3

New Approaches for High Throughput *In Vivo* Exposure Screening

Rapid turn-around of high-quality pharmacokinetic data in animals has long been recognized as a critical, yet potentially rate-limiting step during drug discovery in the pharmaceutical industry. In particular, the recent advent of combinatorial chemistry, which has dramatically increased the number of compounds synthesized during discovery, has triggered a reevaluation of the conventional one-at-a-time approach as primary *in vivo* exposure screening (Kubinyi, 1995 and Tarbit and Berman, 1998). Recent advances in liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)-based quantitative analytical techniques have made it possible to increase the throughput of *in vivo* exposure screening to a significant extent, and several innovative experimental approaches have been introduced to facilitate it. Those include:

1. N-in-1 (cassette or cocktail) dosing.
2. Postdose pooling (or cocktail analysis).
3. AUC estimation from one pooled sample.
4. Continuous sampling.

Depending on study needs and assay capability, a researcher can use only one of the above approaches or combine them for exposure screening in animals. Brief backgrounds and study design strategies for each method are discussed below.

3.1. N-IN-1 (CASSETTE OR COCKTAIL) DOSING

The N-in-1 approach implies administration of a mixture of several compounds in one dosing vehicle to animals as opposed to dosing individual compounds in one vehicle to individual animals at a time. Modern analytical methods such as LC/MS/MS allow the simultaneous measurement of concentrations of multiple compounds coexisting in biological samples with little method development time

(Beaudry *et al.,* 1998 and Olah *et al.,* 1997). There are several important factors to be considered for the N-in-1 dosing with LC/MS/MS (Frick *et al.,* 1998

1. *Number of compounds*

- Mass spectrometry sensitivity. The number of compounds dosed should be detrmined based on assay sensitivity and selectivity as the more compounds that are included, the less sensitive the assay will be. The problem of sensitivity is further compounded because as more drugs are combined in one dose vehicle, a smaller amount of any individual drug is usually given.

- Drug–drug interaction. Any carrier or enzyme-mediated pharmacokinetic process such as cytochrome P450-mediated metabolism is potentially subject to drug–drug interaction, depending on compounds and their concentrations, and the possibility of drug–drug interaction increases as the number of compounds dosed increases.

2. Grouping of compounds

- Compounds with similar physicochemical properties. Compounds with similar aqueous solubility and ionizability (acid or base) are preferable for grouping in the same vehicle, primarily in order to maintain homogeneity and reproducibility of a dosing formulation and sample preparation for assay.

- Compounds with minimal interference in mass spectrometry assay. Preferably, compounds producing the same product (or daughter) ion should be avoided in the same dosing regimen, when the multiple reaction monitoring (MRM) mode is used for the mass spectrometry assay. Compounds in which molecular weights differ by 16 (potential oxidative metabolite) from other compounds should be also avoided because similar fragmentation patterns of potential oxidative metabolites of the other compounds can interfere the MS assay. Compounds best ionized in a different ion mode (positive *vs*. negative ion mode) of MS should not be combined.

3. Dose

- Constant and low dose level. The total dose of compounds should be held constant and as low as possible to minimize potential drug–drug interaction during the various stages of drug absorption and disposition.

4. Reference compound

- Reduce study variability. The inclusion in each study of a reference compound with known exposure profiles as a potential indicator of drug–drug interaction and a biological internal standard minimizes both intra-/interanimal and experimental variabilities among studies. Changes in exposure profiles of this compound might indicate potential drug–drug interactions among compounds examined and/or dosing errors. A similar approach has been used to assess the activities of metab-

Table 3.1. Advantages and Disadvantages of N-in-1 Dosing as Compared to Dosing with Individual Compounds for *In Vivo* Exposure Screening

Advantages	Disadvantages
Rapid screening with more compounds	Needs LC/MS/MS for analysis
Fewer samples for assay	Potential drug–drug interaction
Fewer animals required for studies	More problematic data analysis

olizing enzymes *in vivo* (Frye *et al.,* 1997) and to estimate the extent of membrane permeability of compounds in Caco-2 cells (Taylor *et al.,* 1997).

3.2. POSTDOSE POOLING (OR COCKTAIL ANALYSIS)

As an alternative to N-in-1 dosing, plasma samples collected from different animals after dosing individual drugs can be combined for assay. This method may be useful if significant drug–drug interactions are expected among compounds when they are dosed in one vehicle, despite the fact that its use requires more animals and resources for the study (Kuo *et al.,* 1998).

3.3. AUC ESTIMATION FROM ONE POOLED SAMPLE

For the extent of drug exposure after oral administration, $AUC_{0\text{-}t_{last}}$ is often considered more relevant than C_{max}. The conventional technique for estimating $AUC_{0-t_{last}}$ is to measure plasma concentrations at each time point and calculate AUC with those individual concentrations at different time points. Another interesting approach to the higher throughput of oral exposure is to prepare one *pooled* sample by combining different aliquots of the individual samples at all time points and to calculate $AUC_{0-t_{last}}$ by multiplying its concentration (C_{pool}) with a sampling time interval between time zero and t_{last} (Hop *et al.,* 1998).

$$AUC_{o-t_{last}} = C_{pool} \cdot t_{last} \tag{3.1}$$

In other words, plasma samples from the same animal at different time points are pooled in a weighted ratio that reflects the size of their respective time interval. The advantages of this method is that far fewer samples have to be analyzed and pooled AUC values can still be calculated for each animal. The disadvantage is that the entire concentration *vs*. time profile for the compound cannot be obtained. Mathematical manipulation to calculate proper fractions ($f_0, f_1, f_2, \ldots, f_{last}$) of the total volume of the pooled sample needed for aliquoting individual samples at each time point ($0, t_1, t_2, \ldots, t_{last}$) is based on the linear trapezoidal rule for estimating AUC, as seen in Table 3.2 (Hamilton *et al.,* 1981).

For comparison of AUC values between the sample pooling method and the

Table 3.2. Fractions of Total Volume of a Pooled Sample Required for Aliquoting Individual Samples at Each Time Point for Estimation of $AUC_{0-t_{last}}$

	Fraction of total volume of a pooled sample required for each sampling time point							
Time[a]	0	t_1	t_2	t_3	t_4	t_5	t_6	t_7
t_1	$\frac{1}{2}$	$\frac{1}{2}$	—	—	—	—	—	—
t_2	$t_1/2t_2$	$t_2/2t_2$	$(t_2-t_1)/2t_2$	—	—	—	—	—
t_3	$t_1/2t_3$	$t_2/2t_3$	$(t_3-t_1)/2t_3$	$(t_3-t_2)/2t_3$	—	—	—	—
t_4	$t_1/2t_4$	$t_2/2t_4$	$(t_3-t_1)/2t_4$	$(t_4-t_2)/2t_4$	$(t_4-t_3)/2t_4$	—	—	—
t_5	$t_1/2t_5$	$t_2/2t_5$	$(t_3-t_1)/2t_5$	$(t_4-t_2)/2t_5$	$(t_5-t_3)/2t_5$	$(t_5-t_4)/2t_5$	—	—
t_6	$t_1/2t_6$	$t_2/2t_6$	$(t_3-t_1)/2t_6$	$(t_4-t_2)/2t_6$	$(t_5-t_3)/2t_6$	$(t_6-t_4)/2t_6$	$(t_6-t_5)/2t_6$	—
t_7	$t_1/2t_7$	$t_2/2t_7$	$(t_3-t_1)/2t_7$	$(t_4-t_2)/2t_7$	$(t_5-t_3)/2t_7$	$(t_6-t_4)/2t_7$	$(t_7-t_5)/2t_7$	$(t_7-t_6)/2t_7$

[a] t_1, t_2, t_3, t_4, t_5, t_6, t_7 are sample time points.

conventional method with individual concentrations, let us assume that plasma samples at 0, 0.25, 0.5, 1, 2, 4, 7, and 24 hr postdose after oral administration of a drug have concentrations of 0, 1, 3, 10, 7, 5, 4, and 1 μg/ml, respectively. If the volume of a single pooled sample is set for 480 l, the volumes of plasma samples at different time points to prepare the pooled sample are 2.5, 5, 7.5, 15, 30, 50, 200, and 170 μl at 0, 0.25, 0.5, 1, 2, 4, 7, and 24 hr, respectively (Table 3.3). Thus, in theory, C_{pool} is equal to 3.338 g/ml, as shown below, based on the fractions (f_i) of the total volume of a pooled sample:

$$\begin{aligned} C_{Pool} &= C_0.f_0 + C_1.f_1 + C_2.f_2 + C_3.f_3 + C_4.f_4 + C_5.f_5 + C_6.f_6 + C_7.f_7 \\ &= 0.0.005 + 1.0.01 + 3{\cdot}0.015 + 10.0.03 + 7{\cdot}0.104 + 5{\cdot}0.104 \\ &\quad + 4.0.417 + 1.0.354 \\ &= 3.338 \text{ g/ml} \end{aligned}$$

where $C_0, C_1, \ldots, C_7$ are the concentrations of the drug at 0, 0.25, ..., 24 hr postdose, respectively. In other words, the measured C_{pool} value should be close to 3.338 (g/ml, and thus $AUC_{0-t_{last}}$ based on C_{pool} is approximately 80.112 g.hr/ml:

$$AUC_{0-t_{last}} = C_{pool}.t_{last} \approx 3.338{\cdot}(24-0) = 80.112 \text{ g·hr/ml}$$

AUC estimated using the conventional method with concentrations of individual

Table 3.3. Fractions of the Volume of a Pooled Sample Required from Individual Samples at Various Times Postdose

	Fractions of the volume of a pooled sample for each sampling time point (hr)							
	0	0.25	0.5	1	2	4	7	24
24	0.25/48	0.5/48	0.75/48	1.5/48	3/48	5/48	20/48	17/48
Fraction (f_i)	f_0	f_1	f_2	f_3	f_4	f_5	f_6	f_7

samples at different time points based on the linear trapezoidal rule is 80.375 μg·hr/ml, which is in good agreement with the value using C_{pool}. This so-called "pooling method" can be combined with N-in-1 dosing to further reduce the number ofsamples required for assay.

3.4. CONTINUOUS SAMPLING METHOD

Instead of intermittent sampling followed by subsequent sample pipetting for a pooled sample, continuous blood withdrawal from animals at a suitable flow rate has been explored to obtain a single sample for each animal (Humphreys *et al.,* 1998). The major advantage of this method over the sample pooling method is a reduction in the time required for sample collection and processing (pipetting). $AUC_{0\text{-}tlast}$ can be calculated by multiplying the concentration of a single sample obtained from continuous withdrawal and the sample withdrawal period:

(3.2) $$AUC_{0-t_{last}} = C_{ss_{cw}} \times P_w$$

where C_{sscw} is a drug concentration of a single sample obtained from the continuous withdrawal and P_w is a withdrawal period.

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4

Absorption

Oral dosing is the most common route for the administration of drugs and most of the drugs given orally are generally designed to show systemic pharmacological efficacy rather than local effects in the gastrointestinal (GI) tract. To achieve desirable systemic exposure levels, i.e., plasma or blood drug concentrations, many pharmacokinetic studies are concerned with the bioavailability of drugs after oral administration. As the drug passes down the GI tract, part of the dose may not be available for absorption owing to poor aqueous solubility, limited membrane permeability, and/or chemical or biological degradation, ie., limited absorption. Drug molecules absorbed into the intestinal membranes can then be further subject to intestinal and/or hepatic elimination before reaching the systemic circulation, i.e., first-pass elimination. A thorough understanding of the quantitative contributions of these two processes during absorption is important for enhancing the oral bioavailability of drugs. In this chapter, various physiological factors and physicochemical properties of drug molecules that are critical for oral absorption, factors affecting the first-pass elimination, and various experimental approaches for assessing oral bioavailability are discussed.

4.1. RATE-LIMITING STEPS IN ORAL DRUG ABSORPTION

For drugs orally dosed in solid dosage forms such as tablets or capsules, there are two distinctive processes during absorption: *dissolution* of solid drug particles to drug molecules in the GI fluid and *permeation* of the drug molecules across intestinal membranes (Fig. 4.1). Depending on the relative magnitude of the rates of these two processes, one of them can be rate-limiting in overall drug absorption.

4.1.1. Dissolution Rate-Limited Absorption

As a prerequisite for oral absorption, the drug must be present in aqueous solution except in the case of pinocytosis or for the lymphatic absorption pathways. When a drug is administered in solid dosage formulations such as tablets, *disintegration* of the dosage form into small solid particles in a suspension should occur prior to the dissolution of the particles. In general, disintegration occurs much faster than

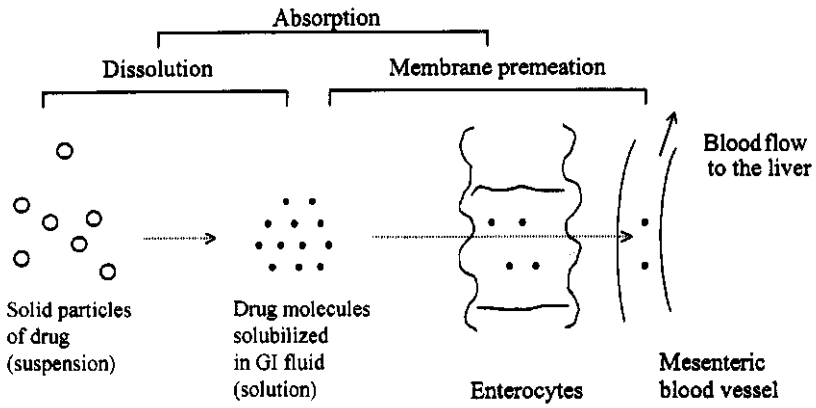

Figure 4.1. Potential rate-limiting steps in drug absorption processes after oral administration of solid dosage forms such as tablets or capsules.

dissolution. For most drugs with high lipophilicity, the rate of absorption can be governed primarily by the dissolution rate of the drug particles. The surface area of the particles, the aqueous solubility of drug, the pH of the GI fluid, and the extent of mixing in the GI tract are the important factors affecting the dissolution rate of solid drug particles.

- Dissolution. The dissolution rate of drug molecules from solid drug particles into a surrounding aqueous medium is a function of the aqueous solubility of the drug, the surface area of the particles, and the dissolution rate constant, and is expressed by the Noyes–Whitney equation:

$$dC/dt = k \cdot S \cdot (C_s - C_t) \tag{4.1}$$

where dC/dt is the rate of dissolution of a solid drug particle; k is the dissolution rate constant; S is the surface area of the dissolving solid drug particle; C_s is the saturation concentration of the drug in the diffusion layer, which can be close to the maximum solubility of drug, as the diffusion layer is considered saturated with drug; and C_t is the concentration of the drug in the surrounding dissolution medium at time t (Fig. 4.2). As a solid drug particle undergoes dissolution, the drug molecules on its surface are the first to diffuse into the solution adjacent to the particle, generating a saturated layer of drug solution that envelops the particle surface. From this drug-saturated solution layer, which is called a diffusion layer, drug molecules dissolve into the surrounding medium, and the layer is continuously replenished with newly diffused drug molecules from the surface of the particle.

The dissolution rate of a drug can be increased by:

1. Increasing the surface area of the particles by reducing particle size (grinding, jet-milling, etc.).
2. Increasing the aqueous solubility of the drug (elevating temperature, changing pH in the case of ionizable drugs, etc.).

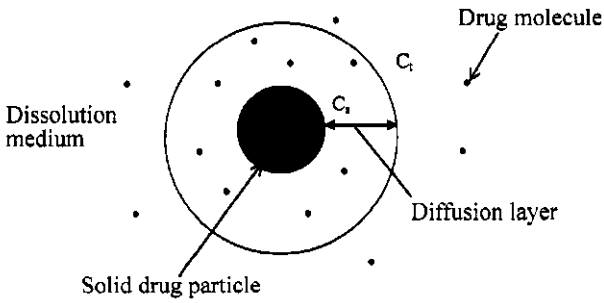

Figure 4.2. Schematic description of drug dissolution from solid drug particles in suspension into surrounding dissolution medium. C_s is the saturation concentration of a drug in the diffusion layer and C_t is the concentration in the surrounding dissolution medium at time t.

3. Increasing the dissolution rate constant (agitating the medium, increasing temperature, decreasing viscosity of the medium, etc.).

4.1.2. Membrane Permeation Rate-Limited Absorption

If the dissolution process is very rapid, the absorption rate of a drug could be dependent primarily on its ability to transport across the intestinal membrane. For highly water-soluble compounds, the membrane permeation can become critical in overall absorption owing to their limited ability to partition into the lipid bilayers of the enterocyte membranes (Fig. 4.3).

4.1.2.1. Permeation

The permeation rate of a drug via the intestinal membrane after oral administration can be expressed as a function of the intestinal membrane permeability (P_{int}), the effective surface area (S_{int}) of intestinal membrane available for permeation of drug molecules, and the concentration of the drug (C_{int}) in the GI fluid:

$$\text{Permeation rate} = P_{int} \cdot S_{int} \cdot C_{int} \tag{4.2}$$

A fraction of oral dose (F_{abs}) absorbed into the portal vein after dosing in an aqueous solution, i.e., no dissolution process, can be estimated using Eq. (4.3) assuming the absence of gut microflora and intestinal metabolism. The utility of Eq. (4.3) to *in vivo* oral drug absorption is rather limited, however, because in most cases, estimates of P_{int} and S_{int} obtained from *in vitro* or *in situ* experiments might not be relevant for *in vivo* conditions:

$$F_{abs} = 1 - e^{-P_{int} S_{int}/Q_{int}} \tag{4.3}$$

4.1.2.2. Permeability

The permeability of a drug reflects how readily the drug molecules pass through membranes. Three major factors governing the permeability of compounds are

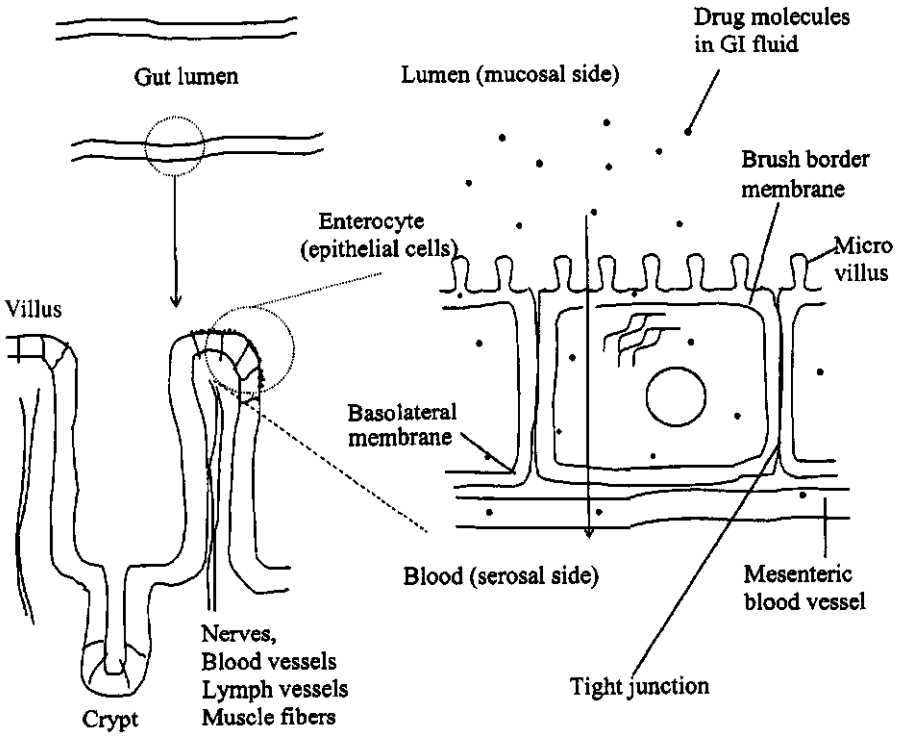

Figure 4.3. Intestinal villus and epithelial cells. The apical surface of the small-intestinal epithelium is covered with villi (0.5–1.0 mm long), which have a single layer of columnar epithelial cells along the surface connected by tight junctions forming barriers between the lumen and serosal capillaries. The villous cells have microvilli (100 nm in diameter), which form brush border membranes, increasing the cell surface area. The average life span of mucosal cells is 2–5 days. The arrow indicates an absorptive direction of drug from the luminal side of the gut to the basolateral side.

lipophilicity, molecular size, and charge. Permeability has a unit of velocity, i.e., distance/time. The product of permeability and surface area available to permeation of a drug can be viewed as distributional clearance with the units of flow rate, i.e., volume/time.

4.2. FACTORS AFFECTING ORAL ABSORPTION

The following is a summary of physiological and physicochemical factors of drugs that affect the rate and extent of oral absorption.

4.2.1. Physiological Factors

(i) Gastric motility and residence time. A small surface area of the stomach may be less favorable for drug absorption compared to the small intestine, the major

absorption site in the GI tract. However, the gastric residence time of a drug can be critical for immediate-release solid dosage forms such as regular tablets and capsules. An important physiological factor dictating the gastric residence time of a drug is gastric motility (Dressman, 1986; Kararli, 1995; Walter-Sack, 1992), which has two distinct modes depending on the presence of food (fasted *vs*. fed states). Gastric motility during the fasted state has three different phases, phases I, II, and III, which repeat periodically (e.g., every 2 hr in humans). Phase I, a quiescent phase, accounts for about half of the fasting cycle period, during which there is little contractile activity. In phase II, irregular contractions start to occur and gradually increase in amplitude and frequency. These progress into a maximal amplitude and frequency of contraction, which is referred to as phase 111. During phase III, the strong contraction can expel the entire gastric content into the small intestine (this process is the so-called *housekeeper wave).* Phase III activity in the stomach is usually associated with the initiation of a *migrating motility complex* (MMC) in the duodenum, which then proceeds to migrate through the small intestine toward the ileum. At the end of phase III activity, stomach motility reverts back to the quiescent phase. When food enters the stomach, ie., in the fed state, the contractions of the stomach return to a level lower than that of phase III. These regular tonic contractions of the stomach propel food toward the antrum while grinding and mixing it with gastric secretions. Implications of gastric residence time on drug absorption should be considered in conjunction with dosage forms. For instance, the gastric residence time of drugs given in liquid dosage forms will depend on the liquid emptying time and the total volume of liquid administered. Since large objects empty only during phase III of the fasted state, the gastric residence time of nondisintegrating solid dosage forms will depend on the frequency of phase III activity, if drug is given in the fasted state, and the time-restoring phase III activity if given in the fed state.

(ii) pH in the gastrointestinal tract. The pH ranges in the human stomach and intestines from the duodenum to the colon are about 1 to 3 and 5 to 8, respectively. For acidic or basic drugs, the un-ionized forms of drugs, if sufficiently lipophilic, are better absorbed than their ionized counterparts. How large a fraction of a drug exists in the un-ionized form in the GI fluid depends on both the drug dissociation constant (K_a) and the pH of the GI fluid. For instance, acidic drugs with pK_a between 4 and 8 exist predominantly as un-ionized forms at the low pH of gastric fluid, may be absorbed in part from the stomach, and can be partially un-ionized in the intestine. Very weak acids ($pK_a > 8$) are essentially un-ionized throughout the entire GI tract. On the other hand, most bases are poorly absorbed in the stomach, as they are largely ionized there at low pH. Weak bases ($pK_a < 5$) are essentially un-ionized throughout the intestine. In some cases, the drug itself can induce changes in the pH of the GI fluid (Dressman *et al.,* 1993).

(iii) Intestinal surface area and transit time. The entire GI tract is lined with a continuous sheet of epithelial cells. The stomach lacks the villus structure of other areas of the GI tract. Owing to the large surface area it offers for absorption by numerous microvilli and relatively long transit time for drug molecules to pass through, the small intestine is considered to be the major site of oral absorption for

most drugs. The colon has a longer transit time than the small intestine; however, the more viscous contents of the colon (low permeability) and the lack of villi (smaller surface area) tend to offset the effects of the longer transit time for drug absorption. In addition, cellular morphology and functions of epithelial cells are quite variable in different segments of the GI tract. Some sites in the GI tract are primarily involved in selective absorption of various nutrients, but not others. Some areas are better equipped for secretion than absorption, while others perform both functions (Hunt and Groff, 1990).

(iv) Food. Food intake stimulates GI secretions including hormones and bile salts, which lower gastric pH, delay stomach emptying, and increase GI transit time. Fluid volume and the quantity of dietary fat in the meal appear to be the primary food-related factors affecting drug absorption. For instance, fluids ingested with the meal can increase the available gastric volume up to as much as 1.5l in humans. The increased secretion of bile salts induced by a fatty meal can enhance the stability of the emulsion phase within the gut lumen, which will increase absorption of lipophilic drugs. The absorption profiles of hydrophilic drugs, however, appear not to be significantly altered when these drugs are given with a fatty diet. Limited studies have suggested that dietary protein could induce an increase in splanchnic and hepatic blood flow, whereas dietary fat does not alter hepatic blood flow (Baijal and Fitzpatrick, 1996; Winne, 1980; Zhi *et al.,* 1995).

(v) Intestinal microflora. There are almost 400 different microorganisms in the GI tract. Some of the microflora residing in the GI tract can metabolize a variety of drugs, which can reduce the amount available for absorption. Hydrolysis of esters and amides, reduction of double bonds, and nitro and diazo groups, dehydroxylation, dealkylation, deamination, acetylation, and esterification are some of metabolic reactions mediated by gut microflora.

(vi) Otherfactors.

1. Wetting of drug particles by the gastric juice or the intestinal fluid.
2. Blood circulation to the site of absorption.
3. Active transporters.
4. Disease state.

4.2.2. Physicochemical Factors of Drugs

(i) Hydrophilicity and lipophilicity. A balance between hydrophilicity and lipophilicity of a drug is important in oral absorption. In general, log $D_{7.4}$ values of compounds between -0.5 and 2 are considered to be optimal for oral absorption.

(ii) Ionizability and charge. The un-ionized form of a drug is better absorbed than its ionized counterpart, and a drug's ionizability is influenced by both the pK_a of the drug and the pH of the GI fluids.

(iii) Chemical stability. Hydrolysis can occur for ester or amide moieties of a drug at acidic or alkaline pH in the GI tract.

(iv) Particle size in suspension. Reduction of particle sizes of solid drug particles usually enhances the dissolution rate of the particles in suspension by increasing their surface area.

(v) Polymorphism of crystalline forms. Many different crystalline (polymorphic) forms of a drug can exist, each of which has a different energy level and different physicochemical properties, including, e.g., melting point, solubility, density, and refractive index. Owing to different kinetic solubilities, dissolution rates can also vary among polymorphic particles. When oral absorption of a drug is limited by dissolution rate, the polymorphic form of the solid drug particles can be important in determining oral bioavailability. In general, an amorphous (metastable) form of a drug has a higher kinetic solubility as compared with crystalline forms. An amorphous form is unstable and converts to the more stable crystalline form with low kinetic solubility during manufacturing and storage, which rather limits its commercial potential.

(vi) Molecular size. Absorption pathways can be affected by the size of drug molecules. Paracellular transport via tight junctions between enterocytes can be an important absorption pathway for small highly water-soluble molecules with molecular weights below 200. As the molecular weight increases, transcellular transport (passive diffusion or active transport) becomes more important.

(vii) Complexation. The rate and extent of oral absorption of many drugs such as tetracycline derivatives and cefdinir, an oral cephalosporin, can be significantly impaired if they form water-insoluble complexes with polyvalent metal ions such as Ca^{+2}, Mg^{+2}, Fe^{+3}, or Al^{+3}, which are often present in food (Hörter and Dressman, 1997).

4.2.3. Effects of the pH and pK_a of a Drug on Absorption (pH-Partition Theory)

Owing to the lipoidal properties of the membrane, passively diffused drugs must undergo partitioning from the aqueous GI fluids into the membrane and eventually into the blood. This concept of absorption by partitioning processes of a drug between water and lipid at different pH is known as the "pH-partition theory" of drug absorption, and it addresses relationships among three different factors affecting partition processes of oral absorption of a compound: the dissociation constant and lipophilicity of the compound and the pH at the absorption site. For an ionizable compound, its un-ionized form is considered to be better partitioning into lipophilic membranes than the ionized counterpart. The ionizability of a compound in aqueous solution is a function of both the dissociation constant (K_a) of the compound and the pH of the surrounding solution. The dissociation constant is often expressed as a pK_a (the negative logarithm of the acidic dissociation constant) for both acidic and basic compounds. The relationship between the pH and pK_a of a compound is described by the Henderson–Hasselbalch equation as shown in Eqs. (4.4) and (4.5).

FOR AN ACIDIC COMPOUND [HA]:

$$PK_a = PH + \log([HA]/[A^-]) \quad (4.4)$$

Concentration of un-ionized compound (acid)

Concentration of ionized compound (salt)

FOR A BASIC COMPOUND [B]:

$$PK_a = PH + \log([BH+]/[B]) \quad (4.5)$$

Ionized (salt)

Un-ionized (base)

A ratio of the concentrations between the un-ionized ([HA] and [B]) and ionized ([A^-] and [BH^+]) forms of acidic and basic compounds in aqueous solution at different pH can be obtained based on these equations. For instance, a ratio between [HA] and [A^-] of an acidic compound with pK_a of 4 in aqueous solution at pH of 7 would be 0.001, which means that the concentration of A^- is 1000-fold greater than that of HA in aqueous solution at pH 7:

Ratio between un-ionized and ionized forms

$$\underset{\downarrow}{pK_a} \quad \underset{\downarrow}{pH} \quad \underset{\downarrow}{\log([HA]/[A^-])}$$

$$4 = 7 + \log([HA]/[A^-])$$

Let us assume that there are a carboxylic acid (R–COOH) with pK_a of 4 and a primary amine ($R\text{-}CH_2\text{-}NH_2$) with pK_a of 9. The ratios between the ionized and the un-ionized concentrations of these compounds in aqueous solution at different pH are summarized in Table 4.1, according to the Henderson–Hasselbalch equation. In the stomach ($pH \approx 2$), a ratio of the ionized (R–COO-) to the un-ionized (R–COOH) forms of the carboxylic acid would be 1:99, i.e., most of the acid is unionized, whereas the primary amine would exist mainly in ionized form ($R\text{–}CH_2\text{–}NH_3^+$) with a negligible amount of the un-ionized ($R\text{–}CH_2\text{-}NH_2$) form. On the other hand, in the intestine ($pH \approx 6$), carboxylic acid exists primarily as the ionized form (ionized:un-ionized = 99:1) and so does the amine (ionized:un-ionized = 99.9:O.l). Therefore, owing to its lower pH, absorption of acidic compounds in the stomach is favored over that in the intestine, although intestinal absorption of compounds, including acids, is quantitatively more important than stomach absorption because of the longer transit time and larger surface area. The pK_a values of several common structural moieties of organic compounds are shown in Table 4.2.

Table 4.1. Ratios of Ionized and Un-Ionized Forms of an Acid (e.g., R–COOH) with pK_a of 4 or a Base (e.g., $R–CH_2–NH_2$) with pK_a of 9 at Different pH

	Ionized: Un-ionized ≈	
pH	Acid ($pK_a = 4$)	Base ($pK_a = 9$)
13		
12	Decreasing	0.1:99.9
11	un-ionized form	1:99
10		10:90
9[a]		50:50
8		90:10
7	99.9:0.1	99:1
6	99:1	99.9:0.1
5	90:10	
4[a]	50:50	
3	10:90	Decreasing
2	1:99	un-ionized form
1	0.1:99.9	

"Note that at the pH value equal to the pK_a of the compounds, the ratios between un-ionized and ionized forms of the compounds become unity. At pH higher than pK_a, there are more ionized molecules than un-ionized molecules for the acid and vice versa for the base.

Table 4.2. The pK_a Values of Common Structural Moieties in Organic Compounds

Chemical moiety	Structure	Acid or base	pK_a[a]
Carboxylic acid	R–COOH	Acid	
	(R: aliphatic)	4–5	
	(R: aromatic)	9–10	
Phenol	$R–C_6H_4OH$	Acid	10
	(R: aliphatic)		
Sulfonic acid	$R–SO_3H$	Acid	< 1
Sulfonamide	$C_6H_5–SO_2–NH–R$	Acid	
	R = H	10	
	R = aromatic or heterocycles		5–7
Hydroxamic acid	R–CO–NHOH	Acid	9
Amine	$R–NH_2$, R_2NH, R_3N	Base	
	(R: aliphatic)		9–10
	(R: aromatic)		4–5
Pyridine	C_5H_5N	Base	5.2
N-oxide	$R_3N–O$	Base	
	(R: aliphatic)		4.6
	$C_5H_5N–O$ (pyridine N-oxide)		0.8
Quaternary Ammonium salt	$R_4–N^+$	Polar cation	Fully ionized Over pH 1 to 13
Alcohol	$RCH_2–OH$	Neutral	
Ether	R–O–R′	Neutral	
Ketone	R–CO–R′	Neutral	
Ester	R–COO–R′	Neutral	
Amide	R–CONH–R′	Neutral	

"The lower the pK_a of an acid, the more acidic the compound, whereas the higher the pK_a of a base, the more basic the compound.

4.2.4. Partition and Distribution Coefficients

Important pharmacokinetic properties of a compound such as metabolism, membrane transport (distribution), and passive absorption can be influenced by several of its physicochemical properties, including lipophilicity [partition (P) and distribution coefficients (D)], molecular weight and surface area (Krarup, 1998; Palm *et al.,* 1996), the ionization state, and the hydrogen-binding capacity (Lipinski *et al.,* 1997). In particular the lipophilic characteristic of a drug has been recognized as one of the important factors governing the extent of protein binding, metabolism, and absorption (Lee *et al.,* 1997; Testa *et al.,* 1997).

The partition coefficient (P, or log P as generally described) of a compound is defined as the ratio of the concentrations of the un-ionized compound in organic and aqueous phases at equilibrium. The partition coefficient can be viewed as an indicator of intrinsic lipophilicity in the absence of ionization or dissociation of the compound. Octanol is the most widely used organic phase for log P measurement of organic compounds.

Distribution coefficient (D, or log D as generally described) is defined as the overall ratio of organic and aqueous phases of a compound, ionized and un-ionized, at equilibrium. When the compound is partially ionized in the aqueous phase, not only the partition equilibrium of un-ionized compound between the aqueous and the organic phases but also the dissociation equilibrium between un-ionized and ionized compound within the aqueous phase will be established. Only the un-ionized form is considered to distribute between the aqueous and the organic phases. These processes are elucidated in the following scheme:

$$[\text{Ionized drug}]_{\text{aq phase}} \overset{\text{Dissociation equilibrium}}{\rightleftharpoons} [\text{Un-ionized}]_{\text{aq phase}} \overset{\text{Partition equilibrium}}{\rightleftharpoons} [\text{Un-ionized}]_{\text{org phase}}$$

Let us consider the partitioning of an organic acid (HA) between organic and aqueous phases at a certain pH. The equilibrium processes of ionized (A^-) and un-ionized (HA) forms between aqueous and organic phases can be described as follows:

$$[A^-]_{\text{aq phase}} \rightleftharpoons [HA]_{\text{aq phase}} \rightleftharpoons [HA]_{\text{org phase}}$$

The partition coefficient (P) and the distribution coefficient (D) of HA can be expressed as

(4.6)
$$P = \frac{[HA]_{\text{org phase}}}{[HA]_{\text{aq phase}}}$$

(4.7)
$$D = \frac{[HA]_{\text{org phase}}}{[HA]_{\text{aq phase}} + [A^-]_{\text{aq phase}}}$$

Since the partition coefficient refers only to equilibrium of un-ionized compound between the phases, it is *pH-independent,* whereas the distribution coefficient is

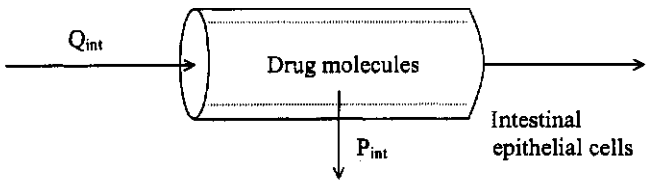

Figure 4.4. Schematic description of permeation of drug molecules via intestinal epithelial cells. Q_{int} and P_{int} represent, respectively, intestinal fluid flow rate (i.e., intestinal fluid volume divided by intestinal transit time) and apparent permeability of drug across intestinal epithelial cells.

pH-dependent because the degree of ionization in the aqueous phase is affected by both the pH and the pK_a of the compound. A rough estimate of log D of a compound at any given pH can be obtained by subtracting one unit from its log P for every unit of pH above or below its pK_a as acid or base, respectively [Eq. (4.8)]. Note that log P is always greater than log D. An estimate of log P of a compound can be obtained through a number of mathematical methods, such as the fragmental constant method developed by Hansch and Leo (1979):

(4.8) $$\boxed{\log D \approx \log P - \Delta|pK_a - pH|}$$

(i) clog P and Mlog P. The clog P and Mlog P of a compound are the log P estimates of the compound calculated with the methods developed by the Medicinal Chemistry Department, Pomona College, CA, and by Moriguchi *et al.* (1992), respectively.

(ii) log D and oral absorption. In general, log $D_{7.4}$ values between −0.5 and 2 are considered to be optimal for oral absorption of compounds. Compounds with log $D_{7.4}$ values smaller than −0.5 or greater than 2 tend to have poor oral absorption owing to limited membrane permeation (low lipophilicity) or poor aqueous solubility (low hydrophilicity), respectively (Smith *et al.,* 1990). The log $D_{7.4}$ is the octanol/aqueous buffer distribution coefficient of compound at pH 7.4 uncorrected for the degree of ionization.

4.3. BIOAVAILABILITY

Bioavailability is considered to be one of the most important pharmacokinetic parameters of any drug developed for extravascular administration.

4.3.1. Definition

Oral bioavailability is a relative term used to describe the rate and extent of absorption after oral administration of a drug compared to that after its administration via a reference route, usually intravenous bolus injection.

UNIT: Bioavailability has no unit. Often, it is expressed as a percent.

4.3.2. Factors Affecting Bioavailability and the First-Pass Effect

Oral bioavailability of a drug is affected by both the extent of its *absorption* into enterocytes from the gut lumen and the extent of its *presystemic elimination* by the intestine and the liver before it reaches the systemic circulation.

4.3.2.1. Incomplete Absorption

Bioavailability can be less than unity, owing to incomplete absorption of a drug from the GI tract. Various physiological and physiochemical factors of a drug that affect intestinal absorption after oral administration have already been discussed.

4.3.2.2. Presystemic Elimination (= First-Pass Effect)

After being absorbed into enterocytes from the gut lumen, drug molecules pass into the portal circulation, and then through the liver and the lung prior to entering the systemic circulation, where blood samples are normally taken. During these absorption processes, a significant portion of a drug can be eliminated by metabolism within the enterocytes, metabolism and/or biliary excretion in the liver, and metabolism in the lung, for the first time, before reaching the systemic circulation. This process is known as the "first-pass or presystemic effect (elimination)." Since elimination by the lung is generally thought to be minimal, the pulmonary first-pass effect after oral administration of a drug is considered to be negligible. Drugs given intravenously also have to first pass through the lung before reaching the systemic circulation. Thus, the first-pass effect in the lung after oral administration is not taken into account for an estimate of bioavailability when drug concentrations after *intravenous* injection are used for exposure comparisons. A schematic illustration of blood circulation and the anatomic arrangement of various organs is shown in Fig. 4.5.

The extent of the presystemic intestinal or hepatic elimination of drug can be affected by:

1. Site of absorption: If the site of absorption of a drug is different from the site of metabolism in the intestine, first-pass intestinal metabolism 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 of the drug in enterocytes will be.
3. Diffusional barrier between the splanchnic bed and the enterocytes: The lower the diffusibility of the drug from the enterocytes to the mesenteric vein, the longer the residence time of the drug within the enterocytes.
4. Mucosal and portal blood flow: Blood in the splanchnic bed can act as a sink to carry drug molecules away from the enterocytes once they are absorbed, which reduces their intracellular residence time in the enterocytes. Factors causing changes in portal blood flow rate can also affect the extent of presystemic hepatic elimination (see Chapter 6).

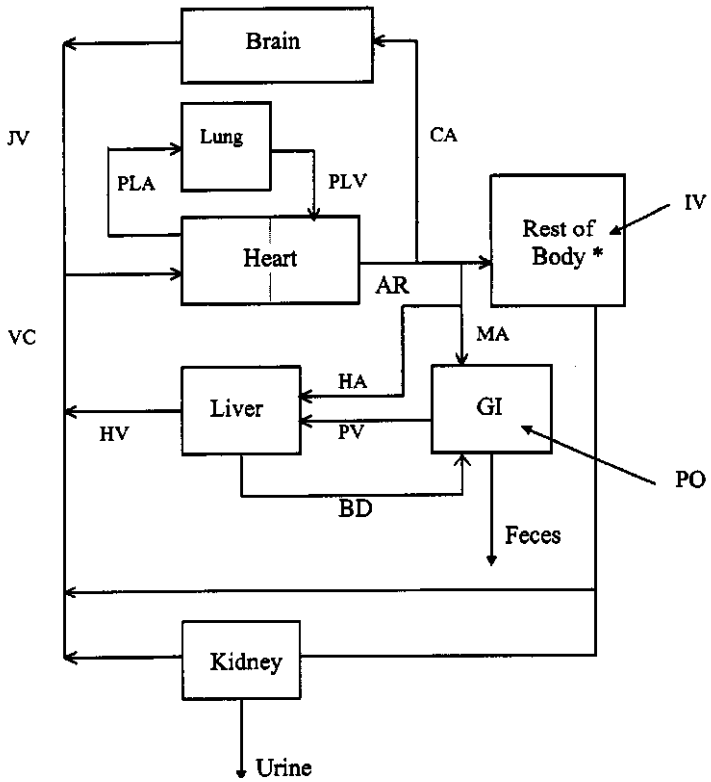

Figure 4.5. Schematic description of the body organs and circulation. AR: aorta, BD: bile duct, CA: carotid artery, GI: gastrointestinal tract, HA: hepatic artery, HV: hepatic vein, IV: intravenous injection, JV: jugular vein, MA: mesenteric artery, PLA: pulmonary artery, PLV: pulmonary vein, PO: oral administration, PV: portal vein, VC: vena cava, *Can be viewed as systemic circulation.

5. Substrate concentrations: Often, concentrations of a drug in certain areas of the intestine or in the mesenteric/portal vein during absorption can be quite high, which can cause nonlinearity in presystemic elimination.

4.3.3. Estimating the Extent of Absorption

4.3.3.1 . Oral Bioavailability

Oral bioavailability (F) of a drug often simply implies the fraction of an orally administered dose that reaches the systemic circulation as unchanged drug and can be estimated by comparing $AUC_{0-\infty}$ of a drug after oral administration to that after intravenous bolus iniection normalized to the dose levels:

(4.9)
$$F = \frac{AUC_{po,0-\infty} \cdot D_{iv}}{AUC_{iv,0-\infty} \cdot D_{po}}$$

where D_{iv} and D_{po} are intravenous and oral doses, and $AUC_{iv,0-\infty}$ and $AUC_{po,0-\infty}$ are the AUC from zero to infinity after intravenous and oral administration, respectively.

(a) Prediction of Oral Bioavailability without Oral Data. If the following four assumptions are met, F of a drug can be estimated from data obtained after intravenous administration alone without oral data using Eq. (4.10):

1. Complete oral absorption.
2. Elimination of the drug occurs via hepatic clearance only.
3. Linear kinetics.
4. Drug concentrations in plasma and blood are the same.

$$F = 1 - \frac{D_{iv}}{Q_h \cdot AUC_{0-\infty,iv}} \tag{4.10}$$

where Q_h is hepatic blood flow rate. The validity of this approach is difficult to prove, however, owing to uncertainty in regard to the above assumptions.

(b) Absolute and Relative Bioavailabilities. Absolute bioavailability is drug availability after administration via routes other than intravenous injection as compared to that after intravenous administration. Relative bioavailability implies drug availability obtained without data from intravenous administration. For example, availability comparisons of a drug between two different oral formulations without intravenous data can be thought of as the relative bioavailability of the two different formulations.

4.3.3.2. Fraction of the Dose Absorbed vs. Fraction of the Dose Eliminated by First-Pass Effects after Oral Administration

The fraction of a dose reaching the systemic circulation as unchanged drug after oral administration can be viewed as the product of the fractions of the dose absorbed into the enterocytes, and subsequently escaping elimination by the enterocytes, the liver, and the lung during the first pass as shown in Eq. (4.11).

$$\boxed{F_s = F_a \cdot F_g \cdot F_h \cdot F_l} \tag{4.11}$$

F_s is the fraction of the dose that reaches the systemic circulation as unchanged drug after oral administration, F_a is the fraction of the dose absorbed into the enterocytes from the intestinal lumen after oral administration, F_g is the fraction of amount of drug absorbed into the enterocytes that escapes the presystemic intestinal elimination, F_h is the fraction of amount of drug entering the liver that escapes the presystemic hepatic elimination, and F_l is the fraction of amount of drug entering the lung that escapes the presystemic pulmonary elimination. Equation (4.11) includes estimates of F_l into F_s. In this case, F_s becomes the bioavailability of a drug after oral administration referred to that after *intraarterial* (not intravenous) admin-

istration at the same sampling site, usually, venous blood (Cassidy and Houston, 1980; Kwan, 1998; Pang, 1986).

When oral bioavailability of a drug is determined compared to exposure after *intravenous* injection, F_1 cancels out because just like the oral dose, the entire intravenous dose also has to first pass through the lung before reaching the systemic circulation. Oral bioavailability of a drug referred to exposure levels after *intravenous* administration (F) is thus only a function of F_a, F_g, and F_h:

(4.12) $$\boxed{F = F_a \cdot F_g \cdot F_h}$$

The methods for estimating F_a, F_g, and F_h separately are discussed below.

(a) Fraction of Dose Absorbed into the Enterocytes from the Intestinal Lumen after Oral Administration (F_a). When oral bioavailability of a drug is poor, information on how much drug is actually absorbed into the enterocytes or into the portal vein after oral administration becomes critical in order to distinguish between the extent of drug absorption and first-pass effects. Study with a radiolabeled drug makes it possible to estimate the actual amount of drug absorbed. There are two different approaches to discern these processes.

(i) Mass balance. Urine, bile, and feces from animals dosed with a radiolabeled drug can be collected over an extended period of time. Total radioactivity of the drug and its metabolites in the urine and bile samples reflects the actual amount of the drug absorbed into the enterocytes from the gut. When the metabolites produced within the enterocytes are released back into the gut lumen and/or the drug is subject to enterohepatic circulation, the total radioactivity found in urine and bile from bile-duct-cannulated animals may be different from the actual amount of drug absorbed in normal animals. Bile-duct-cannulation surgery can also alter animal physiology (liver function, blood protein contents, etc) and the absorption profile of a drug.

(ii) AUC or radioactivity comparison in urine. A ratio of dose-normalized AUC values of total radioactivity, or total radioactivity found in urine between oral and intravenous administration of a radiolabeled compound, approximates to the fraction of the dose absorbed after oral dosing. This method requires intravenous data.

(b) Fraction of the Amount of a Drug Absorbed into the Enterocytes that Escapes Presystemic Intestinal Elimination (F_g). It is difficult to estimate F, experimentally; however, a product of F_a and F_g ($F_a \cdot F_g$, the fraction of a dose absorbed into the portal blood after oral administration) can be estimated. If F_a is measured experimentally, e.g., from a mass balance study with a radiolabeled compound, F_g can be calculated by dividing $F_a \cdot F_g$ by F_a.

(c) Fraction of the Dose Absorbed into the Portal Blood after Oral Administration ($F_a \cdot F_g$). The following four different approaches can be used to estimate $F_a \cdot F_g$. Advantages and disadvantages of each method are summarized in Table 4.3.

Table 4.3. Advantages and Disadvantages of Different Methods for Estimating $F_a \cdot Fg$

Methods	Advantages	Disadvantages
AUC comparison between P.O. and I.P. administration	More accurate estimate of $F_a \cdot F_g$ in the presence of systemic intestinal metabolism of drug	Needs P.O. and I.P. data Experimental difficulties in I.P. dosing Difficult to validate the assumptions (see the text)
AUC comparison between P.O. and I.V. administration	Extra information available when F is known	Needs P.O. and I.V. data Difficult to validate the assumptions (see the text)
Mass balance (Fick's principle)	Needs P.O. data only	Surgical difficulties and complications associated with the serial bleeding from the portal vein Difficult to measure portal blood flow rate Underestimation of $F_a \cdot F_g$ in the presence of systemic intestinal metabolism
Clearance method	More accurate estimation of $F_a \cdot F_g$ in the presence of systemic intestinal metabolism of drug	Needs P.O. and I.V. data Surgical difficulties and complications in serial bleeding from the portal vein Difficult to validate the assumptions (see the text)

(i) AUC comparison between oral and intraportal vein administration. $F_a \cdot F_g$ can be estimated by comparing dose-normalized AUC values after oral and intraportal vein [or intraperitoneal (I.P.)] administration of a drug (Cassidy and Houston, 1980):

$$F_a \cdot F_g = \frac{AUC_{po,0-\infty} \cdot D_{ip}}{AUC_{ip,0-\infty} \cdot D_{po}} \tag{4.13}$$

where D_{ip} and D_{po} are doses after intraportal vein and oral administration, and $AUC_{ip,0-\infty}$ and $AUC_{po,0-\infty}$ are $AUC_{0-\infty}$ in the systemic plasma after intraportal vein and oral administration, respectively. This approach is valid only when the extent of hepatic clearance is the same for intraportal vein and oral dosing.

(ii) AUC comparison between oral and intravenous administration. In the absence of systemic intestinal metabolism of a drug, $F_a \cdot F_g$ can be estimated from the plasma drug concentration–time profiles after intravenous and oral administration without portal blood sampling after oral administration, based on the following assumptions: (1) linear kinetics (administration route-independent clearance); (2)

hepatic clearance is the only elimination pathway after intravenous administration, although there can be intestinal first-pass elimination after oral administration; and (3) blood drug concentrations are equal to plasma drug concentrations:

$$F_a \cdot F_g = \frac{Q_h \cdot AUC_{po} \cdot D_{iv}}{(Q_h\text{-}Cl_s) \cdot AUC_{iv} \cdot D_{po}} \tag{4.14}$$

where Q_h is hepatic blood flow rate.

(iii) Mass balance. The amount of drug in the portal vein after oral administration is the sum of the amount of the newly absorbed drug into the portal vein from the gut and the amount of drug coming from the mesenteric artery supplying blood to the intestine:

Amount of drug in the portal vein over a short period of time (dt) — *Amount of newly absorbed drug over dt* — *Amount of drug coming from the mesenteric artery over* dt

$$Q_{pv} \cdot C_{po,pv}(t) \cdot dt = A(t) + Q_{pv} \cdot C_{po,sys}(t) \cdot dt \tag{4.15}$$

where Q_{pv} is portal vein blood flow rate, and hence $Q_{pv}\,dt$ represents the total volume of blood flowing through the portal vein over a short period of time, dt, from time t. A(t) is the amount of drug newly absorbed from the intestine showing up in the portal vein over dt. $C_{po,pv}(t)$ and $C_{po,sys}(t)$ are the drug concentrations in the portal blood and systemic circulation (usually venous blood) at time t after oral administration, respectively. Drug concentrations in the systemic circulation can be used instead of those in the mesenteric artery for estimating the amount of a drug in the mesenteric artery, because in most cases they can be assumed to be the same in both regions. Figure 4.6 illustrates the relationships among different drug concentrations at various anatomical locations after oral administration.

Integrating Eq. (4.15) from 0 to ∞ gives an estimate of the total amount of drug absorbed into the portal vein (A_a) after oral administration:

$$A_a = Q_{pv} \cdot (AUC_{po,pv} - AUC_{po,sys}) \tag{4.16}$$

where $AUC_{po,pv}$ and $AUC_{po,sys}$ are, respectively, $AUC_{0-\infty}$ of a drug in the portal vein blood and systemic blood (or plasma when both plasma and blood concentrations are the same) after oral administration (Fujieda *et al.,* 1996). Therefore, $F_a \cdot F_g$ can be expressed as

$$F_a \cdot F_g = \frac{Q_{pv} \cdot (AUC_{po,pv} - AUC_{po,sys})}{D_{po}} \tag{4.17}$$

- Portal blood sampling. Portal blood samples can be collected from portal-vein-cannulated animals for serial bleeding or individual animals at different time

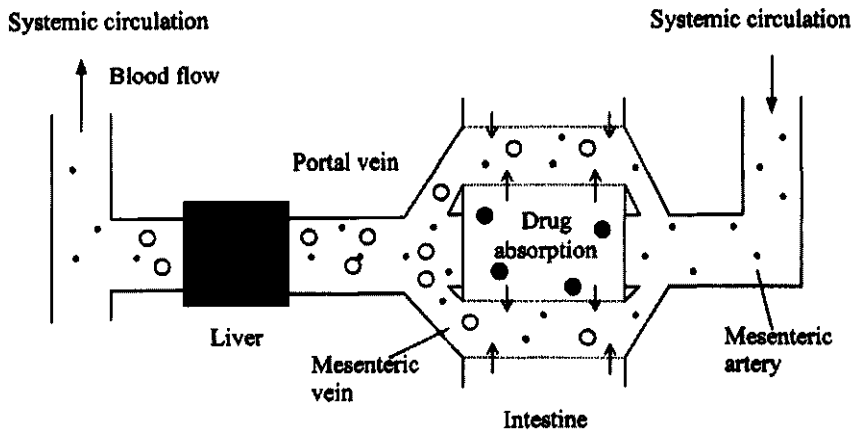

Figure 4.6. Schematic description of relationships among newly absorbed drug molecules (0) from drug particles or molecules (●) in the intestine and previously absorbed drug molecules (•) coming from the systemic circulation via the mesenteric artery. A difference in drug concentrations between the portal vein and the mesenteric artery is due to the newly absorbed drug.

points by terminal bleeding to avoid portal-vein cannulation (see Appendix C). Portal-vein-cannulation surgery may cause some physiological changes in, e.g., portal blood flow rate or the amount of albumin in the blood, which may affect drug disposition profiles, whereas sampling from individual animals at different time points by terminal bleeding may result in a large interanimal variability in exposure.

- Limitations of the mass balance method. (1) The estimate of A_a can vary depending on the portal blood flow rate used (the values published in the literature are often used). (2) A, can be underestimated when a drug is subject to systemic intestinal metabolism. Since the previously absorbed drug returning to the mesenteric artery from the systemic circulation is subject to intestinal metabolism during vascular intestinal transit prior to reaching the portal circulation, the difference between the portal vein and systemic blood concentrations underestimates the true concentration of newly absorbed drug into the portal vein. The clearance method is more suitable for estimating $F_{a.}F_g$, when significant systemic intestinal elimination of the drug is suspected.

(iv) Clearance method. The clearance method was derived based on a three-compartment model (systemic blood, intestine, and liver compartments). Important assumptions for the model include: (1) linear and route-independent kinetics, (2) intestinal and/or hepatic clearance only, and (3) instantaneous and homogeneous distribution of the drug within the compartments. Estimates for A_a and $F_{a.}F_g$ can be obtained using the following equations (Kwon, 1996):

(4.18) $$\boxed{A_a = Cl_b \cdot AUC_{po,pv}}$$

(4.19) $$F_a \cdot F_g = \frac{Cl_b \cdot AUC_{po,pv}}{D_{po}}$$

where Cl_b is the systemic blood clearance (or systemic plasma clearance when blood and plasma concentrations are the same).

NOTE: HOW TO DETECT SYSTEMIC INTESTINAL METABOLISM OF DRUG. For some drugs, intestinal metabolism can play an important role in the elimination of a drug from the systemic circulation. One of the following findings can be indicative of the presence of systemic intestinal metabolism of a drug: (1) $F_a \cdot F_g$ estimated using Eq. (4.17) is smaller than F estimated using Eq. (4.9), which is an impossible outcome in the absence of systemic intestinal elimination. (2) $F_a \cdot F_g$ estimated using Eq. (4.17) is significantly smaller than the estimate using Eq. (4.13) or Eq. (4.19). When a drug is subject to substantial systemic intestinal metabolism, $F_a \cdot F_g$ based on the AUC comparison or the clearance methods becomes more accurate than the mass balance method.

(d) The Fraction of the Amount of a Drug Entering the Liver after Oral Administration that Escapes Presystemic Hepatic Elimination (F_h).

(i) A UC comparison between intraportal and intravenous administration. F_h can be estimated by comparing dose-normalized AUC values of systemic plasma (or blood) drug concentration profiles after intraportal and intravenous administration (Cassidy and Houston, 1980):

(4.20) $$\boxed{F_h = \frac{AUC_{ip} \cdot D_{iv}}{AUC_{iv} \cdot D_{ip}}}$$

(ii) Clearance method. F_h can be also calculated according to the clearance method (Kwon, 1996):

(4.21) $$F_h = \frac{AUC_{po,pv} - AUC_{po,sys}}{AUC_{po,pv}}$$

(e) Thefraction of the Amount of Drug Entering the Lung after Oral Administration that Escapes the Presystemic Pulmonary Elimination (F1). F, can be estimated by comparing dose-normalized AUC in systemic blood after intravenous and intraarterial administration of drug:

(4.22) $$\boxed{F_1 = \frac{AUC_{iv} \cdot D_{ia}}{AUC_{ia} \cdot D_{iv}}}$$

AUC_{ia} is $AUC_{0-\infty}$ in systemic blood after intraarterial administration, and D_{ia} is

the intraarterial dose. In the absence of pulmonary metabolism, AUC_{ia} and AUC_{iv} should be the same.

(f) Relationships between F_s *and* F_a, F_g, F_h *and* F_l. F_s can be expressed as a function of $F_a \cdot F_g$, F_h, and F_l with corresponding AUC comparisons [Eqs. (4.13), (4.20), and (4.22), respectively], assuming the same dose, as follows:

$$F_s = \underset{\downarrow}{\frac{AUC_{po}}{\cancel{AUC_{pv}}}} \cdot \underset{\downarrow}{\frac{\cancel{AUC_{pv}}}{\cancel{AUC_{iv}}}} \cdot \underset{\downarrow}{\frac{\cancel{AUC_{iv}}}{AUC_{ia}}} = \frac{AUC_{po}}{AUC_{ia}} \qquad (4.23)$$

($F_a \cdot F_g$ over the first ratio, F_h over the second, F_l over the third)

When AUC_{ia} and AUC_{iv} are the same, i.e., no pulmonary elimination, F_s becomes equal to F, oral bioavailability (AUC_{po} divided by AUC_{iv}).

(g) Oral Absorption Pathways Avoiding Intestinal or Hepatic First-Pass Effects

(i) Lymphatic delivery in the GI tract. The lymph from the GI tract is collected in the thoracic lymph duct without passing through the liver, before entering the bloodstream. Therefore, the drug absorbed via lymphatic vessels in the GI tract can avoid hepatic first-pass effects, although presystemic elimination of the drug by enterocytes may still occur (Muranishi, 1991). The actual amount of drug delivered via lymphatic pathways can be rather limited owing to the slow flow rate of lymph (see Chapter 13).

(ii) Rectal administration. Blood vessels from the lower part of the rectum connect with the inferior vena cava instead of merging into the portal vein, so that a drug administered in a suppository via a rectal route can avoid hepatic first-pass effects.

4.3.4. Estimating the Rate of Absorption

The rate of drug absorption after oral administration can be assessed from plasma drug concentration–time profiles with curve-fitting or moment analysis of *in vivo* data. *In vitro* or *in situ* experiments such as Caco-2 cell permeation or intestinal perfusion studies can also provide information regarding the rate of intestinal absorption of an orally dosed drug.

4.3.4.1. In Vivo Experiments

The absorption rate constant (k_a), which reflects how fast drug molecules transport across intestinal epithelial cells and reach the systemic circulation after oral dosing, can be estimated by curve-fitting or moment analysis.

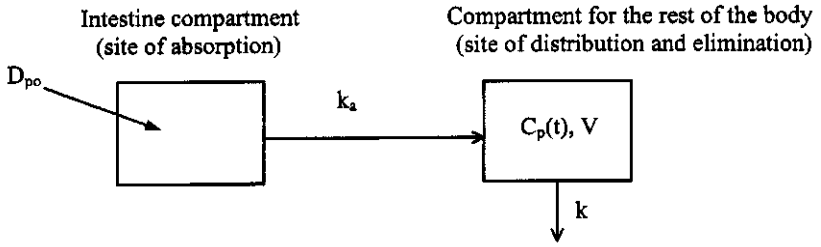

Figure 4.7. Two-compartment model for drug absorption and disposition. Cp(t): plasma concentration at time **t** after oral administration, D_{po}: oral dose, k_a: absorption rate constant, k: elimination rate constant, V: apparent volume of distribution.

(a) Curve Fitting. The value of k_a can be estimated by fitting a proper compartmental model to the plasma drug concentration–time profile after oral administration of a drug, with the method of residuals or a nonlinear least-square regression program such as WinNonlin® (Pharsight, NC).

(i) Bateman equation. The equation most often used for estimating k, is based on a two-compartment model for the intestine and the rest of body (Fig. 4.7), according to which, the time course of $C_p(t)$ after oral administration of a drug can be described as in Eq. (4.24), which is known as the Bateman equation:

$$C_p(t) = \frac{k_a \cdot F \cdot D_{po}}{V \cdot (k_a - k)} \cdot (e^{-k \cdot t} - e^{-k_a \cdot t}) \tag{4.24}$$

where k is the elimination rate constant and V is the apparent volume of distribution of the compartment for the rest of the body; k_a can be estimated by fitting Eq. (4.24) to the exposure profiles of the drug after oral administration with F, V, and k estimated from intravenous studies using a nonlinear regression computer program. It is important to note that k_a estimated with Eq. (4.24) is valid only when the following assumptions are met: (1) first-order absorption and elimination rates, (2) homogeneous behaviors of the intestine for drug absorption, and (3) a one-compartment model for the rest of the body (a monoexponential decline of the plasma drug concentration–time profile on a semilog scale after intravenous bolus injection).

(ii) Method of residuals. The method of residuals is used to estimate k_a of a drug based on the assumption that its rate of absorption is much faster than its rate of elimination from the body, i.e., $k_a \gg k$. In this case, during the terminal phase after oral dosing, e^{-kat} in Eq. (4.24) becomes much smaller than $e^{-k \cdot t}$, so that $C_p(t)$ during the terminal phase $[C_p^{exp}(t)]$ can be approximated as

$$C_p^{Exp}(t) = \frac{k_a \cdot F \cdot D_{po}}{V \cdot (k_a - k)} \cdot e^{-k \cdot t} \tag{4.25}$$

Subtracting E0q. (4.24) from (4.25) yields

$$C_p^{Exp}(t) - C_p(t) = \frac{k_a \cdot F \cdot D_{po}}{V \cdot (k_a - k)} \cdot e^{-k_a \cdot t} \quad (4.26)$$

A plot of $C_p^{exp}(t) - C_p(t)$, the "residuals" of $C_p^{exp}(t)$ and $C_p(t)$ *vs.* time plots, becomes a straight line on a semilog scale and from its slope (= $-k_a/2.303$), k_a can be estimated with curve-fitting as illustrated in Fig. 4.8.

The method of residuals is useful only when all the assumptions that were applied to the Bateman equation are satisfied and $k_a \gg k$. An estimate of k_a from the Bateman equation or the method of residuals is an apparent value reflecting the entire absorption process, including disintegration and dissolution rates from dosage forms (if the drug is not administered in solution) and transport rates passing through the intestine and the liver during absorption.

(iii) C_{max} *and* t_{max} The highest drug concentration after oral administration (C_{max}) and t_{max} the time at which C_{max} is observed, can be derived from the Bateman equation:

$$C_{max} = \frac{F \cdot D_{po}}{V} \cdot e^{-k \cdot t_{max}} \quad (4.27)$$

$$t_{max} = \frac{\ln(k_a/k)}{k_a - k} \quad (4.28)$$

As seen in Eqs. (4.27) and (4.28), both C_{max} and t_{max} are affected by k_a as well as by k. If two different formulations of the same drug (and hence, different k_a values, but the same k) are compared, the formulation with the faster absorption (a greater k_a) would produce a higher C_{max} with an earlier t_{max}.

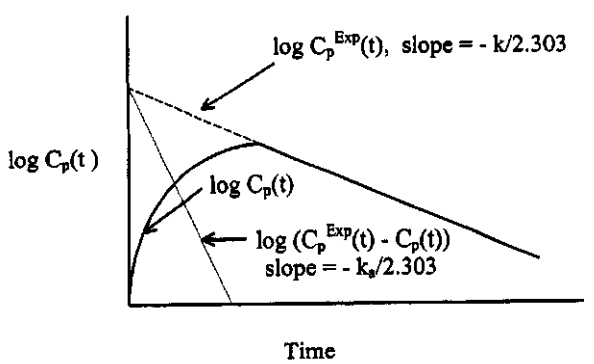

Figure 4.8. The method of residuals to estimate the absorption rate constant (k_a) of a drug after oral administration. A semilogarithmic plot of the difference between $C_p^{exp}(t) - C_p(t)$ exhibits a straight line with a slope of $-k_a/2.303$. $C_p^{exp}(t)$ represents a plasma drug concentration–time plot extrapolated from the terminal phase of $C_P(t)$ to the origin.

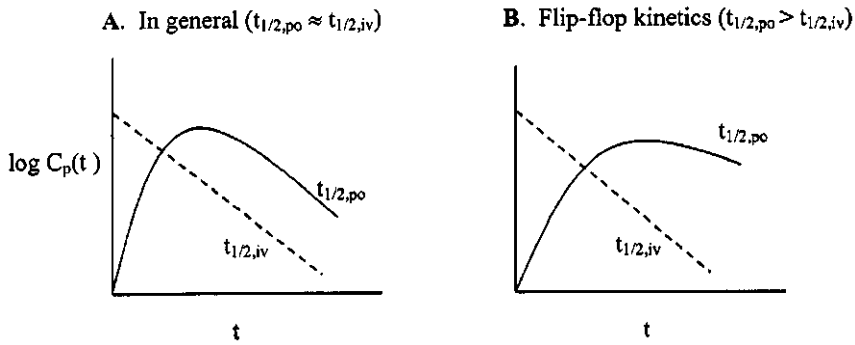

Figure 4.9. Plasma drug concentration *vs* . time profiles of hypothetical drugs after intravenous (----) or oral (——) administration on a semilogarithmic scale. In most cases, the absorption rate constant of drug after oral administration is much greater than the elimination rate constant evidenced by similar terminal half-lives between oral and intravenous administration (A). When the absorption rate constant is substantially smaller than the elimination rate constant, the terminal half-life of the drug after oral administration ($t_{1/2.po}$)becomes longer than that after intravenous administration ($t_{1/2.iv}$)(B).

(iv) Flip-flop kinetics. In a series of two consecutive, irreversible first-order rate processes such as absorption of a drug from the intestine and its subsequent systemic elimination, either step can be rate-limiting in the overall elimination process. In general, k_a of a drug after oral administration is greater than k so that elimination of the drug from the body after oral administration is governed primarily by how fast it can be removed once it enters the systemic circulation. In this case (e.g., $k_a > 3 \times k$), a plasma concentration–time profile after oral dosing exhibits a terminal half-life ($t_{1/2,po}$) similar to that after intravenous injection ($t_{1/2,iv}$). However, when k_a is much smaller than k (e.g., $k > 3 \times k_a$), drug disappearance from the body becomes governed by the rate of absorption rather than by the rate of elimination, and $t_{1/2po}$ becomes longer than $t_{1/2,iv}$. This phenomenon is called "flip-flop kinetics" (Fig. 4.9).

(b) Moment Analysis.

(i) Mean absorption time. The value of k_a can also be estimated using moment analysis from the mean residence time (MRT), since the MRT of a drug after oral administration (MRT_{po}) includes the time required for absorption (mean absorption time, MAT) and MRT after intravenous administration (MRT_{iv}):

(4.29) $$\boxed{MRT_{po} = MAT + MRT_{iv}}$$

and k_a is the reciprocal of MAT:

(4.30) $$\boxed{k_a = \frac{1}{MAT} = \frac{1}{MRT_{po} - MRT_{iv}}}$$

and

$$MRT_{po} = \frac{AUMC_{0-\infty,po}}{AUC_{0-\infty,po}} \quad \text{and} \quad MRT_{iv} = \frac{AUMC_{0-\infty,iv}}{AUC_{0-\infty,iv}}$$

where $AUMC_{0-\infty,\,iv}$ and $AUMC_{0-\infty,\,po}$ are the areas under the first-moment curve of plasma drug concentration *vs.* time, i.e., AUC of the product of concentration and time *vs* .time profile from zero to infinity after intravenous and oral administration, respectively (see Chapter 2).

(ii) MRT_{disint}, MRT_{diss} and MRT_{abs}. Various MRT values for different steps of oral absorption of a drug can be calculated by moment analysis with exposure levels of the drug dosed in different formulations (Fig. 4.10). For instance, the difference in the MRT estimate after the administration of a tablet and of a suspension is the MRT for the disintegration process of the tablet (MRT_{disint}) to particles in suspension. A difference in the MRT after administration of a suspension and a solution is the MRT for the dissolution process of the solid drug particles in suspension (MRT_{diss}) to drug solution. A difference of MRT between an oral solution and an intravenous injection is the MRT for the absorption process of the drug molecules in solution (MRT_{abs})into the systemic circulation (Tanigawara *et al.,* 1982).

For example, MRT_{po} $(= AUMC_{0-\infty,\,po}/AUC_{0-\infty,\,po})$ of a drug determined after oral administration of a tablet is the sum of MRT_{disint}, MRT_{diss}, MRT_{abs}, and MRT_{iv}. Thus, MAT $(= MRT_{po}\text{-}MRT_{iv})$ is the sum of MRT_{disint}, MRT_{diss}, and

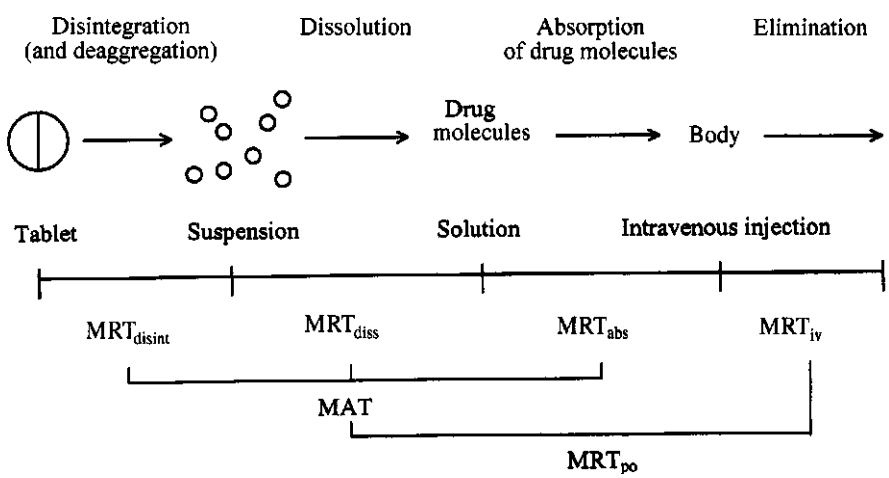

Figure 4.10. Relationships among different mean residence time (MRT) estimates reflecting various absorption processes after oral administration of drug in different oral dosage forms. MAT: mean absorption time of drug after oral administration, MRT_{abs}: MRT for the absorption of the drug molecules in solution, MRT_{disint}: MRT for the disintegration of the solid dosage form such as tablet, MRT_{diss}: MRT for the dissolution of the solid drug particles in suspension, MRT_{po}: MRT after oral administration, MRT_{iv}: MRT after intravenous administration.

MRT_{abs}. MRT_{po} after oral administration of a suspension would include, therefore, MRT_{diss}, MRT_{abs}, and MRT_{iv}, and its MAT is then the sum of MRT_{diss} and MRT_{abs}.

4.3.4.2. In Situ or In Vitro Experiments

In general, most *in situ* or *in vitro* absorption or transport studies, such as intestinal perfusion or Caco-2 cell experiments, are performed with a drug solution rather than solid or suspension formulations. An estimate of the absorption rate constant obtained from *in situ* or *in vitro* studies, therefore, reflects only the membrane permeation process via enterocytes during absorption. It should be noted that the membrane permeation rate of a drug across the enterocytes becomes important in overall absorption only when disintegration and/or dissolution rates of dosage forms are significantly faster than the membrane permeation rate of drug molecules.

(a) Intestinal Perfusion. In situ intestinal perfusion studies are usually conducted with single-pass perfusion as opposed to recirculating perfusion of a drug solution through an isolated intestine segment under steady state conditions. Two different types of studies can be performed, depending on the site of sample collection. Samples can be collected from the inlet and outlet of perfusate only, in which case the rate and extent of the disappearance of the drug from the gut lumen can be determined. Blood samples in the mesenteric vein from the isolated intestine segment can be obtained in addition to perfusate samples. Analyses of these samples can provide information not only on drug disappearance from the gut lumen but also on drug appearance in the mesenteric vein, which is more relevant to actual drug absorption. As the studies are performed under steady state conditions, the effects of nonspecific adsorption of a drug to perfusion apparatus and tubes or intestinal membranes during perfusion on its drug disappearance from the lumen or its appearance in mesenteric vein can be ignored (Raoof *et al.,* 1998).

(i) Drug disappearance from the intestinal lumen. The rate and extent of drug disappearance from the lumen of the isolated intestine at steady state can be determined by measuring inlet and outlet drug concentrations of the perfusate. Drug disappearance from the perfusate can be due to transport of the drug into the enterocytes and/or metabolism by gut microflora inside the intestinal lumen. The apparent absorption rate constant ($k_{a,app}$), which reflects how fast drug molecules disappear from the perfusate flowing through the intestinal segment, can be estimated from the following equation:

$$k_{a,pp} = \frac{\overbrace{Q \cdot (C_{in,ss} - C_{out,ss})}^{\text{Disappearance rate of drug in the intestinal lumen}}}{C_{in,ss} \cdot V} \tag{4.31}$$

where $C_{in,ss}$ and $C_{out,ss}$ are the drug concentrations in a perfusate solution entering

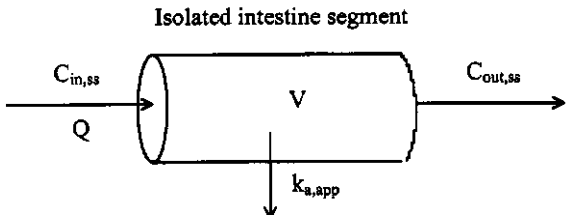

Figure 4.11 . Schematic description of isolated intestine single-pass perfusion. $C_{in,ss}$: inlet drug concentration at steady state, $C_{out.ss}$: outlet drug concentration at steady state, $k_{a,app}$: apparent absorption rate constant, Q: perfusate flow rate, V: volume of the isolated intestine segment.

and leaving the isolated intestine segment at steady state, respectively, and Q and V are the perfusate flow rate and the volume of the intestine segment used in the experiment, respectively. A schematic description of an intestinal perfusion study is illustrated in Fig. 4.11.

If it is assumed that the disappearance of a drug from the intestine is a linear process, the relationship between steady state perfusate drug concentrations and effective permeability (P_{eff}) of the drug disappearing from the intestinal lumen can be expressed as

$$C_{out,ss}/C_{in,ss} = e^{-P_{eff}\cdot(2\pi\cdot r\cdot L)/Q} \tag{4.32}$$

where r and L are the radius and length of the gut lumen, respectively, P_{eff} is the effective permeability of a drug as it is transported from the intestinal lumen into the enterocytes, and may overestimate the true intestinal permeability of the compound in vivo owing to underestimation of the surface area of the gut lumen with $2\pi\ r\cdot L$.

The relationship between $k_{a,app}$ and P_{eff} is

$$k_{a,app} = \frac{Q(1 - e^{-P_{eff}\cdot(2\pi\cdot r\cdot L)/Q})}{V} \tag{4.33}$$

Unstirred water layer and permeability: The unstirred water layer (UWL), sometimes called the aqueous boundary layer, surrounds the surfaces of the brush border membranes of enterocytes. The permeability of a drug across the UWL can be affected by both the thickness of the boundary layer and the flow rate of the perfusate through the intestine. The rate of permeation of the compound in solution from the gut lumen into the splanchnic blood can be limited by the transport of drug molecules across the UWL or the enterocyte membranes (Fig. 4.12) (Amidon *et al.,* 1988; Zimmerman *et al.,* 1997). The effective permeability (P_{eff}) of the GI wall is considered to be a function of both the permeability of UWL (P_{aq}) and the permeability of the enterocyte membranes (P_m):

$$P_{eff} = \frac{P_{aq}\cdot P_m}{P_{aq} + P_m}$$

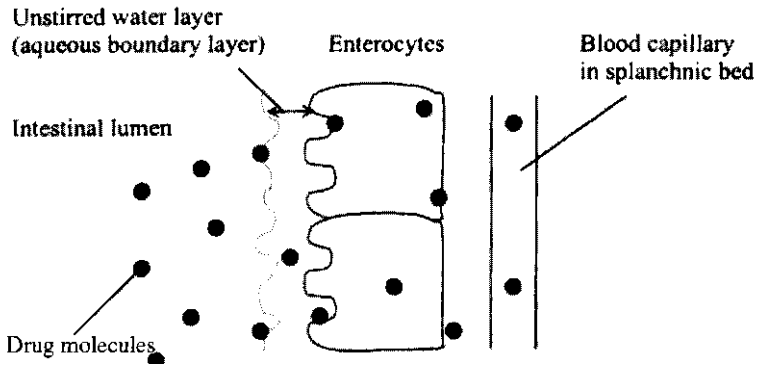

Figure 4.12. Schematic description of an unstirred water layer of enterocyte surfaces.

(ii) Drug appearance in the mesenteric vein. In addition to collection of inlet and outlet perfusate, blood samples taken from the mesenteric vein can provide an estimate of the absorption rate constant more relevant to actual drug absorption and information on the presystemic intestinal metabolism *in vivo*. To maintain a constant blood flow in the mesenteric vein and to avoid mixing of blood from the systemic circulation during sample collection, fresh blood is usually replenished into the mesenteric artery at a constant rate. A schematic description of sample collection from the mesenteric vein during intestinal perfusion of a drug solution is illustrated in Fig. 4.13. The equation describing mass balance during the study is as follows:

Amount of drug perfused into intestine ($Q \cdot C_{in,ss}$)

= Amount of drug leaving from the intestine ($Q \cdot C_{out,ss}$

+ Amount of drug absorbed into the mesenteric vein ($Q_{mv} \cdot C_{mv,ss}$)

+ Amount of drug eliminated by chemical instability in perfusate, intestinal microfloral metabolism, and/or first-pass metabolism by enterocytes

where $C_{in,ss}$ and $C_{out,ss}$ are the drug concentrations in a perfusate solution entering and leaving the intestine segment at steady state, respectively, $C_{mv,ss}$ is the blood drug concentration in the mesenteric vein at steady state, and Q and Q_{mv} are the perfusate and mesenteric blood flow rates, respectively.

Based on this relationship, the following information on absorption can be obtained:

FRACTION OF DRUG ABSORBED INTO MESENTERIC VEIN (F_{mv}):

$$F_{mv} = \frac{Q_{mv} \cdot C_{mv,ss}}{Q \cdot C_{in,ss}} \tag{4.35}$$

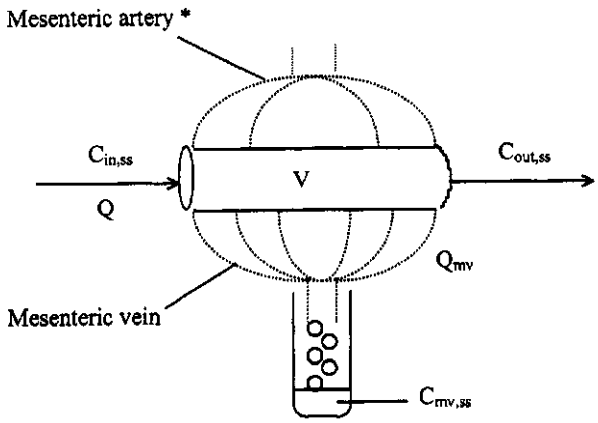

Figure 4.13. Schematic description of intestinal perfusion with blood collection from the mesenteric vein. $C_{in.ss}$: inlet drug concentration at steady state, $C_{out,ss}$: outlet drug concentration at steady state, $C_{mv,ss}$: blood drug concentration in mesenteric vein at steady state, Q: perfusate flow rate, Q_{mv}: mesenteric blood flow rate, V: volume of the isolated intestine, *In order to maintain blood flow in the mesenteric vein, fresh blood should be infused into the mesenteric artery at a constant rate.

APPARENT ABSORPTION RATE CONSTANT ($k_{a,app}$)

(4.36)
$$k_{a,app} = \frac{Q_{mv} \cdot C_{mv,ss}}{C_{i,ss} \cdot V}$$

where $C_{i,ss}$ is an average concentration within the intestine segment;

$$C_{i,ss} = (C_{in,ss} - C_{out,ss})/\ln (C_{in,ss}/C_{out,ss})$$

V is the volume of the intestine segment used in the experiment.

(b) Caco-2 *Ce lls.* Most drugs that are given orally are absorbed across the enterocytes primarily by passive diffusion. In order to transport from the intestinal lumen into the mesenteric vein, drug molecules must diffuse through a series of different physiological barriers, including the mucus gel layer (unstirred water layer), the intestinal epithelial cells, the lamina1 propia, and the endothelium of the intestinal capillary. Among these, it is the single layer of epithelial cells that has been recognized as the most significant barrier.

One of the most commonly used cells to investigate drug transport via enterocytes in humans is the Caco-2 cell line (Artursson, 1991). This cell line is derived from a human colon carcinoma and is distinguished from other cell lines of the same origin by its capability for spontaneous differentiation into monolayers of polarized enterocytes under conventional cell culture conditions (Artursson and Karlsson; 1991; Hidalgo *et al.,* 1989; Rubas *et al.,* 1993). The transport study can be performed from apical (luminal) to basal (blood) sides in an absorptive direction by

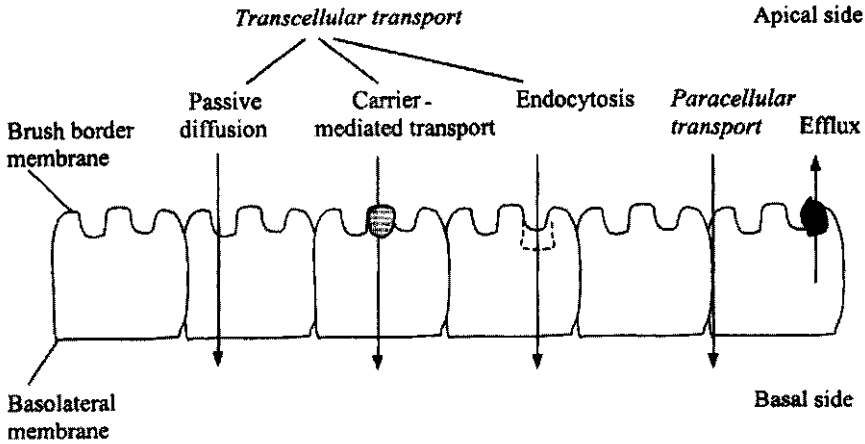

Figure 4.14 . Various membrane transport mechanisms in enterocytes.

placing a drug solution in the apical side and collecting samples from the basal side at different incubation time points, or vice versa, depending on study needs (Fig. 4.14). Apparent membrane permeability (P_{app}) of a drug in Caco-2 cell experiments can be determined as follows:

$$P_{app} = \frac{\text{Amount of drug in acceptor side at time t}/\Delta t}{\text{Surface area of cell layer} \times \text{Concentration of drug in donor side at time 0}} \quad (4.37)$$

where Δt is an incubation period. In most cases, P_{app} is expressed in cm/sec.

(i) Direction of transport. The chambers where drug solution and blank buffer are placed are known as the donor and acceptor sides, respectively. When the apical side (A) of the Caco-2 cells is the donor side, the experiment is performed in an absorptive direction from the gut lumen to the mesenteric vein, whereas when the drug solution is placed in the basal side (B), drug efflux from the mesenteric vein to the gut lumen can be studied (Fig. 4.14). If transport of the drug is mediated solely by passive diffusion, P_{app} estimates must be the same regardless of the direction of the transport. However, when the drug is subject to active transport (Table 4.4) or efflux mechanisms such as P-glycoprotein (Gatmaitan and Arias, 1993; Leveque and Jehl, 1995) in the brush border membranes of the enterocytes, P_{app} values measured from A to B can be greater or smaller than those from B to A, respectively.

(ii) Validation and establishment of Caco-2 cell systems. When the Caco-2 cell line is newly established, a thorough validation of the cell integrity (confluence) and functionality (expression of active transporters and enzymes) is important because of a large variability in cell culture systems depending on study conditions. To assess the integrity of the monolayers, one or both of the following methods can be used.

Table 4.4. Carrier-Mediated Transport Systems in the Intestinal Epithelial Membranes[a]

Locations	Transporters	Substrates
Brush border membrane	Amino acid transporters	Amino acids and amino acid-mimetic compounds such as leucine, lysine, glutamate, L-dopa
	Oligopeptide transporters	Peptidomimetic compounds such as angiotensin converting enzyme (ACE) inhibitors, renin inhibitors, some β-lactam antibiotics
	Monocarboxylic acid transporter	Carboxylic acids such as salicylic acid, benzoic acid
	Glucose transporter	*p*-nitrophenyl-β-D-glucopyranoside
	Bile acid transporter	Taurocholic acid
	Phosphate transporter	Fosfomycin
	Membrane electric potential-dependent transport	Disopyramide, tyramine
	Proton antiporter	Tetraethylammonium, N-methylnicotine amide
	P-glycoprotein[b]	Cyclosporin-A, verapamil, vinblastine
Basolateral membrane	Amino acid transporters	
	Phosphate transporter	

[a]Data taken from Tsuji and Tomai (1996) and Zhang *et al.* (1998).
[b]P-glycoprotein, a multidrug resistance gene product, acts as a barrier to intestinal absorption of numerous xenobiotics by effluxing them out of cytoplasm of enterocytes and/or pumping right back into the intestinal lumen during their uptake into the cells (see Chapter 9, Gatmaitan and Arias, 1993, Hunter and Hurst, 1997; Leveque and Jehl 1995; Wacher *et al.*, 1996).

• Permeation of mannitol via the Caco-2 cell monolayers. Mannitol is known to transport via a paracellular pathway only, and a rate of flux from the donor to the acceptor side greater than 0.5%/hr in Caco-2 cells may indicate that the cells have been damaged and are not suitable for transport studies. P_{app} values of mannitol lower than 10^{-6} cm/sec indicate that the integrity of the monolayer of cells is well maintained. Propranolol is another control compound used for transcellular passive diffusion. P_{app} values of propranolol are usually greater than 10^{-5} cm/sec.

• Transepithelial electrical resistance. The development of a tight junction can be monitored by measuring transepithelial electrical resistance (TEER) across the Caco-2 cells. TEER values of intact Caco-2 cell monolayers range between 200 and 500 $\Omega^* cm^2$ (Hidalgo, 1996).

To assess the functionality of the cells, transport studies with known substrates for active transporters (Table 4.4) can be conducted. In general, it takes approximately 3 weeks after seeding for the full expression of active transporters in cell membranes. Other factors important in cell culture are the passage number of cells, material and surface area of inserts, compositions of incubation buffers, and amount of organic solvent used in studies. In general, less than 1% (v/v) of acetonitrile or methanol or 0.5% (v/v) of dimethylsulfoxide (DMSO) in incubation buffers can safely be used for study.

(iii) Relationship between P_{app} *values and the extent* of *absorption in humans in vivo.* General rules governing the relationship between P_{app} values of compounds determined from Caco-2 cell studies and the extent of absorption of compounds in humans (Artursson and Karlsson, 1991) are summarized in Table 4.5, provided that dissolution and/or intestinal metabolism of the compounds do not affect drug absorption to any significant extent.

(iv) Relationship between P_{app} *and* $k_{a,app.}$ A pharmacokinetic relationship between P_{app} from Caco-2 cell studies and $k_{a,app}$ values from isolated intestinal perfusion experiments can be viewed as follows:

$$P_{app} = \frac{k_{a,app} \cdot V}{S} \tag{4.38}$$

where S and V are, respectively, the surface area and volume of the intestine available for absorption of the drug after oral administration. Equation (4.38) is an oversimplification of the true relationship between P_{app} and $k_{a,app}$ as it ignores experimental differences between *in vitro* and *in vivo* conditions; however, a linear relationship between P_{app} and $k_{a,app}$ has been reported among structurally similar compounds with similar absorption profiles (Cutler, 1991; Kim *et al.,* 1993).

(v) Membrane transport mechanisms in enterocytes. There are basically four different types of membrane transport processes across enterocytes including: (1) passive diffusion, (2) carrier-mediated transport (facilitated diffusion and active transport, (3) paracellular transport, and (4) endocytosis (pinocytosis). Figure 4.14 illustrates the various transport mechanisms in enterocytes.

- Passive diffusion. For most drugs, passive diffusion is a predominant membrane transport mechanism. Un-ionized, lipophilic molecules can diffuse better across membranes than ionized, hydrophilic molecules. Diffusion is a nonsaturable and concentration-gradient-dependent process, which does not require transport carriers or metabolic energy consumption.

- Carrier-mediated transport. Carrier-mediated transport can be divided into two different types, i.e., facilitated diffusion and active transport, which are carrier-mediated transport processes without and with ATP consumption, respectively.

Facilitated diffusion: Some compounds diffuse down electrochemical gradients across membranes more rapidly than expected from simple diffusion based on their physicochemical properties. This "facilitated" diffusion process mediated by carrier systems is saturable and stereospecific. Like simple diffusion, the facilitated diffusion is also a concentration-gradient-dependent process; ie., once the concentrations between membranes reach equilibrium, apparent net transport of compounds via the facilitated diffusion ends. Facilitated diffusion is distinguished from active transport in that it does not require energy consumption.

Active transport: Active transport is saturable and differs from passive diffusion or facilitated diffusion in that solutes can be transported against thermodynamic equilibrium by consuming ATP. A few active transport systems identified at the

Table 4.5. Guidelines for the Relationship between P_{app} Values of Compounds Determined from Caco-2 Cell Studies and the Extent of Absorption of Compounds in Humans[a]

P_{app} of compounds in Caco-2 cells (cm/sec)	Extent of oral absorption in human
$>10^{-5}$	Well absorbed (>70%)
10^{-6}–10^{-5}	Moderately absorbed (20–70%)
$<10^{-6}$	Poorly absorbed (<20%)

[a]Data taken from Artursson and Karlsson (1991).

brush border and basolateral membranes of the intestinal epithelium are summarized in Table 4.5.

- Paracellular transport. In general, transport of small hydrophilic compounds with molecular weight smaller than 200 across the intestinal membrane occurs mainly via tight junctions between adjacent enterocytes. The extent of paracellular transport of compounds can be examined by performing transport studies in the absence and presence of divalent cations such as Ca^{+2}, which neutralize the inside-negatively charged paracellular channel. In general, paracellular transport is considered to be a minor absorption pathway.

- Endocytosis. Endocytosis is the process in which cells take up macromolecules such as proteins or polysaccharides by ingesting parts of their membranes to generate endocytotic vesicles enclosing a minute volume of extracellular fluid and its contents. There are two different types of this membrane engulfment process, i.e., phagocytosis and pinocytosis. Phagocytosis occurs only in specific cells such as macrophages and involves the ingestion of large particles such as viruses or cell debris, whereas pinocytosis occurs in all cell types and leads to the uptake of extracellular fluid and its contents.

(c) *Other In Vitro Systems for Intestinal Drug Absorption.* Various *in vitro* models utilizing isolated cells, membrane vesicles, cell culture systems other than Caco-2 such as HT-29, T84, MDCK, and excised tissues including isolated intestinal segments, Ussing chambers, everted sacs, intestinal rings, and stripped and unstripped mucosal sheets have been investigated for drug absorption to varying degrees (Hillgren, 1995).

4.4. ENTEROHEPATIC CIRCULATION

The liver secretes bile into the duodenum via the bile duct. Bile contains bile salts, which act as surfactants to promote the absorption of lipophilic substances including dietary components and drugs. Approximately 90% of the bile excreted into the intestine is reabsorbed and return to the liver for secretion. Drug can be

excreted from hepatocytes into bile in unchanged and/or metabolized forms. Some metabolites excreted into the intestine via bile can be converted back to the parent drug by enzymatic or chemical reactions in the intestine. For instance, glucuronide conjugates of drugs can be deconjugated to the parent drug by β-glucuronidase produced by intestinal microflora. The unchanged drug or deconjugated metabolites back to the parent drug can be reabsorbed into the portal circulation, a part of which will reach the systemic circulation, and the rest become subject to further metabolism in the liver and/or subsequent biliary excretion (Tabata *et al.,* 1995). Nearly all drugs undergo enterohepatic circulation to a certain extent (Fig. 4.15).

4.4.1. Recognizing Enterohepatic Circulation

Enterohepatic circulation (EHC) of a drug is more apparent in animals having a gall bladder, such as mice, ferrets, dogs, monkeys, and humans, because of the distinctive "hump(s)," i.e., a transient increase, in drug exposure profiles around mealtimes. This is due to the pulsatile release of bile containing drug accumulated in the gall bladder into the duodenum upon food intake followed by subsequent reabsorption of the drug from the intestine. It is, however, common not to observe humps in an exposure profile even if a significant portion of the drug is subject to EHC, and this can be due in part to an insufficient number of data points. There are also examples in dogs where transient increases in exposure have been reported without EHC; e.g., delay in gastric emptying time or changes in the viscosity or pH of the GI tract can also cause the transient increases in exposure profiles (Mummaneni *et al.,* 1995; Reppas *et al.,* 1998).

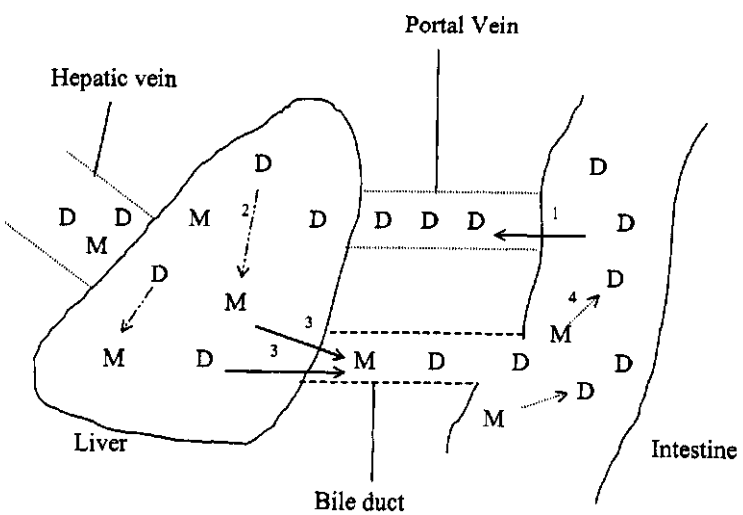

Figure 4.15. Schematic description of enterohepatic circulation of drug. D: drug, M: metabolite—Step 1: Absorption of drug molecules into the portal vein from the gut lumen. Step 2: Biotransformation of the drug to the metabolite by metabolizing enzymes. Step 3: Excretion of the drug and the metabolite into bile. Step 4: Conversion of the metabolite to the parent drug by intestinal microflora in some cases.

In animals without a gall bladder such as rats, the "hump(s)" in an exposure profile after meals may not be apparent for the drug subject to EHC owing to the continuous secretion of bile regardless of food intake. However, transient increases in plasma drug concentration profiles can be also observed in those animals. For example, when deconjugation of glucuronide conjugates of a drug excreted in bile by intestinal microflora occurs in a certain region of the intestine, subsequent reabsorption of the drug can produce a hump in the systemic exposure.

4.4.2. Pharmacokinetic Implications of Enterohepatic Circulation

1. Enterohepatic circulation should be viewed as a part of the distributional processes rather than the elimination processes.
2. In the presence of extensive EHC, the plasma exposure of a drug subject to EHC tends to be higher and is sustained longer than that of a drug with no EHC. As a result, a drug subject to EHC tends to exhibit a lower systemic clearance and a larger volume of distribution with a longer terminal half-life than one with no EHC.
3. It is often difficult to have an accurate estimate of oral bioavailability when the drug is subject to extensive EHC.
4. Biliary excretion of a drug seems to be a more important elimination pathway in laboratory animals such as the rat and the dog, than in the human. Therefore, care should be taken in extrapolating biliary excretion data of compounds obtained in animals to humans. Pharmacokinetic significance of EHC in the extent and duration of exposure of a drug is unclear in humans owing to the fact that there is limited information on the biliary excretion of drugs and their metabolites in humans *in vivo*.

4.4.3. Physicochemical Properties of Compounds for Biliary Excretion

Biliary excretion of compounds is thought to be mainly via carrier-mediated processes. Important physicochemical properties of compounds exhibiting relatively high biliary excretion are lipophilicity, molecular weight, and charge (Hirom *et al.,* 1974).

1. Lipophilicity ($\log P > -2$).
2. Molecular weight (>300 or 500 in rats or humans, respectively, for appreciable biliary excretion).
3. Charge: there appear to be separate biliary secretory mechanisms for acidic, basic, and neutral compounds.

4.4.4. Measuring Clearance in the Presence of Enterohepatic Circulation

Owing to the elevated exposure level of a drug from EHC, the systemic clearance (Cl_s) subject to EHC estimated by the intravenous dose ($D_{iv)}$ divided by AUC after intravenous injection from zero to infinity ($AUC_{0-\infty,iv)}$ may be lower

than the sum of true individual organ clearances. In addition, a reliable determination of AUC from t_{last}, the last time point at which a quantifiable plasma drug concentration can be measured, to infinity ($AUC_{t_{last}-\infty}$) for the estimate of $AUC_{0-\infty, iv}$ using conventional curve-fitting may be difficult owing to the hump(s) in exposure caused by EHC. For a reliable estimate of $AUC_{t_{last}-\infty}$ of a drug subject to EHC after intravenous injection, renal clearance (Cl_s) is utilized.

1. Collect the first urine sample up to t_{last} and the second urine sample from t_{last} to time t in the same animals, at which excretion of a drug in the urine is considered to be completed.
2. Calculate Cl_r of the drug by dividing the amount of drug excreted unchanged in the urine from time 0 to t_{last} ($A_{e,0-t_{last}}$) by $AUC_{0-t_{last}}$. Since Cl_r is constant regardless of the shape of the plasma drug concentration–time profile, an estimation of $AUC_{t_{last}}$ can be obtained by dividing the amount of drug excreted from t_{last} to time t ($A_{e,t_{last}-1}$) in the urine by Cl_r:

$$Cl_r = A_{e,0-t_{last}}/AUC_{0-t_{last}}$$

Therefore,

$$AUC_{t_{last}-\infty} = A_{e,t_{last}-t}/Cl_r$$

3. Cl_s can be estimated by dividing D_{iv} by $AUC_{0-\infty}$ which is the sum of $AUC_{0-t_{last}}$ and $AUC_{t_{last}-\infty}$.

4.4.5. Investigating Enterohepatic Circulation

Various experimental approaches to study the presence and extent of EHC of a drug in animals have been introduced:

1. Comparison of plasma exposure profiles between intact and bile-duct-cannulated animals: If exposure levels of a drug in intact animals are higher than those in bile-duct-cannulated animals, especially during the later stages of disposition, this may indicate that the drug undergoes EHC.
2. Donation of bile from one animal to another: The bile duct of one animal (donor animal) is surgically connected to the duodenum of another animal (recipient animal), and the bile duct of the recipient animal is cannulated for bile collection. After administration of a drug to the donor animal, blood and bile samples from the recipient animal are analyzed. If the drug is found in samples from the recipient animal, it is indicative of EHC.
3. Gut sterilization: When the conversion of conjugate metabolites to the parent drug by intestinal microflora and its subsequent absorption cause a transient increase in an exposure profile, the effects of microflora can be studied by sterilizing the gut lumen with pretreatment of animals with nonabsorbable antibiotics such as lincomycin.

4.5. FECAL EXCRETION OF DRUGS AND COPROPHAGY

Fecal excretion can be an important elimination pathway of xenobiotics from the body. Several important factors contributing to the extent of fecal elimination of drug are as follows:

1. Incomplete absorption: After oral administration of a drug, the nonabsorbed portion can be excreted in feces as unchanged. A significant portion of many macromolecules and ionized compounds at physiological pH may be excreted unchanged in feces after oral administration.
2. Biliary excretion: The biliary excretion of a drug is perhaps the most important factor for its fecal excretion.
3. Intestinal secretion: Drugs can be secreted from mesenteric blood into the intestinal lumen through enterocytes mainly by passive diffusion. The secretion into the intestinal lumen can become an important elimination pathway for certain lipophilic drugs only when other elimination processes are slow. Oral administration of activated charcoal or a fatty diet can facilitate the intestinal secretion of lipophilic drugs.

Most rodents such as rats and rabbits need to feed on their own feces as part of their normal diet, which is known as coprophagy. It is, therefore, sometimes necessary to restrict coprophagy by putting tail cups on the animals or conducting experiments in metabolic cages to separate feces, in order to avoid reintake of the drug in the feces.

4.6. LYMPHATIC ABSORPTION

The lymphatic system is an important vascular network for maintaining body water homeostasis in addition to blood circulation, and the whole GI tract is equipped with lymphatic vessels as well as blood vessels. The intestinal lymphatics are the major absorption pathway for many lipophilic nutrients including fats, lipid-soluble vitamins, and cholesterol. The orally dosed drug molecules have to be transported across enterocytes before entering either blood or lymphatic vessels. The lymph from the GI tract is collected into the thoracic lymph duct without passing through the liver before entering into the bloodstream. Therefore, the drug absorbed via lymphatic vessels in the GI tract can avoid hepatic first-pass effect, although it still has to undergo presystemic intestinal elimination. The lymphatic absorption of compounds is generally considered a minor absorption pathway even for highly lipophilic compounds (Muranishi, 1991).

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