# Part Two
# Physicochemical and Biological Studies of Membrane Permeability and Oral Absorption

# 5
# Physicochemical Approaches to Drug Absorption

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### Abbreviations

| | |
|---|---|
| 1D, 2D, 3D | One-, two-, three-dimensional |
| ACD | Advanced Chemistry Development (software, vendor) |
| ADME | Absorption, distribution, metabolism, and excretion |
| BBB | Blood–brain barrier |
| BMC | Biopartitioning micellar chromatography |
| BNN | Bayesian neural networks |
| Caco-2 | Adenocarcinoma cell line derived from human colon |
| CHIs | Chromatographic hydrophobicity indices |
| CNS | Central nervous system |
| DCE | 1,2-Dichloroethane |
| DMPK | Drug metabolism and pharmacokinetics |
| DMSO | Dimethyl sulfoxide |
| FaSSIF | Fasting-state simulated artificial intestinal fluid |
| HB | Hydrogen bonding |
| HDMs | Hexadecane membranes |
| HSA | Human serum albumin |
| HTS | High-throughput screening |
| IAM | Immobilized artificial membrane |
| ILC | Immobilized liposome chromatography |
| IUPAC | International Union of Pure and Applied Chemistry |
| MAD | Maximum absorbable dose |
| MEKC | Micellar electrokinetic chromatography |
| M&S | Modeling and simulation |
| NMR | Nuclear magnetic resonance |
| PAMPA | Parallel artificial membrane permeation assay |
| PASS | Prediction of activity spectra for substances |
| PBPK | Physiologically based pharmacokinetic modeling |
| P-gp | P-glycoprotein |
| PK | Pharmacokinetic(s) |

| | |
|---|---|
| PPB | Plasma protein binding |
| PSA | Polar surface area (Å$^2$) |
| QSAR | Quantitative structure–activity relationship |
| QSPR | Quantitative structure–property relationship |
| RP-HPLC | Reversed-phase high-performance liquid chromatography |
| SPR | Surface plasmon resonance |
| TLC | Thin-layer chromatography |
| UWL | Unstirred water layer |
| WDI | World Drug Index |

**Symbols**

| | |
|---|---|
| $A_D$ | Cross-sectional area (Å$^2$) |
| Brij35 | Polyoxyethylene(23)lauryl ether |
| Clog *P* | Calculated logarithm of the octanol/water partition coefficient (for neutral species) |
| CLOGP | Daylight/Biobyte computer program for the calculation of log *P* |
| *D* | Distribution coefficient (often in octanol/water) |
| diff(log $P^{N-I}$) | Difference between log $P^N$ and log $P^I$ |
| Δlog *P* | Difference between log *P* in octanol/water and alkane/water |
| $k_a$ | Transintestinal rate absorption constant (min$^{-1}$) |
| $K_a$ | Dissociation constant |
| Elog *D* | Experimental log *D* based on an HPLC method |
| log *D* | Logarithm of the distribution coefficient, usually in octanol/water at pH 7.4 |
| log $D_{7.4}$ | Logarithm of the distribution coefficient, in octanol/water at pH 7.4 |
| log *P* | Logarithm of the partition coefficient, usually in octanol/water (for neutral species) |
| log $P^I$ | Logarithm of the partition coefficient of a given compound in its fully ionized form, usually in octanol/water |
| log $P^N$ | Logarithm of the partition coefficient of a given compound in its neutral form, usually in octanol/water |
| MW | Molecular weight (Da) |
| *P* | Partition coefficient (often in octanol/water) |
| $P_{app}$ | Permeability constant measured in Caco-2 or PAMPA assay (cm/min) |
| p$K_a$ | Ionization constant in water |
| PPB% | Percentage plasma protein binding |
| *S* | Solubility (mg/ml) |
| SITT | Small intestinal transit time (4.5 h = 270 min) |
| SIWV | Small intestinal water volume (250 ml) |
| *V* | Volume (ml or l) |
| $V_{dss}$ | Volume of distribution at steady state (l/kg) |

## 5.1 Introduction

An important part of the optimization process of potential leads to candidates suitable for clinical trials is the detailed study of the absorption, distribution, metabolism, and excretion (ADME) characteristics of the most promising compounds. Experience has learned that physicochemical properties play a key role in drug metabolism and pharmacokinetics (DMPK) [1–5]. In 1995, 2000, and 2004, specialized but very well-attended meetings were held to discuss the role of log *P* and other physicochemical properties in drug research and lead profiling, and the reader is referred to the proceedings for a highly recommended reading on this subject [4, 6, 7].

The molecular structure is at the basis of physicochemical, DMPK, and safety/toxicity properties as outlined in Figure 5.1. Measurement and prediction of physicochemical properties are relatively easy compared to those of DMPK and safety properties, where biological factors come into play. However, DMPK and toxicity properties depend to a certain extent on the physicochemical properties of compounds as these dictate the degree of access to biological systems such as enzymes and transporters.

The change in work practice toward high-throughput screening (HTS) in biology using combinatorial libraries has also increased the demand for more physicochemical and ADME data. There has been an increasing interest in physicochemical hits and leads profiling in recent years, using both *in vitro* and *in silico* approaches [8–11]. This chapter will review the key physicochemical properties, both how they can be measured and how they can be calculated in some cases. Chemical stability [12] is beyond the scope of this chapter, but is obviously important for a successful drug candidate.

The need and precision of a particular physicochemical property for decision making in a drug discovery project depend on the stage in the drug discovery process (see Figure 5.2). While calculated simple filters may be sufficient in library design,

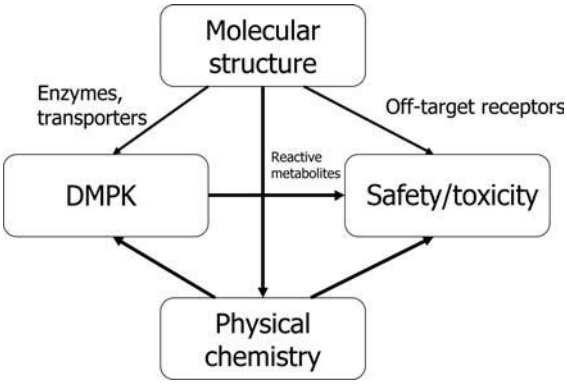


**Figure 5.1** Dependency of DMPK and safety/toxicity properties on structural and physicochemical properties.

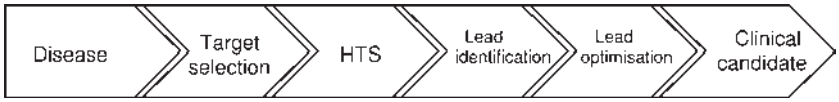


**Figure 5.2** The drug discovery process.

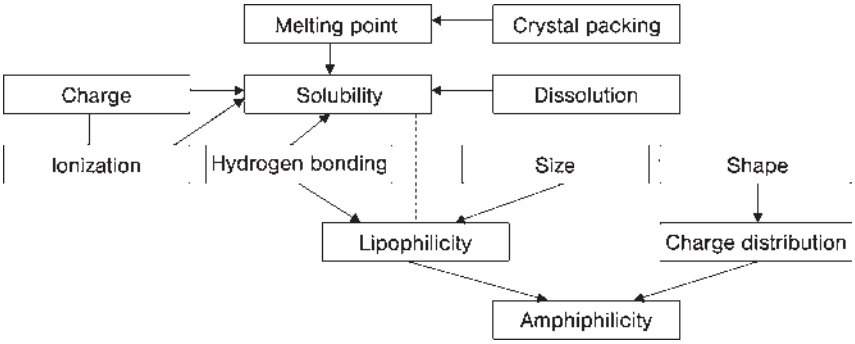


**Figure 5.3** Dependencies between various physicochemical properties.

more experimental data are required in lead optimization. Striking the right balance between computational and experimental predictions is an important challenge in cost-efficient and successful drug discovery.

Physicochemical properties are considerably interrelated as visualized in Figure 5.3. The medicinal chemist should bear in mind that modifying one often means changing other physicochemical properties and hence indirectly influencing DMPK and safety profile of the compound.

## 5.2 Physicochemical Properties and Pharmacokinetics

### 5.2.1 DMPK

The study of DMPK has changed from a descriptive to a much more predictive science [3]. This is driven by great progress in bioanalytics, development of *in vitro* assays and *in silico* modeling and simulation (M&S), and much better basic understanding of the processes. Thus, and fortunately, ADME-related attrition has lowered from about 40% in 1990 to about 10% in 2005 [13].

### 5.2.2 Lipophilicity, Permeability, and Absorption

As an example of the role of physicochemical properties in DMPK, the properties relevant to oral absorption are described in Figure 5.4. It is important to note that

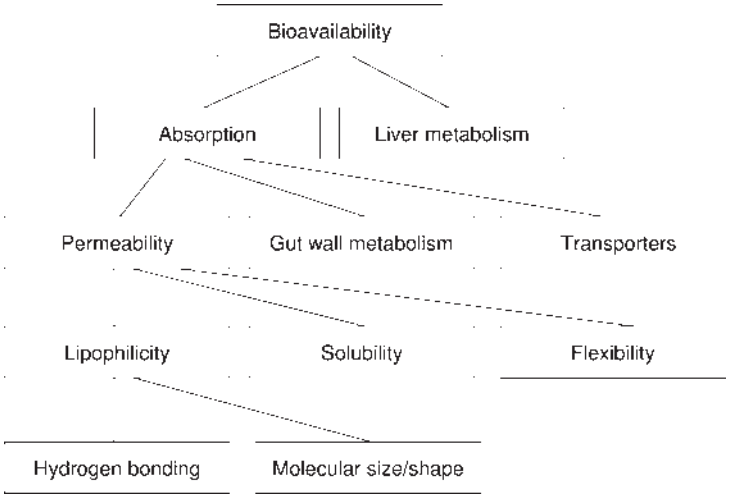


**Figure 5.4** Importance of physicochemical properties on permeability, absorption, and bioavailability [16] (copyright Elsevier).

these properties are not independent but closely related to each other. Oral absorption is the percentage of drug taken up from the gastrointestinal lumen into the portal vein blood. The processes involved are a combination of physicochemical and biological processes (transporters, metabolizing enzymes). The transfer process through a membrane without any biological component is often called permeability. It can be mimicked in an artificial membrane such as the PAMPA (parallel artificial membrane permeation assay) setup (see Section 5.8.1). However, *in vivo* permeability cannot be measured in isolation from biological events. All so-called *in vitro* measures for permeability are nothing else than different types of lipophilicity measures. In plotting oral absorption (percentage or fraction) against any "permeability" or lipophilicity scale (see Figure 5.5), one observes a trend indicating that higher permeability or lipophilicity leads to better absorption. Often a plateau is observed too, indicating that such relationships are in fact nonlinear and can be approached by

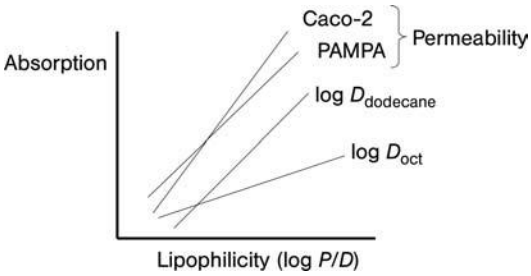


**Figure 5.5** Relationships between oral absorption and permeability/lipophilicity. In reality, these relationships are most likely sigmoidal, that is, more complex than these trends indicate.

a sigmoidal function. Several lipophilicity scales can be related to each other via a Collander (Equation 5.1) or an extended Collander relationship (Equation 5.2) by adding a parameter for the difference in hydrogen bonding (HB) between the two solvent systems. The equivalent for relating, for example, PAMPA scales to each other, or PAMPA with Caco-2, has been published as well [14, 15].

$$\log P_1 = a \log P_2 + b, \tag{5.1}$$

$$\log P_1 = p \log P_2 + q\,\mathrm{HB} + r. \tag{5.2}$$

Instead of using surrogate measures for oral absorption with a lipophilicity or permeability assay *in vitro*, oral absorption can also be estimated *in silico* by using human oral absorption data from the literature [16]. These data are rather sparse because oral absorption is not systematically measured in clinical trials. The data are also skewed toward high absorption compounds. In addition, interindividual variability is important, about 15%. Of course, absorption can also depend on dose and formulation. Therefore, early estimates are only rough guides to get the ballpark right.

### 5.2.3
### Estimation of Volume of Distribution from Physical Chemistry

The distribution of a drug in the body is largely driven by its physicochemical properties and in part for some compounds by the contribution of transporter proteins [17]. By using the Oie–Tozer equation and estimates for ionization ($pK_a$), plasma protein binding (PPB), and lipophilicity ($\log D_{7.4}$), quite robust predictions for the volume of distribution at steady state ($V_{dss}$), often within twofold of the observed value, can be made [18].

### 5.2.4
### Plasma Protein Binding and Physicochemical Properties

Although the percentage of binding to plasma proteins (PPB%) is an important factor in pharmacokinetics and is a determinant in the actual dosage regimen (frequency), it is not important for the daily dose size [3]. The daily dose is determined by the required free or unbound concentration of drug required for efficacy [3]. Lipophilicity is a major driver of PPB% [19, 20]. The effect of the presence of negative (acids) or positive (bases) charges has different impacts on binding to human serum albumin (HSA), as negatively charged compounds bind more strongly to HSA than would be expected from the lipophilicity of the ionized species at pH 7.4 [19, 20] (see Figure 5.6).

## 5.3
## Dissolution and Solubility

Each cellular membrane can be considered as a combination of physicochemical and biological barriers to drug transport. Poor physicochemical properties may some-

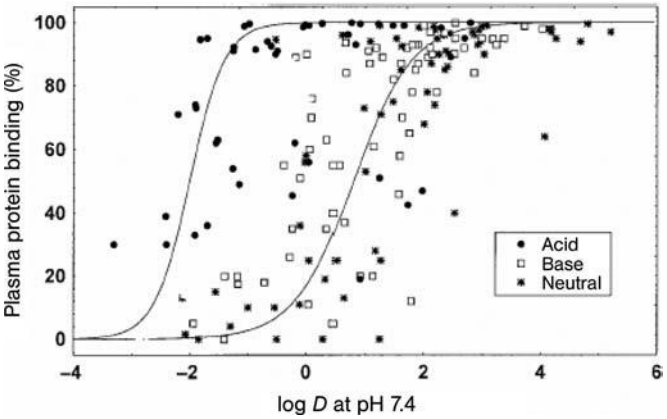


**Figure 5.6** Relationships between percentage human plasma protein binding (hPPB%) and octanol/water log $D_{7.4}$ [20]. Note the about 2 log units downshift of the sigmoidal relationship for acids as compared to neutrals and basics (copyright Springer–Kluwer).

times be overcome by an active transport mechanism. Before any absorption can take place at all, the first important properties to consider are dissolution and solubility [21a]. Many cases of solubility-limited absorption have been reported and therefore solubility is now seen as a property to be addressed at early stages of drug discovery [21b]. Only compound in solution is available for permeation across the gastrointestinal membrane. Solubility has long been recognized as a limiting factor in the absorption process leading to the implementation of high-throughput solubility screens in early stages of drug design [22–24, 136, 137]. Excessive lipophilicity is a common cause of poor solubility and can lead to erratic and incomplete absorption following oral administration. Estimates of desired solubility for good oral absorption depend on the permeability of the compound and the required dose, as illustrated in Table 5.1 [137]. The incorporation of an ionizable center, such as an amine or similar function, into a template can bring a number of benefits including water solubility.

The concept of maximum absorbable dose (MAD) relates drug absorption to solubility via Equation 5.3 [25, 26]:

$$\mathrm{MAD} = S \times k_a \times \mathrm{SIWV} \times \mathrm{SITT}, \tag{5.3}$$

where $S$ is the solubility (mg/ml) at pH 6.5, $k_a$ is the transintestinal absorption rate constant ($\mathrm{min}^{-1}$), SIWV is the small intestinal water volume (ml), assumed to be about 250 ml, and SITT is the small intestinal transit time (min), assumed to be 4.5 h = 270 min.

Dissolution testing has been used as a prognostic tool for oral drug absorption [27]. A Biopharmaceutics Classification Scheme (BCS) has been proposed under which drugs can be categorized into four groups according to their solubility and permeability properties [28]. Because both permeability and solubility can be further dissected into more fundamental properties, it has been argued that the principal

**Table 5.1** Desired solubility needed for expected doses [137].

| Dose (mg/kg) | Permeability (μg/ml) | | |
|---|---|---|---|
| | **High** | **Medium** | **Low** |
| 0.1 | 1 | 5 | 21 |
| 1 | 10 | 52 | 210 |
| 10 | 100 | 520 | 2100 |

properties are not solubility and permeability, but rather molecular size and hydrogen bonding [29]. The BCS has been adopted as a regulatory guideline for bioequivalence studies.

### 5.3.1
### Calculated Solubility

As a key first step toward oral absorption, considerable effort went into the development of computational solubility prediction [30–37]. However, partly due to a lack of large sets of experimental data measured under identical conditions, today's methods are not robust enough for reliable predictions [38]. Further fine-tuning of the models can be expected as new high-throughput data become available to construct such models. Models will be approximate since they do not take into account the effect of crystal packing, ionic force, type of buffer, temperature, and so on. Solubility is typically measured in an aqueous buffer only partly mimicking the physiological state. More expensive FaSSIF solutions have been used to measure solubility, which in some cases appear to give better predictions in physiologically based pharmacokinetic (PBPK) modeling than solubility data using a simpler aqueous buffer [39].

## 5.4
## Ionization ($pK_a$)

For decades, it was assumed that molecules can cross a membrane only in their neutral form. This dogma, based on the pH-partition theory, has been challenged [40, 138]. Using cyclic voltammetry, it was demonstrated that compounds in their ionized form pass into organic phases and might well cross membranes in this ionized form [41].

The importance of drug ionization using cell-based methods such as Caco-2 in the *in vitro* prediction of *in vivo* absorption was discussed [42]. It was observed that when the apical pH used in Caco-2 studies was lowered from 7.4 to 6.0, a better correlation was obtained with *in vivo* data, demonstrating that careful selection of experimental conditions *in vitro* is crucial to have a reliable model. Studies with Caco-2 monolayers also suggested that the ionic species might contribute considerably to overall drug transport [43].

Various ways a charged compound may cross a membrane by a "passive" mechanism have been described [40]. These include ion (trans- and/or paracellular), ion pair, or protein-assisted transport (using the outer surface of a protein spanning a membrane).

Therefore, a continued interest exists in the role of p$K_a$ in oral absorption, which is often related to its effect on lipophilicity and solubility. Medicinal chemists can modulate these properties through structural modifications [44]. Various methods to measure p$K_a$ values have been developed [44–47] and considerable databases are now available.

The difference between the log $P$ of a given compound in its neutral form (log $P^N$) and its fully ionized form (log $P^I$) has been termed diff(log $P^{N-I}$) and contains series-specific information and expresses the influence of ionization on the intermolecular forces and intramolecular interactions of a solute [41, 48, 49].

### 5.4.1
### Calculated p$K_a$

A number of approaches to predict ionization based on structure have been published (for a review see Ref. [50]), and some of these are commercially available. Predictions tend to be good for structures with already known and measured functional groups. However, predictions can be poor for new innovative structures. Nevertheless, p$K_a$ predictions can still be used to drive a project in the desired direction, and rank order of the compounds is often correct. More recently, training algorithms have also become available, which use in-house data to improve the predictions. This is obviously the way forward.

## 5.5
## Molecular Size and Shape

Molecular size can be a further limiting factor in oral absorption [51]. The Lipinski's rule-of-5 proposes an upper limit of MW 500 as acceptable for orally absorbed compounds [136]. High molecular weight (MW) compounds tend to undergo biliary excretion. High MW is a necessary but not sufficient condition for biliary excretion. Substrates of the excretion transporters must also be anionic, that is, resemble the natural substrates, which are biliary acids. Size and shape parameters are generally not measured but rather calculated. A measured property is the so-called cross-sectional area, which is obtained from surface activity measurements [52].

### 5.5.1
### Calculated Size Descriptors

Molecular weight is often taken as the size descriptor of choice, while it is easy to calculate and is in the chemist's mind. However, other size and shape properties are equally simple to calculate and may offer a better guide to estimate potential for

permeability. Thus far, no systematic work has been reported investigating this in detail. Cross-sectional area $A_D$ obtained from surface activity measurements has been reported as a useful size descriptor to discriminate compounds that can access the brain ($A_D < 80\,Å^2$) from those that are too large to cross the blood–brain barrier (BBB) [52]. Similar studies have been performed to define a cutoff for oral absorption [53].

## 5.6 Hydrogen Bonding

Molecular size and hydrogen bonding have been unraveled as the two major components of log *P* or log *D* [54–56]. It was found that hydrogen-bonding capacity of a drug solute correlates reasonably well with passive diffusion. $\Delta\log P$, the difference between octanol/water and alkane/water partitioning, was suggested as a good measure for solute hydrogen bonding [55, 57, 58]. However, this involves tedious experimental work, and it appeared that calculated descriptors for hydrogen bonding could most conveniently be assessed, particularly for virtual compounds.

### 5.6.1 Calculated Hydrogen-Bonding Descriptors

Considerable interest is focused on the calculation of hydrogen-bonding capability in the design of combinatorial libraries for assessing the potential for oral absorption and permeability [16, 59–62]. A number of different descriptors for hydrogen bonding have been discussed [63], one of the simplest being the count of the number of hydrogen bond forming atoms [64].

A simple measure of hydrogen-bonding capacity, originally proposed by van de Waterbeemd and Kansy [65], is the polar surface area (PSA), defined as the sum of the fractional contributions to surface area of all nitrogen and oxygen atoms and hydrogen atoms attached to these. PSA was used to predict the passage of the blood–brain barrier [66–68], flux across a Caco-2 monolayer [69], and human intestinal absorption [70, 71]. The physical explanation is that polar groups are involved in desolvation when they move from an aqueous extracellular environment to the more lipophilic interior of membranes. PSA thus represents, at least, part of the energy involved in membrane transport. PSA depends on conformation, and the original method [65] is based on a single minimum energy conformation. Others [70] have taken into account conformational flexibility and coined a dynamic PSA, in which a Boltzmann-weighted average PSA is computed. However, it was demonstrated that PSA calculated for a single minimum energy conformation is in most cases sufficient to produce a sigmoidal relationship to intestinal absorption, differing very little from the dynamic PSA described above [71]. A fast calculation of PSA as a sum of fragment-based contributions has been published [72], allowing these calculations to be used for large data sets such as combinatorial or virtual libraries. The sigmoidal relationship can be described by $A\% = 100/[1 + (\mathrm{PSA}/\mathrm{PSA}_{50})^{\gamma}]$,

where $A\%$ is percentage of orally absorbed drug, $PSA_{50}$ is the PSA at 50% absorption level, and $\gamma$ is a regression coefficient [73].

Poorly absorbed compounds have been identified as those with a PSA > 140 Å$^2$. Considering more compounds, considerably more scatter was found around the sigmoidal curve observed for a smaller set of compounds [71]. This is partly due to the fact that many compounds not only show simple passive diffusion but are also affected by active carriers, efflux mechanisms involving P-glycoprotein (P-gp) and other transporter proteins, and gut wall metabolism. These factors also contribute to the considerable interindividual variability of human oral absorption data. A further refinement in the PSA approach is expected to come from taking into account the strength of the hydrogen bonds, which in principle already is the basis of the HYBOT approach [60–62].

## 5.7 Lipophilicity

### 5.7.1 log *P* and log *D*

Octanol/water partition (log *P*) and distribution (log *D*) coefficients are widely used to estimate membrane penetration and permeability, including gastrointestinal absorption [74, 75], BBB crossing [57, 66], and correlations to pharmacokinetic properties [1]. The two major components of lipophilicity are molecular size and hydrogen bonding [54], each of which has been discussed above (see Sections 5.5 and 5.6).

According to published IUPAC recommendations, the terms hydrophobicity and lipophilicity are best described as follows [76]:

- *Hydrophobicity* is the association of nonpolar groups or molecules in an aqueous environment, which arises from the tendency of water to exclude nonpolar molecules.
- *Lipophilicity* represents the affinity of a molecule or a moiety for a lipophilic environment. It is commonly measured by its distribution behavior in a biphasic system, either liquid–liquid (e.g., partition coefficient in 1-octanol/water) or solid–liquid (retention on reversed-phase high-performance liquid chromatography (RP-HPLC) or thin-layer chromatography (TLC) system).

The intrinsic lipophilicity (*P*) of a compound refers only to the equilibrium of the unionized (neutral) drug between the aqueous phase and the organic phase. It follows that the remaining part of the overall equilibrium, that is, the concentration of ionized drug in the aqueous phase, is also of great importance in the overall observed partition ratio. This in turn depends on the pH of the aqueous phase and the acidity or basicity ($pK_a$) of the charged function. The overall ratio of drug, ionized and unionized, between the phases has been described as the *distribution coefficient* (*D*) to distinguish it from the intrinsic lipophilicity (*P*). The term has become widely used in recent years to

describe, in a single term, the *effective (or net) lipophilicity* of a compound at a given pH taking into account both its intrinsic lipophilicity and its degree of ionization. The distribution coefficient ($D$) for a monoprotic acid (HA) is defined as

$$D = \frac{[\mathrm{HA}]_{\mathrm{organic}}}{[\mathrm{HA}]_{\mathrm{aqueous}} + [\mathrm{A}^-]_{\mathrm{aqueous}}}, \tag{5.4}$$

where [HA] and [$A^-$] represent the concentrations of the acid in its unionized and dissociated (ionized) states, respectively. The ionization of the compound in water is defined by its dissociation constant ($K_a$) as

$$K_a = \frac{[\mathrm{H}^+][\mathrm{A}^-]}{[\mathrm{HA}]}, \tag{5.5}$$

sometimes referred to as the Henderson–Hasselbalch relationship. The combination of Equations 5.4–5.6 gives the pH distribution (or "pH-partition") relationship:

$$D = \frac{P}{1 + (K_a/[\mathrm{H}^+])}, \tag{5.6}$$

more commonly expressed for monoprotic organic *acids* in the form of Equations 5.7 and 5.8:

$$\log\left(\frac{P}{D} - 1\right) = \mathrm{pH} - \mathrm{p}K_a \tag{5.7}$$

or

$$\log D = \log P - \log(1 + 10^{\mathrm{pH} - \mathrm{p}K_a}). \tag{5.8}$$

For monoprotic organic *bases* ($BH^+$ dissociating to B), the corresponding relationships are

$$\log\left(\frac{P}{D} - 1\right) = \mathrm{p}K_a - \mathrm{pH} \tag{5.9}$$

or

$$\log D = \log P - \log(1 + 10^{\mathrm{p}K_a - \mathrm{pH}}). \tag{5.10}$$

From these equations, it is possible to predict the effective lipophilicity (log $D$) of an acidic or basic compound at any pH value. The data required to use the relationship in this way are the intrinsic lipophilicity (log $P$), the dissociation constant ($pK_a$), and the pH of the aqueous phase. The overall outcome of these relationships is the effective lipophilicity of a compound, at physiological pH, which is approximately the log $P$ value minus one unit of lipophilicity; for every unit of pH, the $pK_a$ value is below (for acids) and above (for bases) pH 7.4. Obviously, for compounds with multifunctional ionizable groups, the relationship between log $P$ and log $D$, as well as log $D$ as a function of pH, becomes more complex [62, 65, 67]. For diprotic molecules, there are already 12 different possible shapes of log $D$–pH plots. Ion pairs (salts), zwitterions, and ampholytes are special cases and both measurement of log $P$/$D$ and their interpretation need special attention [44, 49].

Traditional octanol/water distribution coefficients are still widely used in quantitative structure–activity relationship (QSAR) and in ADME/PK studies. However, alternative solvent systems have been proposed [77]. To cover the variability in biophysical characteristics of different membrane types, a set of four solvents has been suggested, sometimes called the "critical quartet" [78]. The 1,2-dichloroethane (DCE)/water system has been promoted as a good alternative to alkane/water due to its far better dissolution properties [79, 80], but may find little use because of its carcinogenic properties.

Several approaches for higher throughput lipophilicity measurements have been developed in the pharmaceutical industry [47] including automated shake-plate methods [81] and immobilized artificial membranes (IAMs) [82]. A convenient method to measure octanol/water partitioning is based on potentiometric titration, called the pH method [83]. Methods based on chromatography are also widely used and include, for example, chromatographic hydrophobicity indices (CHIs) measured on immobilized artificial membranes [19, 84]. Another chromatography-based method is called Elog *D* giving log *D* values comparable to shake-flask data [85].

### 5.7.2 Calculated log *P* and log *D*

A number of rather comprehensive reviews on lipophilicity estimation have been published and are recommended for further reading [86–88]. Owing to its key importance, a continued interest is seen to develop good log *P* estimation programs [89–91]. Most log *P* approaches are limited due to a lack of parameterization of certain fragments. For the widely used CLOGP program, a version with the ability to estimate missing fragments has become available [92].

With only few exceptions, most log *P* programs refer to the octanol/water system. Based on Rekker's fragmental constant approach, a log *P* calculation for aliphatic hydrocarbon/water partitioning has been reported [93]. Another more recent approach to alkane/water log *P* and log *D* is based on the program VolSurf [94]. It is believed that these values may afford a better prediction of uptake in the brain. The group of Abraham investigated many other solvent systems and derived equations to predict log *P* from structure for these solvent systems, which are also commercially available [91].

log *D* predictions are more difficult as most approaches rely on the combination of estimated log *P* and estimated p$K_a$. Obviously, this can lead to error accumulation and errors of 2 log units or more can be found. Some algorithms, however, are designed to learn from experimental data so that the predictions improve over time. An interesting approach is also the combination of a commercial log *D* predictor with proprietary descriptors using a Bayesian neural network (BNN) approach [95].

Often ignored is the fact that log *P*/*D* is a conformation-dependent property [161], which has elegantly been demonstrated with the molecular lipophilicity potential (MLP) descriptor [87]. The MLP algorithm allows to calculate virtual log *P* values in conformational space.

**Table 5.2** *In vitro* models for membrane permeability.

| Permeability model | References |
|---|---|
| Solvent/water partitioning | |
| Octanol/water distribution | [49] |
| Chromatography | |
| IAMs | [105–109] |
| ILC | [111] |
| MEKC | [113] |
| BMC | [114] |
| Vesicles | |
| Phospholipid vesicles | [129] |
| Liposome binding | [117, 118] |
| Transil particles | [120–122] |
| Fluorosomes | [123] |
| SPR biosensor | [125, 126] |
| Colorometric assay | [124] |
| Artificial membranes | |
| Impregnated membranes | [69] |
| PAMPA | [96–102] |
| Filter IAM | [100–102] |
| HDM | [103, 104] |
| Other | |
| Surface activity | [128] |
| Cell-based assays | |
| Caco-2 | [73, 75] |
| MDCK | [150] |

## 5.8 Permeability

An overview of permeability assays is presented in Table 5.2. As discussed earlier in this chapter, these permeability scales are correlated to each other, as well as the various lipophilicity scales, via extended Collander equations.

### 5.8.1 Artificial Membranes and PAMPA

When screening for absorption by passive membrane permeability, artificial membranes have the advantage of offering a highly reproducible, high-throughput system. Artificial membranes have been compared with Caco-2 cells and found to behave very similar for passive diffusion [69]. This finding was the basis for the development of the parallel artificial membrane permeation assay for rapid prediction of transcellular absorption potential [96–99]. In this system, the permeability through a membrane formed by a mixture of lecithin and an inert organic solvent on a hydrophobic filter support is assessed. While not completely predictive for oral absorption in humans,

PAMPA shows definite trends in the ability of molecules to permeate membranes by passive diffusion, which may be valuable in screening large compound libraries. This system is commercially available [100], but can easily be set up in-house. Further optimization of the experimental conditions has been investigated concluding that predictability increases when a pH of 6.5 or 5.5 is used on the donor side [101, 102]. It was also demonstrated that the effect of a cosolvent such as dimethyl sulfoxide (DMSO) could have a marked effect depending on the nature, basic or acidic, of the compound [102]. Stirring of the donor compartment to limit the contribution of the unstirred water layer (UWL) appears to be important to get meaningful results. There have been so far no reports in the literature about using PAMPA data in a drug discovery project.

A similar system based on polycarbonate filters coated with hexadecane, also called hexadecane membranes (HDMs), has been reported [103, 104]. Thus, this system consists of a 9–10 μm hexadecane liquid layer immobilized between two aqueous compartments. Also, here it was observed that in this setup for lipophilic compounds, the diffusion through the unstirred water layer becomes the rate-limiting step. To mimic the *in vivo* environment permeability, measurements were repeated at different pH values in the range 4–8, and the highest transport value was used for correlation with the percentage absorbed in humans. This gives a sigmoidal dependence, which is better than when taking values measured at a single pH, for example, 6.8.

#### 5.8.1.1 *In Silico* PAMPA

The experimental $P_{app}$ data have been used to build predictive models. However, since PAMPA is already a model, an *in silico* model based on this is a model of a model. The predictability for *in vivo* permeability or absorption of such *in silico* PAMPA model can be questioned (see Equation 5.11), since it is two steps from reality.

$$\text{model} \times \text{model} = \text{random}. \tag{5.11}$$

### 5.8.2 IAM, ILC, MEKC, and BMC

Immobilized artificial membranes are another means of measuring lipophilic characteristics of drug candidates and other chemicals [105–109]. IAM columns may mimic membrane interactions better than the isotropic octanol/water or other solvent/solvent partitioning system. These chromatographic indices appear to be a significant predictor of passive absorption through the rat intestine [110].

A related alternative is called immobilized liposome chromatography (ILC) [111, 112]. Compounds with the same log *P* were shown to have very different degrees of membrane partitioning on ILC depending on the charge of the compound [112].

Another relatively new lipophilicity scale proposed for use in ADME studies is based on micellar electrokinetic chromatography (MEKC) [113]. A further variant is called biopartitioning micellar chromatography (BMC) and uses mobile phases of Brij35 (polyoxyethylene(23)lauryl ether) [114]. Similarly, the retention factors of 16 beta-blockers obtained with micellar chromatography using sodium dodecyl sulfate

as micelle-forming agent correlate well with permeability coefficients in Caco-2 monolayers and apparent permeability coefficients in rat intestinal segments [115].

Each of these scales produces a lipophilicity index related but not identical to octanol/water partitioning.

### 5.8.3 Liposome Partitioning

Liposomes, which are lipid bilayer vesicles prepared from mixtures of lipids, also provide a useful tool for studying passive permeability of molecules through lipid. This system, for example, has been used to demonstrate the passive nature of the absorption mechanism of monocarboxylic acids [116]. Liposome partitioning of ionizable drugs can be determined by titration and has been correlated with human absorption [117–119]. Liposome partitioning is only partly correlated with octanol/water distribution and might contain some additional information.

A further partition system based on the use of liposomes, and commercialized under the name Transil [120, 121], has shown its utility as a lipophilicity measure in PBPK modeling [122]. Fluorescent-labeled liposomes, called fluorosomes, are another means of measuring the rate of penetration of small molecules into membrane bilayers [99, 123]. Similarly, a colorometric assay amenable to high-throughput screening for evaluating membrane interactions and penetration has been presented [124]. The platform comprises vesicles of phospholipids and the chromatic lipid-mimetic polydiacetylene. The polymer undergoes visible concentration-dependent red–blue transformations induced through interactions of the vesicles with the studied molecules.

### 5.8.4 Biosensors

Liposomes have been attached to a biosensor surface, and the interactions between drugs and the liposomes can be monitored directly using surface plasmon resonance (SPR) technology. SPR measures changes in refractive index at the sensor surface caused by changes in mass. Drug–liposome interactions have been measured for 27 drugs and compared with fraction absorbed in humans [125]. A reasonable correlation is obtained, but it is most likely that this method represents just another way of measuring "lipophilicity." The throughput was 100 substances/24 h, but further progress seems possible. In more recent work using this method, it is proposed to use two types of liposomes to separate compounds according to their absorption potential [126].

## 5.9 Amphiphilicity

The combination of hydrophilic and hydrophobic parts of a molecule defines its amphiphilicity. A program has been described to calculate this property and

calibrated against experimental values obtained from surface activity measurements [127]. These values can possibly be used to predict effect on membranes leading to cytotoxicity or phospholipidosis, but may also contain information, yet not unraveled, on permeability. Surface activity measurements have also been used to make estimates of oral absorption [128].

## 5.10 Drug-Like Properties

The various properties described above are important for drugs, particularly for those given orally. The important question arises whether such properties of drugs are different from chemicals used in other ways. This has been subject of a number of studies [130, 131, 162]. Using neural networks [132, 133] or a decision tree approach [134], a compound can be predicted as being "drug-like" with an error rate of about 20%. A further approach to predict drug-likeness consists of training of the program PASS [135], which was originally intended to predict activity profiles and thus is suitable to predict potential side effects.

From an analysis of the key properties of compounds in the World Drug Index (WDI), the now well-accepted rule-of-5 has been derived [136, 137]. It was concluded that compounds are most likely to have poor absorption when the molecular weight is more than 500, the calculated octanol/water partition coefficient (Clog *P*) is more than 5, number of H-bond donors is more than 5, and the number of H-bond acceptors is more than 10. Computation of these properties is now available as a simple but efficient ADME screen in commercial software. The rule-of-5 should be seen as a qualitative absorption/permeability predictor [138], rather than a quantitative predictor [139]. The rule-of-5 is not predictive for bioavailability as sometimes mistakenly assumed. An important factor for bioavailability in addition to absorption is liver first-pass effect (metabolism). The property distribution in drug-related chemical databases has been studied as another approach to understand "drug-likeness" [140, 141].

Other attempts have been made to try to define good leads [142, 143]. In general, lead-like properties are lower/fewer than drug-like properties. Thus, MW < 350 and Clog $P < 3$ should be good starting points for leads [142]. A rule-of-3 has been proposed [143] for screening small fragments, which says the good lead fragments have MW < 300, Clog $P < 3$, H-bond donors and acceptors less than 3, and rotatable bonds less than 3.

Similarly, in a study on drugs active as central nervous system (CNS) agents, using neural networks based on Bayesian methods, CNS-active drugs could be distinguished from CNS-inactive ones [144]. A CNS rule of thumb says that if the sum of the nitrogen and oxygen (N + O) atoms in a molecule is less than 5, and if the Clog $P - (N + O) > 0$, then compounds are likely to penetrate the blood–brain barrier [145]. Another "rule" is that the PSA should be less than 90 $Å^2$, the MW should be less than 450, and the log *D* at pH 7.4 should be between 1 and 3 [146]. In designing CNS drugs, it is important to distinguish BBB penetration and CNS efficacy. The CNS efficacy is a subtle balance between permeability, effect of BBB transporters, lipophilicity, and free fraction in blood and brain [147].

These aforementioned analyses point to a critical combination of physicochemical and structural properties [148], which to a large extent can be manipulated by the medicinal chemist. This approach in medicinal chemistry has been called property-based design [2]. Properties in this context mean physicochemical as well as pharmaco- and toxicokinetic properties. These have been neglected for a long time by most medicinal chemists who in many cases in the past had the quest only for strongest receptor binding as ultimate goal. However, this strategy has changed dramatically, and the principles of drug-like compounds are now being used in computational approaches toward the rational design of combinatorial libraries [149] and in decision making on acquisition of outsourced libraries.

## 5.11 Computation Versus Measurement of Physicochemical Properties

### 5.11.1 QSAR Modeling

Calculation of many different 1D, 2D, and 3D descriptors for building predictive QSAR models for physicochemical (and ADMET) properties is possible by using a range of commercially available software packages, such as ACD, Sybyl, Cerius2, Molconn-Z, HYBOT, VolSurf, MolSurf, Dragon, MOE, BCUT, and so on. Several descriptor sets are based on quantification of 3D molecular surface properties [151, 152], and these have been explored for the prediction of, for example, Caco-2 permeability and oral absorption [16]. It is pointed out here that a number of these "new" descriptors are often strongly correlated to the more traditional physicochemical properties. An aspect largely neglected so far is the concept of molecular property space that looks at the conformational effects on physicochemical properties [153].

Numerous QSAR tools have been developed [152, 154] and used in modeling physicochemical data. These vary from simple linear to more complex nonlinear models, as well as classification models. A popular approach more recently became the construction of consensus or ensemble models ("combinatorial QSAR") by combining the predictions of several individual approaches [155]. Or, alternatively, models can be built by running the same approach, such as a neural network of a decision tree, many times and combining the output into a single prediction.

To build robust predictive models, good-quality training set and sound test set are required. Criteria for a good set include sufficient coverage of chemical space, good distribution between low- and high-end values of the property studied, and a sufficiently large number of compounds. Models can be global (covering many types of chemistry) or local (project specific). There are many reasons why predictions can fail [156], and medicinal chemists need to be aware of these. There is also a difference between a useful model and a perfect model. The latter does not exist!

In-house physicochemical data collections are growing rapidly through the use of HTS technologies [157]. Therefore, the need for rapidly building and updating is also increasing. Systems for automatic and regular updating of QSAR predictive models have been reported [158] and we expect these to become more widespread. A consequence of regularly updated *in silico* models is that the predicted values will change too. This will require adapted ways of working in projects using more dynamic data generation and interpretation tools.

### 5.11.2
### *In Combo*: Using the Best of Two Worlds

In modern drug discovery, speed and cost control, in addition to high quality, are important. *In silico* virtual screening for drugability [159] is a good first step in library design and compound acquisition. Once compounds have been made for a targeted project, a well-balanced approach using both *in silico* predictions and *in vitro* screening will be a good strategy to guide the program in a cost-efficient manner. New experimental data can be used to update predictive models regularly so that the ongoing projects can benefit from the latest local and global models available [158, 160].

## 5.12
## Outlook

Physical chemistry plays a key role in the behavior of drugs. Measurement of the key properties has been automated and industrialized to high throughput. The data can be and are used to build robust predictive models, which are used in design building in the required compound quality. These can in turn also be used to limit the use of experiments when not strictly needed. This is of course compound saving and more cost-effective. Predictive models for physicochemical, DMPK/ADME, and toxicity/safety properties are thus great tools in virtual screening, prioritization, decision making, and guiding projects [162].

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# 6
# High-Throughput Measurement of Physicochemical Properties

*Barbara P. Mason*

### Abbreviations

| | |
|---|---|
| ADME | Absorption, distribution, metabolism, and excretion |
| BBB | Blood–brain barrier |
| BSA | Bovine serum albumin |
| Caco-2 | Human colon adenocarcinoma cell line used as a permeation/absorption model |
| CHI | Chromatography hydrophobicity index |
| DMSO | Dimethyl sulfoxide |
| FCS | Fetal calf serum |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (zwitterionic buffer) |
| HTS | High-throughput screening |
| MDCK | Madin–Darby canine kidney |
| MW | Molecular weight |
| PAMPA | Parallel artificial membrane permeability assay |
| PBS | Phosphate buffered saline |
| RP-HPLC | Reverse-phase high-performance liquid chromatography |
| TFA | Target factor analysis |
| UWL | Unstirred water layer |

### Symbols

| | |
|---|---|
| $\log D_{\mathrm{pH}}$ | Logarithm of the distribution coefficient in octanol/water at pH described in subscript |
| $\log P$ | Logarithm of the partition coefficient in octanol/water |
| ml | Milliliter |
| $\mathrm{p}K_{\mathrm{a}}$ | Ionization constant in water |

| | |
|---|---|
| $p_sK_a$ | Ionization constant in cosolvent/water |
| $S$ | Solubility |
| $P_a$ | Apparent permeability (accounting for UWL effects) |
| $P_e$ | Effective permeability (not accounting for UWL effects) |
| %R | Percentage of the compound retained in the membrane |

## 6.1 Introduction

Information about the fundamental properties of a series of compounds, such as permeability, solubility, lipophilicity, and $pK_a$, is extremely useful to the medicinal chemist in drug discovery. It provides insights into the behavior of compounds, which can be directly applied to planning modifications of structures and scaffolds to improve their behavior. With the introduction and development of high-throughput screening (HTS), combinatorial chemistry, and more recently fragment library approaches, the number of compounds requiring such profiling in some, if not all, physicochemical parameters showed a massive increase while the amount of compound available for study was greatly reduced and very often present as a 10 mM DMSO stock solution. It is, therefore, not a surprise that there have been many significant advances in the technology and methods for measuring these parameters in a high-throughput capacity. It should be noted that this chapter will not make any comment as to the definition of high throughput; where it is feasible to run the methods discussed in microtiter plate format or generate a large number data of points at a time on a given set of compounds, this will be classed as "high throughput." Some background theory and mathematics will be introduced where appropriate, more detailed, explanations and derivations are available, and references to these will be given.

## 6.2 Positioning of Physicochemical Screening in Drug Discovery

It has always been tempting to drive medicinal chemistry forward on the basis of selectivity and potency alone; however, this has seen the downfall of many projects. While there is a strategic need in drug discovery to identify compounds that will be readily absorbed and distributed around the body, it could be argued that it is more important to identify those series of compounds that *will not*, so that a judicious choice can be made as to whether they are sufficiently potent to make it worth tackling via formulation or to modify the series appropriately. It is prudent to determine which compounds may be prone to these problems as early in the discovery process as possible. Another advantage of screening physicochemical properties early is that it will give invaluable information in sample handling or assistance in the understanding and explanation of outliers in the biological screening assays.

A typical project lifecycle can be described as having five main stages: (i) target identification, (ii) target validation, (iii) high-throughput screening, (iv) hit to lead, and (v) lead optimization. (The exact number, description, and transition from one stage to another are particular to each organization.) Medicinal chemistry typically plays a role in stages (iii)–(v) by providing a huge number of compounds for protein/enzyme 384-well-style assays in HTS, fewer compounds for cell-based functional assays (although the number of compounds being produced at this stage is still significant, particularly in the major pharmaceutical companies) in the hit-to-lead stage, and finally a handful of compounds per project for multiple disease state models in the lead-optimization stage.

## 6.3 "Fit for Purpose" Versus "Gold Standard"

The challenge then for physical chemists is to provide physicochemical profiling at correct times during this project lifecycle on these varying numbers and quantities of compounds, and to be able to produce data sufficiently quickly, so that lessons can be learnt and judgments are made before the project moves forward. Physicochemical profiling needs to work closely with DMPK and pharmacology to provide a comprehensive data package for the compounds where they have been measured under conditions that are relevant to the assay systems used to generate the potency and selectivity information; in other words, the assays need to be "fit for purpose." Conversely, scientists working on development and preformulation under regulatory guidelines will require a different set of measurements, generated by using recognized, industry standard methods that are capable of producing "gold standard" data.

Advances in instrumentation and computing power have meant that analytical data are both more precise and more reproducible than probably any other measurement made and particularly when compared with biological data. It should be possible with the liquid handling and plate-moving robots available to increase throughput significantly and still provide this high-quality data. This is indeed true; however, the high-quality data presuppose that the samples used for data generation are also of high quality. This is a major assumption, particularly in HTS and library analysis. For any technique, which does not involve a chromatographic separation step, it will not be possible to distinguish an analyte from a contaminant, causing complications in the processing of the data and leading to false positives if its presence is not known. Samples containing highly soluble or highly absorbing impurities, even at relatively low concentration levels, will have a profound effect on the data. To be 100% certain of the accuracy and precision of one's calibration curves, solid material is the starting point of choice. However, this is not a practical option for high-throughput profiling due to the massive pressure that this would place on compound logistics for even a small company. For the majority of high-throughput methods, the compounds are stamped out in microtiter plates and are present as DMSO stock solutions of supposedly known concentration, 10 mM being common.

Although it may appear that to start all assays from a standard 10 mM DMSO stock solution will simplify the profiling processes, there are a number of issues that should be taken into account. The presence of this organic solvent may mask or modify important physical properties that will need to be factored into the information before it can be used for any real benefit. It is very important to ensure that the compound is stable in the solvent and therefore QC checks should be carried out before the assay screens commence to have a time zero point from which to reference. If this is carried out using an HPLC–MS method, then it will have the added benefit of confirming compound purity and validity at the outset. It should also be considered that it is possible that the compounds are not present as 10 mM DMSO stock solutions [1]. This will need to be known and calibration curves adjusted to ensure that any quantitative calculations are correct.

High-throughput physicochemical profiling does have a valid place in drug discovery, and there are four fundamental properties to be measured: aqueous solubility, p$K_a$, lipophilicity, and permeability [2]. The types of data generated using traditional "gold standard" methods are vital for physicochemical profiling. Such methods include shake-flask log *P*, thermodynamic solubility, and potentiometric p$K_a$, providing a means by which compounds can be compared and their behavior and characteristics can be described. While these methods are not slow, they cannot be used for many hundreds or even thousands of compounds at a time produced during HTS and hit-to-lead stages because they use much larger quantities of compound than that are typically available and are not always flexible enough to accommodate the varying conditions that a compound will be exposed to as it passes through a long cascade of assays.

## 6.4 Solubility

Solubility measurements are made to determine an intrinsic property, which influences the absorption potential of a compound [3]. Even though solubility itself does not directly dictate the absorption of a drug, it is important to consider solubility in relation to permeability and potency. In addition, in medicinal chemistry projects, there are other issues to consider that may also be affected by poor solubility, particularly insolubility under screening assay conditions.

### 6.4.1 "Thermodynamic" Versus "Kinetic"

Traditional "shake-flask" or gold standard solubility methods start from solid material vigorously mixed with an aqueous buffer until equilibrium is reached between undissolved and dissolved materials. This may take only a minute or 72 h or more and is compound specific.

These equilibrium solubility conditions are defined as being "thermodynamic" – the most stable species is in solution at equilibrium and not necessarily the fastest

dissolving. Starting from solid means that forces involved in the crystal lattice need to be overcome before the compound will dissolve. Dissolution is rate limited and therefore a kinetic process, but it needs to occur before the final solubility is reached at true thermodynamic equilibrium.

Assays starting with a compound predissolved in an organic solvent (typically DMSO) tend to have shorter incubation times, do not include time course measurements, and therefore the position of equilibrium is not determined, and for highly soluble species the compound may not be present in excess. This type of measurement is typically referred to as "kinetic."

In the hit-to-lead stage of drug discovery, compounds are generally only available in solvents such as DMSO for a number of reasons:

- Stock solutions allow ease of compound storage and distribution and as such they are particularly amenable to plate-based formats.
- They aid poorly soluble compounds in becoming more accessible to the aqueous environment of bioassays.
- DMSO stock solutions are typically the vehicle of choice for all but a few selected *in vivo* experiments, and subsequently, DMSO is present to some degree in almost all early-stage screens.

Attempting to measure a thermodynamic solubility of compounds which will then be used under these screening conditions, will not necessarily give a useful picture of the compounds' performance. They will not reflect the more "transient" nature of the compound that is present in nonequilibrium systems. The presence of organic solvents changes the dielectric constant of an aqueous solution and thus helps to solvate lipophilic compounds in particular, and will give an increased solubility for some series of compounds across the Biopharmaceutics Classification System (BCS) [4]. This is an important consideration in the ultimate use of the solubility data, and a lack of full solubility of the analyte at the test concentration will lead to an underestimation of the compound's true activity. Measuring the solubility of the compounds in close approximation to the assay conditions to which they will be exposed will be more relevant. Indeed, if sensitivity is not an issue, then the quantities, concentrations, and incubation conditions used should reflect those available in the discovery assays.

Another important consideration is that of batch-to-batch variability. Typically, in medicinal chemistry laboratories, compounds are synthesized in large numbers rather than in large quantities. Should a compound prove to be sufficiently interesting, it is resynthesized and reanalyzed. It is likely that the compounds will have been purified by column chromatography, dried down from organic solvents or freeze-dried. No effort will have been spent on creating homogeneous crystals because the medicinal chemist has other priorities. For each of these resyntheses, these compounds will be complex amorphous solids of unspecified crystal (or noncrystal) form. Furthermore, compounds stored for any length of time, even under "optimum" conditions of temperature, light, humidity, and inert atmosphere, are subject to deterioration. This may be where the sample is decomposing or where the crystal form changes and new polymorphs are formed. These species may have vastly

different lattice energies and it is clear that determining the "true" thermodynamic solubility of each of these batches may, and probably will, give very different data that cannot be used for comparison studies.

### 6.4.2 Methods of Measuring High-Throughput Solubility

Solubility solution conditions are important. The pH–solubility profile is a function of the intrinsic solubility of the neutral form, with the solubility of the ionized species (protonated for bases and deprotonated for acids) being typically much higher than that of the neutral species. Therefore, $pK_a$ as well as concentration of DMSO present in the final incubated solution needs to be considered.

There are a variety of methods for determining solubility in a relatively high-throughput manner but of these, two methods occur most often. These can be classified as "supernatant concentration" and "precipitate detection."

### 6.4.3 Supernatant Concentration

The supernatant concentration method uses small volumes of stock solution added to wells containing aqueous buffer in a microtiter filter plate of the type available from Millipore Inc. The solution is incubated for a given amount of time (typically in the range of 1–24 h depending on the requirements of the laboratory) and then filtered or centrifuged. The supernatant is analyzed by UV plate reader or HPLC and the concentration of dissolved species is calculated by reference to a calibration curve. This is often either a three- or a four-point curve prepared from serial dilutions of the stock solution using a solvent such as 80% v/v acetonitrile/water in which the compound is fully soluble.

It is a relatively simple exercise to automate this method using liquid handling robotic hardware, and with integrated plate moving arms, filter manifold systems, plate shakers, centrifuges, and plate readers, the throughput that these types of systems can achieve may only be limited by the plate storage capacity and budget. A UV plate reader such as a Molecular Devices Spectramax 190, reading a scan for each well of a 96-well plate from 200 to 400 nm with 1 nm increments, takes approximately 20 min. For laboratories, where only a single wavelength is required, this is reduced to seconds.

According to Beer's law, path length is a fundamental property of the absorbance of the sample as shown in Equation 6.1:

$$A = Ecl, \tag{6.1}$$

where $A$ is absorbance, $c$ is the concentration, $l$ is the path length, and $E$ is the molar extinction coefficient.

Using a UV plate reader, correction factors for path length are included in the instrument; however, it is critical that the path length is the same in the incubation and calibration samples. The actual path length itself is determined by the depth of

sample in the well, that is, the sample volume. Owing to differences in the solubility of varying samples on the plate, it is likely that the quantity of precipitate present in the wells will vary randomly. It will therefore not be possible to filter a predetermined specified volume of supernatant at the end of the incubation period. For this reason, it is recommended that the samples are filtered into a receiver plate and an aliquot is transferred to a UV plate for analysis.

### 6.4.4 Measuring Solubility Across a pH Range

The p$K_a$ of a molecule, and therefore the pH of the aqueous environment of the solubility assay, is extremely important. Each group carrying out these measurements will have chosen carefully their incubation conditions. High-throughput platforms give the option of carrying out the solubility measurements at a range of pH values; however, this will bring another level of complexity to the assay. Although it will reduce the number of individual compounds that can be analyzed per plate, it will allow an "on-the-fly" visualization of the effects of p$K_a$ (and therefore % ionized) and pH on the solubility. If a UV plate reader is used, then selecting a single analytical wavelength per compound ($\lambda_{max}$, for instance) is not an appropriate option since there may be different molar extinction coefficients for the ionized and neutral species should there be an ionization event at or near the chromophore that causes a spectral change.

Following Beer's law given in Equation 6.1, for a given wavelength, equivalent concentrations of species with a different molar extinction coefficient will not have an equivalent absorbance. Under these circumstances, the use of calibration curves determined from compounds dissolved in organic solvents and therefore present only in the neutral form will not be appropriate for the determination of the concentration of a species that is ionized.

This problem can be overcome by determining a scan of the samples across a range of wavelengths, typically 200–400 nm and selecting the wavelength at which the extinction coefficient is equivalent for all species, the *isosbestic point* (DMSO absorbance will cause interference if scanning is done at wavelengths much lower than 230 nm), to plot the calibration curves and thus calculate the concentration of the analyte present.

To determine the isosbestic point, two calibration samples are required in addition to those of the calibration line. These two will contain sufficient aqueous buffer to cause the pH to shift to the extremes of the pH range of the assay, for example, pH 3 and pH 9, but not enough to cause poorly soluble compounds to precipitate out of solution. As shown in Figure 6.1, the spectra for pH 3 and pH 9 have equivalent absorbances at 315 nm, and therefore this is the isosbestic point and should be chosen for analysis. For situations where there is no clear isosbestic point, a wavelength should be selected from a region of homology.

Setting the concentration in the two spiked solutions to be the same as that of one of the calibration lines will give three spectral lines for qualitative comparison, which will provide additional information about the behavior of the compound. In Figure 6.1, the spectral profiles under purely organic conditions and organic solvent

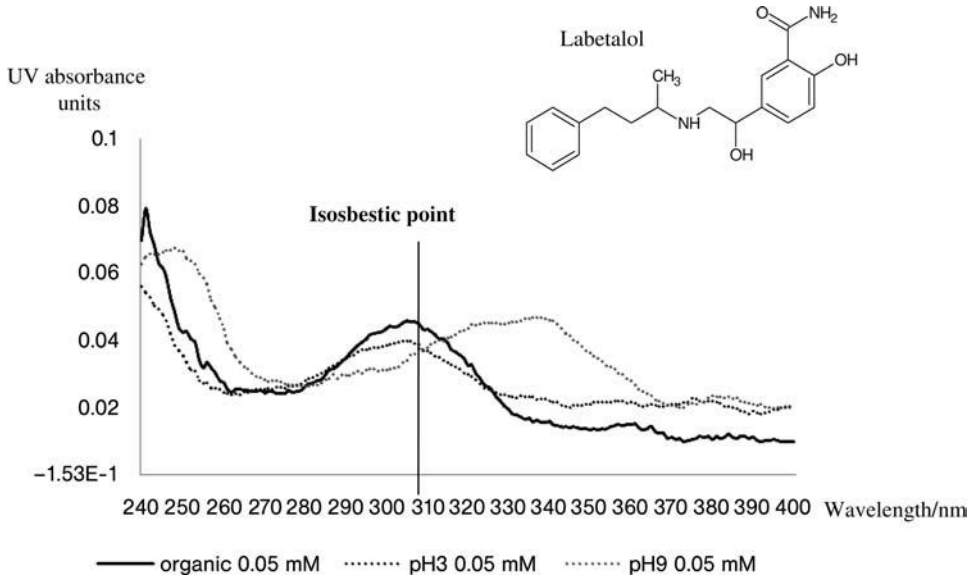


**Figure 6.1** Plot of labetalol UV absorbance spectra showing pH 3, pH 9, and organic calibration lines at 0.05 mm.

spiked with pH 9 buffer are equivalent whereas that spiked with pH 3 buffer shows a different profile. This indicates that under basic conditions the compound is in its neutral form, while under acidic conditions it is in its ionized form. Comparison of these calibration spectra with those from the solubility supernatant samples will determine the pH range at which the ionization occurred. Figure 6.2 shows that labetalol has an acidic ionization event between pH 3 and pH 5 and that the acidic

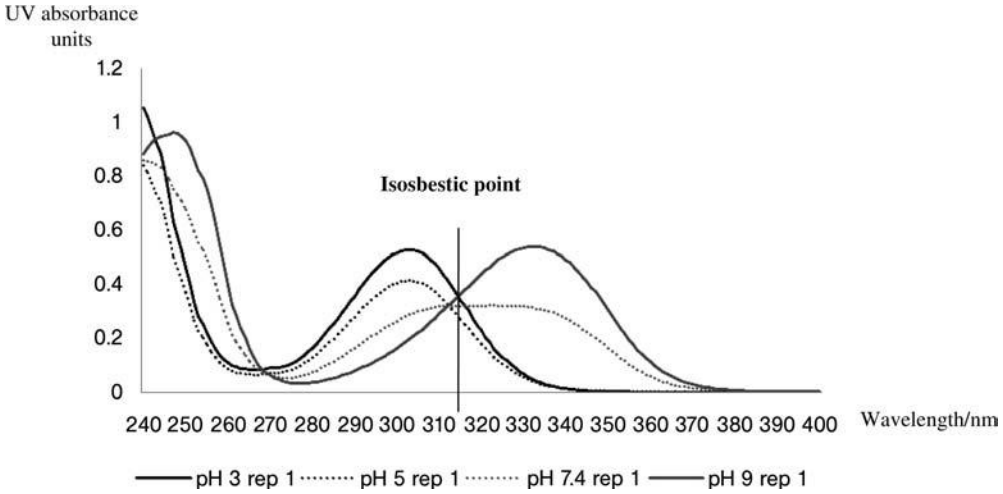


**Figure 6.2** Plot of labetalol solubility lines at pH 3, pH 5, pH 7.4, and pH 9.

center is close to the chromophore, in this instance, the phenolic OH group. In situations where there is more than one possible ionization site, this can be extremely helpful in the assignment of p$K_a$ values.

Another major benefit to medicinal chemists of providing pH profile data is the early identification of stability issues – if the solubility and calibration spectra do not match, it will indicate that degradation of the compound may be occurring.

### 6.4.5
### Supernatant Concentration Methods from Solid Material

Methods, which start from DMSO solutions, are relatively easily modified to allow miniaturized shake-flask measurements to be made. A major consideration is the logistics of weighing out the compounds to an accuracy high enough to make the analysis from solid material valid. There are a number of robotic platforms commercially available, which can incorporate automated weighing stations (Zinsser Analytic Inc.) for use with a very large number of compounds. An extra step will be required in the method to transfer an aliquot of the incubation samples to a filter plate to remove the supernatant for analysis, although centrifugation will negate this, and subsequently determine the concentration of the sample against a calibration curve by UV plate reader either at a single wavelength or from a scan, which again depends on whether multiple pH values or HPLC detection is used.

### 6.4.6
### Precipitate Detection

Precipitate detection methods typically use light scattering techniques such as nephelometry, flow cytometry, and turbidity measurements to determine the amount of the precipitate formed during the incubation process. A major advantage with these types of techniques is the availability of particle size distribution and aggregation information.

Bevan [5] measured precipitation in microtiter plates by light scattering directly using a BMG NEPHELOstar plate-based nephelometer. Small volumes of concentrated DMSO stock solutions are added to wells containing aqueous buffer and this is then serially diluted across the wells of the microtiter plate and allowed to equilibrate. The concentration of the resulting precipitate is determined by nephelometry. For compounds that are poorly soluble, the wells are turbid and produce a higher degree of scattering. Plotting turbidity versus concentration will give the maximum concentration dissolved, which is the quoted solubility value.

Dehring *et al*. [6] have used the same nephelometric technology to determine kinetic solubility on a high-throughput robotic platform with good comparison of data with that obtained in a lower throughout method using flow injection analysis (FIA). Fligge and Schuler [7] used a fast nephelometric method in conjunction with liquid chromatography/mass spectrometry (LC/MS) detection method using the same 384-well microtiter plates as used in the LC/MS hit validation without the need for further sample preparation.

### 6.4.7
### Other Methods of Measuring Solubility

Using the Sirius GLpKa instrument and CheqSol technology provides an elegant method for determining the solubility of ionizable molecules by using pH-metric titration. An acid or base titrant is added to precipitate the sample, which is detected by using D-PAS (a quartz fiber dip probe measuring UV absorbance). Small volumes of acid or base are added to the system to cycle the solution between sub- and supersaturated states close to the equilibrium. Equilibrium solubility is calculated using mass and charge balance equations. This method is particularly useful for poorly soluble compounds and reduces the analysis time for equilibrium solubility from potentially greater than 24 h to around 1 h per sample [8]. (For compounds that do not supersaturate, Bjerrum curve analysis is used.) pION Inc. provides the pSol Gemini instrument for measuring equilibrium solubility using an alternative pH-metric titration method whereby titrations are assumed to establish equilibrium typically over a 12 h period. The company has developed a μSol instrument for measuring high-throughput solubility on a Tecan robotic platform from DMSO stock solutions using UV detection.

Recent studies by Seadeek [9] and Sugano [10] discuss how the crystal form and solubility can be monitored together to assess the crystallinity of the precipitate.

## 6.5
## Dissociation Constants, $pK_a$

It is not easy to find a definition of dissociation constant, $pK_a$, which is not cumbersome and confusing, and yet the extent of ionization, of which this is a measure, is of fundamental importance. Biologically active molecules tend to be either fully or partially charged at physiological pH with the charged functionality often being required for the biological activity, as well as physicochemical properties such as solubility. Knowledge of the dissociation constant and the protonation equilibria plays an important role in the understanding of absorption, transport, and receptor binding. From the Henderson–Hasselbalch equation given in Equation 6.2,

$$\begin{array}{l} \underset{\text{Protonated base}}{BH^+} \rightleftharpoons H^+ + \underset{\text{Deprotonated(neutral)base}}{B} \\ \underset{\text{Protonated(neutral)acid}}{HA} \rightleftharpoons H^+ + \underset{\text{Deprotonated acid}}{A^-} \end{array} \qquad pK_a = pH + \log_{10}\frac{[\text{protonated}]}{[\text{deprotonated}]}, \tag{6.2}$$

for an aqueous solution of a compound with one ionizable group, the acid dissociation constant, or ionization constant, $pK_a$, being equal to the pH at which 50% of the compound is in its ionized form (deprotonated for acids and protonated for bases) and 50% is in its neutral form. (This has to be extended using equilibrium equations for multiprotic molecules and Avdeef [11] has shown the derivation of

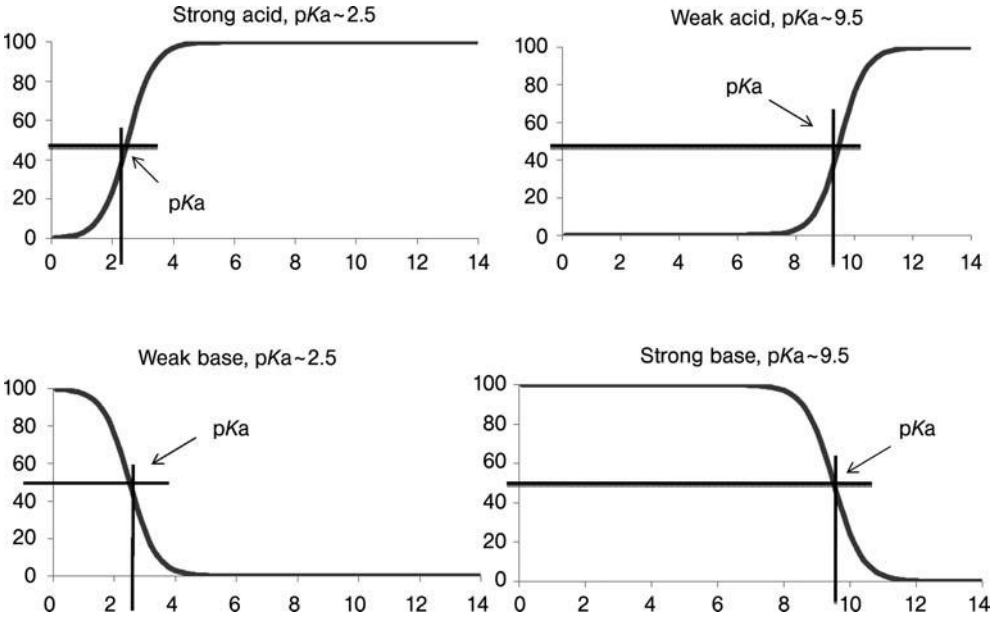


**Figure 6.3** Plots of % ionized for a strong acid and weak base with $pK_a \sim 2.5$, weak acid and strong base with $pK_a \sim 9.5$.

these equations.) Rearranging this equation allows the calculation of % ionized as shown in Equation 6.3:

$$\begin{aligned} \%\ \text{Ionized for bases} &= \frac{100}{1+10^{(pK_a-pH)}}; \\ \%\ \text{Ionized for acids} &= \frac{100}{1+10^{(pH-pK_a)}}. \end{aligned} \tag{6.3}$$

Figure 6.3 shows plots of pH versus % ionized for a strong acid and weak base with $pK_a \sim 2.5$ and weak acid and strong bases with $pK_a \sim 9.5$, respectively.

### 6.5.1 Measuring $pK_a$

To measure $pK_a$, the compound is exposed to a changing pH and some characteristics of the molecule that change as a function of pH, for example, solubility, absorbance, and conductivity, are measured. Potentiometric titrimetry in aqueous solution is the traditional method that has been used. $pK_a$ is determined from the shape of a titration curve [12] derived from plotting electromotive force ($E$ or emf) or pH versus the volume of reagent added. The aqueous analyte solution is either preacidified to pH 3 using 0.5 M hydrochloric acid and titrated to pH 12 using 0.3 M potassium hydroxide solution, or prebasified and titrated with acid and monitored using a glass electrode. It is very important for all $pK_a$ measurements to ensure that the ionic strength of the system remains constant so that the activity coefficients of

all the species involved also remain constant. The system is generally maintained under an inert atmosphere to avoid the risk of $CO_2$ contamination. This method is not amenable to sparingly soluble compounds and highly depends on sample purity. It also needs a relatively large amount of sample, $>5 \times 10^{-4}$ M solution is required to be able to detect a significant change in the shape of the titration curve compared to a blank, and typical sample volumes in the region of 5 ml, although 100 µl volumes are now possible using microelectrodes. The reagent (acid or base) must be added in a stepwise manner, and the protonation equilibria need to be achieved after each addition before measurements can be taken, with 20–40 measurements required in the pH range 3–11. A measurement of this type will take typically 20–40 min per compound. However, this is the most precise method and is generally used for high-quality determinations. Dual-phase potentiometry using direct titration with base followed by back titration with acid in the presence of octanol provides both log *P* and p$K_a$ values. Sirius Analytical's GLpKa instrument allows the automation of this procedure, which reduces the time sufficiently to allow 30–40 titrations per day [13–17].

Owing to increased sensitivity, hybrid potentiometric/UV spectroscopic techniques are useful for reducing the sample concentration required, typically using less than $10^{-5}$ M solutions. A UV absorbance spectrum is measured at each pH but the samples must have a UV chromophore, which demonstrates a spectral change due to ionization. This means that the ionization site must be part of or in close proximity (up to 4 atoms) to the chromophore. Sirius GLpKa instrument with D-PAS attachment uses a fiber optic dip probe in a titration cell and while the samples are titrated across the pH range, the multiwavelength UV spectra are obtained at each pH. The p$K_a$ values are calculated using target factor analysis (TFA) [16] but again the limiting step is electrode stability. The new Fast D-PAS software allows this technology to be used in a high-throughput mode by making measurements in a linear buffer solution with each titration taking 2 min. This method is particularly suitable for samples that are not stable or are poorly soluble since these measurements can be made before precipitation occurs. It is also possible to determine p$K_a$ using pH gradient titration and this is the principle behind the Sirius Profiler SGA instrument. The samples are injected into a flowing pH gradient created by mixing an acidified and basified buffer together using calibrated syringe pumps ensuring that the pH varies linearly with time. The pH can be determined from the time elapsed eliminating the need to wait for stabilization of an electrode. UV spectroscopy is again used to monitor changes in the absorbance of the compound as a function of pH. This reduces the analysis time to ~4 min per cycle giving a throughput of more than 200 compounds per day.

### 6.5.2
### p$K_a$ Measurements in Cosolvent Mixtures

Poor water solubility is still a problem for all these methods, but it can be overcome to some extent by using a mixture of solvents, although the presence of organic modifiers causes the pH scale to shift and may cause the p$K_a$ to change. The

dissociation equilibria are governed by electrostatic interactions as well as by solute–solvent interactions. As shown in Equation 6.2, during the dissociation of uncharged acids, charged species are created. In this instance, the electrostatic interactions become very important, as the corresponding $pK_a$ increases with decreasing polarity of the solution. During the dissociation of cationic species, there is no change in the number of charges and therefore the permittivity of the solution does not change. In this situation, the solute–solvent interactions are more important than the electrostatic interactions and there is a small error on the $pK_a$.

Apparent $pK_a$ values measured in the presence of cosolvents are therefore different from those measured in purely aqueous systems and will not give a true indication of the % ionized of the species. One-unit error in $pK_a$ calculation will carry through to a 1-unit error in log $D_{pH}$ if calculated from a measured log $P$ value. This is particularly important where the $pK_a$ is close to the pH of the region of absorption, which can lead to errors in predicting behavior. The Yasuda–Shedlovsky technique uses the measurement of the apparent $pK_a$ ($p_sK_a$) in a cosolvent mixture and extrapolates back to 100% aqueous. This works well and is well documented [18, 19]; however, it is sample expensive requiring at least three experiments per sample and is therefore not appropriate for a high-throughput setting. Recent work has been published by Völgyi *et al.* [20] demonstrating the use of a universal cosolvent system allowing a single-point measurement against a general calibration curve to determine $pK_a$ in an aqueous environment by using the Yasuda–Shedlovsky plot.

### 6.5.3
### $pK_a$ Measurements based on Separation

Many different types of chromatographic methods have been used to determine $pK_a$ value, such as ion-exchange chromatography, gas chromatography, and RP-HPLC, is well placed for high-throughput analysis. A review by Hardcastle *et al.* [21] explains the theory behind the calculations and the derivation of equations for the determination of $pK_a$ value. It has been demonstrated that the correct determination of pH of the mobile phase is key to the determination of $pK_a$ value of an analyte from chromatographic retention [22, 23]. However, this is the $pK_a$ value of the analyte in the mobile-phase system and not in a purely aqueous environment. The dielectric constant of cosolvent/water mixtures is less than that of water alone and therefore the extent of ionization and the associated ionization equilibria are suppressed:

$$p_sK_a \text{ acids} > pK_a \text{ acids and } p_sK_a \text{ bases} < pK_a \text{ bases.}$$

While this does not give a "true" $pK_a$ value without using the Yasuda–Shedlovsky approach, it does give the dissociation constant for the analyte in the chromatographic system, which will help determine a generic system for the separation of complex mixtures. There are a number of benefits of using chromatographic retentions and capacity factor as a tool for determining $pK_a$ over the potentiometric

methods. Sample requirements are very small and poor aqueous solubility is no longer a problem. Since it is a separation technique, the purity of the compound does not interfere with the analysis, and fast methods significantly reduce analysis time and increase throughput. However, due to stability issues, the pH range of the mobile phase can be limited and therefore the range of p$K_a$ values that can be determined and the precision of the values are not generally as great as those determined by potentiometry.

Capillary electrophoresis has been used for over a decade to determine accurate p$K_a$ values [24, 25] requiring only small amounts of analyte at very low concentrations. It does not require the quantitative determination of the solute or titrant concentrations and since it is a separation technique, impurities do not present a problem, and nonaqueous solvents can be used for poorly soluble compounds [26]. This technique relies solely on migration times. The effective mobility of an ion, $m_{eff}$, is related to the fraction of ionized species present and therefore the p$K_a$ can be determined, provided the equilibrium is fast with respect to the separation time. A review by Poole *et al.* [27] presents model equations for p$K_a$ determination for compounds with up to three ionization centers. A single peak will be observed for all interconverting species, which will depend on the properties of the electrolyte solution. The weakness of capillary electrophoresis as a high-throughput method is its requirement for multiple buffer systems, since while the analysis is rapid the number of channels available is reduced. There are no special instrument requirements for this system, provided there is an effective thermostating of the column since the equilibrium constants depend on temperature. A commercial instrument is now available from Advanced Analytical [28] using a 96-capillary array separation cassette and diode array detection designed to be used in a 96-well microtiter plate format. Twelve electrolyte solutions of differing pH are used to analyze samples simultaneously allowing a throughput of around 16 samples per hour across a pH range of 2–12. Lišková and Šlampová [29] give details of practical considerations with respect to buffers across the pH range 2–12, and it has been shown that placing the system under pressure during the electrophoretic separation reduces migration times and is good across the pH range 2.5–10.5 [30] as it increases the throughput for nonparallel systems. These methods still rely on the presence of a chromophore since they use UV detection, and due to the very small path lengths, concentrations of about 10 μM are to be recommended to achieve a high enough signal for detection. It is possible to use mass spectrometric detection coupled to capillary electrophoresis, although modification of the electrolytes used will be necessary for compatibility with the mass spectrometer. Wan *et al.* [31] describe a method of simultaneous measurement of a pooled sample comprising 1 μl of 10 mm DMSO stocks of each up to 56 compounds. The presence of 5% DMSO neither did influence the effective mobilities of the samples, and therefore had minimal effect on p$K_a$ values determined, nor did it interfere with peak identification. The use of pressure assistance and volatile buffers meant that the total cycle time for these 56 compounds was less than 150 min, although throughput will depend on the resolution of the mass spectrometer. The main disadvantage of this procedure was that compounds that were too similar in mass (a difference of <2 Da) could be misidentified.

## 6.6 Lipophilicity

Lipophilicity is the major driving force for binding drugs to a receptor target. If a compound is too lipophilic, it will be retained longer, have a wider distribution and greater nonspecific binding, and potentially be more readily metabolized. It is a means of estimating a molecule's affinity for a lipid, nonaqueous environment. There are many routes by which a drug can be absorbed by passage through membranes and tissues, but transport by passive diffusion is the most common route [32]. For this to occur, the drug must be lipophilic enough to pass from an aqueous environment into the lipid core of the membranes but not so highly lipophilic that it is retained there.

Measurement of lipophilicity is well documented and a large database of measured log *P* values is available [33]. Lipophilicity is defined as the behavior of a compound in a biphasic system, solid/liquid or liquid/liquid, and it is usually expressed by the octanol/water partition coefficient (*P*) or the distribution coefficient (*D*) with octanol and water traditionally forming the biphasic system. Hansch and Fujita [34–36] recommended the use of the partition coefficient logarithm (log *P*) to model the biological partition behavior of drug molecules. An octanol/water system was chosen because it was known that water-saturated *n*-octanol forms into near-spherical clusters: the OH groups of ~16 octanol molecules coordinate around a core of water molecules with the hydrocarbon chains pointing outward. This produces a phase with some of the characteristics of a phospholipid membrane bilayer: the regions where the lipophilic character predominates (as in the core of the lipid membrane) adjacent to a region with a high degree of polar character (e.g., at the membrane surface) [37]. However, an octanol/water system cannot completely model the combination of charge and polarity, which exists in the phospholipid head groups of biological membranes, since it is not sensitive to the hydrogen-bond donor characteristics of the solutes.

### 6.6.1 log *P* Versus log $D_{pH}$

The difference between log *P* and log $D_{pH}$ is often a cause for confusion. log *P* is the $\log_{10}$ of the partition coefficient and is the extent to which the *neutral species* has an affinity for the organic environment relative to that of the aqueous environment. log $D_{pH}$ is the $\log_{10}$ of the distribution coefficient and is similar in that it is also a measure of the extent of the affinity for organic over aqueous except that it is *all species present at a given pH* that are measured. For compounds with no ionizable groups or those where the test pH is sufficiently far away from the p$K_a$ of the ionizable groups so that they are in their neutral form, log *P* and log $D_{pH}$ are equivalent. If the test pH of the log $D_{pH}$ assay is changed, the extent of ionization of the molecule will also change. It is therefore imperative that the test pH is always quoted for log *D*, often as a subscript with the nomenclature log $D_{pH}$. (although, it is not uncommon for the pH value to be found as a superscript with the subscript being used to designate the solvent). This is summarized in Figure 6.4.

$$\text{Partition } (P) = \frac{[\text{ single species in organic phase}]}{[\text{ single species in aqueous phase}]} \qquad \text{Distribution } (D) = \frac{[\text{ unionised + ionised }]_{\text{organic}}}{[\text{ unionised + ionised }]_{\text{aqueous}}}; \text{ where } [\ ] = \text{concentration}$$

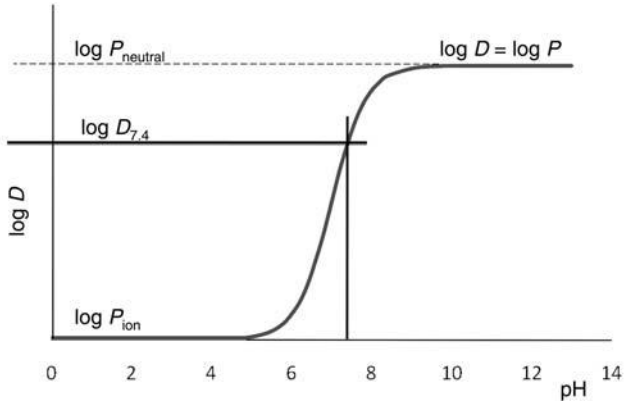


**Figure 6.4** Summary of the difference between log $P$ and log $D_{pH}$ with a plot of log $D$ versus pH for a base.

log $D_{pH}$ is arguably a more useful descriptor for ionizable compounds, but, since it depends on pH, values determined at differing pH values should never be compared. A major advantage of log $D_{pH}$ is that it can be measured very easily since no account of the species present is made. It is extremely amenable to high-throughput technologies, and a number of different methods have been studied. Some are true partition experiments while others use validated chromatographic systems that reference retention time and capacity factor to known lipophilicity values and are a good surrogate for log $P$ measurements.

### 6.6.2 Measuring Lipophilicity

The shake-flask method has been described as the "gold standard" – it has been the method of choice for literature publications against which other methods of lipophilicity determinations have traditionally been calibrated, although other methods, now commonly used, are capable of generating the same quality of data. The shake-flask method starts from solid material incubated in a biphasic solution of aqueous and octanol, and the relative amounts in each layer are determined. There are, however, a number of problems with this method. For example, microemulsions can be formed, which prevent the two layers from separating, and these can be stable for days. The upper and lower ranges that are achievable can cause detection problems; for example, log $D_{7.4} = 4$ contains 10 000 times more samples in the octanol layer causing saturation of the detector, while the quantity present in the aqueous layer may be below the detection limit. Using mass balance equations can accommodate this but the systems must be well validated.

Using potentiometric methods, log *P* is calculated from the difference between the apparent p$K_a$ ($_sK_a$) values measured in a biphasic system such as octanol/water. The first such method was developed by Sirius Analytical as a basis for its GLpKa instrument. The solid sample is dissolved in a biphasic system, acidified or basified, and titrated with base or acid under controlled conditions. The resulting titration curve is compared with a simulated curve produced from $_sK_a$ values (and other variables), which are systematically varied until the two curves match as close as possible. This process is known as refinement [38]. The major drawback of this method is that it can be used only for ionizable molecules and is not appropriate for lipophilic weak bases with a low p$K_a$ or weak acids with a high p$K_a$ if the values shift to $_sK_a$ values outside the measurement range of 2–12. It can, however, be used over a wide range of phase ratios since the pH is measured without phase separation. There are a number of reviews that explain the methodologies and mathematics behind the pH-metric method of determining log *P* [39–41].

### 6.6.3
### High-Throughput log $D_{7.4}$ Measurements

"Miniaturizing" the shake-flask method so that it is transferable to a liquid-handling robotic system is relatively straightforward. The samples can be dispensed into tubes in a microtiter plate format and the two phases are added using any of the standard instruments available. To ensure a thorough mixing of the two phases, the tubes need to be sealed tightly and the plates inverted before, and shaken vigorously during, the incubation period. Once the incubation is complete, the phases need to be allowed to separate fully and this can be achieved most effectively by centrifugation. Using disposable tips and with careful teaching of the aspirate heights, it is possible to sample from the two layers, which are then analyzed and the ratios of the concentration calculated. The use of RP-HPLC analysis allows fast and simple measurements, although it is important to ensure that the octanol and aqueous layers are alternated to avoid a buildup of octanol on the column.

### 6.6.4
### High-Throughput log $D_{7.4}$ Versus Shake-Flask log $D_{7.4}$

In a high-throughput setting, it is beneficial to have a generic method that is applicable to all the compounds being analyzed. If this is not the case, adapting methods can take up valuable time and resources. For this reason, it is most common for the volumes used in the octanol and aqueous layers to be the same. However, in the high-throughput partition experiment detailed above, the samples are present as liquids in DMSO, and adding equal volumes of octanol and water to the sample aliquot will lead to one phase being present in excess. DMSO preferentially partitions into the aqueous layer rather than the octanol and therefore the total volume of DMSO aliquot plus aqueous buffer must be the same as that for the octanol for the two volumes to be equivalent. This assay has been validated by UCB (unpublished data) against the shake-flask method and holds up very well, $R^2$ is 0.9. Figure 6.5

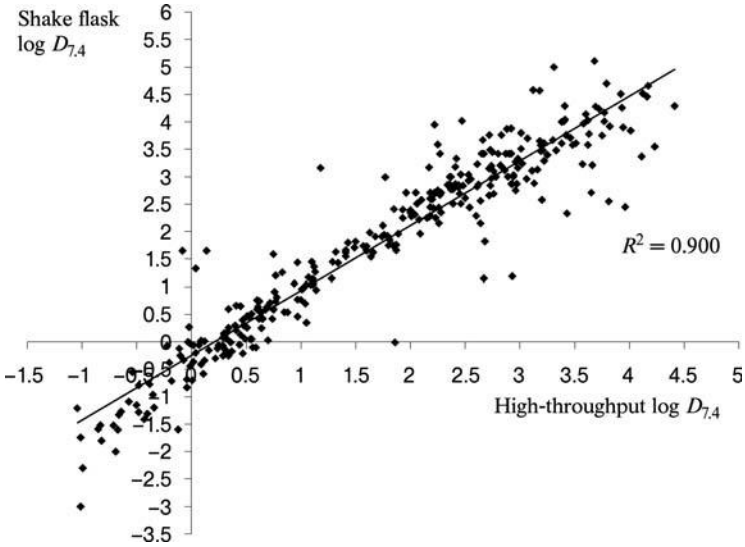


**Figure 6.5** Correlation of high-throughput log $D_{7.4}$ measurements from DMSO stock solutions with shake-flask log $D_{7.4}$ measurements from solid material.

shows the correlation between two sets of data for ~300 relatively diverse compounds consisting of commercially available drugs and a range of compounds from a number of medicinal chemistry projects.

There is a loss of correlation at the extremes of the lipophilicity ranges and this can in some way be explained by the presence of DMSO in the high-throughput assay. The relative amount of DMSO present is so low that it will have a negligible effect on the final lipophilicity value. However, for compounds, which are very highly lipophilic, their value may be slightly reduced due to the apparent pulling of the compound into the aqueous layer by the DMSO rather than the compound partitioning into the octanol where it has greater affinity.

### 6.6.5
### Alternative Methods for Determining High-Throughput log $D_{pH}$

Chromatographic retention time gives a direct measure of the extent of a compound's interaction with the stationary phase. This will therefore relate to its distribution between the two phases. A review by Gocan *et al.* [42] describes the use of chromatography to determine lipophilicity. There are direct methods where sets of compounds with a diverse range of well-characterized lipophilicities are analyzed by RP-HPLC to create a calibration curve constructed from retention times and capacity factors versus lipophilicity against which unknown compounds can be measured [43–47]. This type of approach is good for high throughput in microtiter plate format, and samples are generally from DMSO stock solutions and therefore do not need weighing. It does not depend on the amount of compound injected onto the column

and is not affected by impurities in the sample or the solvent vehicle used. Different types of stationary phase such as immobilized artificial membrane (IAM), human serum albumin (HSA), and α-acid glycoprotein (AGP) [48] can be used depending on the partition and is not restricted to octanol/water partitioning. These methods, however, do not allow direct comparative studies between methods without the use of standards.

Sirius Analytical has developed a commercially available Profiler LDA instrument in which a proprietary stationary phase is coated with octanol. Octanol-saturated mobile phase is recirculated to maintain the octanol content of the stationary phase as constant. This system gives a dynamic range that covers $-1 < \log P < 5$.

Chromatographic hydrophobicity index (CHI) was introduced by Valkó *et al.* in 1997 [49] where an index is derived from the compound's retention time in a fast-gradient RP-HPLC system. The gradient is produced by changing the proportions of buffer and acetonitrile in the mobile phase. The sample is injected onto the column with a low percentage of acetonitrile so that it preferentially binds to the stationary phase. The gradient is then increased until the sample dissolves when the percentage of acetonitrile present in the mobile phase is high enough and the sample will elute from the column. The system is calibrated using retention times for a set of standards with known CHI values. Constants can be used to calculate CHI for the unknown samples, which are normally in the range of 0–100 to give the approximate percentage of acetonitrile required to produce an equal distribution of the compound between mobile and stationary phases.

Using microemulsion electrokinetic chromatography (MEEKC) [50], microemulsions are made from a combination of aqueous buffer, *n*-butanol, heptane, and a surfactant placed inside a fused silica capillary. DMSO stock solutions are diluted with buffer and a highly lipophilic marker, dodecaphenone. This is injected at the anode end of the capillary with a UV detector placed at the cathode end. An electric field is applied, which produces negatively charged droplets of organic solvent. Neutral solutes present in the aqueous phase migrate with the endosmotic flow while those present in the organic phase will migrate at the speed of the charged droplets. This method is suitable only for compounds that are electrically neutral at the pH of the buffer so that bases are run in pH 10 buffer and acids in pH 3 buffer. This method, therefore, does not allow for a single method to be used for all compounds.

## 6.7 Permeability

It is not uncommon for drug compounds to be able to perform very well in a variety of microtiter plate-based assays, but when transferred to *in vivo* assays, they cannot reach the therapeutic target site. The molecule must permeate through a number of cell membranes made up of phospholipid bilayers, which can increase the passage of highly charged polar molecules. Among the most common means by which a molecule can cross such a membrane are transcellular routes such as passive diffusion, carrier-mediated active transport, and metabolic enzymes, paracellular

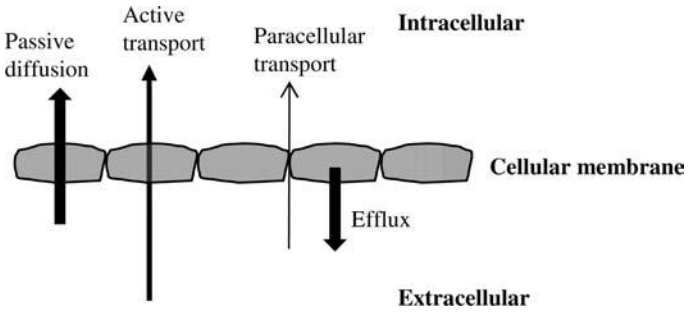


**Figure 6.6** Routes by which a drug can cross a membrane.

transport where the molecules diffuse through the tight junctions between the cells, and ATP-dependent efflux mechanisms where the drug is pumped out back, as described in Figure 6.6.

Cultured cells such as Caco-2 (human colon adenocarcinoma) or MDCK (Madin–Darby canine kidney) have long been used to give some measure of the permeation rate. Caco-2 cells express peptide transporters such as PEPT1 [51] and efflux systems such as P-gp [52]. The Caco-2 cell monolayer *in vitro* permeability assay mimics most of the transport pathways in the gastrointestinal tract and has therefore gained broad acceptance as a surrogate marker for estimating *in vivo* drug absorption potential. Krishaa *et al.* [53] and Faassen *et al.* [54] describe in detail the Caco-2 experiment methods. Although these methods remain the key benchmark assays, they are expensive to run, highly variable, and relatively unamenable to a high-throughput environment due to long membrane culture time and the requirement for multiple time point measurements.

The advantage of cell culture models is that they are able to measure active transport processes across the cell membranes and not just the interaction of a drug with a lipid bilayer. They can also be used to study passive and active transport routes; indeed, much of the knowledge as to the active transport mechanisms in the intestine has been derived from cell culture studies. Despite the predominant route being passive diffusion, the research into transport mechanisms indicates that there are a large number of drugs that are used as substrates for active transporter and efflux systems, and it must therefore be appreciated that multiple transport routes may be involved in the intestinal drug transport.

Since the majority of drugs are absorbed by passive diffusion, an assay that measures the rate of permeation through a simple artificial membrane, which mimics this, may be useful; however, how much so as a gastrointestinal tract membrane mimic will be determined by the membrane composition. (Fisher *et al.* [55] provide a review of the molecular parameters that govern passive diffusion.) A major step in the advancement of noncell-based permeability screens designed to overcome many of these issues was the PAMPA (parallel artificial membrane permeability assay) first published by Kansy in 1998 [56] where a concentrated, negatively charged phospholipid bilayer membrane is supported on a filter in a 96-well plate. This technique has been investigated and modified many times and has been widely implemented in

high-throughput screening cascades across the industry and has found favor as a tool for the rank ordering of compounds. PAMPA data are frequently correlated with those from cell-based assays and indeed also with *in vivo* absorption data with some success in terms of $R^2$ and the numbers of outliers, although how relevant it is to "predict a prediction" remains a matter for individual groups to decide, with similar conclusions being arguably possible from calculated properties such as polar surface area and number of hydrogen-bond donors and acceptors. It is worth noting that there is a lack of published evidence to suggest that PAMPA has been instrumental in the design of a drug candidate or in driving a project forward in the same way as the log *P* or solubility has been on a compound-by-compound basis.

### 6.7.1
### Permeability and Lipophilicity

According to Fick's first law of diffusion, the passive diffusion of a drug across a membrane is directly proportional to the membrane–water partition coefficient, provided the interior of the membrane is homogeneous and the concentration of the drug on the "receiver" side of the membrane is much less than that on the "donor" side of the membrane, although in practice this linearity does not hold over a very wide range of lipophilicities due to issues such as the presence of aqueous pores in oily membranes, membrane retention of lipophilic molecules, p$K_a$ effects, aggregation of the solute, and the unstirred water layer (UWL).

Both lipophilicity values, log *P* and log $D_{pH}$, are a ratio between two immiscible phases determined once the system has reached equilibrium and is therefore a thermodynamic system. Permeability values, however, are rates of passage through the membranes and conditions are carefully selected to ensure that the system does not reach equilibrium. This is a kinetic system and depends on many variables such as incubation time, membrane composition, stirring rate, pH, and buffer composition. For this reason, it is extremely difficult to make reliable group-to-group comparisons. Although the rank ordering of compounds should be the same, it is unlikely that the absolute values would be the same, making validation of new methods very difficult. As a result, substituting permeability values for lipophilicity values and vice versa should be carried out with caution while correlation of the one with the other has been shown to be reasonably successful [57]. Faller *et al.* [58] have demonstrated the use of PAMPA technology for determining lipophilicity and Chen *et al.* [59] described a variation using polymer-plasticized polyvinylchloride.

### 6.7.2
### Cell-Based Assays

For cell-based assays, all of the instruments used need to be maintained in a sterile environment if growing the cells is to be automated. These assays are labor intensive, expensive, and not generally well suited to high throughput. The cells need to be cultured for around 21 days (depending on the cell line). Bellman *et al.* [60] presented details of a high-throughput Caco-2 cell-based method for measuring permeability

using LC/MS detection. Using 5 μM sample concentration in the donor (apical) wells, the authors measured both apical-to-basolateral (A–B) and basolateral-to-apical (B–A) directions by taking 100 μl samples from both the donor and the acceptor (basolateral) at time $t = 0$ and after 90 min incubation and replacing with 200 μl of 50% acetonitrile/water. A generic HPLC method was used with ESI mass spectrometry detection. They demonstrated that it is possible to measure all the samples in duplicate with a throughput of 20 samples in 24 h. This is an increase in throughput but it still does not satisfy the needs of a department requiring analysis of much greater numbers of compounds.

### 6.7.3
### Noncell-Based Assays: Chromatographic Methods

Chromatography is easily automated and using short retention times hundreds of compounds can be analyzed quickly by methods developed to model intestinal permeability. The stationary phase consists of either immobilized liposomes or immobilized phospholipids. These methods are excellent for screening purposes as they require only small amount of compound, and automation is straightforward. A variety of columns are commercially available and the historical concerns for column stability have been largely overcome. Permeability is related to the retention time of the compound on the stationary phase designed to mimic lipid bilayers [61, 62], which is due to electrostatic interactions between the drug and the lipid surface and partitioning into and across the lipid phase. Retention on the column therefore does not always reflect transport across the membrane [63]. It has been suggested that the correlation from these methods with drug permeability shows no improvement over the use of log *P* [64]. Zhue *et al.* [65] present a good data set with human fraction absorbed, Caco-2 permeability data, and log *P* values for 92 compounds, which are useful for validation studies.

### 6.7.4
### Noncell-Based Assays: Parallel Artificial Membrane Permeability Assay

In contrast to cell-based assays, noncell-based permeability assays using artificial membranes supported on filters are fast, flexible, cheap, and fully automatable. They are therefore ideally placed for use in high throughput. There have been many variations of this assay in terms of the fine details of the experiment and these will be discussed in due course; however, the basic principles remain the same, based on a 96-well microtiter plate format.

A "sandwich" is formed from two plates, a donor plate and a receiver plate. The donor plate is usually of a specially designed geometry, available from pION Inc. for use with its Evolution system and GUT box or from Millipore Inc. These plates are machined to minimize vortexing during shaking so that the buffer solution remains in contact with the lower surface of the receiver plate at all times without the formation of a meniscus. The receiver is a 96-well microfilter plate. The filters are generally 125 μm thick with 0.45 μm pores and 0.3 $cm^2$ cross-sectional area with 70%

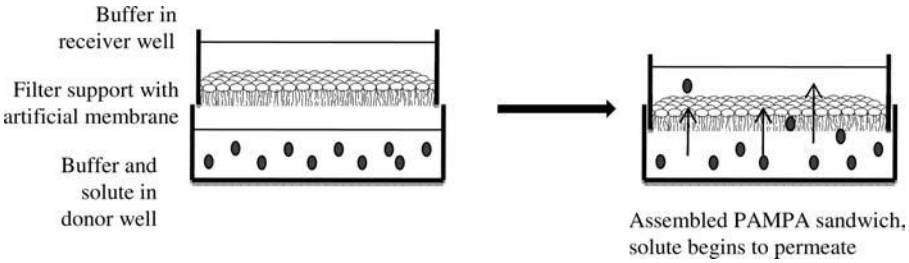


**Figure 6.7** Schematic representation of PAMPA system.

porosity onto which is placed a solution of the artificial lipid membrane. BD Biosciences has produced a commercially available PAMPA plate system, which is a 96-well insert with a PVDF filter plate precoated with structured layers of phospholipids and has a matched receiver microplate [66].

The samples as DMSO stock solutions are added to the donor wells and are diluted with buffer. The receiver plate is placed on top of the donor wells, the lipid solution is added carefully to ensure that there is complete coverage of the filter support, and then the buffer solution for the receiver system is added. The resulting sandwich is covered to avoid evaporation and is shaken. Figure 6.7 shows a schematic representation of the construction and incubation of the PAMPA sandwich system.

After the samples have been incubated for the chosen time, the plates are separated and aliquots removed from both donor and receiver wells and the concentration of the solute determined. This is most commonly carried out by using HPLC, LC/MS, or UV plate reader detection.

#### 6.7.4.1 Membrane Composition

There have been many variations in the composition of artificial membranes used in a PAMPA-style analysis and each has been tailored to specific investigations. Sugano *et al.* [67, 68] investigated a variety of phospholipid membrane systems and experimental conditions and were able to show that by modifying the membrane it was possible to improve the predictive power of PAMPA.

Di *et al.* [69] describe the use of a PAMPA for the prediction of passage of a drug across the blood–brain barrier by the modification of the membrane using porcine polar brain lipid and have demonstrated the ability to determine which compounds will be most likely to be CNS positive and CNS negative.

#### 6.7.4.2 Suggestions for PAMPA

The following recommendations are a good starting point for a gastrointestinal, noncell-based artificial membrane permeability assay [70]:

- *Donor wells*: pH 6 and pH 7 (ionization-maintained sink), 5–10 mm bile acid, such as taurocholic acid or glycocholic acid to solubilize lipophilic molecules (binding-maintained sink).
- *Receiver wells*: pH 7.4 phosphate buffer containing 3% w/v bovine serum albumin (BSA) added to receiver wells (binding-maintained sink).

- The artificial membrane composed of phosphatidyl choline, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and cholesterol.
- DMSO final concentration <10%.

In cell-based assays, membrane integrity is monitored using conductivity measurements but is not amenable to high throughput, and alternatives are needed. One approach may be to perform the assay in triplicate, rejecting the data where two points are not in good agreement. This would reduce throughput and does not remove all doubts as to the quality of the data.

Alternatively, a well-validated poorly permeable compound is included in the analyte solutions as an internal standard. A compound such as theophylline has an effective permeability of $\sim 0.12 \times 10^{-6}$ cm/s (exact value will depend on assay conditions). If the compound were seen to be permeating significantly faster than this effective permeability, it could be concluded that the membrane had been compromised. This approach would mean that a detection method based on separation, such as HPLC, would be needed. Upon the inclusion of a second internal standard, which was known to be highly soluble, such as verapamil, effective permeability $16 \times 10^{-6}$ cm/s would enable monitoring of the incubation time to ensure that equilibrium had not been reached for highly permeable compounds.

A third method of checking the membrane integrity is to monitor the appearance of DMSO in the receiver wells due to damaged membranes by analyzing down to 200 nm. This could be automated, would not result in the need for separation of the analyte before detection, and would not reduce throughput.

#### 6.7.4.3 Considerations in the Calculation of Permeability from PAMPA Data

The equations used to calculate permeability from PAMPA data are derived from Fick's law and assume that equilibrium between the donor and the receiver wells has not been achieved. The equations also assume that the membrane has been fully saturated by the sample upon leaving the donor well before permeation into the receiver well commences. For most compounds, this saturation time is extremely short and does not generally present a problem. Membrane retention also needs to be considered. This will tend to be higher for more lipophilic compounds and has sometimes been seen up to 90%. The consideration, or otherwise, of membrane retention will dictate which set of equations should be used and whether the resulting permeability value is termed the apparent permeability as is used in cell-based determinations or the effective permeability.

Permeability is a kinetic process, and is quoted as a rate. In cell-based assays such as Caco-2, a number of time points are generally taken from both the donor (apical) wells and the receiver (basolateral) wells. Because of this, retention on the membranes is not determined and the permeability values quoted are "apparent," $P_a$. The main benefit of PAMPA type assays is their usefulness as high-throughput tools and therefore taking time point measurements will create a bottleneck. Measurements of the donor and receiver wells are made at the end of the incubation period and referenced back to the starting concentration of the solute in the donor wells.

Membrane retention values can be determined using mass balance and inclusion of this gives the "effective" permeability, $P_e$. These are shown in Equations 6.4–6.6:

$$P_e = \frac{-2.303}{At}\frac{V_R V_D}{V_R + V_D}\log_{10}\left[1-\left(\frac{V_R + V_D}{(1-R)V_D}\right)\left(\frac{C_R}{C_D}\right)\right], \tag{6.4}$$

where

$$R = \frac{[C_e-(C_D + C_R)]}{C_e}. \tag{6.5}$$

$$P_a = \frac{V_R V_D}{AtV_R + V_D}\ln\left[1-\left(\frac{C_R}{C_D}\right)\right]. \tag{6.6}$$

Effective permeability ($P_e$) and apparent permeability ($P_a$) can be determined using Equations 6.4 and 6.5 and Equation 6.6, respectively, where $A$ is the membrane area ($cm^2$), $t$ is the incubation time (s), $V_R$ is the volume of the receiver well ($cm^3$), $V_D$ is the volume of the donor well ($cm^3$), $C_R$ is the concentration of solute in the receiver well at time $t$ ($mol/cm^3$), $C_D$ is the concentration of the solute in the donor well at time $t = 0$ ($mol/cm^3$), and $C_e$ is the concentration of the solute at equilibrium ($mol/cm^3$).

The distinctions between these two have been discussed here only briefly. Avdeef [71] has published more detailed explanations and derivations of the equations to be used.

### 6.7.5 Sink Conditions

The term "sink" when referred to *in vitro* permeability systems means any process that significantly lowers the concentration of the neutral species from the receiver wells. *In vivo* sink conditions can be thought of as the continued removal of the permeating species due to the continuous flow of blood. There are three methods of introducing sink conditions into an *in vitro* system:

(1) *Physically-maintained sink conditions:* In cell-based assays, an aliquot is removed for analysis from the receiver wells to make time point measurements. This is replicated in Caco-2 but not in PAMPA.

(2) *Ionization-maintained sink conditions:* Owing to the dependence of ionization on pH, weak acids will be more permeable in a gradient system where the pH of the donor is below that of the acceptor, while weak bases will be more permeable in an iso-pH system. Uncharged species will show the same results using either system. If the pH gradient is wide enough, a neutral compound will become ionized once it reaches the receiver wells.

(3) *Binding-maintained sink conditions:* The presence of serum proteins in the receiver wells, such as 3% w/v BSA, will bind the neutral compound once it crosses the membrane.

The pION Inc. method uses "double-sink" conditions, both ionization and binding maintained.

### 6.7.6
### Unstirred Water Layer

The passive transport of compounds across a membrane is the combination of diffusion through the membrane and the regions of undisturbed solution, the unstirred water layer, on either side of the membrane. The solute samples are present in the bulk solution, which, upon stirring, move through the bulk to the interface of the bulk solution and the UWL. *In vivo*, the gastrointestinal UWL is in the region of 30–100 μm due to an efficient mixing near the surface of the endothelium [72], while in the Caco-2 system it is >1000 μm. Diffusion laws govern the progress of the solute through the UWL that can be reduced in size by increased stirring but will never actually be removed. The UWL is virtually the same for drugs of a comparable size and can be determined by measuring the transport of the compounds from the donor to the receiver wells without addition of the artificial membrane to the filter supports, by determining the stirring rate dependence of the permeability of the compounds (a technique used by pION Inc. in its Gut Box technology, where the shaking of the system can be preset according to desired UWL) or by determining the pH dependence of the effective permeability.

### 6.7.7
### Surface Properties for the Determination of Permeability

Surface tension measurements have been shown to correlate with ADME properties [55, 73–75]. The Kibron Delta8 instrument is a multichannel tensiometer that studies the dependence of surface activity on the solution composition. The technology is based on the determination of the maximum force exerted by surface tension on a wetting probe as it is withdrawn from the liquid/air interface. The main forces acting on the probe are the buoyancy due to the volume of the liquid displaced by the probe and the mass of the meniscus adhering to the probe. The maximum pull force is recorded when the buoyancy force reaches a minimum – just before the meniscus breaks. This instrument uses a microtiter plate-based format and can measure a plate in around 2 min. Across the plate is a series of 8 samples at 12 concentrations, starting from DMSO stock solutions. This allows prediction of the passive diffusion through membranes from plots of the critical micelle concentration versus the concentration of the onset of surface activity ($C_o$) by calibration against drugs with known permeabilities. This technique can be used for passage through the blood–brain barrier or the gastrointestinal tract [76].

## 6.8
## Data Interpretation, Presentation, and Storage

A major consideration in setting up a high-throughput screen is the data collection, processing, interpretation, and dissemination. It is particularly important to ensure that there is sufficient time and resources dedicated to studying the data. Discrete

numbers are useful for QSAR, QSPR work, correlation, and method validation but they can be overwhelming, particularly for the nonexpert user. A clear and unambiguous method of visualizing the data is needed. This is often done by binning the data as well as providing a numerical value. If this approach is adopted for all of the assays, it is possible to produce the data in a report-style format with expert interpretation, which gives a package of data showing how all the properties of a given series of compounds interrelate. This is particularly useful for reference purposes. Freeman [77] demonstrated how this approach provides a valuable tool for the use of high-throughput physicochemical profiling.

## 6.9 Conclusions

This chapter has provided only a brief introduction to the field of high-throughput physicochemical screening. It is intended to demonstrate that with ingenuity it is possible to automate or increase throughput of these techniques. High-throughput measurement of physicochemical parameters has a valid place in drug discovery, its exact positioning within the timelines of a project will depend on the individual organization and its particular needs. With careful thought, it is possible to analyze a large number of compounds using very little sample and generate a package of data, which will help to drive projects forward. The assay conditions must be relevant to the individual questions being posed and the data should be "fit for purpose." However, if budgets are generous and compound numbers are high enough, there is a danger that too much data could be generated – a case of diluting the information through shear volume of data – and measurements should not be made "just because they can" be made. Screening for all physicochemical properties is a compromise, high throughput with a low predictive potential or low-throughput with a high predictive potential. However, using multiple data points, pH for instance, rather than large compound numbers and devoting sufficient time to interpretation and education may tip the balance of this compromise toward a higher predictive power.

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# 7
# An Overview of Caco-2 and Alternatives for Prediction of Intestinal Drug Transport and Absorption

*Anna-Lena Ungell and Per Artursson*

### Abbreviations

| | |
|---|---|
| 2/4/A1 | Conditionally immortalized cell line derived from fetal rat intestine |
| ABC | ATP-binding cassette |
| ATCC | American-type culture collection |
| BCRP | Breast cancer-resistance protein (ABCG2) |
| BSA | Bovine serum albumin |
| Caco-2 | Adenocarcinoma cell line derived from human colon |
| cDNA | Complementary DNA |
| CYP | Cytochrome P450 |
| DMSO | Dimethyl sulfoxide |
| ECACC | European Collection of Cell Cultures |
| ER | Efflux ratio (transport basolateral-to-apical divided by transport a pical-to-basolateral) |
| hCE-1 | Human carboxyesterase-1 |
| hCE-2 | Human carboxyesterase-2 |
| HT-29 | Pluripotent adenocarcinoma cell line derived from human colon |
| IEC-18 | Rat intestinal epithelial cell line |
| LLC-PK1 | Pig kidney epithelia cell line |
| MDCK | Madin–Darby canine kidney epithelial cell line |
| MDR1 | Multidrug resistance protein 1 (ABCB1) |
| MRP | Multidrug resistance-associated protein family 1–6 (ABCC1–6) |
| MTX | Methotrexate |
| OATP1B1 | Organic anion-transporting polypeptide (SLC21A6; OATP2) |
| PEPT1 | Oligopeptide transporter (solute carrier family 15, member 1 (SLC15A1)) |
| T84 | Colon carcinoma cell line derived from human colon |

**Symbols**

| | |
|---|---|
| Clog $P$ | Predicted octanol/water partitioning coefficient |
| $F_a$ | Fraction of the oral dose absorbed |
| $P_{app}$ | Apparent permeability coefficient |

## 7.1 Introduction

One of the limiting factors for the successful therapeutic application of new oral drugs is their transport across one or several membrane(s) into the system, for example, over the intestinal membrane into the systemic circulation, into the cell interior to the target receptor, or into the central nervous system (CNS). The drug transport across a biological membrane can be influenced by a number of factors such as solubility, membrane partitioning, metabolism, and active transport processes [1–3]. To obtain high-quality and useful predictions of the transport processes, highly standardized *in vitro* models, suitable for screening of a large number and variety of drug molecules, are used.

Complementary to experimentally based *in vitro* screening assays are *in silico* predictions of intestinal drug permeability and absorption from molecular structures. These methodologies, which are treated elsewhere in this book, are very time efficient and have a large capacity for virtual screening of entire chemical libraries in early drug discovery. However, they are based on simplistic approximations regarding, for example, membrane partitioning and active transport mechanisms and therefore do not describe the biological complexity of the model in sufficient detail as compared to cell-based *in vitro* models, such as Caco-2 monolayers and alternative cell models, for prediction of intestinal permeability and drug absorption.

## 7.2 Cell Cultures for Assessment of Intestinal Permeability

Cell culture-based models are the most commonly used methods for studying the mechanisms of passive and active drug transport, and interactions with epithelial proteins, such as transporters and enzymes. These are both simple and quick to use, and still reflect most of the different mechanisms involved in the absorption process. Almost 20 years ago, Caco-2 cells grown on permeable supports were introduced as an experimental tool for mechanistic studies of the intestinal drug transport [4, 5]. At the same time, it was suggested that the Caco-2 model was suitable for screening the intestinal drug permeability and predicting the oral absorption potential of new drug substances [5]. Several factors spurred the development of Caco-2 and similar cell models. These included (1) the awareness that inferior pharmacokinetic properties, including insufficient drug absorption, remained the major reason for the failure of new drug candidates in the clinical

phase [6]; (2) the insight that drug absorption across biological barriers is a fairly complex process involving several pathways and that it can therefore not easily be delineated in experimental animals [7]; and (3) the introduction of combinatorial chemistry in drug discovery [8] .

As with all new techniques that are rapidly embraced by the scientific community, the initial enthusiasm and in some cases uncritical use of Caco-2 cells unraveled the limitations of this *in vitro* [7] and other similar models, for example [9]. A period of critical evaluation followed and today the majority of researchers using these models are aware not only of their advantages but also of their limitations. MDCK (Madin–Darby canine kidney) cells are another well-used cell line, which has been compared with the Caco-2 for the use as intestinal permeability [10]. For the screening of a large number of compounds, these two cell cultures provide extremely useful tools for both preclinical screening and for mechanistic purposes and have routinly been used in the drug industry even for cassette dosing and for analyzing large combinatorial libraries [11, 12].

### 7.2.1 Caco-2

The main reasons for the popularity of the Caco-2 cell line are that the cells are easy to maintain in culture, and that they develop unusually high degree of differentiation spontaneously under standard culture conditions. The cells exhibit a good reproducibility, robustness, and functional properties of human intestinal epithelial cells. The model has proved capable of predicting the oral absorption of a variety of drug compounds (see Ref. [13]). The Caco-2 cell line originates from a human colon adenocarcinoma [14] and can be obtained from American-type culture collection (ATCC) or the European Collection of Cell Cultures (ECACC). It is a polyclonal cell line, that is, it consists of a heterogeneous population of cells [15], which means that the properties of the cells may change with time in culture. The cells should therefore be used within a limited number of passages, especially for screening purposes over a long period of time. The heterogeneous properties of the cells may be one explanation for the differences in morphology, paracellular permeability, and expression of enzymes and transporters that have been reported from different research groups [16–22]. The cell culture protocol therefore must be standardized and validated by time during screening, and each laboratory has to provide its own standardization [23–25]. Many clones of Caco-2 cells with partly different properties have been derived, but it is beyond the scope of this chapter to cover the vast literature on the physiology of Caco-2 cells.

Caco-2 cells form tight junctions and express many of the brush border enzymes (hydrolases) that are found in the normal small intestine, for example, alkaline phosphatase, sucrase, and amino peptidases [26–29]. Cytochrome P450 (CYP450) isoenzymes and some phase II enzymes (e.g., glutathione-*S*-transferases, sulfotransferase, and glucuronidase) have been identified [29–33] in these cells; however, the level of CYP expression (e.g., CYP3A4) is low in the original cells under standard cell culture conditions [34].

A large number of transport proteins have been identified in Caco-2 cells. Among the efflux transporters, the MDR-1 gene product P-glycoprotein (P-gp) is the most extensively investigated [35–37]. Several different efflux transporters have been identified in the Caco-2 cell line at mRNA level [23, 24, 38–40], and some of these have been verified also at protein and functional levels [41]. Taipalensuu *et al*. showed that the normal Caco-2 cells do not overexpress the efflux transporter P-gp in comparison with human jejunal biopsies [38]. A genetically related protein, BCRP, has also been discussed recently, but this protein seems to be expressed less in cell lines such as Caco-2 than in the human jejunum [38, 39]. The multidrug resistance-related (associated) protein family, MRPs, has also been identified in Caco-2 cells [38–40, 42]. Of the 8 to 10 different MRPs that have been proposed to exist, 6 have been identified in Caco-2 cells, referred to as MRP1–6 [38, 39, 42]. In addition, transport systems for glucose [43, 44], amino acids [45–48], dipeptides [49–51], vitamins [52], and bile acids [53, 54], which are normally found in the small intestinal enterocytes, have been characterized in the Caco-2 model. The expression of the active transport systems is time dependent and may vary with nutritional conditions [55, 56]. Therefore, culture conditions can dramatically alter the biological characteristics and transport properties of Caco-2 cell monolayers [57–60]. As the Caco-2 cell model expresses many important intestinal transporters, it can be used to study not only the passive transport mechanisms but also the mechanisms involving active drug transport. Owing to the complexity of this model of drug transport, there has been reports delineating both pros and cons for the use of it [7, 61–63].

### 7.2.2 MDCK Cells

The MDCK cell line is also frequently used by pharmaceutical companies to monitor intestinal drug transport, despite the fact that the cell line originates from the dog kidney [10, 64, 65]. An advantage with this cell line as compared to Caco-2 cells is that it differentiates more rapidly. There are two distinct subclones of MDCK cells: MDCK Strain I that forms very tight monolayers and MDCK Strain II that forms monolayers with more leaky tight junctions. Irvine and coworkers reported that the correlation to oral fraction absorbed ($F_a$) based on 55 different compounds was comparable in the Caco-2 and MDCK model systems [10], although it should be pointed out that due to species differences, the endogenous expression of canine transporters in the renal MDCK monolayers is likely to be very different from that of the human intestinal transporters in Caco-2 cells. Thus, while MDCK monolayers may be useful for estimations of passive epithelial transport, but they may not be applicable to mechanistic studies of human drug transport or for predicting active uptake or efflux across the human intestinal epithelium. In the normal MDCK cell line, a low level of P-gp has been identified [65], while uptake transporters such as renal organic cation transporters have been explored in mechanistic studies [66].

In general, the inherent expression of canine transport proteins in MDCK cells is low. This fact together with the seemingly correct sorting of transport proteins to the right location in the plasma membrane has made these cells a popular choice for the

stable expression of transport proteins of human origin. Furthermore, MDCK cells overexpressing transport proteins maintain cell–cell contact via tight junctions, a feature sometimes lost upon the manipulation of differentiated epithelial cell lines. MDCK cells overexpressing human P-gp (MDR1) have been a useful tool for investigating the contribution of P-gp to transepithelial transport [67–69]. However, it appears that the MDR1-MDCK cells, like many other stably transfected cell lines, tend to form multilayers and in addition, are not as well polarized as MDCK cells [67, 70]. An alternative cell line for assessing P-gp involvement is MDR1-transfected LLC-PK1 (pig kidney) cells [71].

In recent years, it has been recognized that the interplay between uptake and efflux transporters may determine the cellular pharmacokinetics of drugs. Thus, a more hydrophilic drug may require an active uptake mechanism to cross the cell membrane and enter the cell. Only from within the cell will such a molecule become accessible to the binding site(s) of an efflux transporter, such as MRP2. Recently, it was shown that cells overexpressing another efflux protein, ABCG2/BCRP, correctly identified binding to the transport proteins for drugs with a lipophilicity that allowed significant partitioning into the cell membrane (Clog $P > 0.5$) [72]. Since the cell line in question did not express uptake transporters, this result indicates that a log partitioning coefficient above 0.5 was needed for passive membrane permeation and access to the ABC-transporter. Thus, both appropriate uptake and efflux transporters need to be inserted into a cell line to reveal the transport mechanism of more hydrophilic efflux substrates [73]. This was elegantly demonstrated for the rather hydrophilic cholesterol-lowering agent pravastatin (log $P < 0.5$), using MDCK cells overexpressing the pravastatin uptake transporter OATP1B1 and the pravastatin efflux transporter MRP2 [74]. Only in double-transfected cells, an efflux of pravastatin via MRP2 could be observed. Numerous research groups working to better model the interplay between different transport proteins have now constructed a large variety of double-transfected cell lines. Extensions of this technique have resulted in MDCK cells overexpressing as many as four transport proteins [75]. It should be noted that the relative expression levels of multiple transgenes may be difficult to control, which may obscure the goal of obtaining more *in vivo*-like cell cultures. Furthermore, it is often difficult to generate double-transfected cell lines that retain the required differentiated properties including an intact paracellular barrier. Caco-2 cells may therefore remain a viable future alternative in some of these situations, provided sufficiently specific substrates or inhibitors can be identified.

### 7.2.3 2/4/A1 Cells

As for active drug transport, there is no quantitative relationship between passive drug permeability in Caco-2 cells *in vitro* and drug transport in the human small intestine *in vivo* [76, 77]. Apart from high-permeability drugs that partition into the cell membranes at comparable, rapid speeds *in vitro* and *in vivo*, compounds with intermediate or low permeability have a lower permeability in the Caco-2 model than

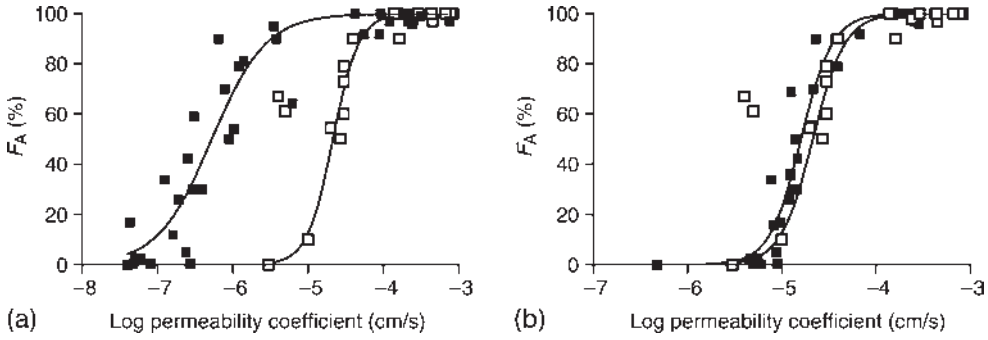


**Figure 7.1** Comparison between the permeability coefficients obtained after *in vivo* perfusion of the human jejunum (open symbols) (data compiled from publications by Lennernäs's laboratory [162, 163]) and Caco-2 cells (filled symbols) (data compiled from publications by Artursson's laboratory [84, 164]) (a) and 2/4/A1 cells [84] (b). There is a quantitative overlap in permeability between completely absorbed drugs in Caco-2 cells and in the human jejunum, while the permeabilities for the incompletely absorbed drugs are approximately two orders of magnitude lower in Caco-2 cells than those in the human jejunum. In contrast, the relationship in 2/4/A1 is almost completely overlapping compared to that in the human jejunum for both completely and incompletely absorbed drugs.

*in vivo*. As shown in Figure 7.1a, this difference increases with a decrease in compound permeability. There are two major reasons for this difference.

First, the paracellular route is tighter in Caco-2 cells than that in the small intestine *in vivo*. Although the average pore radius of the tight junctions in the human small intestine is around 8–13 Å [78], the corresponding radius in Caco-2 cells is lower. As low-permeability drugs are generally more polar than high-permeability drugs, they tend to distribute more slowly into the cell membranes. However, at least a fraction of the drugs are transported through the water pores of the tight junctions, via the paracellular pathway. If this pathway is narrower, as in Caco-2 cells, the permeability will become lower than that *in vivo*. We recently proposed a solution to this problem by exploiting a more leaky cell culture model established from the rat fetal intestine, 2/4/A1 [77, 79]. This cell line, which has paracellular permeability comparable to that of the human small intestinal epithelium *in vivo*, gives a better quantitative relationship with human permeability data generated in the Loc-I-Gut perfusion technique [77] (Figure 7.1b). It is impossible to speculate upon the relative contribution of the paracellular pathway and the possible increased absorptive surface area to the passive transport of low- permeability drugs [80]. Here, we can only conclude that it is possible to mimic human small intestinal permeability to drugs by using a more leaky cell culture model (such as 2/4/A1) than the Caco-2 model. As an alternative, correction factors for the low paracellular permeability in Caco-2 cells and other membrane models have been introduced [81, 82]. Interestingly, the 2/4/A1 cell line seems not to express functional (drug) transporting proteins [83], which makes it an interesting alternative in studies of passive permeability. Indeed, recent data from our laboratory suggest that 2/4/A1 cells better predict the human absorption of intermediate- to low-permeability drugs than do Caco-2 cells [84]. Another advantage of the 2/4/A1 cell line is that a relatively large amount of low-permeability drugs is

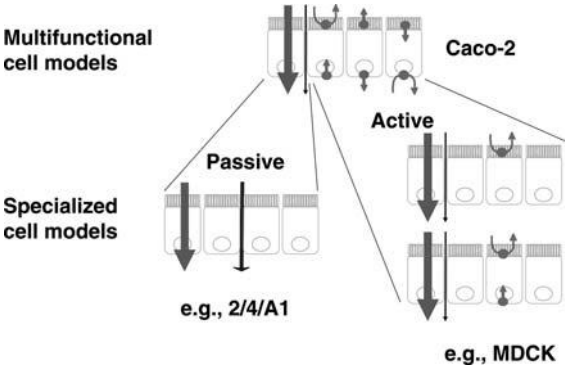


**Figure 7.2** Studies of specific drug transport routes in multifunctional cell models such as Caco-2 cells may be complicated, as there is a lack of specific substrates for many drug transporters. Therefore, specialized cell models that accommodate mainly passive (2/4/A1) or selected active (MDCK-MDR1) transport pathways are preferred in some cases.

transported, thus eliminating the need for expensive analysis equipment such as an LC/MS/MS system. However, it must be pointed out that 2/4/A1 cells have poorly differentiated morphology and lack many of the enzyme systems and transporters that are present in Caco-2 cells and in the normal human small intestinal epithelium [83]. Thus, 2/4/A1 cells seem best applicable to investigating passive transport properties of drugs without the consideration of active transport mechanisms. Moreover, the culture conditions are very specific and more demanding than the relatively straightforward procedures used for Caco-2 and MDCK cells, and their use in the pharmaceutical industry has hitherto been limited. A simple serum-free culture procedure for 2/4/A1 cells has recently been developed, but it remains to be seen if this technique will make 2/4/A1 cells a more attractive alternative in drug discovery settings [85, 98] (Figure 7.2).

### 7.2.4 Other Cell Lines

HT29 is another well-studied human colon carcinoma cell line [15, 28]. When grown under standard culture conditions, the cells form multilayers of undifferentiated cells. However, under modified culture conditions, HT29 cells differentiate into polarized monolayers of absorptive and/or, interestingly, mucus-secreting goblet cells, depending on the chosen conditions. Several permanently differentiated clonal cell lines have been established from HT29 cells. The mucus-layer-producing variants have attracted some interest for two reasons: the mucus layer covering the intestinal epithelium *in vivo* may limit the absorption of some drugs and Caco-2 and MDCK cells lack this barrier. Mucus-producing clones such as HT29-H and HT29-MTX (methotrexate-induced cells) have been used for the development of mucus-layer-containing cell culture models [86–89]. Cocultures of Caco-2 cells and HT29-H

and HT29-MTX have also been investigated, but these have not yet found wide application in drug discovery [88, 90].

Another human colonic cancer cell line is T84, which forms monolayers that are even tighter than those of the Caco-2. It has been described as resembling a colonic crypt cell phenotype. Hence, these cells have been used mainly in studies of epithelial ion secretion and are generally not considered to be adequate for drug transport studies, particularly with respect to carrier-mediated processes [13, 91, 92]. The rat intestinal epithelial cell line IEC-18 has been evaluated as a model to study small intestinal epithelial permeability. This cell line, which forms very leaky monolayers, was proposed to be a better model than the Caco-2 monolayers for evaluating the small intestinal paracellular permeation of hydrophilic molecules [93]. Importantly, the leaky tight junctions of the IEC-18 cells are a result of an undeveloped paracellular barrier lacking the perijunctional actin belt. In addition, the IEC-18 cells have minute expression of transporters [91, 93].

## 7.3 Correlation to Fraction of Oral Dose Absorbed

Many academic and industrial laboratories have shown that the drug permeability measured in Caco-2 cell monolayers can be used to predict the oral absorption of drugs in humans. Various data sets have therefore been used to establish correlations between Caco-2 permeability and the fraction absorbed orally in humans [5, 18]. Taken together, these studies show good predictability, though with a relatively wide variation in the appearance of correlation profiles between different laboratories [18]. Initially, the good relationship between the passive drug transport across Caco-2 cells and the absorbed fraction after oral administration to humans [5] may be surprising, given that oral drug absorption is influenced by many factors besides drug permeability, such as drug solubility, dissolution, active transport, and, in some cases, presystemic metabolism. The first study with Caco-2 cells was performed under highly controlled conditions on registered drugs that did not have solubility problems and that were largely passively transported. In addition, their metabolism could be accounted for [5]. Similar good results are obtained when the same parameters are strictly controlled in expanded data sets.

However, many drug discovery scientists initially were disappointed when the experimental in-house compounds gave relationships with a much larger scatter than that reported in the original publication [7]. There are several contributing factors to this difference. Discovery compounds have generally been neither characterized nor optimized with regard to chemical stability, metabolism, solubility, or dissolution rate. Another difference is that Caco-2 predictions of oral drug absorption using small data sets for standardization are generally carried out manually by multiple samples at different time points, and full attention is also given to, for example, mass balance issues and the contribution from active transport. Nevertheless, there are several reports describing the usefulness of Caco-2 permeability data also obtained in automated systems, in predictions of oral absorption, for example [94–96], and,

when combined with metabolic stability data, also bioavailability [97]. In the screening setting, binning of permeability values in up to three categories, predicting high, intermediate, and low absorption after oral administration is commonly used. When an analogous series of compounds is tested, permeability ranking is an alternative. Recently, ranking of incompletely absorbed drugs ($F_a < 30\%$) was used to compare the performance of Caco-2 that expresses functional transporters and 2/4/A1 cells that lack functional transport proteins. Both passively and actively transported compounds were included in the study. Both cell lines generated good results,with a slight advantage for 2/4/A1 cells, suggesting that the passive permeability route dominates also in the case of many compounds that are at least partly transported via active transport mechanisms across the intestinal epithelium [98]. Recently, an independent study came to the same conclusion regarding the 2/4/A1 cells [62].

In the drug industry, Caco-2 cells have often been used to rank compounds in analogous compound series and libraries or to estimate $F_a$ in humans early in the screening process. When such data sets are used, Caco-2 cell permeability measurements provide the opportunity to establish structure–permeability relationships for quite different analogous series of drugs. Several examples of the latter case have been published. For example, these include a series of conventional drugs [99–102], peptides, and peptide mimetics [103–107] as well as compounds generated in high-throughput drug discovery [108, 109]. Although most of these structure–permeability relationships have been established for passive membrane permeability, there are also examples of structure–permeability relationships for a series of drugs that are absorbed via an active transport mechanism [110–115].

## 7.4 Cell Culture and Transport Experiments

Drug absorption experiments are easy to perform in cell culture models, such as Caco-2. Comprehensive step-by-step protocols for the determination of drug permeability and prediction of drug absorption in Caco-2 monolayers has recently been published [25, 116]. The outlined principles are applicable also to the other cell culture models reviewed above. Briefly, the drug is added to the apical (mucosal) side and the appearance of the drug on the basolateral side (serosal) is followed by time. The model also permits experiments to be carried out in the reverse direction, that is, from the basolateral side to the apical side. The monolayers should be agitated during the experiments, not only to produce more reproducible results but also to reduce the effects of aqueous boundary layers adjacent to the epithelial membrane [117]. Without correct stirring conditions being maintained during the experiments, the measured permeability values for rapidly transported compounds will be significantly underestimated. The experiments should preferably be performed under "sink" conditions (e.g., the drug concentration on the receiver side should be less than 10% of the concentration on the donor side during an experiment) to avoid bias by backdiffusion of significant amount of compound from the receiver chamber and

to maintain a "constant" applied drug concentration gradient during the course of the experiment. The following Equation 7.1 is generally used for the calculation of the apparent permeability coefficient ($P_{app}$):

$$P_{app} = \frac{(dQ/dt)}{(A \times C_{d0})}, \tag{7.1}$$

where $dQ/dt$ is the rate of appearance of drug on the receiver side, $C_{d0}$ is the initial drug concentration on the donor side, and $A$ is the surface area of the filter membrane.

This equation for calculation of $P_{app}$ is easily improved by taking into account the change of donor concentration ($C_d$) during the experiment, which affects the concentration gradient and the driving force for passive diffusion (Equation 7.2):

$$P_{app} = \frac{k \times V_r}{A}, \tag{7.2}$$

where $k$ is the change in drug concentration in the receiver chamber ($C_{r-ti}/C_{d-ti}$) per unit time, $C_{r-ti}$ is the concentration on the receiver side at the end of each time interval, $C_{d-ti}$ is the average of the donor concentration determined at the beginning and at the end of each time interval, $V_r$ is the volume of the receiver chamber, and $A$ is the surface area of the filter membrane. By using this method of calculation, a more accurate determination of the $P_{app}$ value is obtained, particularly for rapidly transported drugs where $P_{app}$ values exceed $10 \times 10^{-6}$ cm/s.

A general equation that does not require sink conditions can also be applied [118, 119] (Equation 7.3). In this "nonsink" analysis, $P_{app}$ is determined by nonlinear curve fitting of

$$C_R(t) = \left[\frac{M}{(V_D + V_R)}\right] + \left\{C_{R,0} - \left[\frac{M}{(V_D + V_R)}\right]\right\} e^{-P_{app}A(1/V_D + 1/V_R)t}, \tag{7.3}$$

where $V_D$ is the volume of the donor compartment, $V_R$ is the volume of the receiver compartment, $A$ is the area of the filter, $M$ is the total amount of substance in the system, $C_{R,0}$ is the concentration of the substance in the receiver compartment at the start of the time interval, and $C_R(t)$ is the concentration of the substance at time $t$ measured from the start of the time interval.

The trend in the industry has been to automate the Caco-2 permeability assay using semi- or fully automated procedures. With such systems throughputs on the order of hundreds of compounds per week are possible. Of particular importance, for good estimation of the permeability coefficient, the compound must be completely dissolved during the transport experiment. Therefore, discovery compounds are often diluted in physiological buffers from stock solutions in DMSO. Twenty-four-well plates with monolayers are usually used for higher analytical precision and compound yield, but 96-well plates for higher throughput are also frequently used. A mixture of several reference compounds is often included on each plate to capture variability between assays by time/passage.

The recovery should be sufficient to assure that reliable $P_{app}$ values are obtained and reported (Equation 7.4). Common limits for recovery are 80–120%. Sometimes,

when lipophilic compounds with assumed high permeability are investigated, a lower recovery may be acceptable. The recovery is calculated according to

$$\text{Recovery}\,[\%] = \frac{\left[C_{\text{D(fin)}} \times V_{\text{D}} + \Sigma(C_{S(t)} \times V_{S(t)}) + C_{\text{R(fin)}} \times V_{\text{R(fin)}}\right] \times 100}{C_{\text{D(0)}} \times V_{\text{D(0)}}}, \tag{7.4}$$

where $C_{\text{D}}$ and $C_{\text{R}}$ are the concentrations on the donor (D) and receiver (R) sides of the monolayer at the start (0) or end (fin) of the experiment, $C_{S(t)}$ denotes the concentrations of the samples withdrawn at different time points $t$, and $V$ is used for each of the respective volumes.

### 7.4.1 Quality Control and Standardization

The variable performance of Caco-2 cells can be minimized by education and training in good cell culture practice [25, 116, 120]. Here, we only note that a major reason for the different results obtained with Caco-2 cells is related to the interval of passage number and ages (time grown on filter) at which the cells are studied. It is therefore important to define a limited number of passages and days that can be used for the experiments. Caco-2 cells obtained from ATCC or from ECACC are normally at passages 20–40. Our experience is that within a predefined and controlled interval of passages, the cells perform very consistently, provided identical cell culture conditions are used. We conclude that in contrast to what is generally believed it is possible to maintain the permeability characteristics of Caco-2 cells over long time periods, at least in the same laboratory.

Another technical limitation of Caco-2 cells is the long culture time required to obtain full differentiation of the cells. It takes 3 weeks to obtain fully differentiated cell monolayers of Caco-2 cells on filter inserts [1, 116, 121]. It has recently been suggested that 2 weeks of culture on filters is sufficient for obtaining a full expression of transporters and integrity [23], but these claims require solid experimental confirmation.

In some screening laboratories, even 2 weeks are considered too long and too demanding to be practical, and culture protocols have been developed to speed up the differentiation process, usually to less than 1 week [122–124]. Today, at least one 3-day system, based on proprietary media supplements and collagen-coated filter inserts, is available (http://www.bdbiosciences.com). Although limited, the published information about the performance of Caco-2 monolayers cultivated under these accelerated protocols suggests that the cells are not fully differentiated and therefore have to be used at a certain time point, for example, on day 3, to obtain reproducible results, as the degree of differentiation may vary from one day to another. Clearly, data from different publications or laboratories should not be mixed without prior harmonization of the experimental protocols. This is underscored by a recent comparative study in which the mRNA expression and function of a number of transport proteins were compared in Caco-2 cells cultivated according to different standard procedures used

in 10 laboratories in the drug industry and universities [125] – although the results were in qualitative agreement, large variations in expression and function were observed between the different laboratories.

### 7.4.2 Optimizing Experimental Conditions: pH

The pH in the lumen of the GI tract *in vivo* in humans is variable; typically, it is pH 1–2 in the stomach, 5–6.5 in the duodenum and proximal jejunum, 6.5–7.5 in the mid-jejunum, and almost up to 8 in the terminal ileum [126]. In the large bowel, the pH varies between 6.5 and 8 from the colon ascendens to the sigmoideum. This bulk pH will affect the solubility and the degree of ionization of the drug and hence regional differences in the concentration of uncharged drug species, which provides a driving force for the drug absorption. The transport across the rate-limiting barrier of the intestinal epithelial cell membrane is, however, affected by another pH, the so-called microclimate or surface pH, which is up to one pH unit lower in parts of the small intestine compared to the bulk pH adjacent to the epithelial cell surface [127]. In the cell culture models, the pH of the apical solution therefore has a direct impact on the transport experiments, as the solution is in direct contact with the membrane [128]. When only transport in the absorptive direction is considered, the cell-based screening model should reflect the gradient under physiological conditions and reflect the absorption across the jejunum (the main part of absorption of most drugs); thus, a pH of 6.5 should be applied to the apical side while the pH at the basolateral side should be kept at 7.4.

The passive permeability of an ionizable compound will obey the pH partition hypothesis. For weak acids, for example, salicylic acid, the dependence on a pH gradient is complex as both the passive diffusion and the active transport process (which in the case of organic anions may be driven by a proton gradient) will depend on the proton concentration in the apical solution [129]. Similarly, for weak bases such as alfentanil, metoprolol, propranolol, or cimetidine, an apical pH of 6.5 will decrease the passive transport toward the basolateral side [130]. Applying this pH gradient during bidirectional transport studies for weak bases will create an efflux ratio, that is due to unequal concentrations of the uncharged drug species on the apical (pH 6.5) and basolateral (pH 7.4) sides, rather than an active efflux mechanism [129, 131]. In conclusion, in early permeability screening where a pH gradient is often used, it can be difficult to distinguish a passive asymmetric uptake or efflux caused by the pH effect on ionization from a true transporter-mediated uptake or efflux. Thus, caution should be exercised in interpreting efflux data obtained from permeability screening using the recommended pH gradient systems.

### 7.4.3 Optimizing Experimental Conditions: Concentration Dependence

Optimizing the permeability measurements to avoid adsorption to plastic, filters, or accumulation within the cell monolayer seems highly relevant for increasing the

predictivity of the screening model in the early screening of highly lipophilic drugs [132]. In general, DMSO solutions are the most commonly used vehicle in the early stages. As the available amount of the compounds is small at this stage, only low concentrations of the drug can be used and the influence of carrier-mediated transport (uptake or efflux) may be overemphasized compared to the *in vivo* situation. This could, for instance, result in a falsely low permeability to compounds that are substrates for efflux transporters at the intestinal membrane as after oral drug administration, these transporters could become saturated at the higher (therapeutic) concentrations obtained in the gut after dissolution of the dosage form.

### 7.4.4
### Optimizing Experimental Conditions: Solubility and BSA

The adsorption of compounds to plastic surfaces and accumulation of compounds within the cell membrane are related to the lipophilicity of the compound. Highly lipophilic drugs most likely have high intrinsic permeabilities, but it may be difficult to make a correct determination due to low recoveries in the *in vitro* system. Many authors have suggested using BSA to improve sink conditions and to reduce the adsorption phenomenon [128, 133, 134]. The effect of the presence of BSA will be determined by both the protein-binding capacity of the drug to be tested and its intrinsic permeability, that is, a high protein-binding and a high-permeability value will increase the impact of BSA in the basolateral chamber. Recently, a new promising methodology was presented that may account for the effect of protein binding on the drug permeability through an indirect procedure [135]. Using this approach, good corrections for the changes in unbound drug concentration were obtained for a small set of drugs, and further studies are needed to show on the general applicability of the methodology. There are several positive factors that favor the use of BSA in the basolateral medium. First, it mimics the *in vivo* situation where the circulating blood provides an excellent base for sink conditions due to a large volume and content of albumin [136]. Second, serum albumin hinders adsorption onto plastic surfaces and filters and thereby reduces the loss of compound in the experimental system, as well as in the different steps of dilution before the analysis of drug content. Third, the accumulation of a lipophilic drug within the cell monolayer is reduced due to maintained sink conditions. Fourth, it seems to be more generally applicable as a solubilizer of lipophilic drugs than detergents such as Cremophor [137].

## 7.5
## Active Transport Studies in Caco-2 Cells

Drug transport studies in Caco-2 cells grown on permeable supports are easy to perform under controlled conditions. This makes it possible to extract information about specific transport processes that would be difficult to obtain in more complex models such as those based on whole tissues from experimental animals. When the mRNA expression of drug transporting proteins in Caco-2 cells was compared with

that in various segments of the human intestine and colon in two independent studies [23, 24], fairly good correlations were obtained. Recently, these studies were expanded to incorporate expression comparisons of transport proteins between human intestinal, liver, and kidney tissues and their respective organotypic cell lines [39]. Again, a good correlation was obtained for Caco-2 cells and the human jejunum while the corresponding comparisons for human liver and kidney gave poor results. Furthermore, comparisons with expression data from rat intestine, increased the scatter significantly, indicating that human cell lines such as Caco-2 are more representative of human than of rat organs with respect to transporter expression [39]. The latter finding is supported by differences in transport parameters for the human ABC transporters MDR1 and MRP2 in human Caco-2 cells and canine MDCK cells [138]. Differences between the Caco-2 and MDCK cell lines have also been reported with regard to the activity of peptide transporters [139].

It can be argued that the abundant expression of transport proteins in Caco-2 cells may obscure the study of a specific transporter, especially if the transporter lacks a specific substrate, as in the case of most efflux transporters of the ABC transporter family [140]. However, the expression of multiple transport systems in Caco-2 may be an advantage in the study of (1) the interplay between several transporters, for example, Refs [141, 142]; (2) the interplay between drug metabolism and drug transport [143–148], and (3) the relative contribution of passive and active transport mechanisms to the overall transport of a drug, for example, Refs [98, 149] . Recently, a large number of new inhibitors and substrates for specific transporters were identified among registered drugs, using efficient screening methods, for example, Refs [72, 150, 151]; but, additional studies are needed to investigate their specificities with regard to the broad collection of drug-transporting proteins.

## 7.6
## Metabolism Studies using Caco-2 Cells

Cell culture models can be used to evaluate the importance of metabolism in gut membranes, both with respect to oxidative metabolism via the cytochrome P450 system and phase-II reactions [30–34, 143, 152]. In general, CYP3A4 activity in the parent clone of Caco-2 is very low or absent. Therefore, if a compound is metabolized by CYP450 3A4 in the intestinal membrane, permeability for that compound across Caco-2 cell monolayers will overpredict the absorbed fraction. Since CYP3A4 is the dominating drug-metabolizing enzyme of the human small intestine, a variety of approaches have been described to enhance its functional activity in Caco-2 cells. For example, Caco-2 cells have been transfected with cDNA encoding for CYP3A4 [153, 154]. Another approach is to treat the Caco-2 cells with dihydroxyvitamin D3 that induces an increased activity of the enzyme [155, 156]. Significant induction of CYP3A4 activity and expression has also been reported in CYP3A4-transfected cells by incubation with 12-*O*-tetradecanoylphorbol-13-acetate and sodium butyrate [157].

The inherent enzymatic activity seems low also in other cell lines used for screening, such as MDCK and LLC-PK1 cells [158]. Both MDCK and LLC-PK1 cells have been transfected with CYP3A4 and MDR1 for studying the concert action between drug metabolism and secretion via efflux transporters [158].

Expression levels and activities of enzymes within the gut should be compared with the levels in the different cellular models before starting screening programs. In addition, the presence of the correct enzyme at the brush border membrane or intracellularly in the cellular models is important if the influence of enzymatic degradation on total transport across the intestinal membrane is evaluated. Caco-2 cells are often used for evaluation of prodrug activation as a model for intestinal bioactivation [159, 160]. A report by Imai *et al.*, however, indicates that in the case of Caco-2 cells, the main carboxyl esterase is identified as the hCE-1 and corresponds to the hepatic variant, while in the human intestine the most abundant carboxyl esterase is the hCE-2 [161]. As the specificity of these two enzymes differs, it was suggested that prediction of human intestinal absorption using Caco-2 cells should be performed carefully in the case of ester- and amide-containing drugs such as prodrugs. In addition, if transfected cell lines such as the MDCK-MDR1 are used in studies of prodrug transport, it is important to know if the cells can activate the drug once it has entered the cell. This is especially important if the drug formed is evaluated to be a potential substrate for the MDR1 efflux mechanism. Thus, without prior knowledge of the enzymes involved, studies can be misleading.

There are also successful studies of metabolism during transport using Caco-2 cells. Hubatsch *et al.* have reported a study of metabolism of a tripeptide (anti-HIV) by brush border enzyme dipeptidylpeptidase IV, and the dipeptide thus formed was then transported via PEPT1. These data gave helpful knowledge regarding both prodrug activation and transport of the inactive dipeptide via the PEPT1 [147].

## 7.7 Conclusions

We conclude that Caco-2 cell cultures remain a versatile and general model to study drug transport mechanisms and screening of drug permeability. Especially important is to have consistency. Ensured high quality during culturing and transport experiments is recommended, as differences can cause major variability among the data acquired. Alternative models that express fewer drug transport pathways may be preferable in situations where specific drug transport mechanisms are to be identified. Therefore, such alternative models to Caco-2 cells are developed in many laboratories for the investigation of, for example, drug transport by specific transport proteins.

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# 8
# Use of Animals for the Determination of Absorption and Bioavailability

*Chris Logan*

### Abbreviations

| | |
|---|---|
| ADME/PK | Absorption, distribution, metabolism, and excretion/pharmacokinetics |
| AUC | Area under the plasma concentration–time curve |
| Caco-2 | Adenocarcinoma cell line derived from human colon |
| DMPK | Drug metabolism and pharmacokinetics |
| GIT | Gastrointestinal tract |
| HPLC | High-pressure liquid chromatography |
| hpv | Hepatic portal vein |
| HTS | High-throughput screen |
| i.v. | Intravenous |
| i.t. | Intratracheal |
| MDCK | Madin–Darby canine kidney cells |
| PAMPA | Parallel artificial membrane permeation assay |
| $P_{app}$ | Apparent permeability coefficient |
| PB/PK | Physiologically based pharmacokinetics |
| PK | Pharmacokinetics |
| p.o. | Oral (per os) |

### Symbols

| | |
|---|---|
| $C_{min,ss}$ | Minimum plasma concentration at steady state |
| $f_u$ | Fraction unbound in plasma |
| $k$ | Elimination rate constant |
| log $D$ | Logarithm of the distribution coefficient in octanol/water (usually at pH 7.4) |
| $\tau$ | (Tau) dosing interval |
| $V_d$ | Volume of distribution |

## 8.1 Introduction

This chapter will review some of the important methods for carrying out *in vivo* absorption and bioavailability studies, as well as attempt to provide an overview of how the information may be used in the drug discovery process. The chapter is aimed at medicinal chemists and thus will focus on the use of animals in discovery phase absorption, distribution, metabolism, and excretion/pharmacokinetic (ADME/PK) studies, rather than the design of studies that are for regulatory submission or part of a development safety package.

### 8.1.1 ADME/PK in Drug Discovery

The need to carry out ADME/PK studies prior to the start of drug development has only recently become widely accepted. The very high failure rate of drug development has been well known for a long time, but the key publication of Prentis *et al*. in 1988 [1] highlighted that a significant proportion of the failures (39%) for the seven major UK pharmaceutical companies could be attributed to "inappropriate pharmacokinetics." In a more recent report [2], the failure rate attributed to the same cause was 25%. Whether this apparent improvement is due to the variability in the reporting system or a very rapid change due to the incorporation of DMPK into discovery is not clear. However, it is often very difficult to attribute a failure to a single cause; is the failure due to the toxicity of the compound or to poor PK, which leads to excessive exposures at the peak concentrations that are necessary to achieve the required pharmacological effect over the whole dosing period? Our own experience, like that of others [3], is that there are often several aspects that contribute to the decision not to progress a development project.

Nonetheless, it is now generally accepted that it is worthwhile "frontloading" projects with ADME/PK and toxicology information in order to improve the chances of compounds achieving registration and becoming "best in class" [4].

The incorporation of ADME/PK into the discovery process has required a complete reevaluation of the approach to the science. Drug discovery can be seen as a cyclical process (Figure 8.1), with chemists making compounds that are screened for biological activity. The biological data are fed back to the chemists who use it to improve the design of the next compounds, which are then used to initiate the next revolution of the cycle. The incorporation of ADME/PK in to drug discovery means that there is now a second, often orthogonal, make/test cycle. For this cycle to be productive, it is essential for it to operate at the same rate as the biological testing, otherwise the chemistry will have moved on, and the ADME/PK data will have been generated on compounds that are no longer of interest.

Of course, as the generation of biological information has moved toward high-throughput approaches, ADME/PK is also needed to aspire to similar expectations. This has led to significant automation and simplification of the ADME screens, as will be seen elsewhere in this book. Even so, few projects have had ready access to truly

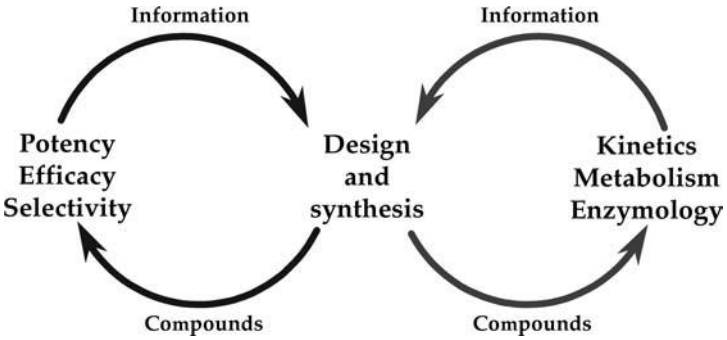


**Figure 8.1** Research optimization process.

high-throughput screening (HTS) ADME/PK, and so it is more usual for DMPK considerations to be taken on when projects are at the "hit-to-lead" stage or later [4].

### 8.1.2
### The Need for Prediction

As ADME/PK has become incorporated into drug discovery, it has become necessary to reconsider the purpose of the studies. If the science is really going to reduce the attrition rate in development, then it is essential for the studies to allow predictions of the PK in man to be made. This means predicting the likely size and frequency of the dose. A review of the top 10 medicines of 1999 (Table 8.1) shows all of them to be once-a-day compounds. It is clear that to be "best in class" and to be able to maintain that position as follow-up compounds come along, it seems probable that a compound will need to be suitable for once-a-day dosing.

Although the pressure to screen large numbers of compounds quickly has led to the rapid development of *in silico* and *in vitro* assays, the sheer number and complexity of the processes involved in determining the disposition of any particular compound mean that *in vivo* studies are still required to provide assurance that the important processes are modeled with sufficient accuracy [4–6], and, indeed, that the potential contribution of processes for which there are no good *in vitro* models (e.g., biliary secretion) are adequately assessed.

Although prediction of ADME/PK in man may be the primary purpose for the preclinical studies, it is also important that potential new drugs have acceptable properties in toxicology species. Without these it can be very difficult to generate adequate safety margins to allow studies in man to start. It is also likely that the development safety assessment program will be difficult and hence slow.

## 8.2
## Consideration of Absorption and Bioavailability

There are two methods of dosing that are of primary interest to medicinal chemists: the oral and intravenous routes. Oral is important because it is generally the most

**Table 8.1** The top 10 best-selling drugs in 1999.

| Product | Indications | 1999 sales [$billion] | Percentage of global sales | Launched | Dosing regime |
|---|---|---|---|---|---|
| Losec (omeprazole) | Duodenal ulcer Gastroesophageal reflux *Helicobacter* infections | 5.7 | 1.9 | 1989 – UK and US | Once daily, except when used as part of combination therapy |
| Zocor (simvastatin) | Hypercholesterolemia<br>Hyperlipoproteinemia<br>Hypertriglyceridemia | 3.9 | 1.3 | 1989 – UK<br>1991 – US | Once daily |
| Lipitor (atorvastatin) | Atherosclerosis<br>Dyslipidemia<br>Hypercholesterolemia | 3.8 | 1.3 | 1997 – UK and US | Once daily |
| Norvasc (amlodipine besilate) | Hypertension | 3.0 | 1.0 | 1990 – UK<br>1992 – US | Once daily |
| Prozac (fluoxetine) | Depression<br>Obsessive–compulsive disorders<br>Panic<br>Post-traumatic stress disorder | 2.9 | 1.0 | 1988 – US<br>1989 – UK | Once daily |
| Ogastro (lansoprazole) | Duodenal ulcer | 2.3 | 0.8 | 1994 – UK | Once daily, except when used as part of combination therapy for *H. pylori* and for hypersecretory conditions. Twice-daily when dose $\geq$20 mg |
| | Gastroesophageal reflux<br>*Helicobacter* infections | | | 1995 – US | |

| | | | | | |
|---|---|---|---|---|---|
| Seroxat (paroxetine) | Depression<br>Obsessive–compulsive disorders<br>Panic<br>Post-traumatic stress disorder | 2.1 | 0.7 | 1991 – UK<br>1993 – US | Once daily |
| Zoloft (sertraline) | Depression<br>Obsessive–compulsive disorders<br>Panic<br>Post-traumatic stress disorder | 2.0 | 0.7 | 1996 – EU and US | Once daily |
| Claritin (loratadine) | Allergy<br>Rhinitis | 2.0 | 0.7 | 1989 – UK<br>1993 – US | Once daily |
| Zyprexa (olanzapine) | Bipolar disorders<br>Gilles de la Tourette's syndrome<br>Psychotic disorders | 1.9 | 0.6 | 1996 – UK and US | Once daily |

Data from Scrip 2001 Yearbook, 17th edition, Table 2.7, p. 69. Sales of top 10 products worldwide 1999.

convenient method of administration for patients and the one most likely to result in high patient compliance. Again, this is confirmed by inspection of Table 8.1, showing the best-selling drugs in 1999. All of the top 10 compounds are for oral administration. Thus, oral administration is likely to be the desired route for any compound to be developed. However, intravenous dosing is also important because it allows determination of both rate of clearance and volume of distribution. These two are usually the primary parameters that determine the half-life. Clearance can be modulated in a series of compounds by altering rates of metabolism, while altering partition properties may change volume. Thus, it is important for medicinal chemists to know how these two parameters vary within their chemical series in order to be able to optimize the chemistry.

The important stages in delivering a drug to its desired target after an oral dose can be summarized as shown in Figure 8.2. Initially the formulation has to be swallowed and survive the transition to the site of absorption – the gastrointestinal tract (GIT). The time required for this to happen will depend on the stomach-emptying time, which in turn will be a function of the fed/fasting state of the subject or animal that is being studied (see for example Ref. [7]). This kind of information can only be obtained from *in vivo* studies.

Once in the GIT, when the drug has been released from the formulation into solution, the process of absorption may begin. In this phase, the compound has to pass across the wall of the GIT. This can be either by passive diffusion, which is commonly thought to be the most predominant route for the majority of drugs with molecular weights below 1000 Da, or it can be by paracellular absorption, or by active uptake. The paracellular route avoids passing through the cells, and instead the drug gains access to the portal blood by either passing through the tight junctions between the cells or through the nonrestrictive junctions. This method of

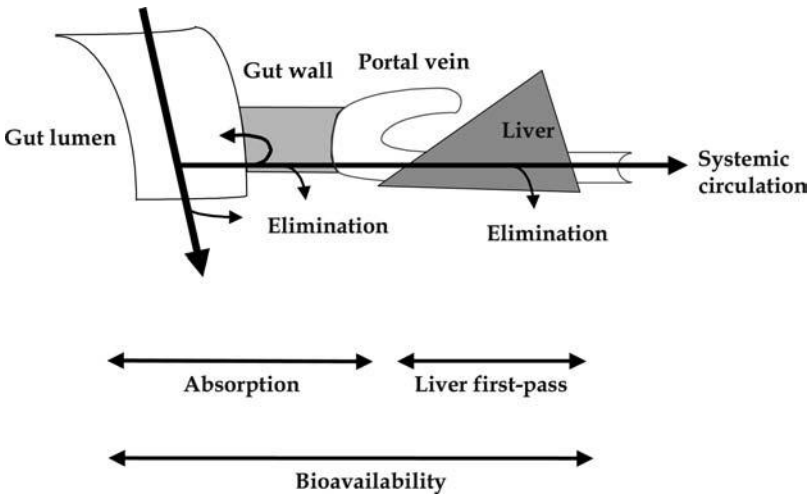


**Figure 8.2** Absorption and bioavailability.

absorption can be important for compounds of a smaller size (and hence lower molecular weight) and higher polarity than the norm. Active uptake mechanisms are most common for naturally occurring compounds such as sugars, amino acids, and di- and tripeptides.

The compound in the portal blood is transported to the liver, which usually is the major site of metabolism for pharmaceuticals. In the liver, there is usually one, or more, of three principal fates for the drug: either metabolism; excretion into the bile; or return to the blood for distribution to the other tissues of the body. These other tissues may also be sites of metabolism or, particularly in the case of the kidney, sites of excretion.

There is often confusion as to the meaning of absorption, as opposed to bioavailability. For the purposes of this chapter, absorption will be taken to mean the processes that are involved in transferring the drug in solution from the site of administration to the venous blood. In the case of oral absorption, this will be to the hepatic portal vein (see Figure 8.2). Bioavailability is the ratio of the AUC after administration by the route of interest and after administration of the same amount of drug direct into the systemic circulation, usually by intravenous injection. Thus, bioavailability, after oral dosing, differs from absorption by also including the effects due to such processes as metabolism and/or biliary secretion during the first pass of the compound through the liver.

Bioavailability is an important parameter in drug-screening cascades. It gives a good indication of the efficiency of the delivery of the compound to the systemic circulation by the chosen route. It can only be measured *in vivo* but, as will be described below, it can be predicted for man using a number of methods.

Measurement of absorption can be complicated by efflux mechanisms. It is clear that many compounds are actively transported back into the GIT, into the bile, or into the urine by efflux proteins. In the case of those in the GIT, these may have an impact on the apparent absorption of a compound. Some understanding of the substrate specificity for one of these proteins, P-glycoprotein, is becoming apparent [8, 9], but currently the understanding is limited. At the moment, there are no published reliable methods either *in vivo* or *in vitro* for predicting the importance of efflux mechanisms for a particular compound in man [10–12].

Absorption studies can be carried out using a variety of dosing routes, and although this chapter will focus on oral dosing, analogous stages can be envisioned after other methods of dosing.

## 8.3 Choice of Animal Species

The main preclinical species used for pharmacokinetic studies are the rat, mouse, and dog. An examination of the Biosys database for 2000 and 2001 shows that of the abstracted papers, 6334 mapped to the subject heading "Pharmacokinetics." Of these, the vast majority (70%) were studies on humans. Studies on rats constituted 14% of the reports, mice 7.5%, and dogs 3.4% (Table 8.2). Nonhuman primates can

**Table 8.2** Numbers of pharmacokinetic studies by animal.

| Species | Total number of studies[a] | Percentage[a] |
|---|---|---|
| All species | 6334 | 100 |
| Human | 4411 | 69.6 |
| Rat | 862 | 13.6 |
| Mouse | 478 | 7.5 |
| Dog | 215 | 3.4 |
| Rabbit | 199 | 3.1 |
| Guinea pig | 38 | 0.6 |
| Hamster | 23 | 0.4 |
| Nonhuman primate | 21 | 0.3[b] |

Numbers of papers abstracted into Biosys Previews and mapped to the subject heading Pharmacokinetics.

[a] Numbers given against individual species sum to more than the total given for all studies as some studies included more than one species.

[b] Many primate studies are on human antibodies that cannot be tested with other species due to problems of antigenicity.

also be important pharmacokinetic models, but ethical and practical considerations severely limit studies in these animals such that, with in the same period, they represented less than 0.5% of the abstracted reports on PK.

The initial choice of the rat as the primary species for pharmacokinetic studies arose because of their use in pharmacology and toxicology studies. However, there is now such a large database of information about the relative pharmacokinetics of the same compounds in rats and man that, as described below, useful predictions to man can be made.

The importance of the mouse as a species for pharmacokinetics will probably increase as genetically modified mice become more important in producing humanized models for *in vivo* pharmacology. The mouse presents a particular challenge to pharmacokineticists because of the very small volumes of blood that can be obtained and the difficulties this presents for bioanalysis. However, there are now published methods for obtaining repetitive samples from mice [13], and this means that, provided a statistically appropriate experimental design is used (essentially a Latin Square – see Ref. [14]), the numbers of animals used in a study can be limited. This same approach can be used for studies in larger animals when the analytical method requires plasma samples that are so large that a complete PK profile cannot be determined in a single animal.

## 8.4 Methods

There are a number of important methods that are worthy of discussion before consideration of how the data are used to predict human ADME/PK.

### 8.4.1 Radiolabels

An approach that can be used in determining ADME/PK parameters that is simple to execute and gives confidence that the whole dose is accounted for is to use a radiolabel. This has been the standard approach for development ADME studies for many years. The common isotopes used are $^{14}C$ or $^{3}H$ (tritium).

Of course, it is important to ensure that the site of labeling is chosen carefully so that it is not readily lost by metabolism. For example, $CH_3-N$ and $CH_3-O$ groups, although perhaps amenable to simple synthetic approaches, are often major sites of metabolism and could lead to significant portions of the dose being converted to $^{14}CO_2$ or $^{3}H_2O$. Even though it is possible to trap and count the exhaled gas, from a practical point of view, these kinds of labels are the poor choices.

The incorporation of $^{14}C$ into compounds at a suitable site often requires extensive and complicated syntheses and thus a relatively long time. This usually means that $^{14}C$-labeled compounds are unsuitable for studies to be carried out during discovery. There are, however, very rapid methods for incorporating $^{3}H$ into compounds. The newer methods, generally involving metal-catalyzed exchange reactions [15–18], in our experience, mean that suitable labels can often be prepared in 2 or 3 weeks. These timescales make the approach viable for discovery support. Additionally, and importantly, these methods can lead to *specific* incorporation of tritium.

There is a general prejudice among drug metabolism scientists against using tritiated compounds. This is because such labels have often given rise to the formation of $^{3}H_2O$. Tritiated water has a remarkably long half-life in the body of between 6 and 9 days [19–22], and this is probably much longer than the half-life of the compound of interest or its metabolites. In any studies, significant production of $^{3}H_2O$ is an unwanted complication. However, we have found that *specifically* labeled compounds often lose only small amounts of radioactivity as $^{3}H_2O$, and most of this can be readily removed by freeze–drying the samples. Hence, it is usually possible to gain comprehensive information about the fate of the bulk of the dose. We have often found the use of a $^{3}H$-labeled compound has significantly improved our knowledge of a compound, and hence its chemical series, and given clear information on the major pathways of clearance or extent of absorption. This then allows the data from *in vitro* screens to be used with greater confidence.

### 8.4.2 *Ex Vivo* Methods for Absorption

#### 8.4.2.1 Static Method

There are several approaches to estimating absorption using *in vitro* methods, notably, Caco-2 and MDCK cell-based methods or using methods that assess passive permeability, for example, the parallel artificial membrane permeation assay (PAMPA) method. These are reviewed elsewhere in this book. The assays are very useful and usually have an important role in the screening cascades for drug

discovery projects. However, as discussed below, the cell-based assays are not without their drawbacks, and it is often appropriate to use *ex vivo* and/or *in vivo* absorption assays.

The simplest *ex vivo* assay consists of isolating segments of the GIT in an anesthetized rat, while leaving the blood and nervous supply intact as far as possible [23]. Hence, the segments continue to receive a blood supply, and any absorbed compound is carried away. The compound of interest is injected into segments, and at the end of the study the isolated segments are collected and analyzed for remaining compound. Absorption is estimated by loss. By injecting the compound into different segments at different times, a time course for the loss may be established. The approach has the advantage of simplicity, but suffers from the need to obtain good recoveries from what is often a difficult matrix to analyze. For poorly absorbed compounds – often the ones for which reliable estimation of absorption is needed – the method is unable to accurately determine small differences.

#### 8.4.2.2 **Perfusion Methods**

Because of these problems, perfusion assays have been developed. Success in predicting absorption in man using *in-situ* single-pass perfusion of the rat intestine has been reported [24–26]. In this model, the animal is anesthetized and a segment of the gut is exposed and cannulated. A formulation of the drug is perfused through the gut segment, and the concentration before and after perfusion is determined. This approach has the advantage of being able to make several estimations of the concentration of the perfusate and of allowing measurements to be made from a cleaner matrix.

For a series of rennin inhibitors, a good correlation between the measured membrane permeability and $\log D$ was found ($r^2 = 0.8$). The model has been validated against a human perfusion model [10], as well as being extended by including molecular weight as a third parameter [27]. A further development of the model is to chronically cannulate the animals so that they can be allowed to recover [28]. This model should minimize any effects of the anesthetic on the absorption process.

Using the single-pass *in-situ* absorption model in the anesthetized rat, a study of nine compounds found a good correlation between rat and man as to whether compounds were subject to active uptake or absorbed by simple passive diffusion [29].

However, because of the significant surgical alterations that are necessary, studies using isolated perfused gut loops do not always accurately predict the results in whole animals, and there can be significant advantages in whole animal models for absorption.

### 8.4.3
### *In Vivo* Methods

There are several possible *in vivo* approaches to the determination of the absorption of a compound after oral dosing. Probably the simplest and most direct is to use a

radiolabel. For the vast majority of studies, this means either a $^{14}C$ or $^{3}H$ label. The approach used can be quite simple: the labeled version of the drug is administered to an animal that is then housed in a "metabolism cage" for the separate, and complete, collection of both urine and feces. The samples of excreta are collected for as long as is necessary to obtain a full recovery of radioactivity. They are then analyzed for radioactive content. At its simplest, it can usually be assumed that, after an oral dose, at least all of the radioactivity that appears in the urine must have been absorbed, thus giving an assessment of the minimum absorption of the compound. Collecting the feces and subjecting them to chromatographic analysis with radiodetection can refine the study. This allows the identification of the proportion of the dose that has been absorbed but then excreted in the bile as metabolites (as opposed to the dose that has not been absorbed and has passed straight through the GIT as the parent compound). This approach should be supported with further studies to ensure that the parent compound is not metabolized directly in the GIT by the microflora. However, it is possible to be misled if the parent compound is absorbed but excreted unchanged in the bile.

Another refinement, that avoids the necessity of developing suitable fecal extraction and chromatographic methods, is to dose the radiolabeled compound by both the i.v. and p.o. routes in two separate studies. Knowing that, by definition, the whole of the i.v. dose must have been bioavailable; a comparison of the proportion of the dose in the urine after the two different routes allows estimation of the percent absorbed. An analogous approach can be used without the use of a radiolabel, when the urine from the two studies is analyzed either for the parent compound or, more usually, for a major common metabolite. Assuming quantitatively identical clearance after both the i.v. and p.o. doses, the ratio of the amounts of analyte in the two experiments gives the absorption.

## 8.5
## *In Vivo* Methods for Determining Bioavailability

### 8.5.1
### Cassette Dosing

Cassette dosing or "*N* into 1" dosing was one of the first techniques used to enhance the throughput of ADME/PK studies. It has the advantage of reducing the number of animals used and increasing the number of compounds that can be tested in a set time. This method involves dosing each animal with several compounds at the same time [30]. The selectivity and sensitivity of analytical methods now available, usually HPLC/mass spectrometry [31], mean that it is possible to analyze for each of the compounds in the presence of others [32, 33]. Although reports on cassettes of up to 22 compounds have been made [34], it is more usual to limit the number to between 3 and 6. There are significant benefits to this approach, as animals are only dosed once and the same number of plasma samples is collected as would be for a single compound study. However, the dose levels must be limited in order to

minimize possible stress to the animals and possible compound–compound interactions.

The potential for the metabolites that are formed to have the same masses as other parent compounds is another factor that limits the number of compounds that may be included in the cassette, as does the potential for drug–drug interactions [35]. Other limitations are the total dose that can be administered without saturating important pathways of metabolism or distribution and the solubility of the compounds in the dosing formulation. However, there is a balance to be achieved as, if the dose of each component given is very low, it is likely that the analytical method will not have sufficient sensitivity to provide an accurate assessment of the pharmacokinetics.

Nonetheless, the approach can provide – both routinely and rapidly – large amounts of pharmacokinetic or other distribution information on several compounds without significantly increasing the burden on the animals, while also minimizing the number of animals used. It is common to include a compound of known pharmacokinetics that acts as a control in each of these studies. This can help in identifying when the coadministered compounds have changed the kinetics. However, such marker compounds will not necessarily highlight problems with compounds that are subject to different clearance mechanisms [35].

### 8.5.2 Semisimultaneous Dosing

An approach that can bring benefits by reducing variability and increasing the speed of generating results is to use "semisimultaneous dosing" pharmacokinetic studies [36]. In these studies, animals are dosed by the two different routes of interest, a short period apart: often 4–6 h and usually less than 48 h. Blood samples are collected in the usual way following the dosing and analyzed for the parent compound. The pharmacokinetic profiles are then constructed, subtracting out, if necessary, any part of the profile from the first dose that is still present during the profile from the second dose [37]. These studies allow both profiles to be determined in the same animals at essentially the same time ("semisimultaneous"). This has the advantage of reducing variability in the pharmacokinetic profiles from the two doses and allowing a more reliable comparison of the two profiles. To ensure that there is not a significant increase in the number of samples that are taken to determine the two profiles, the samples can be withdrawn through an indwelling catheter or the total number of venepunctures restricted to the same number that would be used for single-dosing studies. The total amount of blood taken need not be significantly greater than is taken in a normal pharmacokinetic study, and so there is little increase in the stress on the animals. These studies have the advantage of eliminating a second procedure for the animals, while retaining the advantage of a crossover design with little chance for significant alteration in the factors that control the pharmacokinetics between the two doses. The approach also generates information more rapidly than when there is a "washout" period between the two doses.

The original proposal of the approach, supported by a Monte Carlo simulation study [36], has been further validated with both preclinical [38, 39] and clinical

studies [40]. It has been shown to be robust and accurate and is not highly dependent on the models used to fit the data. The method can give poor estimates of absorption or bioavailability in two sets of circumstances: (i) when the compound shows nonlinear pharmacokinetics, which may happen when the plasma protein binding is nonlinear, or when the compound has cardiovascular activity that changes blood flow in a concentration-dependent manner; or (ii) when the rate of absorption is slow, and hence "flip-flop" kinetics are observed, that is, when the apparent terminal half-life is governed by the rate of drug input.

### 8.5.3
### Hepatic Portal Vein Cannulation

The use of hepatic portal vein-cannulated animals can be helpful in determining specific causes of poor bioavailability. After oral dosing, the total bioavailability of a compound is normally calculated as

$$\text{Bioavailability} = \frac{\text{AUC}_{\text{po}}}{\text{AUC}_{\text{iv}}} \times \frac{\text{Dose}_{\text{iv}}}{\text{Dose}_{\text{po}}}, \tag{8.1}$$

where AUC is the area under the drug concentration–time curve to infinite time and p.o. and i.v. indicate oral or intravenous routes. The oral bioavailability can also be considered from the perspective of loss at different stages of the process of reaching the systemic circulation, that is,

$$F_{\text{oral}} = (1-f_{\text{G}})(1-f_{\text{H}})(1-f_{\text{abs}}), \tag{8.2}$$

where $f_{\text{abs}}$ is the fraction not absorbed from the GIT, and $f_{\text{G}}$ and $f_{\text{H}}$ are the fractions of drug cleared (e.g., metabolized) in the gut wall and the liver, respectively. It is possible to measure the relative contributions of these processes by carrying out dosing and or sampling of the hepatic portal vein [41] in addition to the normal methods of p.o. and i.v. dosing coupled with i.v. sampling. Thus,

$$f_{\text{G}} = \frac{1-\text{AUC}_{\text{po}}}{\text{AUC}_{\text{hpv}}} \tag{8.3}$$

and

$$f_{\text{H}} = \frac{1-\text{AUC}_{\text{hpv}}}{\text{AUC}_{\text{iv}}}. \tag{8.4}$$

These multiple input experiments can be carried out in a crossover fashion.

## 8.6
## Inhalation

There are many ways of administering compounds to man or preclinical safety species, and it is not possible to review them all within the scope of this chapter. However, the inhalation route is worthy of some consideration as it can be important.

This is usually when the target organ is the lung, in diseases such as asthma or chronic obstructive pulmonary disorder (COPD), or when the lung may be a suitable route of administration for the systemic delivery of macromolecular peptide or protein biopharmaceuticals – compounds that would neither survive passage through, nor be absorbed from, the GIT [42, 43]. The absorption of these molecules is thought to occur by diffusion in the conducting airways [44] and by diffusion and transcytosis in the alveolar region of the lungs [42]. Even with the lower metabolic activity in the lung [45], direct administration can be a useful way of delivering compounds to their site of action, while limiting systemic side effects.

However, it is rarely possible to carry out inhalation studies during the research phase. Compared with intratracheal (i.t.) dosing, inhalation dosing is perhaps physiologically more similar to the clinical dosing method, is noninvasive, results in lower dose rates, and may well provide more even and representative distribution within the lungs. Nonetheless, i.t. instillation is often a worthwhile alternative as it allows accurately quantified doses to be administered and does not require the complex dosing systems needed in inhalation studies. Inhalation dosing invariably leads to significant oral exposure, either due to direct ingestion of the aerosol or by the animal grooming particles from its pelt after dosing has finished (see Ref. [46] and references cited therein). Although, i.t. administration has been shown to produce a very nonuniform distribution within the lungs, it has also been possible to obtain remarkably consistent, dose-proportional absorption over a wide range of doses (up to two and four orders of magnitude) [47], suggesting that absorption from the lung will not necessarily be saturated. Compounds given by the i.t. route can give rise to pharmacokinetics that closely mimic those of an i.v. dose [48, 49] with apparently very rapid and extensive absorption. However, i.t. dosing can also give indications of differing rates of absorption from the lung, depending on the compound and its physicochemical properties [45, 47, 50] or formulation [51, 52]. It has been reported that for a series of drugs, the absorption after aerosol administration was approximately twice as fast as through i.t. dosing [53], suggesting that absorption from the deeper alveolar region may be more rapid than that from the tracheobronchial region of the lung.

Although the usual animal model for i.t. studies is the rat [45, 47, 48, 54], studies on dogs [50, 54], rabbits [49], and guinea pigs [55] have also been reported.

A detailed review of i.t. dosing has recently been published [46], which provides practical details of the technique.

## 8.7 Relevance of Animal Models

### 8.7.1 Models for Prediction of Absorption

Measurement of the fraction absorbed, as described elsewhere in this book, can be carried out using *in vitro* systems. However, for Caco-2 cells, for example, the

relationship between the apparent rate of permeability that is measured and the percentage of the dose absorbed in man is often very steep. Thus, small changes in the measured rate of permeation may result in a compound with low human absorption being predicted to have good absorption [6, 56]. Other model systems, such as those based on the use of gut tissue in Ussing chambers, are highly dependent on the supply of good-quality tissue. Because of these kinds of issues, *in vivo* models can have significant advantages over the *in vitro* systems. Although the rate of absorption can be highly variable, the extent has often been shown to be similar between species including man (see for example Ref. [57] and references cited therein), and this similarity has recently been analyzed and the correlation between percentage dose absorbed in rat and man shown to be reliable and quantitative [58]. The relationship was analyzed for a group of 64 drugs, which covered a wide range of physical properties (acids, bases, neutrals, and zwitterions) and molecular weights (138–1202 Da). Also included were compounds for which absorption may involve carrier-mediated mechanisms. Excluded were compounds thought to be unstable in the GIT or which are affected by particle size or are polymorphic. The ratio between percent absorbed in human and rat was found to be very close to 1, with a correlation coefficient of 0.97.

The other principal preclinical PK model – the dog – is not thought to be such a useful model for prediction of absorption in man because of larger pore size and greater pore frequency in the paracellular pathway of dog compared with rat [59].

### 8.7.2
### Models for Prediction of Volume

Estimation of the volume of distribution in man may be carried out in a number of ways. These methods have recently been reviewed by Obach *et al.* [60], who carried out a wide-ranging evaluation of a large number of different ways of predicting the human pharmacokinetics of 50 compounds that entered development at Pfizer. One of the simplest methods was reported to be the most reliable. It is based on the assumption that the free fraction of drug in the plasma in dog and human and the volume of distribution are proportional, that is, free $V_{d(human)} =$ free $V_{d(dog)}$. This allows a prediction for $V_d$ in man to be generated:

$$V_{d(\text{predicted in man})} = f_{u(man)} \times V_{d(dog)}/f_{u(dog)} \tag{8.5}$$

Both human and dog volumes are in units of $\text{L kg}^{-1}$, and $f_u$ is the fraction of the drug unbound in plasma. The method was found to predict within twofold for about 80% of the compounds, which spanned about three orders of magnitude in their $V_d$. Although the dog has been recommended as the best model for predicting volume in man [60], there are also reports indicating that the rat may also be a suitable model [61].

## 8.8 Prediction of Dose in Man

### 8.8.1 Allometry

One of the most frequently used methods for predicting human pharmacokinetics from animal data is allometry. This technique was initially used to explain the relationship between body size and organ weights in animals [62–67]. The approach is based on finding a correlation between a physiological and the pharmacokinetic parameter of interest. Generally, the relationship takes the form of

$$y = a \times B^x, \tag{8.6}$$

where $y$ is the dependent variable, for example, clearance; $B$ is the independent variable, for example, body weight, brain size or maximum life span; and $a$ and $x$ are the allometric coefficient and exponent, respectively.

The allometric coefficient and exponent are determined empirically and are not thought to have any physiological correlate.

The drawback of this approach is that it is essentially empirical and does not allow for differences in metabolic clearance between the species, that is, it assumes that clearance is proportional to blood flow. This works well for compounds that are highly extracted in the liver and/or where passive renal clearance is the major pathway [5, 68]. An approach for compounds that are actively secreted into the urine has also been proposed [69], though the precise values of some of the physiological scaling factors have been questioned [70].

Unfortunately, when clearance is largely metabolic and low, allometry can significantly overpredict the human value [71]. Recent investigations have attempted to address this by combining allometric approaches with *in vitro* metabolism data [5].

A recent debate on allometric scaling has suggested that a great deal of further work is necessary before allometry can be used with confidence in a prospective manner. It is claimed that it is not possible to know in advance when allometry will not be suitable, and indeed the accuracy of the predictions may not be as reliable as assumed [72–74].

### 8.8.2 Physiologically Based Pharmacokinetics

Another method of predicting human pharmacokinetics is physiologically based pharmacokinetics (PB/PK). The normal pharmacokinetic approach is to try to fit the plasma concentration–time curve to a mathematical function with one, two, or three compartments, which are really mathematical constructs necessary for curve fitting, and do not necessarily have any physiological correlates. In PB-PK, the model consists of a series of compartments that are taken to actually represent different tissues [75–77] (Figure 8.3). In order to build the model, it is necessary to know the size and perfusion rate of each tissue, the "partition coefficient" of the compound

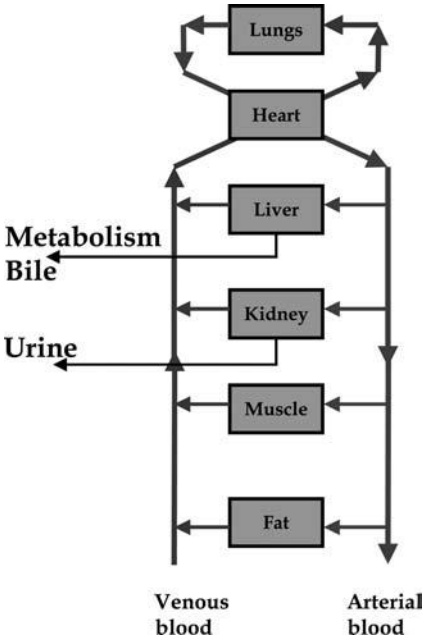


**Figure 8.3** Physiological pharmacokinetic model.

between each tissue and blood, and the rate of clearance of the compound in each tissue. Although different sources of errors in the models have been described [78–80], these kinds of models are extremely appealing to kineticists because they lead to a fuller understanding of the factors that determine the pharmacokinetics of any compound. However, they require many experimental determinations to be made for each compound, and thus they are unlikely to become the method of choice during the routine design, make/test cycle (see Figure 8.1).They may however, become an important contributor to the decision about the suitability of a compound to progress into development.

### 8.8.3
### Prediction of Human Dose

As stated in the Section 8.1, one of the principal purposes of carrying out DMPK studies during the discovery phase is to reduce the failure rate during development. For DMPK, this logically means predicting the pharmacokinetics that will be observed and hence the dose that will be required in man when clinical studies are carried out.

It is possible to predict the steady-state minimum plasma concentration (Figure 8.4) using the equation

$$C_{\mathrm{min,ss}} = \frac{f_{\mathrm{a}} \cdot \mathrm{Dose}}{V(\mathrm{e}^{k\tau}-1)}, \tag{8.7}$$

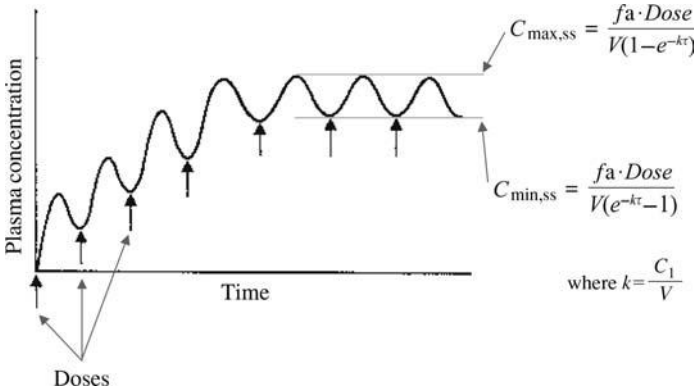


**Figure 8.4** Dose prediction.

where $C_{min,ss}$ is the minimum plasma concentration at steady state; $f_a$ is the fraction absorbed in man; Dose is the dose (in mg kg$^{-1}$); $V$ is the volume of distribution at steady state (in l kg$^{-1}$); $k$ is the elimination constant (this is given by clearance divided by volume); and $\tau$ is the dosing interval given in h$^{-1}$.

The equation is an approximation, adapted from that for intravenous dosing [81], corrected by addition of a term for absorption. Essentially it assumes instantaneous absorption of the dose, but for compounds with reasonable physicochemical and PK properties that are expected to be suitable for once-a-day dosing, this approximation makes little difference to the predicted value of $C_{min,ss}$. Use of the relationship can provide a simple approach for estimating the required dose in man for a compound in the discovery phase.

Equation 8.7 can be rearranged to allow the prediction of the dose and dose interval, provided that the following can be estimated: human potency, absorption, clearance, and volume.

Estimation of the potency can be made in several ways and will be highly dependent on the nature of the target. If a purified system is used, it is normal to correct for the effect of plasma protein binding (which can be measured directly in human plasma) as it is usual for the effect to be proportional to the unbound concentration [82]. This can be used to set a value for the minimum plasma concentration at steady state.

As described above, it will be normal to assume that the dose interval is 24 h, that is, once-a-day dosing. Absorption can be estimated with good confidence in the rat (see Section 8.1). Clearance is the sum of the predicted hepatic, renal, biliary, and extrahepatic clearance. Hepatic clearance can be derived from *in vitro* studies with the appropriate human system, using either microsomes or hepatocytes. We prefer to use an approach based on that described by Houston and Carlile [83]. Renal clearance can be predicted allometrically (see Section 8.8.1). The other two potential methods of clearance are difficult to predict. To minimize the risks, animal studies can be used to select compounds that show little or no potential for clearance by these routes. As volume can be predicted from that measured in the dog, after correction for human

and dog plasma protein binding (see Section 8.2), it is possible to make predictions for all of the important parameters necessary.

We believe that this approach brings together the best combination of *in vitro*, *in vivo*, and allometric approaches and can provide useful estimates of likely human doses, provided that sufficient attention is paid to the errors associated with all of the measurements [4].

## 8.9 Conclusions

The purpose of this chapter has been to illustrate the potential role of animal studies in ADME/PK in drug discovery. Given that one of the major objectives for ADME/PK is to predict PK in man, it must be concluded that much work is still to be done in the development of reliable and accurate models. Although, quite rightly, many studies have focused on *in silico* and *in vitro* approaches, there is still a general agreement that, with current knowledge, we are still highly dependent on animal models [3, 5, 6, 84]. Indeed, their use in predicting important parameters such as absorption and volume of distribution has been highlighted in this chapter.

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# 9
# *In Vivo* Permeability Studies in the Gastrointestinal Tract of Humans

*Niclas Petri and Hans Lennernäs*

### Abbreviations

| | |
|---|---|
| BCRP | Breast cancer-resistant protein |
| CYP3A4 | Cytochrome P450 3A4 |
| HBD | Number of hydrogen-bond donors |
| hPEPT1 | Oligopeptide carrier for di- and tripeptides |
| LNAA | Large neutral amino acid |
| MRP | Multidrug-resistant protein family |
| P-gp | P-glycoprotein |
| PSA | Polar surface area |

### Symbols

| | |
|---|---|
| $CL_{int}$ | Intrinsic clearance |
| Clog *P* | Logarithm of the calculated octanol/water partition coefficient (for neutral species) |
| $P_{eff}$ | Effective intestinal permeability |
| $F$ | Bioavailability |
| $F_a$ | Fraction dose absorbed |
| $E_G$ | Gut wall extraction |
| $E_H$ | Hepatic extraction |
| log $D_{6.5}$ | Logarithm of the distribution coefficient in octanol/water at pH 6.5 |
| MW | Molecular weight |
| $Q_h$ | Hepatic blood flow |

## 9.1 Introduction

The predominant way of delivering drugs to the systemic circulation to generate pharmacological and clinical effects is the oral route. Self-administration of drugs to

the gastrointestinal (GI) tract is considered to be safe, efficient, and easily accessible with minimal discomfort to the patient in comparison with other routes of drug administration. The design and composition of the pharmaceutical dosage formulation, as well as the physicochemical properties of the drug itself, will certainly affect *in vivo* performance and hence the therapeutic outcome. Bioavailability ($F$) of drugs after oral administration is determined by several factors such as solubility and dissolution, transit time, GI stability, intestinal permeability, and first-pass extraction in the gut and/or by the liver [1–4]. Among these factors, effective intestinal permeability ($P_{eff}$) is a major determinant of fraction dose absorbed ($F_a$) [1, 3, 5]. It is a recognized fact that some drugs may be transported by multiple mechanisms, passive diffusion, and various carrier-mediated transporters via both absorptive and secretory routes (Figure 9.1) [1, 3, 6–8]. The expression and functional activity of intestinal transport proteins and enzymes are currently under examination, and the future will reveal the extent to which various transporters contribute to intestinal absorption and presystemic metabolism of drugs (Figure 9.1) [9]. Undoubtedly, such knowledge will increase our understanding of the mechanisms underlying the variability between individuals and regulation of responses to drugs, from both genomic and nongenomic perspectives [9, 10]. In spite of the fact above, the *in vivo*

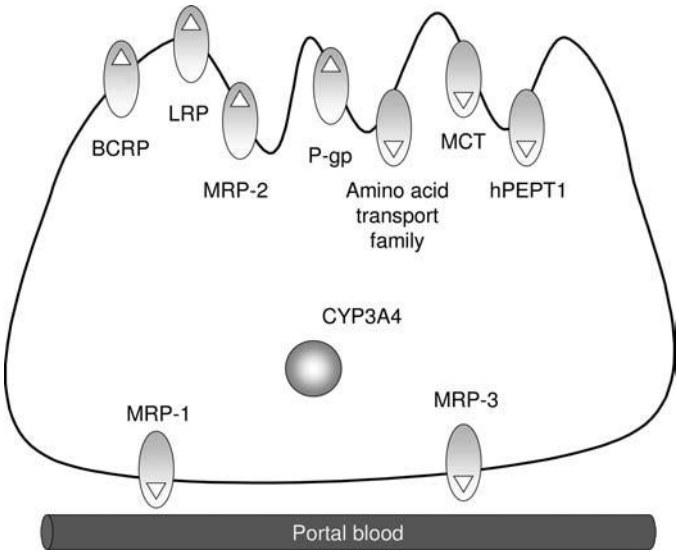



**Figure 9.1** $P_{eff}$ of drugs *in vivo* may be affected by several parallel transport mechanisms in both absorptive and secretory directions. A few of the most important transport proteins that may be involved in the intestinal transport of drugs and their metabolites across intestinal epithelial membrane barriers in humans are displayed. P-gp, P-glycoprotein; BCRP, breast cancer-resistant protein; LRP, lung-resistant protein; MRP1–5, multidrug-resistant protein family; hPEPT1, oligopeptide carrier for di- and tripeptides; MCT, $H^{(+)}$-monocarboxylic acid cotransporter. CYP3A4 is an important intracellular oxidation CYP P450 enzyme; approximately 50–60% of all used drugs are substrates for this enzyme.

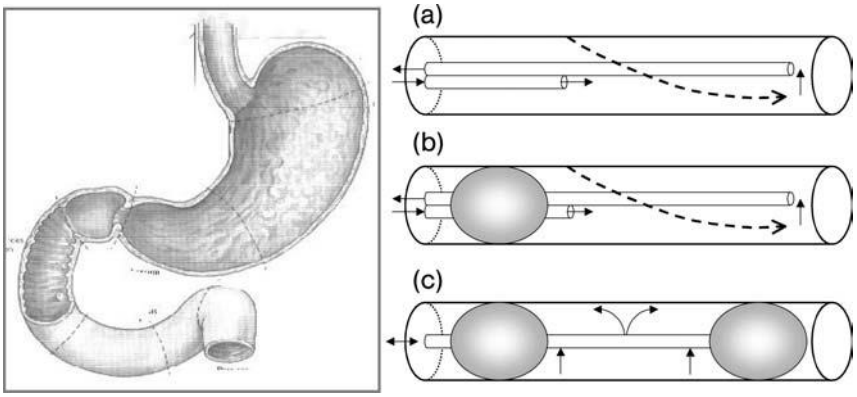



**Figure 9.2** Schematic diagram of three different perfusion methodologies for human use: (a) open; (b) semiopen; and (c) double balloon. For the open and semiopen, the hydrodynamics is best described by the parallel-tube model (see the dotted line for the concentration profile over the intestinal length). The well-stirred model is the best hydrodynamic model for the double-balloon perfusion technique.

measured drug transport permeability ($P_{eff}$) represents the total transport (i.e., the macroscopic transport rate) of all parallel processes.

Direct measurements of intestinal absorption, secretion, and metabolism of drugs in humans are possible by using regional intestinal perfusion techniques [6, 11, 12]. In general, three different clinical tools have been employed in the small intestine: (i) a triple-lumen tube including a mixing segment, (ii) a multilumen tube with a proximal occluding balloon, and (iii) a multilumen tube (Loc-I-Gut) with two balloons occluding a 10 cm long intestinal segment (Figure 9.2) [5, 6, 11, 13–15] . The advantages and disadvantages of various intestinal perfusion techniques are discussed elsewhere [3, 16]. In Figure 9.3, the complete Loc-I-Gut concept is displayed [11, 13]. This intestinal perfusion technique has been widely applied to investigate drug absorption, presystemic metabolism, drug dissolution, *in vitro–in vivo* correlation, drug–drug interactions, variability between individuals, GI physiology, and disease mechanisms (Figure 9.4) [3, 11, 16–36]. The Loc-I-Gut approach makes it possible to investigate and predict integrated *in vivo* processes in the human intestine, where genetic, biochemical, physiological, pathophysiological, and environmental influences may all affect the transport/metabolism of drugs [3, 11]. In addition, the Loc-I-Gut technique has been used to establish an *in vivo* human permeability database for the proposed Biopharmaceutical Classification System (BCS) for oral immediate-release products (Figure 9.5) [1, 31, 37]. Human *in vivo* $P_{eff}$ values obtained under physiological conditions provide the basis for establishing *in vitro–in vivo* correlations, which can be used to make predictions about oral absorption as well as to set bioequivalence standards for drug approval [1, 16, 21, 28, 29, 31]. Recently, this single-pass perfusion approach has been used for measurements of the expression and function of enzymes and transporters in human-shed (harvested) enterocytes in

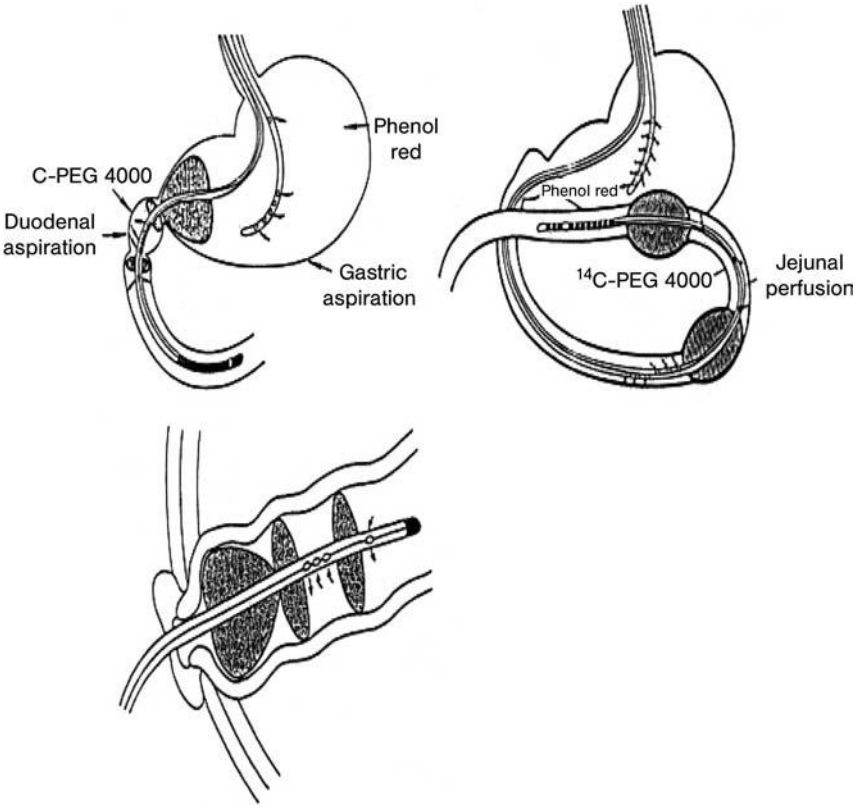



**Figure 9.3** The total Loc-I-Gut concept. Left: a perfusion system of the duodenal segment. Center: the tube system with double balloons allows a segmental single-pass perfusion of jejunum. Below: a perfusion system of the small intestinal stomi.

combination with measurements related to transport and presystemic metabolism in the same individuals [38, 39]. This body of data clearly indicates that our understanding of intestinal absorption, secretion, and metabolism of drugs has been significantly increased through the application of intestinal perfusion techniques. The purpose of this chapter is to describe how human *in vivo* perfusion studies continue to provide important information on oral drug delivery and help summarize reports based on these techniques.

## 9.2
## Definitions of Intestinal Absorption, Presystemic Metabolism, and Absolute Bioavailability

The most useful pharmacokinetic variable for describing the quantitative aspects of all processes influencing the absorption (fraction dose absorbed, $F_a$) and first-pass

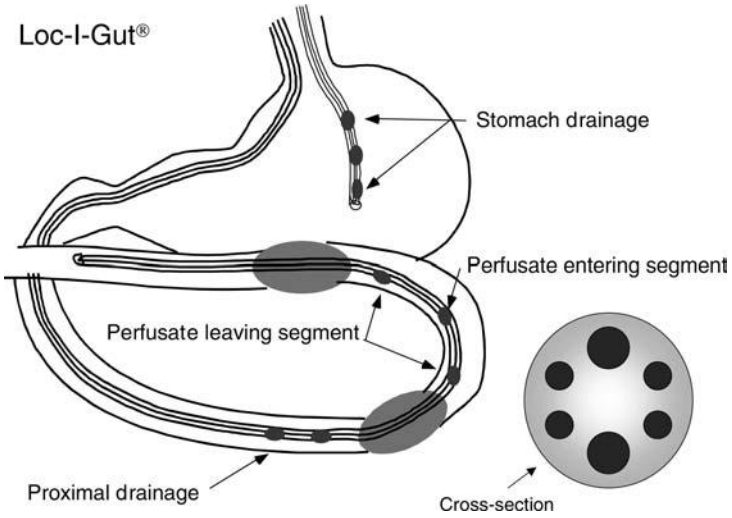



**Figure 9.4** Loc-I-Gut is a perfusion technique for the proximal region of the human jejunum. The multichannel tube is 175 cm long and is made of polyvinyl chloride with an external diameter of 5.3 mm. It contains six channels and is provided distally with two 40 mm long, elongated latex balloons, placed 10 cm apart, each separately connected to one of the smaller channels. The two wider channels in the center of the tube are for infusion and aspiration of perfusate. The two remaining peripheral smaller channels are used for administration of marker substances and/or for drainage. At the distal end of the tube, a tungsten weight aids the passage of the tube into the jejunum. The balloons are filled with air when the proximal balloon has passed through the ligament of Treitz. Gastric suction is performed using a separate tube. $^{14}$C-PEG 4000 is used as a volume marker to detect water flux across the intestinal barrier.

metabolism and excretion ($E_G$ and $E_H$) in the gut and liver is the absolute bioavailability ($F$) [40]. This pharmacokinetic parameter defines the fraction of the dose that reaches the systemic circulation and is used in the evaluation of the pharmacological profile and safety of oral pharmaceutical products in various clinical situations. Bioavailability depends on three major factors: $F_a$, the first-pass extraction of the drug in the gut wall ($E_G$), and the liver ($E_H$) (Equation 9.1) [2–4, 15, 35]:

$$F = F_a \cdot (1-E_G) \cdot (1-E_H). \quad (9.1)$$

Several factors may affect $F_a$ and $E_G$ of drugs. These can be divided into three general categories: (i) pharmaceutical factors; (ii) physicochemical factors of the drug molecule itself; and (iii) physiological, genetic, biochemical, and pathophysiological factors in the intestine [3, 5–8, 11, 15, 27, 32, 41–46]. According to scientific and regulatory definitions, $F_a$ is the fraction of the dose transported (absorbed) across the apical cell membrane into the cellular space of the enterocyte [3, 11, 16, 25–31, 47, 48]. Once the drug has reached the intracellular site, it may be subjected to CYP P450 metabolism, predominantly by CYP3A4, as well as other enzymatic steps [2–4, 15, 34, 35, 38, 49]. The enzymatic capacity of the small intestine to metabolize drugs can be expressed in pharmacokinetic terms as $E_G$ [40]. It is important to emphasize that CYP3A4 is not expressed in the colon [50, 51] and that drug metabolism by colonic microflora

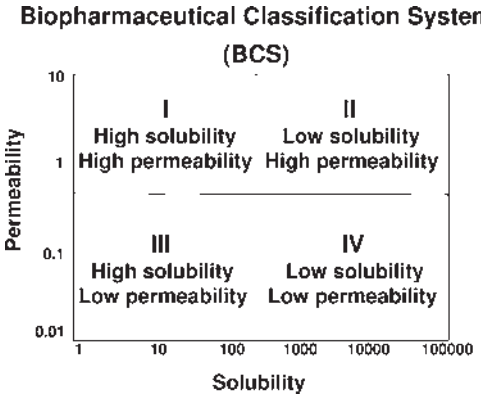



**Figure 9.5** The Biopharmaceutics Classification System provides a scientific basis for predicting intestinal drug absorption and identifying the rate-limiting step based on primary biopharmaceutical properties such as solubility and $P_{eff}$. The BCS divides drugs into four different classes based on these two parameters. Drug regulation aspects related to *in vivo* performance of pharmaceutical dosage forms have been the driving force in the development of BCS. BCS-based guidelines for industry are mainly used to indicate when bioavailability/bioequivalence (BA/BE) studies can be replaced by *in vitro* bioequivalence testing (www.fda.gov/cder/guidance/3618fnl.htm).

may play a crucial role in colonic drug absorption, especially with regard to drugs given in extended-release dosage forms, which may be subjected to predominantly hydrolytic and other reductive reactions [52, 53]. The fraction that escapes metabolism in the small intestine ($1 - E_G$) may undergo additional metabolism and/or biliary secretion in the liver ($E_H$) before reaching the systemic circulation. $E_H$ depends on blood flow ($Q_h$), protein binding ($f_u$), and the intrinsic clearance of enzymes and/or transporters ($CL_{int}$) [40]. Recently, it has also been recognized that membrane transport into hepatocytes must be included in models for predicting and explaining liver extraction.

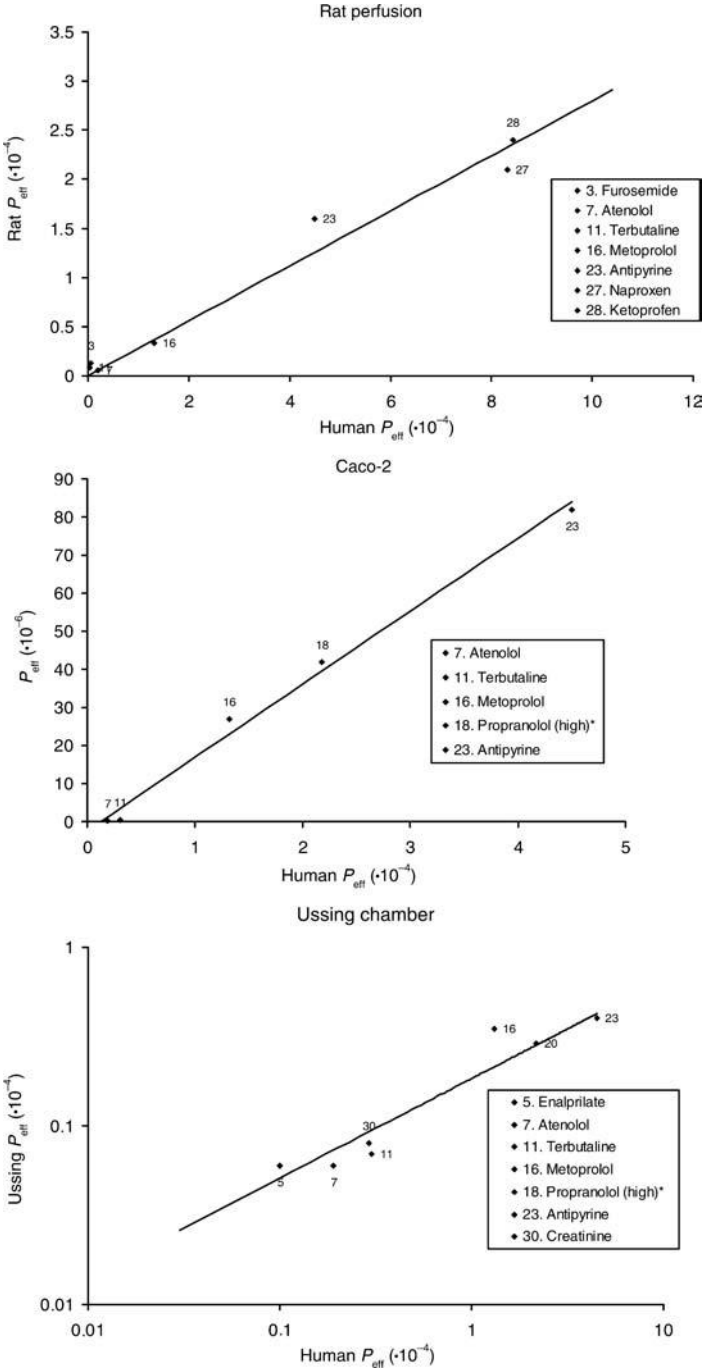
## 9.3 Methodological Aspects of *In Vitro* Intestinal Perfusion Techniques

Clinical studies of $P_{eff}$, secretion, and metabolism of various compounds such as drugs, environmental pollutants, and nutrients are rarely performed *in vivo* in humans even if experimental techniques are available (Figures 9.2–9.4) [3, 11, 13, 16, 17, 24–31]. Direct measurements of compound transport and metabolism in mesenteric and portal veins in humans are not possible for obvious reasons. Perfusion techniques, however, present great possibilities to measure intestinal processes. Over the past 70 years, different *in vivo* intestinal perfusion techniques have been developed and the importance of this work has been clearly demonstrated [3, 5, 6, 11, 13–16, 25–31]. The fundamental principle of an *in vivo* intestinal perfusion experiment is that $P_{eff}$ is calculated from the rate at which the compound disappears

from the perfused intestinal segment. The accurate determination of $P_{eff}$ requires knowledge of the hydrodynamics, perfusion rate, and surface area of the perfused intestinal segment [3, 11, 16, 25–31]. Fluid hydrodynamics depends on the perfusion technique applied, flow rate, and GI motility [11, 30]. The major advantages of using $P_{eff}$ as the absorption parameter are that first it is possible to measure regardless of the transport mechanism(s) across the intestinal mucosa; second, it predicts $F_a$; and finally, it can be used to assess *in vitro–in vivo* correlations that validate the use of different intestinal absorption models [21, 22, 28, 29] commonly applied in drug discovery and preclinical development (Figure 9.6). Such *in vivo* studies of intestinal absorption and function provide a fully comprehensive profile of the integrated response to drugs in humans, by taking genetic, biochemical, physiological, pathological, and environmental factors into account [54]. We have established a good correlation between $P_{eff}$ determined *in vitro* and historical data on $F_a$ for a large number of structurally diverse drugs (Figures 9.7 and 9.8).

The enterocyte is the most common cell type (>90%) in the small intestinal barrier, which also contains a significant number of lymphocytes, mast cells, and macrophages. The intestinal $P_{eff}$ for passive transcellular diffusion is considered to reflect the diffusion across the complex apical membrane into the cytosol, which is situated close to the cytoplasmic leaflet of the apical enterocyte membrane [3, 5, 7, 11, 16, 25–31, 47, 49, 55]. Consequently, intestinal perfusion models that measure the disappearance of the drug from the perfused segment directly describe its quantitative uptake into epithelial cells. The apical enterocyte membrane is very complex and is thought to represent the rate-limiting step in diffusion across this barrier. In addition, it has been speculated that the exofacial leaflet is responsible for the low permeability of the apical membrane [47, 55, 56]. Molecular dynamics simulations have identified four separate regions in the membrane, although the biological membrane containing multiple components may be considered more complex [56]. More studies are required to establish the role of bilayer asymmetry and membrane proteins in determining the unique permeability properties of the barrier imposed by the epithelial apical membrane [47, 55, 56].

Assessing the effect of intestinal metabolism on $P_{eff}$ as a membrane transport rate parameter is a methodological issue [7, 26, 34, 35, 49]. An evaluation of its influence has to include a study to establish which enzyme(s) is (are) involved and the site of metabolism in relation to the site of the measurements. Intracellular metabolism in the enterocyte, for example, by CYP3A4 and di- and tripeptidases, does not occur in the vicinity of the outer leaflet of the apical membrane and is therefore not considered to affect $P_{eff}$ determined by the disappearance approach (single-pass perfusion) [7, 15, 26, 34, 35, 38, 49]. However, drug metabolism in the lumen and/or at the brush border will directly interfere with the determination of $P_{eff}$ because in this case the drug is metabolized before it is absorbed [57, 58]. It has also been suggested that intracellular metabolism may indirectly affect $P_{eff}$ by providing a further sink boundary condition across the apical membrane. However, we have shown that specific inhibition of enterocyte CYP3A4 by ketoconazole does not change the $P_{eff}$ of *R*/*S*-verapamil, which suggests that sink condition *in vivo* is provided by the highly perfused mesenteric blood vessels (Figure 9.9) [34, 35].


Rat perfusion
Rat $P_{eff}$ ($\cdot 10^{-4}$)
Human $P_{eff}$ ($\cdot 10^{-4}$)
3. Furosemide
7. Atenolol
11. Terbutaline
16. Metoprolol
23. Antipyrine
27. Naproxen
28. Ketoprofen
Caco-2
$P_{eff}$ ($\cdot 10^{-6}$)
Human $P_{eff}$ ($\cdot 10^{-4}$)
7. Atenolol
11. Terbutaline
16. Metoprolol
18. Propranolol (high)*
23. Antipyrine
Ussing chamber
Ussing $P_{eff}$ ($\cdot 10^{-4}$)
Human $P_{eff}$ ($\cdot 10^{-4}$)
5. Enalprilate
7. Atenolol
11. Terbutaline
16. Metoprolol
18. Propranolol (high)*
23. Antipyrine
30. Creatinine

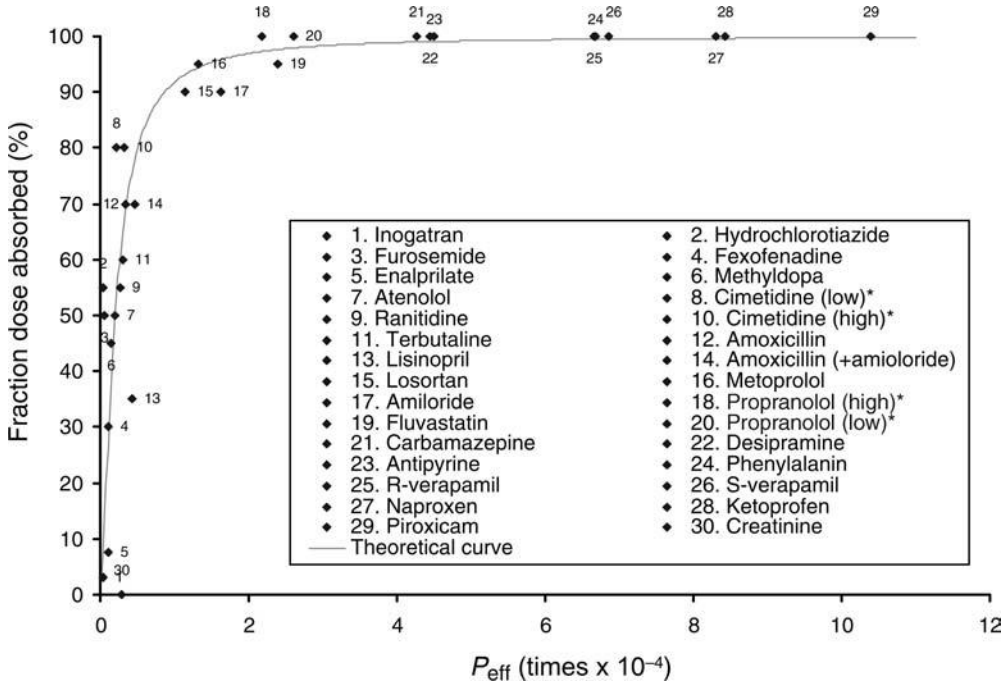



**Figure 9.7** *In vivo* $P_{eff}$ values in humans can be determined using Loc-I-Gut. These values correlate closely to $F_a$ of oral doses for a large number of drugs from different pharmacological classes and which are thus structurally diverse.

## 9.4
## Paracellular Passive Diffusion

Enterocytes are connected by negatively charged tight junctions, and the intracellular space formed is considered to be the paracellular route [59, 60]. The available surface area for paracellular intestinal absorption has been estimated to be about 0.01% of the total surface area of the small intestine [59, 60]. The quantitative importance of the paracellular route for macroscopic intestinal absorption of hydrophilic compounds

**Figure 9.6** Human *in vivo* permeability is one of the cornerstones of the BCS. Correlation of these measurements with fraction dose absorbed and permeability values from other permeability models make it feasible to classify drugs according to BCS and to define bioequivalence regulation for pharmaceutical product approval. These human *in vivo* $P_{eff}$ values were determined using a regional double-balloon perfusion approach (Loc-I-Gut) (Figure 9.4). The use of $P_{eff}$ as the absorption parameter has several important advantages. First, it is possible to measure $P_{eff}$ regardless of transport mechanism (s) across the intestinal mucosa, and second, it predicts $F_a$ and can be used to assess *in vitro–in vivo* correlations that validate the use of different intestinal absorption models [21, 22, 28, 29] commonly applied in discovery and preclinical development.

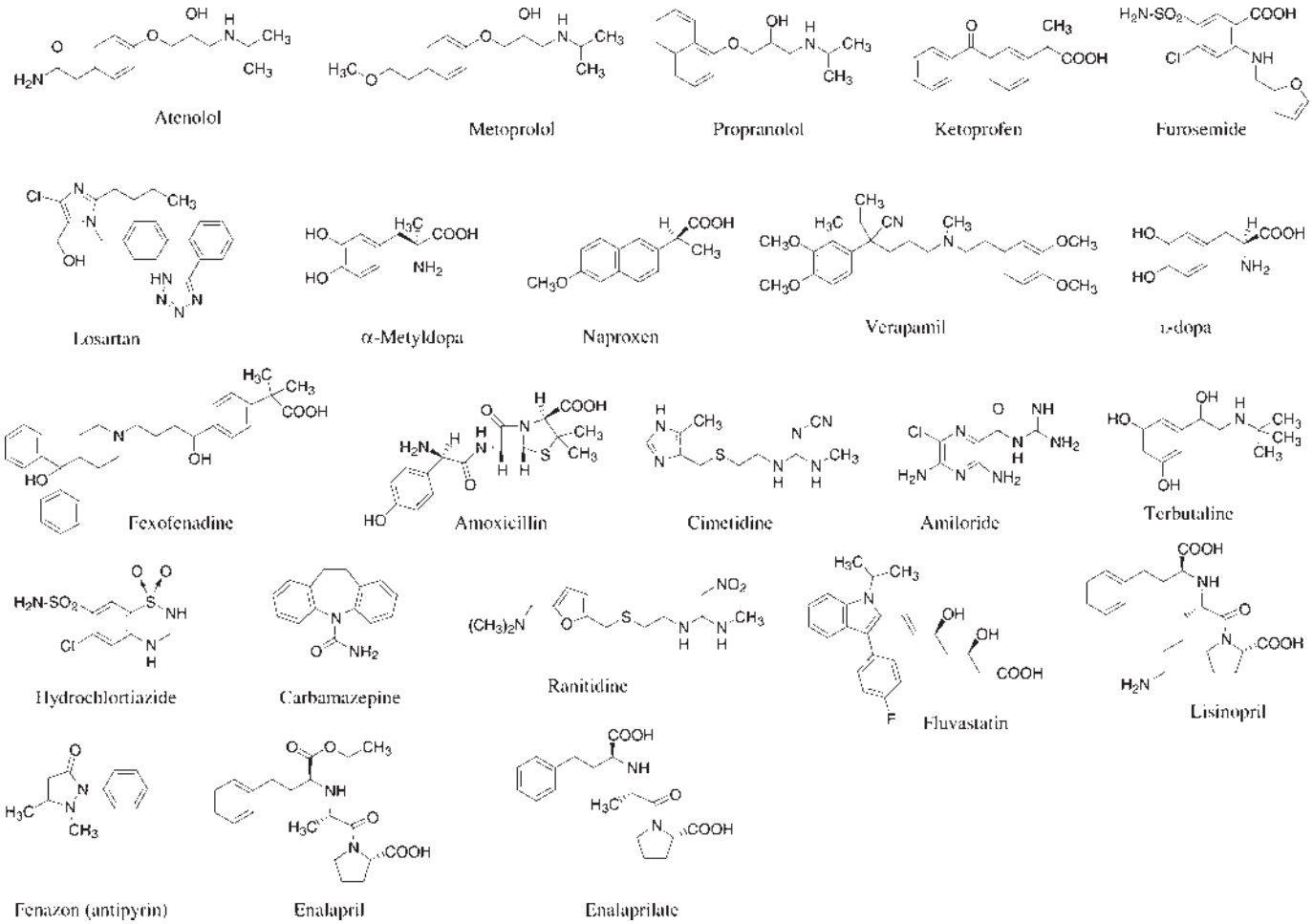



**Figure 9.8** Chemical structures of drugs for which human *in vivo* $P_{eff}$ values have been determined.

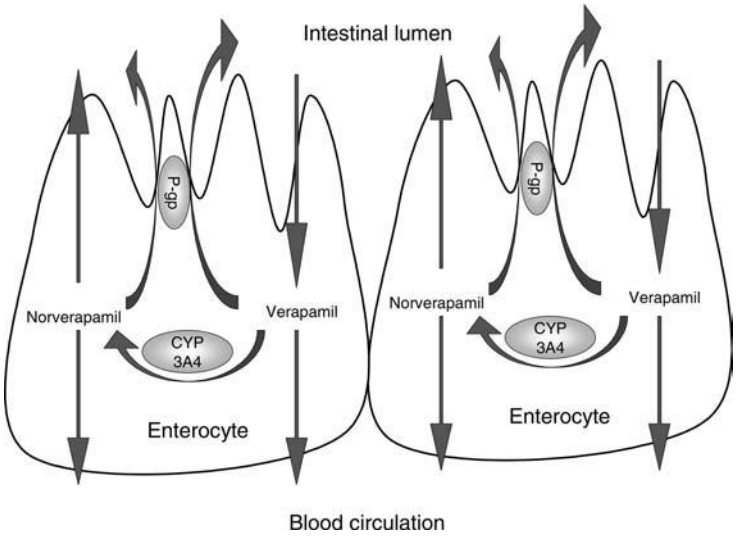



**Figure 9.9** Schematic illustration of P-gp transport and CYP3A4 metabolism of *R*/*S*-verapamil in the human jejunum. It is assumed that the drug has to be absorbed before it can be metabolized by CYP3A4 inside the human enterocyte. Both parent drug and its formed metabolite, *R*/*S*-norverapamil, may be transported into the blood circulation as well as back into the intestinal lumen.

*in vivo* is not yet fully clear. Several *in vitro* investigations have demonstrated that the paracellular route is important for intestinal absorption of various hydrophilic compounds [48, 61–63]. However, *in vivo* studies have suggested that this route contributes only slightly to overall intestinal absorption of drugs [20, 23, 25, 27, 64, 65]. Other *in vitro* studies have suggested that the tight junctions between enterocytes are regulated by nutrients to induce solvent drag and thereby increase intestinal absorption [62, 66–68].

In our assessment of the paracellular hypothesis, we have assumed that water transport during solvent drag is largely paracellular. On the basis of this assumption, we have suggested that compounds with a molecular weight (MW) of over approximately 200 Da (radius > 4.0 Å) are too large to traverse the intercellular space between enterocytes and are therefore not sensitive to solvent drag in terms of quantitative absorption [20, 23, 25, 27]. This hypothesis is supported by our observation that in humans, small hydrophilic compounds such as urea (MW 60 Da, molecular radius 2.6 Å) and creatinine (MW 113 Da, molecular radius 7.2–8.0 Å) are affected by solvent drag, whereas other hydrophilic compounds with an MW more than 180 Da, such as D-glucose (MW 180 Da), antipyrine (MW 188 Da), L-dopa (MW 197 Da), terbutaline (MW 225 Da), atenolol (MW 266 Da), and enalaprilate (MW 348 Da) remain unaffected [20, 23, 25, 27]. Further evidence to support the hypothesis that fairly hydrophilic drugs undergo passive transcellular transport includes the observations that atenolol (logarithm of the distribution coefficient in octanol/water at pH 6.5 ($\log D_{6.5}$) < −2, molecular polar surface area (PSA) 88 $Å^2$, number of hydrogen-bond

donors (HBD) 4) inhibits the efflux mediated by P-glycoprotein (P-gp) and that terbutaline (log $D_{6.5} < -1.3$, PSA 76 Å$^2$, HBD 4) is extensively metabolized in the gut wall during first-pass extraction following oral administration. Both these compounds have been suggested to be largely absorbed by the paracellular route due to their hydrophilic properties [61]. However, atenolol has been reported to decrease the basal apical transport of celiprolol, a P-gp substrate that does not undergo CYP3A4 metabolism, in Caco-2 cells [69]. This transport inhibition arises due to competition for the binding sites of P-gp, which are suggested to be located at the transmembrane region of P-gp. Terbutaline undergoes extensive sulfate conjugation after oral administration, which appears to predominately occur in the gut wall [70, 71]. This particular conjugation enzyme is located in the cytosolic fraction of the enterocyte, indicating that terbutaline is transported via the transcellular route despite its hydrophilic properties and low $P_{eff}$. Our human *in vivo* perfusion data together with the evidence accumulated from studies on atenolol and terbutaline support the hypothesis that small and fairly hydrophilic drugs are mainly absorbed via the transcellular route if passive diffusion is the predominant intestinal absorption mechanism. In addition, Soergel suggests that the intestinal mucosa is nearly impermeable to paracellular transport of hexoses, while Amelsberg *et al*. hypothesize that paracellular absorption in mammals is unlikely to make a major contribution to small intestinal absorption of bile acids (i.e., of MW 500–600 Da) [60, 72].

## 9.5 Transcellular Passive Diffusion

Previously, the unstirred water layer (UWL) adjacent to the intestinal lining was considered to be the rate-limiting step for intestinal $P_{eff}$ of high-permeability compounds [27, 73]. However, several *in vivo* studies clearly report that the thickness of this UWL is significantly less than what was previously assumed, since there is an instantaneous mixing of intestinal fluids [43, 74]. For example, in 1995, Fagerholm and Lennernäs observed no significant changes in estimated $P_{eff}$ of two high-permeability compounds, D-glucose ($10 \times 10^{-4}$ cm/s) and antipyrine ($4 \times 10^{-4}$ cm/s), or UWL thickness over a fourfold range of perfusion rates (1.5–6.0 ml/min) when using the Loc-I-Gut technique in humans [73]. It is thus currently accepted that the epithelial membrane controls the transport rate for both low- and high-permeability compounds regardless of the transport mechanism *in vivo* [43, 73, 74].

The main intestinal absorption mechanism for drugs *in vivo* is considered to be passive transcellular membrane diffusion with the rate-limiting step imposed by the apical membrane [3, 27, 36, 61, 75]. As most drugs are fairly lipophilic in nature, this mode of absorption is most frequent. For instance, in a pharmacokinetic database of 472 drugs, 235 (50%) had a log *P* value of more than 2 and 379 (80%) had a log *P* value of more than 0 [76]. Even if a drug is a substrate for an intestinal transport protein, passive diffusion will probably be the main absorption mechanism if the drug has suitable lipophilic physicochemical properties. However, intestinal carrier-mediated membrane transport will dominate for hydrophilic drugs and polar metabolites if

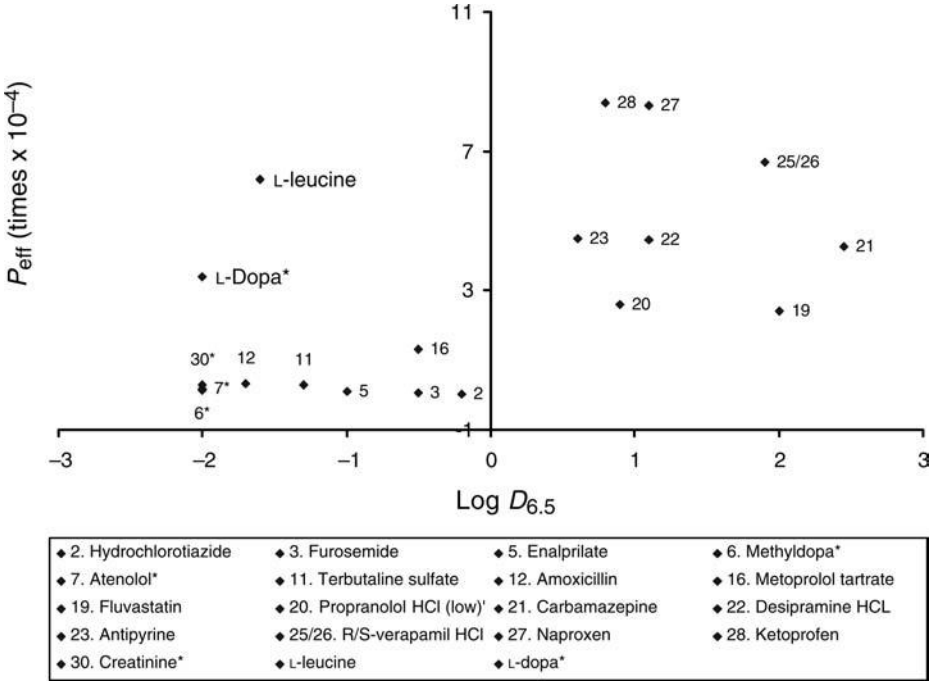



**Figure 9.10** Correlation between *in vivo* $P_{eff}$ determined using the Loc-I-Gut technique in humans and the octanol/buffer pH 6.5 partitioning coefficients for a large number of drugs. Drugs with octanol/buffer pH 6.5 partitioning coefficients higher than zero are highly permeable and well absorbed in humans ($F_a > 90\%$).

they are substrates for any transporter, since passive membrane diffusion is then expected to be slow for such compounds [31, 36].

In a detailed multivariate data analysis report on the relationship between compound structure and permeability, we have shown that the *in vivo* jejunal human $P_{eff}$ for 22 compounds with diverse structures, as determined by the Loc-I-Gut technique, correlated well with both experimentally determined lipophilicity values using a pH-metric technique and calculated molecular descriptors [36] (Figures 9.8, 9.10 and 9.11). Seven of the compounds were omitted from the final analysis as their transport was either carrier mediated (amoxicillin, D-glucose, L-leucine, L-dopa, and α-methyldopa) or mediated via the paracellular route (urea and creatinine). The remaining 15 drugs were included in the multivariate analysis for passive membrane diffusion even if some (verapamil, losartan, furosemide, and fluvastatin) have been considered to be substrates for efflux proteins, such as P-gp and multidrug-resistant protein (MRP), located in the enterocyte membrane [7, 36, 46, 77]. The relationships shown in Figures 9.7, 9.10 and 9.11 strongly suggest that the dominant intestinal absorption mechanism for these drugs is probably passive transcellular diffusion, which is supported by the fact that for these drugs, there is a linear relationship between the fraction dose absorbed and the clinical dose range. The theoretical

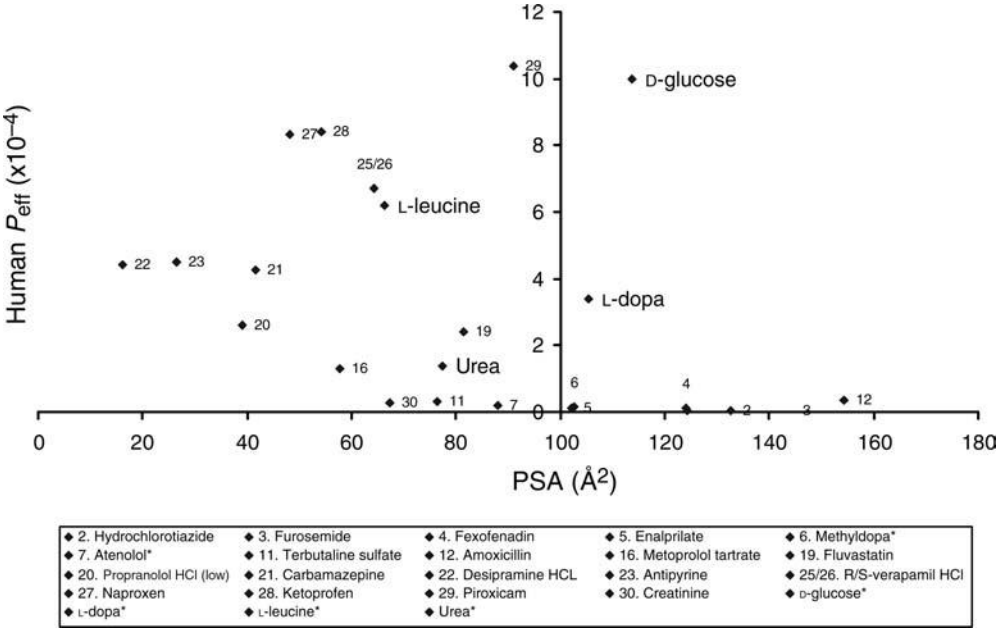



**Figure 9.11** Correlation between *in vivo* $P_{eff}$ determined using the Loc-I-Gut technique in humans and the PSA for a large number of drugs. Drugs with PSA less than 100 Å$^2$ are highly permeable and well absorbed in humans ($F_a > 90\%$).

models based on these *in vivo* permeability data from healthy volunteers can be used to predict passive intestinal membrane diffusion in humans for compounds that fit into the defined property space [36]. We used one of the models obtained from this multivariate analysis to predict the log $P_{eff}$ values for an external validation set consisting of 34 compounds. A good correlation was found with the absorption data of these compounds, which, together with the observation that *in vivo* intestinal absorption of many drugs is dominated by passive diffusion [36], further validates our *in vivo* permeability data set.

In accordance with its polar nature, furosemide (log $D_{6.5}$ −0.5, PSA 124 Å$^2$, HBD 4) has a low jejunal $P_{eff}$ of $0.05 \pm 0.014$ cm/s (as measured using the Loc-I-Gut technique) and is classified as a low-permeability compound according to the BCS [1, 16]. Interestingly, after oral administration, absorption and bioavailability (35–75%) of furosemide are highly variable [78, 79]. Several hypotheses have been advanced to account for this high variability, such as active intestinal secretion, low passive diffusion, and highly pH-dependent dissolution and permeability [80]. The data shown in Figures 9.10 and 9.11 suggest that drugs with octanol/buffer partitioning coefficients higher than zero and a PSA less than 100 Å$^2$ will be highly permeable ($P_{eff} > \approx 1.0 \times 10^{-4}$ cm/s and $F_a > 90\%$) across the human jejunum. The data further imply a strong influence of pH on physicochemical properties that will most certainly alter passive $P_{eff}$ *in vivo*. At pH 7.4, 6.5, and 5.5, the experimentally determined

partitioning coefficients for furosemide were −0.9, −0.5, and 0.4, respectively [36]. In addition, uncharged furosemide has an experimentally determined partitioning coefficient (i.e., log *P* value) of 2.53 ± 0.01. Finally, a recent *in vitro* study shows that active intestinal secretion is important for the transport of furosemide across a Caco-2 monolayer [80]. However, these *in vitro* results must be confirmed *in vivo* before any conclusions regarding the mechanisms underlying the intestinal absorption of furosemide in humans are drawn.

## 9.6 Carrier-Mediated Intestinal Absorption

Most nutrient absorption occurs in the proximal jejunum. Accordingly, a very large number of carrier proteins, channels, and enzymes are expressed in this highly absorptive part of the GI tract. Carrier-mediated intestinal absorption of drugs is the dominant absorption mechanism of any drug with a reasonably high affinity for any intestinal transport protein, whereas passive permeability plays a relatively small role due to the polar nature of the compound [3, 27, 36, 61, 75]. The intestinal epithelium is polarized, and many transport proteins are located and maintained in the apical membrane due to the tight junctions, which prevent the diffusion of proteins within the membrane. Tight junctions also prevent backflow of nutrients from the basal side of the enterocytes into the gut lumen.

The oral bioavailability of poorly absorbed drugs has been increased by targeting them at nutrient transport systems [7]. Two nutrient absorption mechanisms, the oligopeptide carrier and the amino acid transport family, are among the most important that may be utilized for drug transport in the absorptive direction. These proteins are expressed to any significant degree only in the small intestine and therefore drugs that are mainly absorbed via these carriers will not be absorbed in the colon. By using the Loc-I-Gut technique in the proximal jejunum in humans, we investigated the transport of six drugs (amoxicillin, cephalexin, enalapril, lisinopril, α-methyldopa, and L-dopa) that are substrates for either of these proteins [3, 16, 31, 81, 82].

The oligopeptide carrier, hPEPT1, is a symport carrier, which transports a substrate with a proton across the apical enterocyte membrane. The oligopeptide carrier gains access to protons on the substrate via a sodium carrier, the $Na^+/H^+$ exchanger, located in the brush border membrane of enterocytes [83]. In humans, the intestinal absorption of amoxicillin decreases from an average value of 72 ± 9 to 45 ± 11% as a consequence of an increase in the oral dose from 500 to 3000 mg [84]. These data confirm that hPEPT1 has a high transport capacity, as amoxicillin is well absorbed despite administration of such large doses. The *in vivo* jejunal $P_{eff}$ for amoxicillin has been reported to be $0.4 \times 10^{-4}$ cm/s at a concentration of 300 mg/l (0.82 mm), which corresponds to an oral dose of 1200 mg [31]. This jejunal $P_{eff}$ value predicts an $F_a$ value of less than 90%, which classifies amoxicillin as a low-permeability drug according to the BCS. A study by Winiwarter *et al.* in 1999 showed that $P_{eff}$ measured *in vivo* was higher than that predicted for amoxicillin from its physicochemical properties (log $D_{6.5}$ −1.7, logarithm of the calculated octanol/water partition coefficient

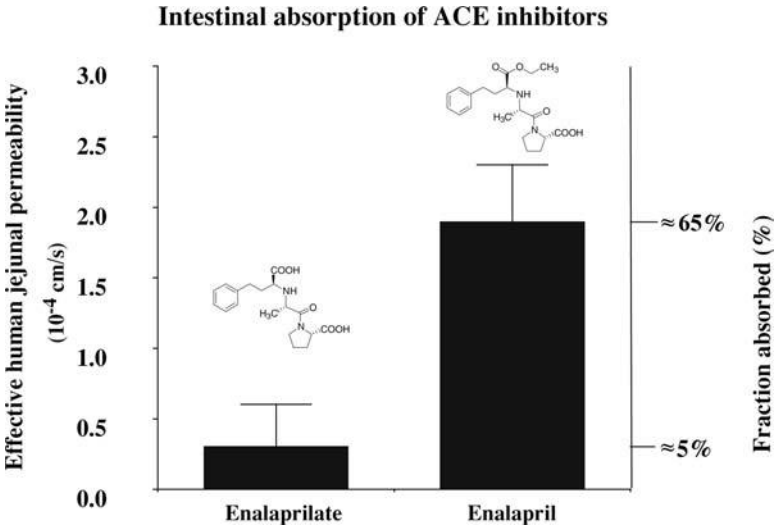



**Figure 9.12** The effective permeability ($P_{eff}$, mean ± SD) of enalapril and enalaprilate in human jejunum *in vivo*. The $P_{eff}$ values predict the fraction dose absorbed for both drugs obtained in pharmacokinetic studies in humans. The higher jejunal $P_{eff}$ of enalapril is most likely due to significantly higher transport through the peptide carrier.

(for neutral species) (Clog *P*) = 0.33, PSA 154 Å$^2$, MW 365 Da) [36]. This observation supports previous pharmacokinetic reports and suggests that the intestinal absorption of amoxicillin is higher (about 50–75%) than that expected for a compound of its low lipophilic and amphoteric nature [84, 85]. The large variability in *in vivo* $P_{eff}$ values for amoxicillin between individuals may be due to polymorphism in the expression of hPEPT1. In addition, nutritional status may contribute to the variability, since it has been reported that transcription of the PEPT1 gene may be activated by dietary amino acids and dipeptides [86]. It has also been reported that the integrated response to a certain stimulus may increase PEPT1 activity by translocation from a preformed cytoplasmic pool [87]. We have reported that both diacid and active forms of enalaprilate have low $P_{eff}$ and, consequently, low $F_a$ in humans (Figure 9.12). The prodrug approach of esterifying enalaprilate to enalapril increased *in vivo* $P_{eff}$, as well as $F_a$, after oral administration (Figure 9.12). This is most likely due to the higher transport activity of hPEPT1 with the esterified prodrug enalapril than with the diacid form, enalaprilate [88]. In the human jejunum, we determined *in vivo* $P_{eff}$ for amoxicillin, cephalexin, enalapril, and lisinopril. These compounds have physicochemical properties that predict low passive diffusion across the human intestine. It has also been shown that in Caco-2 cells *in vitro* permeability is low, which is in accordance with the poor expression of hPEPT1 in that absorption model. Passive diffusion is low due to the polar nature of the compounds [89]. However, $P_{eff}$ *in vivo* has been reported to be significantly higher. This is in accordance with a higher

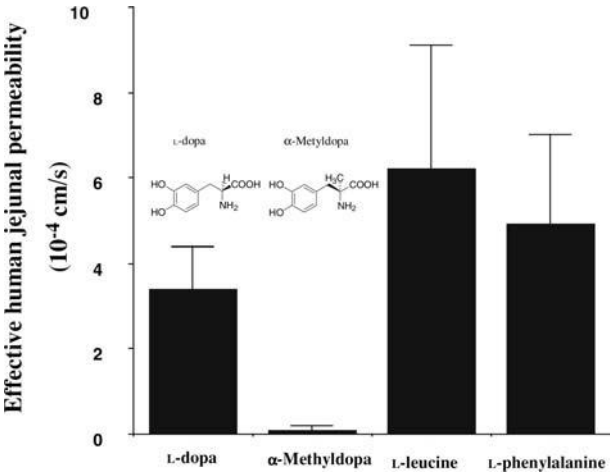



**Figure 9.13** The effective permeabilities ($P_{eff}$, mean ± SD) of L-dopa, α-methyldopa, L-leucine, and L-phenylalanine in human jejunum *in vivo*. The $P_{eff}$ values are determined using a single-pass perfusion technique in human jejunum *in vivo* at the following concentrations: 2.5, 6.7, 40, and 0.06 mm for L-dopa, α-methyldopa, L-leucine, and L-phenylalanine, respectively.

expression of hPEPT1, which is the main mechanism for intestinal absorption of these drugs.

$P_{eff}$ for drugs transported by the amino acid transporter for large neutral amino acid (LNAA) was determined *in vivo* in healthy volunteers for L-dopa and α-methyldopa. α-Methyldopa was classified as a low-$P_{eff}$ drug ($0.1 \pm 0.1 \times 10^{-4}$ cm/s at a perfusate concentration of 6.0–6.5 mm) (Figure 9.13) [16, 36, 81]. The corresponding *in vivo* $P_{eff}$ for L-dopa was about 30 times higher ($3.4 \pm 1.0 \times 10^{-4}$ cm/s at a luminal concentration of 2.0–2.5 mm) (Figure 9.13) [3, 16, 36]. The difference in *in vivo* $P_{eff}$ between α-methyldopa and L-dopa is probably due to a lower affinity of the LNNA transporter for α-methyldopa in addition to lower transport capacity of the LNNA transporter for α-methyldopa. The low *in vivo* $P_{eff}$ of α-methyldopa indicates that passive diffusion for this compound is also low, which is in accordance with its physicochemical properties (MW 211 Da, log $D_{6.5} < -2$, PSA 103 Å$^2$, HBD 5) [36]. Figure 9.13 illustrates that a small change in the chemical structure of a substrate for the LNAA transporter significantly alters its *in vivo* permeability. This is due to the narrow substrate specificity of this carrier protein. Two nutrient substrates for this carrier family, L-leucine and L-phenylalanine, have high *in vivo* $P_{eff}$ in humans even if in our study, they were determined at very different perfusate concentrations (Figure 9.13). This observation confirms that the amino transport family also exhibits high *in vivo* transport capacity in the human jejunum.

The rate and extent of intestinal absorption of cimetidine have been widely discussed, and $F_a$ for this drug has been estimated at around 75% [90, 91]. It has been reported that cimetidine is a substrate for both P-gp and/or organic cation transporters (OCNT1 and OCNT2) [82, 92]. We determined the human jejunal *in vivo*

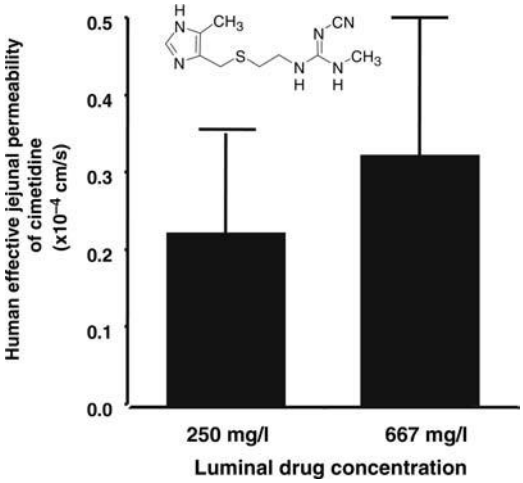



**Figure 9.14** The effective permeabilities ($P_{eff}$, mean ± SD) of cimetidine in human jejunum at two clinically relevant luminal concentrations. The rate and extent of intestinal absorption of cimetidine have been widely discussed, and $F_a$ for this drug has been estimated at around 75% [90, 91]. It has been reported that cimetidine is a substrate for both P-gp and/or organic cation transporters (OCNT1 and OCNT2) [82, 92]. We determined the human jejunal *in vivo* $P_{eff}$ at two different clinically relevant concentrations to investigate saturation in any carrier-mediated transport across the intestinal epithelium. No difference in $P_{eff}$ values between the two concentrations was noted, and, together with the observation that human permeability *in vivo* is similar to permeability in the Caco-2 model (with low expression of carrier proteins), this suggests that passive diffusion is the dominant mechanism even for cimetidine [82].

$P_{eff}$ at two different clinically relevant concentrations to investigate saturation in any carrier-mediated transport across the intestinal epithelium (Figure 9.14). No difference in $P_{eff}$ between the two concentrations was noted, and, together with the observation that human permeability *in vivo* is similar to permeability in the Caco-2 model (with low expression of carrier proteins), this suggests that passive diffusion is the dominant mechanism even for cimetidine [82]. If, on the other hand, organic cation transporters dominated, human intestinal *in vivo* permeability would be expected to be significantly higher than the Caco-2 permeability as the expression of both OCNT1 and OCNT2 is higher in the human small intestine than in the Caco-2 model [82].

## 9.7 Jejunal Transport and Metabolism

Cytochrome P450 (EC 1.14.14.1) enzymes are well known for their ability to metabolize the majority of drugs, detoxify environmental pollutants, and activate some classes of carcinogens [93]. The most highly expressed subfamily is CYP3A, which includes the isoforms CYP3A4, CYP3A5, CYP3A7, and CYP3A43 [93, 94]. The most abundant isoform is CYP3A4, which accounts for 30% of the total P450 content

in the liver and about 70% of the total P450 content in the intestine [93–95]. The CYP3A4 isoform is one of the most important enzymes for oxidative drug metabolism. About 50–60% of clinically used drugs are metabolized by this particular isoenzyme [93, 96]. Despite the higher enzymatic capacity in the liver than in the small intestine, it has been shown that the human intestine contributes significantly to the first-pass extraction of drugs metabolized by CYP3A4 [4, 97–99]. The apical recycling hypothesis has been proposed to account for this high level of gut wall extraction, despite a relatively low intestinal CYP3A4 activity compared to that in the liver. This hypothesis suggests that P-gp and CYP3A4 act synergistically to prolong the intracellular residence time and thereby repeatedly expose the drug to the CYP3A4 enzyme [49, 77, 100, 101]. In addition, the process may support the active transport of formed metabolites toward the intestinal lumen, which may prevent product inhibition of the enzyme [102–104]. Further evidence to support the metabolism–efflux interplay is provided by the close vicinity of P-gp and CYP3A4 in the enterocyte [100, 105] and by the overlapping substrate specificity [106]. However, the validity of this elegant hypothesis *in vivo* is uncertain as CYP3A4 substrates, such as midazolam and felodipine, undergo extensive gut wall metabolism even though they are not subjected to any intestinal efflux [46, 107].

The intestinal epithelium has a carrier-mediated efflux system for limiting the uptake of xenobiotics, which is in turn mediated by ATP-binding cassette (ABC) transport proteins [3, 7, 108–110]. These proteins are also expressed in numerous cell types in tissues such as the liver, kidney, testes, placenta, and blood–brain barrier and may play a role in the pharmacokinetics of drugs [108]. The multidrug resistance transporter gene MDR1 (HUGO nomenclature: ATP-binding cassette transporter gene ABCB1) encodes P-gp and is the most extensively studied, but other multidrug transporters such as multidrug-resistant protein family (MRP1–6) and breast cancer-resistant protein (BCRP) are also under investigation [46, 111, 112]. Under normal circumstances, these proteins restrict the entry and increase the excretion of agents from the cells where they are expressed [108]. Despite extensive research on the effect of efflux proteins on intestinal drug absorption, relatively few examples in humans have been reported [6, 107, 113, 114]. One notable example of their clinical significance, however, was reported in 1999 by Greiner *et al.*, who showed that the plasma concentration time profile (i.e., bioavailability) of oral digoxin was significantly lower during rifampin treatment, a finding that was attributed to increased expression of intestinal P-gp [8]. It has also been shown that a polymorphism in exon 26 (C34 35T) can result in decreased intestinal expression of P-gp, along with increased oral bioavailability of digoxin [115, 116]. Similarly, atorvastatin (80 mg once a day) has been shown to affect the steady-state pharmacokinetics of digoxin in humans [117]. $C_{max}$ and plasma AUC have been reported to increase by 20 and 15%, respectively. Renal clearance was unaffected, which suggests that this drug–drug interaction is due to increased intestinal absorption and/or decreased biliary secretion of digoxin, mediated through P-gp inhibition [117]. This was also confirmed *in vitro* using Caco-2 cells, where atorvastatin decreased digoxin secretion by 58%, equivalent to the extent of inhibition observed with verapamil, a well-known P-gp inhibitor [117, 118]. Recently, several clinical studies have also claimed that inhibition of intestinal efflux

(especially of P-gp) was the major cause of increased bioavailability when certain drugs were coadministered [100, 119]. In many of these studies, however, it is likely that inhibition of CYP3A4 accounts for most of the increased bioavailability, whereas the role of cellular efflux *in vivo* at the intestinal level remains unclear. The reason for this overinterpretation of the role of enterocyte efflux activity on intestinal drug absorption may be due to the overlapping specificities of both substrates and inhibitors for both CYP3A4 and P-gp [106]. Additional factors may include saturation of the efflux carrier due to high drug concentration in the intestinal lumen and/or a fairly high passive permeability component [34, 35].

Direct *in vivo* assessment of the quantitative importance of gut wall metabolism and transport of drugs and metabolites in humans is difficult and has consequently not been attempted often [3, 6, 11, 12, 15, 16, 23, 25–32, 34, 35, 81]. The most direct *in vivo* approach to investigating these processes in drugs with variable and incomplete bioavailability has been shown to be single-pass intestinal perfusion or an instillation approach (Figure 9.2) [3, 6, 11, 12, 15, 16, 25–32, 34, 35, 81]. In general, traditional pharmacokinetic studies are limited in their capacity to distinguish intestinal extraction from hepatic extraction, as discussed by Lin *et al*. [120]. However, measured values of metabolic extraction of *R*/*S*-verapamil in the human gut (≈50%) and liver (≈50%), using the steady-state single-pass perfusion approach and the instillation technique, have been reported to be similar [15, 34, 35] and are also in agreement with findings from traditional pharmacokinetic studies [2, 121].

A single-pass perfusion approach using the Loc-I-Gut technique was applied for a direct *in vivo* assessment in the human jejunum. *R*/*S*-Verapamil ($\log D_{6.5}$ 2.7, octanol/$H_2O$, pH 7.4, MW 455 Da) was used as the model compound for CYP3A4- and P-gp-mediated local intestinal kinetics [2, 34, 35, 122] (Figures 9.7 and 9.9). $P_{eff}$ values for both enantiomers at each concentration used (4.0, 40, 120, and 400 mg/l) were $2.5 \times 10^{-4}$, $4.7 \times 10^{-4}$, $5.5 \times 10^{-4}$, and $6.7 \times 10^{-4}$ cm/s, respectively (Figure 9.15) [34, 35]. The luminal concentration in the upper part of the small intestine after oral administration of a 100 mg dose of verapamil in an immediate-release dosage form is expected to reach 400 mg/l [1, 34, 35]. The three other perfusate concentrations represent fractions of the dose when 30, 10, and 1%, respectively, remain to be absorbed [34, 35]. The increase in *in vivo* jejunal $P_{eff}$ of *R*/*S*-verapamil, along with its increased luminal perfusate concentration, is in accordance with a saturable efflux mechanism mediated by P-gp (Figure 9.15). Furthermore, there was no difference in $P_{eff}$ between the *R*- and *S*-forms of verapamil at any luminal concentration, which suggests that efflux transport cannot discriminate between the two forms of verapamil. However, the measured *in vivo* jejunal $P_{eff}$ ($>2.0 \times 10^{-4}$ cm/s) was sufficient at all four perfusate concentrations to predict complete intestinal $F_a$ following oral dosing (Figures 9.7 and 9.15) [34, 35]. Together with the fact that $P_{eff}$ and $F_a$ are excellent, as predicted from the physicochemical properties of *R*/*S*-verapamil, this suggests that passive diffusion is the dominating transport mechanism for this drug in the human intestine [34–36].

Ketoconazole, a well-known potent inhibitor of CYP3A4 metabolism and a less potent P-gp modulator, acutely inhibited CYP3A4 metabolism but did not affect the $P_{eff}$ of *R*/*S*-verapamil when they were coperfused through the human jejunal

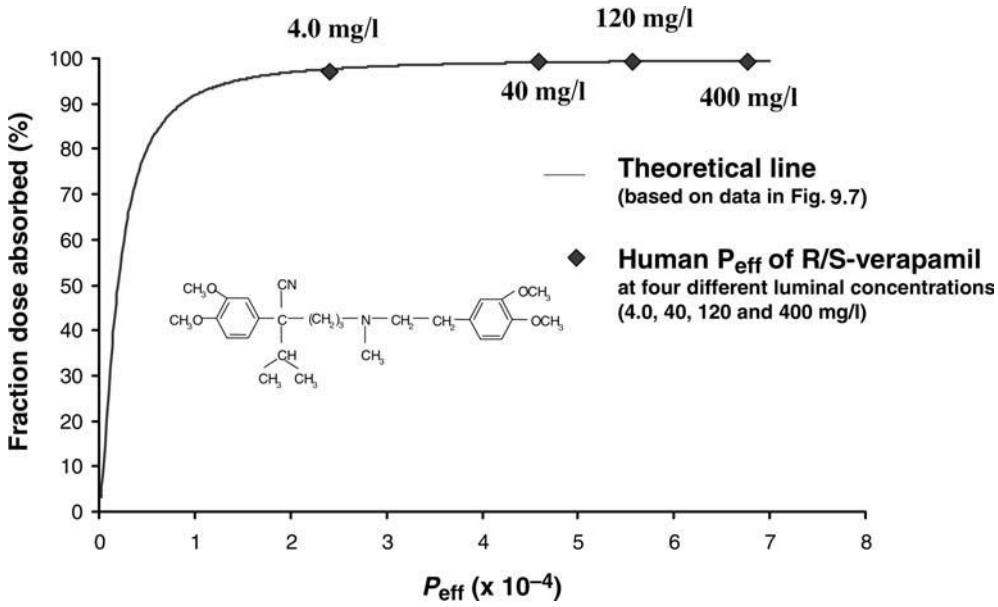



**Figure 9.15** The effective permeability ($P_{eff}$, mean ± SD) of *R*/*S*-verapamil in human jejunum at two clinically relevant luminal concentrations. $P_{eff}$ for both enantiomers at each of the concentrations (4.0, 40, 120, and 400 mg/l) was $2.5 \times 10^{-4}$, $4.7 \times 10^{-4}$, $5.5 \times 10^{-4}$, and $6.7 \times 10^{-4}$ cm/s, respectively (Figure 9.15) [34, 35]. The luminal concentration in the upper part of the small intestine after oral administration of a 100 mg dose of verapamil in an immediate-release dosage form [1, 34, 35] is expected to reach 400 mg/l. The three other perfusate concentrations represent fractions of the dose when 30, 10, and 1%, respectively, remain to be absorbed. The increased *in vivo* jejunal $P_{eff}$ of *R*/*S*-verapamil along with its increased luminal perfusate concentration is in accordance with a saturable efflux mechanism mediated by P-gp. However, the measured *in vivo* jejunal $P_{eff}$ ($>2.0 \times 10^{-4}$ cm/s) was sufficient at all four perfusate concentrations to predict complete intestinal $F_a$ following oral administration.

segment at 40 mg/l (ketoconazole) and 120 mg/l (*R*/*S*-verapamil)(Figures 9.9 , 9.15, and 9.16) [35]. This confirms that *in vivo*, ketoconazole is a less potent inhibitor of P-gp than CYP3A4 in humans and that even if a significant proportion of verapamil is transported by passive diffusion, increased $P_{eff}$ would be expected for P-gp inhibition [35, 123–125]. It also demonstrates that intracellular metabolism has no effect on apical drug permeability (Figures 9.9 and 9.16). In this regard, it has been proposed that intracellular CYP3A4 metabolism may provide a more pronounced concentration gradient across the apical enterocyte membrane, which theoretically should increase $P_{eff}$ [120, 126, 127]. Since verapamil is transported mainly via passive diffusion and is subjected to extensive CYP3A4 metabolism in the gut, it is considered to be a good model drug to investigate this issue in humans [2, 15, 34–36]. However, inhibition of small intestinal metabolism did not result in decreased jejunal $P_{eff}$. In addition, if intracellular CYP3A4 metabolism had a pronounced effect on jejunal $P_{eff}$, *S*-verapamil would have been expected to have significantly higher

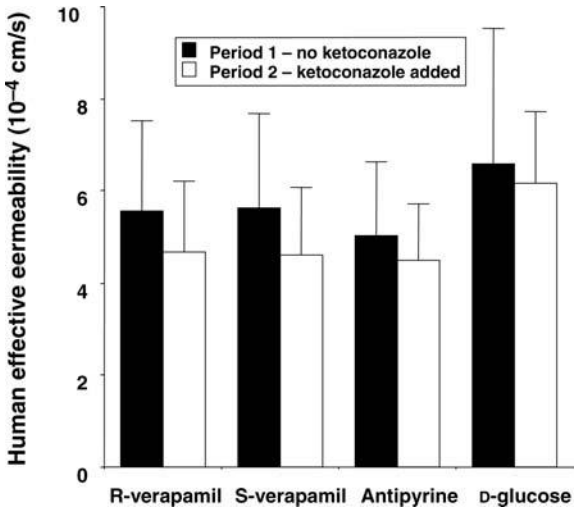



**Figure 9.16** The absence of an acute effect of ketoconazole on $P_{eff}$ (mean ± SD) of *R*/*S*-verapamil, antipyrine, and D-glucose in humans. The data suggest that the extensive intracellular metabolism in the human enterocyte mediated by CYP3A4 on substrates such as *R*/*S*-verapamil has no direct or indirect effect on $P_{eff}$ in humans. In addition, the similarity in $P_{eff}$ values between *R*- and *S*-verapamil further supports the hypothesis that intracellular metabolism has no effect on $P_{eff}$, as stereoselective CYP3A4 metabolism (*S*-verapamil is more readily metabolized) is not reflected in *in vivo* $P_{eff}$.

$P_{eff}$, as it is more extensively metabolized than *R*-verapamil in the gut wall. However, we have shown with the Loc-I-Gut technique that the human jejunal $P_{eff}$ for *S*- and *R*-verapamil are identical, though *S*-verapamil was subjected to a significantly higher degree of intestinal first-pass metabolism [35]. Altogether, this suggests that extensive CYP3A4 metabolism in enterocytes in humans does not affect drug permeability across the apical membrane by increasing the concentration gradient across the apical enterocyte membrane (Figures 9.9 and 9.15). A more plausible explanation is that the concentration gradient *in vivo* across the intestinal barrier is provided by extensive mesenteric blood flow. This emphasizes an important methodological aspect of intestinal perfusion techniques in which $P_{eff}$, based on the disappearance rate from a perfused segment, is not affected by extensive intracellular metabolism.

Jejunal permeability and intestinal and hepatic extraction of fluvastatin, a drug completely metabolized by CYP2C9, were investigated in humans by using the Loc-I-Gut technique [32]. It was shown that the contribution of the intestine to the total first-pass effect is negligible for fluvastatin. This observation is consistent with the observation that CYP2C enzymes are expressed at low levels in enterocytes [8, 128]. When tested at clinically relevant concentrations *in vitro* using Caco-2 cells, the efflux ratio of fluvastatin was approximately 5 and was probably mediated by MRP2. The human *in vivo* $P_{eff}$ was high ($2.4 \pm 1.4 \times 10^{-4}$ cm/s), which demonstrates that despite significant *in vitro* intestinal efflux, fluvastatin is completely absorbed across the intestine *in vivo* (Figure 9.7) [3, 16, 32]. This is most likely due to the significant

contribution of passive $P_{eff}$ to the overall absorption of fluvastatin, which is in accordance with its lipophilic properties (acid p$K_a$ 4.3, log $D_{6.5}$ 2.0, PSA 81 Å$^2$, HBD 3) (Figures 9.10 and 9.11) [32, 36]. A similar pattern has been shown for many drugs. For instance, jejunal $P_{eff}$ for cyclosporine, a well-known CYP3A4 and P-gp substrate, has been reported to be high and predicts complete intestinal absorption even if the drug is subjected to significant efflux in *in vitro* cell models [2, 15, 34, 35, 129, 130]. Altogether, these directly determined *in vivo* human $P_{eff}$ values suggest that in humans, drugs with a high passive $P_{eff}$ contribution to the overall absorption rate will be completely absorbed from the gut even if they are substrates (such as verapamil, fluvastatin, losartan, and cyclosporine) for one and/or several efflux transporters. The values also suggest that drugs with a sufficiently high lipophilicity will be absorbed from the gut mainly by passive transcellular diffusion. Finally, *in vivo* data suggest that for many drugs, the effect of intestinal active efflux on $F_a$ is limited even if they are efflux substrates, which is in accordance with the limited number of reports supporting its clinical significance [8, 115, 116]. This may be due to a high contribution of passive diffusion, as well as due to the fact that these efflux proteins located in the intestine may be easily saturated owing to high concentrations adjacent to the intestinal membrane of the orally administered drug.

Model drugs and direct *in vivo* methods are needed to perform accurate investigations of the clinical significance and the pharmacogenetics of transporters and their influence on pharmacokinetics. Such model compounds should not be metabolized, which would make the assessment of the role of transporter possible. For instance, digoxin and fexofenadine have been suggested to be model compounds to assess the phenotype for P-gp significance [8, 115, 116, 131]. We investigated the effect of ketoconazole on the measured $P_{eff}$ for fexofenadine because concomitant oral administration of these drugs led to an increase in $C_{max}$ and AUC, which is consistent with inhibition of P-gp-mediated transport [132, 133]. Fexofenadine has indeed been shown to be a substrate for P-gp in Caco-2 and L-MDR1 cells, and its disposition is altered in knockout mice lacking the gene for Mdr1a [134, 135]. It was therefore expected that ketoconazole would increase jejunal $P_{eff}$ and plasma AUC of fexofenadine when added to the jejunal perfusion, but this did not occur. On the contrary, jejunal $P_{eff}$ remained low (0.1–0.2 $\times$ $10^{-4}$ cm/s) and variable, which, according to the BCS, classifies it among the low-permeability compounds (Figures 9.5 and 9.7) [1]. The reported absence of an effect of ketoconazole on fexofenadine permeability means that further *in vivo* studies are needed to fully understand the interaction between fexofenadine and ketoconazole. It also means that we must increase our understanding of transport mechanisms before we can conclude that fexofenadine is an appropriate *in vivo* probe for P-gp activity in humans. Our understanding of the expression of transporters and their functional activity in different human tissues is at a nascent stage, and there is a need for more *in vivo* pharmacokinetic data to validate *in vitro* methods of studying both quantitative and qualitative aspects of drug transport [107].

Glaeser *et al.* have shown that the majority of shed human enterocytes collected from an intestinal perfusion were still functionally active and did not show signs of apoptosis [38]. On the basis of a validation of the Loc-I-Gut system for the study of

gene expression during perfusion, changes in mRNA levels in shed enterocytes before and after perfusion of a 10 cm long jejunal segment were studied in parallel to $P_{eff}$, metabolism of selected compounds in the gut wall, and the excretion of their metabolites back into the lumen (Figure 9.4) [39]. Sulforaphane and quercetin-3,4′-diglucoside were rapidly effluxed back into the lumen as sulforaphane-glutathione and quercetin-3′-glucuronide conjugates, respectively. Gene expression analysis in exfoliated enterocytes showed a $1.8 \pm 0.5$-fold (range 1.1–2.3) induction of glutathione transferase A1 (GST) mRNA and a $2.1 \pm 1.3$-fold (range 0.7–4.0) induction of UDP-glucuronosyl transferase 1A1 (UGT1A1) mRNA after only 90 min exposure to these two compounds. The technique demonstrates its applicability to the study of intracellular process and their relationships to changes in gene expression (Figure 9.4) [38, 39].

## 9.8 Regional Differences in Transport and Metabolism of Drugs

Regional differences in transport and metabolism of drugs, especially in the colon, have not been investigated thoroughly in humans by using intestinal perfusion techniques. Therefore, there is a need to develop clinical techniques that make it possible to directly investigate the transport and metabolism *in vivo* of drugs in various regions of the GI tract. This would certainly improve our understanding of regional differences in the transport and metabolism of drugs, an understanding that is crucial for the development of orally controlled release systems, which have received increased attention as they create new therapeutic opportunities. In addition, regional absorption and metabolism may also influence the local effect of a drug, which is targeted at a certain region where diseases such as inflammatory bowel disease (IBD) and colon cancer are localized [136].

Investigations of regional differences in permeability and metabolism have been performed by using various animal models [22, 29, 75, 102, 109, 112, 137]. Animal tissues, mainly rat specimens, are widely used in the Ussing chamber to investigate transport of drugs across specific regions of the intestine [29, 75, 138], whereas studies in human tissues are few due to the limited availability of tissue specimens.

Regional differences in functional activity of P-gp have only been reported in a few cases and are mainly based on animal studies [22, 75, 109, 112, 137]. For instance, one of the few systemic kinetic analyses of efflux activity showed that marked differences exist along the different regions of the rat GI tract [112]. Maximal transporter activity varied over a fourfold to fivefold range in the order ileum > jejunum > colon. Earlier studies have claimed that MDR1 mRNA levels should be highest in the human colon [129]. Recent investigations, however, indicate that P-gp is more readily expressed in the small intestine than in the colorectal region [50, 109]. Interestingly, in 2002, Nakamura *et al.* reported that MDR1, MRP1, and CYP3A mRNA levels were higher in human duodenal tissue than in normal colorectal and colorectal adenocarcinoma tissues [50]. However, reported levels of MDR1 mRNA and P-gp-mediated efflux activity in the small intestine and colon are inconsistent, which may be

attributed to differences in species, methodology and study designs in the various reports [107, 109, 112, 129, 137, 144].

It has also been shown *in vitro* by using rat small intestinal and colonic tissues in an Ussing chamber that low-permeability drugs (BCS classes III–IV) have an even lower permeability in the colon, whereas high-permeability drugs (BCS classes I–II) show a slightly higher permeability in the colon when passive diffusion is the dominant mechanism (Figure 9.5) [75]. This $P_{eff}$ pattern has also been shown to be relevant for small and large intestinal specimens from humans using the Ussing chamber model [104]. A regional difference in permeability for five different compounds has been reported from a study that applied an open triple-lumen tube and perfused an 80 cm long segment of human jejunum and ileum (Figure 9.2) [139]. $P_{eff}$ for hydrochlorotiazide, atenolol, furosemide, and cimetidine, all of which are classified as low-permeability drugs according to the BCS, decreased in the ileum in comparison with the jejunum [3, 139]. This *in vivo* observation is in agreement with the regional Ussing chamber studies in rats for low-permeability compounds [75]. Salicylic acid, which is highly permeable, was well absorbed throughout the small intestine. The small intestinal regional permeability pattern has also been demonstrated for ranitidine (low permeability) and paracetamol and griseofulvin (two high-permeability compounds) by using a similar open intestinal perfusion technique [140–142]. *In vivo* permeability measurements of drugs in the colonic/rectal region in humans are difficult, which probably explains the limited amount of published data. However, we developed and validated a new technique for the perfusion of a defined and closed segment in the colon/rectum [143]. We observed that the permeability of antipyrine in the rectal region was high and D-glucose was not absorbed, which is in accordance with the fact that passive diffusion is the dominant drug absorption mechanism in this specific intestinal region. However, we found the present technique valuable for studying drug absorption from the human rectum, which encouraged us to investigate the influence of a penetration enhancer, sodium caprate, on the rectal absorption of phenoxymethyl penicillin and antipyrine [12]. The data suggest that sodium caprate alone has a limited effect on the permeability *in vivo* across the rectal epithelium when it is presented in a solution. Interestingly, there was a correlation between $P_{eff}$ for sodium caprate and the individual plasma AUC and $C_{max}$ of phenoxymethyl penicillin, which indicates that the permeability of the enhancer in the tissue upon which it should act is crucial to achieving an effect.

## 9.9 Conclusions

We have emphasized the need for more *in vivo* studies to deconstruct the dynamic interplay between mechanisms of drug transport and metabolism in the human intestine. There is also a need to further develop *in vivo* techniques to directly measure these processes in various regions along the GI tract in humans and to relate the findings to physiological/pathophysiological conditions.

This will increase our knowledge of the important transport mechanisms and will provide *in vivo* data leading to the development and validation of rapid and more reliable *in vitro* intestinal models.

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