The initial rates of metabolism of a compound of interest by purified or recombinant P450 isozymes are measured. If a particular P450 isoform causes a faster initial disappearance rate compared to the other P450 isoforms tested, the metabolism of the compound may be mediated predominantly by that isoform (Guengerich *et al.,* 1996). The information from this type of study does not address the quantitative contribution of the particular isoform to the overall metabolism of the compound by cytochrome P450s.

8.5.3. Important Factors in Drug Metabolism Experiments

There are several important factors to be considered for *in vitro* and *in situ* drug metabolism studies: concentrations of enzymes, e.g., microsomal protein concentrations or the number of hepatocytes, concentrations of substrates and cosubstrate(s),

effects of organic solvents for compounds with low aqueous solubility, and assay sensitivity, among others.

8.5.3.1. Substrate Concentrations for Metabolism Studies

In order to make a meaningful extrapolation of *in vitro* findings to *in vivo* situations, *in vitro* metabolism experiments should be conducted at physiologically or toxicologically relevant drug concentrations. Important differences between *in vivo* and *in vitro* metabolism in terms of drug concentrations are summarized in Table 8.7.

The most physiologically relevant concentrations of substrates and cosubstrates in an incubation buffer for *in vitro* metabolism studies would be those that can produce unbound drug concentrations adjacent to metabolizing enzymes similar to those within cells *in vivo.* In practice, it is almost impossible to accurately measure

Table 8.7. Differences between *In Vivo* and *In Vitro* Metabolism Experimentsin Terms of Drug Concentrations

Factor	*In vivo*	*In vitro*
Drug concentrations[a]	0.01–10 μM (a typical range of total therapeutic drug concentration in plasma)	1–1000 μM
Protein binding	Can be extensive	Moderate at a typical microsomal protein concentration ($\leqslant$ 1 mg/ml) used for *in vitro* experiments
Unbound drug concentration	Can be substantially lower than total drug concentration in plasma	Lower than total concentration, but not to the extent observed *in vivo*
Concentration *vs.* time profile	Continuously changing	Fixed initial concentrations
Duration of drug exposure	Long	Short
Responsible metabolizing enzymes[b]	A few enzyme systems with high affinity and low capacity	Potentially more enzyme systems involved depending on experimental conditions used due to higher substrate concentrations (in a closed system)
Effects of other elimination pathways on drug concentrations	Effects of biliary excretion, renal elimination, and intestinal secretion	Cannot be examined

[a] In most cases, drug concentrations for *in vitro* metabolism studies are higher than *in vivo* unbound (or even total) therapeutic drug concentrations, mainly owing to assay limitations associated with smaller sample volumes obtained from *in vitro* studies. This can cause physiologically irrelevant extrapolation of *in vitro* data to *in vivo* in the rate and route of metabolism.

[b] It is more likely that a few (or even single) metabolizing enzyme(s) with high affinity and low capacity would be mainly responsible for biotransformation of a drug over a low therapeutic concentration range *in vivo.* At higher drug concentrations used in *in vitro* studies enzyme systems, which may not be important *in vivo,* can have significant effects on drug metabolism. Besides, further elimination of primary metabolites produced in a closed system such as microsomes, in which many phase I and II metabolizing enzyme acivities are absent, can be hindered.

the unbound substrate concentrations available to the metabolizing enzymes in endoplasmic reticulum or cytosol in hepatocytes *in vivo.* Several *in vitro* techniques, such as digitonin treatment of hepatocytes, for estimating unbound drug concentrations within hepatocytes have been reported in the literature; however, none of them appears to be reliable because of experimental difficulties and/or unrealistic assumptions. In addition, various active transport systems located in the sinusoidal membrane of hepatocytes can make predicting an intracellular drug concentration even more complicated.

For metabolism studies with primary hepatocytes or liver slices, unbound drug concentrations in sinusoidal blood (blood within sinusoids of the liver) available to hepatocytes *in vivo* would be the most desirable. It is also difficult to measure or make any reliable estimate of sinusoidal unbound drug concentrations from systemic plasma drug concentrations, because the former can be significantly different from the latter owing to metabolic activities in the liver. Therefore, the assumption that unbound drug concentrations in plasma are equal to those in the sinusoidal blood and thus to those adjacent to the metabolizing enzymes within hepatocytes may not be valid.

As a result of these difficulties in determining approprate drug concentrations, it is desirable to conduct *in vitro* metabolism studies over a wide range of substrate concentrations, e.g., 0.01–100 μM, especially for investigational drugs, for which little or no clinical pharmacology or safety data are available. This range of drug concentrations is likely to cover most of the therapeutic exposure levels of the drug *in vivo.* It is equally important to realize that in many cases the selection of drug concentrations used in *in vitro* studies is also affected by the experimental conditions, such as drug availability, aqueous solubility of the drug in an incubation buffer, and assay sensitivity (Rodrigues, 1994).

8.5.3.2. Effects of Organic Solvents on In Vitro P450 Metabolism Studies

For compounds with poor aqueous solubility, water-miscible organic solvents such as dimethyl sulfoxide are often used to enhance solubility of compounds in aqueous incubation media for *in vitro* metabolism studies. In general, compounds are dissolved into those organic solvents as stock solutions at high concentrations and then diluted to proper concentrations for the studies in aqueous incubation media. It has been found that the organic solvents used for solubilizing lipophilic compounds can have significant inhibitory effects on the activity of metabolizing enzymes. The inhibitory effects of three widely used organic solvents, i.e., dimethyl sulfoxide, methanol, and acetonitrile, on the activity of cytochrome P450s in isolated human liver microsomes are summarized below (Chauret *et al.,* 1998; Kawalek and Andrews,1980).

(a) Dimethyl Sulfoxide. Although dimethyl sulfoxide (DMSO) is considered to be a good universal organic solvent for solubilizing lipophilic compounds, it appears not to be an optimal solvent for *in vitro* cytochrome P450-mediated metabolism studies using human liver microsomes. It has been reported that DMSO showed significant inhibitory effects (10–60%) on the activities of several P450 isoforms (CYP2C8/9, 2C19, 2D6, 2E1, and 3A4) even at low levels (0.2% v/v).

(b) Methanol. Methanol exhibited no measurable inhibitory effects on the catalytic activities of CYP1A2, 2A6, 2C19, 2D6, and 3A4 at 0.5–1%. However, significant inhibition has been reported on the activities of CYP2C9 and 2E1 at the same concentration range.

(c) Acetonitrile. Acetonitrile (ACN) appears to be the most suitable organ solvent among the three, as long as its concentration is kept at a relatively low level. At up to 1% ACN, no significant inhibition was noted on the activities of CYP1A2, 2A6, 2C8/9, 2C19, 2D6, 2E1, and 3A4. It is important to note that the effects of an organic solvent described above can vary with the experimental conditions, such as the types and concentrations of substrates, the integrity of microsomes used, and protein concentrations of microsomes, among others. In general, less than 0.2%, 0.5%, and 1% (v/v) of DMSO, methanol, and acetonitrile, respectively, are recommended for solubilization of lipophilic substrates for *in vitro* microsome studies, in order to minimize their inhibitory effects on the activities of cytochrome P450s.

8.6. PHYSIOLOGICAL AND ENVIRONMENTAL FACTORS AFFECTING DRUG METABOLISM

Physiological factors affecting the rate and pathway of drug metabolism include, e.g., species, genetics, gender, age, hormone, disease, and pregnancy. Environmental factors include, among others, diet, smoking, heavy metals, pollutants, and insecticides.

8.6.1. Physiological Factors

8.6.1.1. Species-Related Differences in Metabolism

There are species-related differences in both quantitative (the same metabolites with different rates) and qualitative (different metabolites via different metabolic pathways) aspects of drug metabolism. Information on species-related differences in the metabolism of investigational drugs is especially critical for drug safety evaluation in both animals and humans, because metabolic profiles in animals can be substantially different from those in humans, and the toxicity caused by the metabolites seen in the one may not be observed in the other and viceversa.

Common laboratory animals, especially rats, metabolize drugs considerably faster than humans; however, variability in drug-metabolizing enzymes is generally considered to be greater in humans than in animals (Nedelcheva and Gut, 1994; Smith, 1991; Soucek and Gut, 1992). Species differences can occur in both phase I and II metabolism, and are considered to be due mainly to evolutionary divergence among various species. For instance, there are significant differences in expression and the extent of activities of cytochrome P450s among different species (Table 8.8). The activity of β-glucuronidase in the intestine is much higher in rats than in humans. The activity of glutathione-S-transferase is substantially higher in mice and

Table 8.8. Differences in Important Cytochrome P450 Isoforms between Humans and Rats

Species	Cytochrome P450 isoforms			
Human	1A1,[a] 1A2[a]	2A6 2B6 2C8, 2C9, 2C10, 2C18, 2C19 2D6 2E1[a]	3A3, 3A4, 3A5, 3A7	4A9, 4A11
Rat[b]	1A1, 1A2	2A1, 2A2, 2A3 2B1, 2B2 2C11, 2C12, 2C13 2D1, 2D2, 2D3, 2D4, 2D5 2E1	3A1, 3A2	4A1, 4A2, 4A3

[a] There are three species-independent cytochrome P450s (the same name across all mammalian species), i.e., CYP1A1, 1A2, and 2E1, of which the genetic regulation is highly conserved among different species.
[b] Rats have approximately 40 different cytochrome P450 isoforms.

hamsters than in rats and humans. Glucuronidation activities are usually higher in rabbits. In general, the metabolism of the old world monkey, notably the rhesus monkey, is the one that resembles human metabolism most closely. In some animals, particular metabolic pathways are deficient (Table 8.9).

8.6.1.2. Genetics-Related Differences in Metabolism

Distinctive population subgroups may exhibit differences in their ability to metabolize certain drugs as compared to the general population. These differences among individuals in the extent of drug metabolism often follow a bimodal distribution pattern as shown in Fig. 8.6, which is indicative of genetic polymorphism in metabolism (pharmacogenetics). Genetic polymorphism (or simply polymorphism) is defined as a Mendelian or monogenic trait that is found in at least two phenotypes (and presumably at least two genotypes) in more than 1–2% of the population. If the frequency is lower than 1–2%, it is called a rare trait.

Table 8.9. Species Defective in Particular Xenobiotic Metabolism

Reactions or enzymes	Defective species
N-hydroxylation of aliphatic amines	Rat, marmoset
N-hydroxylation of arylacetamide	Guinea pig
N-acetylation of primary amines	Dog, guinea pig
Glucuronidation	Cat
Sulfation	Pig, opossum
Mercapturic acid formation	Guinea pig
Epoxide hydrolase	Mouse

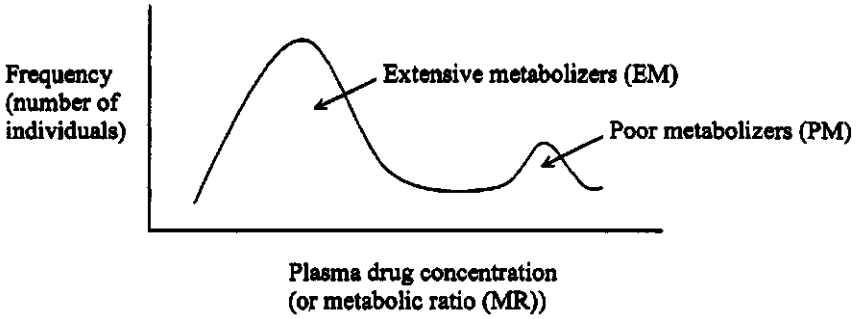


Figure 8.6. Metabolic polymorphism (a bimodal distribution of phenotypes in populations).

(a) Extensive Metabolizer and Poor Metabolizer. The poor metabolizer (PM) is an individual with deficient metabolic ability for a certain drug owing to genetic defects in a particular metabolizing enzyme(s), usually resulting in a higher exposure of the drug as compared to the normal population [extensive metabolizers (EM)].

Several metabolizing enzymes are known to exhibit polymorphism in humans. Table 8.10 summarizes the percentage of poor metabolizers in different populations who do not express the corresponding CYP isoforms in the liver. For instance, plasma exposure levels of certain drugs such as debriosquine metabolized by CYP2D6 are substantially higher in approximately 5–10% of Caucasians, as compared to the rest of population, which corresponds to the extent of polymorphic distribution of the poor CYP2D6 metabolizers in Caucasians. Genetic polymorphism becomes important in therapeutic monitoring, especially when drug elimination occurs mainly via a single metabolic pathway subject to polymorphism. Careful dosage monitoring should be implemented to avoid any adverse effects (idiosyncratic responses) of the drug at the high exposure levels produced in poor metabolizers (Daly *et al.,* 1993; Daly, 1995; Smith *et al.,* 1994).

(b) Phenotyping and Genotyping. For polymorphic phenotyping to identify the P450 isoform or N-acetyltransferase responsible for metabolism of a drug, a metabolic ratio (MR) between the amount of parent drug and certain metabolite(s)

Table 8. 10. Important Metabolizing Enzymes Exhibiting Polymorphism in Humans

Enzymes	% Population as poor metabolizers	Known substrates
CYP2C18	2–3% of Caucasians	S-mephenytoin
CYP2C19	2–3% of Caucasians and 20% of Asians	—
CYP2D6	5–10% of Caucasians and 1–2% of Asians	Debriosquine, spartein, dextrometorphan
CYP3A5	80% of Caucasians	Midazolam
N-acetyl-transferase	50% of Caucasians and <25% of Asians	Isoniazid, sulfametazine

excreted in the urine produced by particular polymorphic enzyme(s) of interest can be measured (Bertilsson, 1995). Estimates of MR are significantly higher in poor metabolizers as compared to those in extensive metabolizers:

$$\text{MR} = \frac{\text{Amount of parent drug excreted in urine}}{\text{Amount of metabolite excreted in urine}} \tag{8.1}$$

Polymorphic genotype screening can be done using a polymerase chain reaction (PCR) followed by specific probes for many of the mutations in genes responsible for defective or absent metabolizing enzymes.

8.6.1.3. Gender-Related Differences in Metabolism

There are significant gender-related differences in cytochrome P450 expression in rats (male rats have a higher metabolic activity than female rats), due mainly to the different patterns of growth hormone secretion (pulsatile in male rats and continuous in female rats, Table 8.11) (Agrawal and Shapiro, 1997). Gender differences in hepatic metabolism have also been seen for many steroid hormones such as androgens, estrogens, and corticosteroids (Skett, 1988). Gender-related differences in metabolism are, in general, not apparent in mice and dogs. Although data are limited and not in complete agreement, CYP3A4 activity appears to be higher (approximately 1.4-fold) in women than in men, whereas the activities of many other metabolizing enzymes (e.g., CYP1A2,2C19, UDPGT) may be higher in men than in women (Gleiter and Gundert-Remy, 1996; Harris *et al.,* 1995; Mugford and Kedderis, 1998).

8.6.1.4. Effects of Aging on Metabolism

It has been found that in rats the activity of cytochrome P450 decreases with age, but there is no change in uridine diphosphate-glucuronosyltransferase activity. In humans, cytochrome P450 activities decrease with age.

8.6.1.5. Effects of Disease on Metabolism

The level of cytochrome P450 isozymes in rats can be increased or decreased by the physiological status of the animals, including starvation (2B1/2, 2E1, 3A2,

Table 8.11. Gender-Related Differences in Cytochrome P450 Expression in Rats

Male specific isoforms	2C11, 2C13, 2C22, 2A2, 3A2
Female specific isoforms	2C12
Male dominant isoforms	2B1, 2B2, 3A1
Female dominant isoforms	1A2, 2A1, 2C7, 2E1
No gender difference	1A1, 2C6

4A1-3), diabetes (2A1, 2C7/11/12/13, 2E1, 3A2), or high blood pressure (2A1, 2C11, 3A1) (Shimojo, 1994). In humans, elevated hepatic cytochrome P450 activity has been reported in diabetic patients. Liver diseases such as cirrhosis and hepatoma can impair hepatic metabolism (George *et al.,* 1995).

8.6.2. Environmental Factors

Important environmental factors affecting drug metabolism in humans include diet, smoking, and pollutants (Baijal and Fitzpatrick, 1996; Guengerich, 1995; O'Mahony and Woodhouse, 1994; Williams *et al.,* 1996). Some of the important relevant chemical effects include:

- Butylated hydroxytoluene (BHT, food additive) inhibits lipid peroxidation.
- Caffeine induces or inhibits oxidative metabolism of some drugs.
- Charcoal-broiled meat [polycyclic aromatic hydrocarbons (PAH)] or cigarette smoking (PAH) induces CYP1A1/2.
- Cruciferous vegetables (cabbage, cauliflower, or brussels sprouts) induces CYP 1 A 1/2.
- Grapefruit juice inhibits CYP3A4.
- Vitamin C induces oxidative metabolism in elderly patients with vitamin C deficiency.

8.7. METABOLITE KINETICS

A thorough understanding of *in vivo* disposition profiles of metabolite(s) is important in assessing a drug's pharmacological and/or toxicological effects. After administration, a drug is excreted in urine or bile, and/or is converted to metabolites, which will be eliminated. The rates of change in the amount of drug metabolites in the body are affected by how fast they are generated (formation rate of the metabolites) as well as how rapidly they are eliminated (elimination rate of the metabolites). To elucidate the pharmacokinetic relationship between these two processes, let us assume simple one-compartment models for both the parent drug and its metabolite. A drug plasma concentration–time profile after intravenous bolus injection is assumed to follow a first-order decline, and the exposure profile of the metabolite, if the metabolite itself is administered intravenously, is also assumed to follow a first-order elimination (Fig. 8.7).

The rate of change in the amount of drug can be described as first-order kinetics of the amount present in the body after intravenous injection:

$$dA(t)/dt = -(k_m + k_{other}) \cdot A(t) = -k \cdot A(t) \tag{8.2}$$

A(t) is the amount of drug in the body at time t after intravenous bolus injection. k, k_m, and k_{other} are the rate constants representing systemic elimination of the drug, metabolism of the drug to the metabolite, and elimination processes of the drug other than metabolism, respectively. From Eq. (8.2), the concentration of a drug in

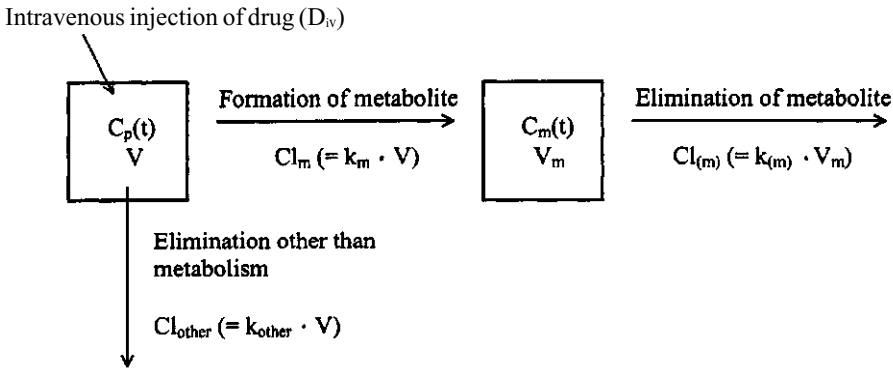


Figure 8.7. Processes affecting concentrations of the parent drug and its metabolite under a linear condition after intravenous administration, assuming one-compartment models for both the drug and its metabolite. $C_p(t)$, $C_m(t)$: concentrations of the drug and its metabolite in plasma at time t, respectively; CI_m and Cl_{other}: metabolic clearance of the drug to produce the metabolite and drug clearance other than metabolism, respectively; $Cl_{(m)}$: systemic clearance of the metabolite, which is the same as the systemic clearance measured after intravenous bolus injection of the metabolite, itself; D_{iv}: intravenous dose of drug; k_m and k_{ther}: rate constants representing the metabolism of the drug to the metabolite and elimination processes of the drug other than metabolism, respectively; $k_{(m)}$: rate constant representing systemic elimination processes of the metabolite, when the metabolite itself is administered intravenously; V and V_m: volumes of distribution of the drug and the metabolite, respectively.

plasma at time t [Cp(t)] can be derived as

$$C_p(t) = (D_{iv}/V) \cdot e^{-k \cdot t} \tag{8.3}$$

where D_{iv} is an intravenous dose, and V is the volume of distribution of the drug. The rate of change in the amount of the metabolite depends on the difference between the rate of formation of the metabolite from the drug and the rate of elimination of the metabolite itself:

Rate of change of amount of metabolite in the body = *Rate of formation of metabolite from the drug* − *Rate of elimination of metabolite from the body*

$$dA_m(t)/dt = k_m \cdot A(t) - k_{(m)} \cdot A_m(t) \tag{8.4}$$

where $A_m(t)$ is the amount of the metabolite in the body at time t after intravenous administration of the drug, and $k_{(m)}$ is the rate constant for the systemic elimination processes of the metabolite. From Eqs. (8.3) and (8.4), the concentration of the metabolite in plasma at time t [$C_m(t)$] can be described as follows:

$$C_m(t) = \frac{k_m \cdot D_{iv}}{V_m \cdot (k_{(m)} - k)} \cdot (e^{-k \cdot t} - e^{-k_{(m)} \cdot t}) \tag{8.5}$$

where V_m is the volume of distribution of the metabolite. Equation (8.5) can be simplified at later time points under two different conditions, depending on the magnitude of k and $k_{(m)}$.

8.7.1. "Formation-Rate-Limited" Metabolite Kinetics

If the elimination rate constant of the drug is much smaller than that of the metabolite, i.e., $k \ll k_{(m)}$, then, the semilogarithmic plasma concentration *vs.* time curve of the metabolite declines in parallel with that of the drug during terminal phases with similar slopes and half-lives (Fig. 8.8A). In this case, the formation of the metabolite from the drug is much slower than the elimination of the metabolite, and becomes rate-determining in overall changes in metabolite concentrations in the body. According to Eq. (8.5), when $k \ll k_{(m)}$, $e^{-k \cdot t} - e^{-k(m) \cdot t}$ approaches $e^{-k \cdot t}$ during later time points, since $e^{-k(m) \cdot t}$ is negligible compared to $e^{-k \cdot t}$. Equation (8.6) describes $C_m(t)$ during the later phase:

$$C_m(t) = \frac{k_m \cdot D_{iv}}{V_m \cdot k_{(m)}} \cdot e^{-k \cdot t} \tag{8.6}$$

The exponential term of $C_m(t)$ during the later time points, $-k \cdot t$ is equal to that of $C_p(t)$ [Eq. (8.3)], and thus terminal half-lives ($t_{1/2}$) of the drug and the metabolite become similar. If the metabolite itself is administered intravenously, its $t_{1/2}$ becomes shorter than that of the parent drug after intravenous injection. Rate constant terms in Eq. (8.6) can be replaced with clearance (Cl) and volume of distribution (V) terms as follows:

$$k_m = CL_m/V \qquad k(m) = Cl_{(m)}/Vm$$

and

$$C_m(t) = \frac{(Cl_m/V) \cdot D_{iv}}{V_m \cdot Cl_{(m)}/V_m} \cdot e^{-k \cdot t}$$

$$= \frac{Cl_m}{Cl_{(m)}} \cdot \underbrace{\frac{D_{iv}}{V} \cdot e^{-k \cdot t}}_{C_p(t)} \quad \text{during the terminal phase} \tag{8.7}$$

Cl_m is the metabolic clearance of the drug to produce the metabolite, and $Cl_{(m)}$ is the systemic clearance of the metabolite, which can be determined following intravenous injection of the metabolite itself. According to Eq. (8.7), the relative extent of plasma concentration levels of the drug [$C_p(t)$] and its metabolite [$C_m(t)$] during the terminal phase depends on the ratio between Cl_m and $Cl_{(m)}$ under the formation-rate-limited metabolite kinetic conditions.

Summary of formation-rate-limited metabolite kinetics:

1. The terminal half-life of the metabolite formed from the drug is similar to that of the drug itself.
2. If the metabolite itself is administered intravenously, its terminal half-life is shorter than that of the parent drug after intravenous injection.

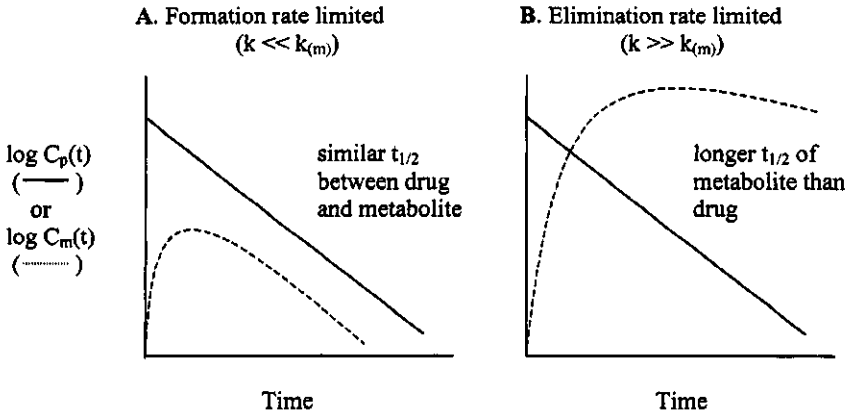


Figure 8.8. Plasma concentration *vs.* time profile of a drug [$C_p(t)$, —] and its metabolite [$C_m(t)$, ---], following intravenous drug administration, based on one-compartment models for both drug and metabolite on a semilog scale. A and B represent the formation-rate-limited and the elimination-rate-limited metabolite kinetics, respectively.

3. Relative plasma concentration levels of the drug and its metabolite during the terminal phase are dependent on the ratio between the metabolic clearance of the drug for producing the metabolite and the systemic clearance of the metabolite.

8.7.2. "Elimination-Rate-Limited" Metabolite Kinetics

The other extreme case is when k is much greater than $k_{(m)}$. In this case, the semilogarithmic plasma concentration *vs.* time curve of the metabolite will show a shallower slope with a longer half-life than the drug during the terminal phase (Fig. 8.8B). This is because when $k \gg k_{(m)}$ $e^{-k \cdot t}$ becomes much smaller than $e^{-k(m) \cdot t}$ at later time points:

$$C_m(t) = \frac{k_m \cdot D_{iv}}{V_m \cdot k} \cdot e^{-k_{(m)} \cdot t} \quad \text{during the terminal phase} \tag{8.8}$$

As indicated in Eq. (8.8), the exponential term of $C_m(t)$ during the terminal phase, $-k_{(m)}t$, becomes greater than that of $C_p(t)$, $-k \cdot t$ [Eq. (8.3)], and thus $t_{1/2}$ of the metabolite is longer than that of the drug. In this case, if the metabolite itself is administered intravenously, its $t_{1/2}$ is also longer than that of its parent drug after intravenous injection. Rate constants in Eq. (8.8) can be replaced with Cl and V terms as follows:

$$C_m(t) = \frac{(Cl_m/V) \cdot D_{iv}}{V_m \cdot (Cl/V)} \cdot e^{-k_{(m)} \cdot t}$$

$$= \frac{Cl_m}{Cl} \cdot \frac{D_{iv}}{V_m} \cdot e^{-k_{(m)} \cdot t} \quad \text{during the terminal phase} \tag{8.9}$$

Summary of elimination-rate-limited metabolite kinetics:

1. The terminal half-life of the metabolite formed from the drug is longer than that of the drug itself.
2. If the metabolite itself is administered intravenously, $t_{1/2}$ of the metabolite is longer than that of drug following intravenous administration.

Exception: As shown above, $t_{1/2}$ of the metabolite produced after administration of the parent drug cannot be shorter than that of the drug, regardless of the route of administration under linear conditions. It is, however, possible to have $t_{1/2}$ of the metabolite shorter than that of the drug during the later time points. This can be due either to product inhibition (Perrier *et al.,* 1973), ie., the metabolite inhibits metabolism of its parent drug or to rapid depletion of the cosubstrate(s) required for conversion of the drug to the metabolite during the early phase.

8.7.3. Pharmacokinetic Properties of Metabolites

In general, both $CI_{(m)}$ and V_m of metabolites tend to be smaller than those of the parent drugs, which appears to be due to the increased hydrophilicity of the metabolites as compared with the parent drugs. The following summarizes the general pharmacokinetic properties of metabolites:

1. Metabolites are more polar and hydrophilic than their parent drugs, and thus more readily excreted in the urine.
2. Metabolites are often acidic (by oxidation and/or glucuronide or sulfate conjugation of the neutral or basic parent drugs).
3. The volume of distribution of the acidic metabolite is usually smaller than that of the neutral or basic parent drug. This is because acidic metabolites tend to be highly albumin-bound, which may restrict their distribution from plasma to other tissues or organs, and the extent of tissue binding of acidic metabolites tends to be less than for their neutral or basic parent drugs.

8.7.4. Estimating Systemic Clearance of Metabolites

One of important concepts in metabolite kinetics is mass balance between the total amount of drug converted to the metabolite and the total amount of metabolite eliminated. Equation (8.4) can be expressed in clearance and concentration terms of a drug and its metabolite:

$$dA_m(t)/dt = Cl_m \cdot C_p(t) - CI_{(m)} \cdot C_m(t) \tag{8.10}$$

Integration of Eq. (8.10) from 0 to ∞ yields

$$\underbrace{\int_0^\infty \frac{dA_m(t)}{dt}\,dt}_{\uparrow\ 0} = Cl_m \cdot \int_0^\infty C_p(t)\,dt - Cl_{(m)} \cdot \int_0^\infty C_m(t)\,dt$$

$$0 = Cl_m \cdot AUC_{0-\infty} - Cl_{(m)} \cdot AUC_{m,0-\infty} \tag{8.11}$$

where $AUC_{0-\infty}$ and $AUC_{m,0-\infty}$ are the AUCs of a drug and its metabolite following drug administration from 0 to ∞, respectively. There is no metabolite in the body at time 0 and ∞; therefore integration of the rate of change in the amount of the metabolite from 0 to ∞ becomes 0. Equation (8.12) represents a mass balance between the amount of drug converted to metabolite and the amount of metabolite eliminated from the body from time 0 to ∞:

MASS BALANCE OF METABOLISM:

Total amount of drug transformed to metabolite from time 0 to ∞ — *Total amount of metabolite eliminated from time 0 to ∞*

(8.12) $$AUC_{0-\infty} \cdot Cl_m = AUC_{m,0-\infty} \cdot Cl_{(m)}$$

This relationship is true regardless of the route of drug administration. Important assumptions include linear kinetics, i.e., drug or metabolite concentration-independent Cl_m and $Cl_{(m)}$, and no effects of the metabolite on the drug elimination mechanism(s). When the drug is also eliminated other than via metabolism (Cl_{other}), Cl_m can be expressed as the difference between the systemic clearance of the drug (Cl_s) and Cl_{other}:

(8.13) $$Cl_m = Cl_s - Cl_{other}$$

It can be difficult to measure Cl_m experimentally for a particular metabolite when there several different metabolites are generated from the drug. $Cl_{(m)}$ of the particular metabolite can be determined following intravenous administration of the metabolite itself.

8.8. INDUCTION OF METABOLISM

Administration of certain xenobiotics sometimes results in a selective increase in the concentration of metabolizing enzymes in both phase I and II metabolism, and thereby in their activities (Barry and Feely, 1990; Okey, 1990; Park, 1987). Enzyme induction becomes important especially when polypharmacy involves drugs with narrow therapeutic windows, since the induced drug metabolism could result in a significant decrease in its exposure and therapeutic effects. In addition, enzyme induction may cause toxicity, associated with increased production of toxic metabolites.

8.8.1. Mechanisms of Induction

- Stimulation of transcription of genes and/or translation of proteins, and/or stabilization of mRNA and/or enzymes by inducers, resulting in elevated enzyme levels.

- Stimulation of preexisting enzymes resulting in apparent enzyme induction without an increase in enzyme synthesis (this is more common *in vitro* than *in vivo).*
- In many cases, the details of the induction mechanisms are unknown. The following two receptors have been identified for CYPlA1/2 and CYP4A1/2 induction: (a) *Ah* (aromatic hydrocarbon) receptor in cytosol, which regulates enzyme (CYP1A1 and 1A2) induction by polycyclic aromatic hydrocarbon (PAH)-type inducers; and (b) peroxisome proliferator activated receptor (PPAR), where hypolipidemic agents cause peroxisome proliferation in rats (CYP4A1 and 4A2); humans have low PPAR and show no effects from hypolipidemic agents.

8.8.2. Characteristics of Induction

- Induction is a function of intact cells and cannot be achieved by treating isolated cell fractions such as microsomes with inducers. Evaluation of enzyme induction is usually conducted in *ex vivo* experiments, ie., treating animals *in vivo* with potential inducers and measuring enzyme activities *in vitro* or in cell-based *in vitro* preparations such as hepatocytes, liver slices, or cell lines. Recent studies have demonstrated that primary cultures of hepatocytes can be used for studying the inducibility of metabolizing enzymes such as P450 under certain incubation conditions(Silva, 1998).
- Enzyme induction is usually inducer-concentration–dependent. The extent of induction increases as the inducer concentration increases; however, above certain values, induction starts to decline.
- In general, inducers increase the content of endoplasmic reticulum within hepatocytes as well as liver weight.
- In some cases, an inducer induces enzymes responsible for its own metabolism (so-called "autoinduction").

8.8.3. Inducing Agents

In general, enzyme inducers are lipophilic at physiological pH and exhibit relatively long $t_{1/2}$ with high accumulation in the liver. There are several different classes of enzyme inducers.

1. Barbiturates: phenobarbitone, phenobarbital.
2. Polycyclic aromatic hydrocarbons (PAH): 3-methylcholanthrene (3-MC), 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD), β-naphthoflavone β(-NF).
3. Steroids: pregnenalone 16-α-carbonitrile (PCN), dexamethasone.
4. Simple hydrocarbons with aliphatic chains: ethanol (chronic), acetone, isoniazid.
5. Hypolipidemic agents: clofibrate, lauric acids.
6. Macrolide antibiotics: triacetyloleandomycin (TAO).
7. A wide variety of structurally unrelated compounds: e.g., antipyrine, carbamazepine, phenytoin, and rifampicin.

Table 8.12 summarizes cytochrome P450 isoforms subject to induction and their corresponding inducing agents in humans.

Table 8.12. A Summary of Inducible Cytochrome P450 Enzymes in Human Liver and Known Inducers[a]

CYP	Inducers
1A1/2	Lansoprazole, omeprazole
2A6	Anticonvulsants, barbiturates, dexamethasone, rifampicin
2B6	Anticonvulsants, rifampicin
2C8, 9, 19	Anticonvulsants, barbiturates, rifampicin
2E1	Ethanol, isoniazid
3A4	Anticonvulsants, barbiturates, dexamethasone, rifampicin

[a]Among human cytochrome P450 isoforms, CYP2D6, 3A5, 4A9, and 4A11 are not inducible.

8.8.4. Time- and Dose-Dependence of Induction

The time course of induction may vary with different inducing agents. Increased transcription of P450 mRNA has been detected in a nucleus as early as 1 hr after administration of phenobarbiton in rats, although the maximum induction may take 2–3 days. Induction is usually reversible, i.e., when the inducer is removed, enzyme levels return to normal. In general, the degree of induction increases with the inducer dose; however, after certain dose levels, induction starts to decline.

8.8.5. Species Differences in Induction

There are significant differences in the inducibility of inducers among different species. Table 8.13 lists some of the important inducers with species-dependent P450 induction.

Table 8.13. Differences in Induction between Human and Laboratory Animals for Cytochrome P450 Enzymes

CYP	Species	Inducibility
CYP1A	Human and dog	Inducible by omeprazole
	Rabbit and mouse	Noninducible by omeprazole
CYP3A	Human and rabbit	Inducible by rifampicin, but not by PCN[a]
	Rat	Inducible by PCN, but not by rifampicin

[a]PCN: pregnenalone 16-α-carbonitrile.

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