Everything you need to know about ADME

5th Edition

cyprotex

AN EVOTEC COMPANY

Contents

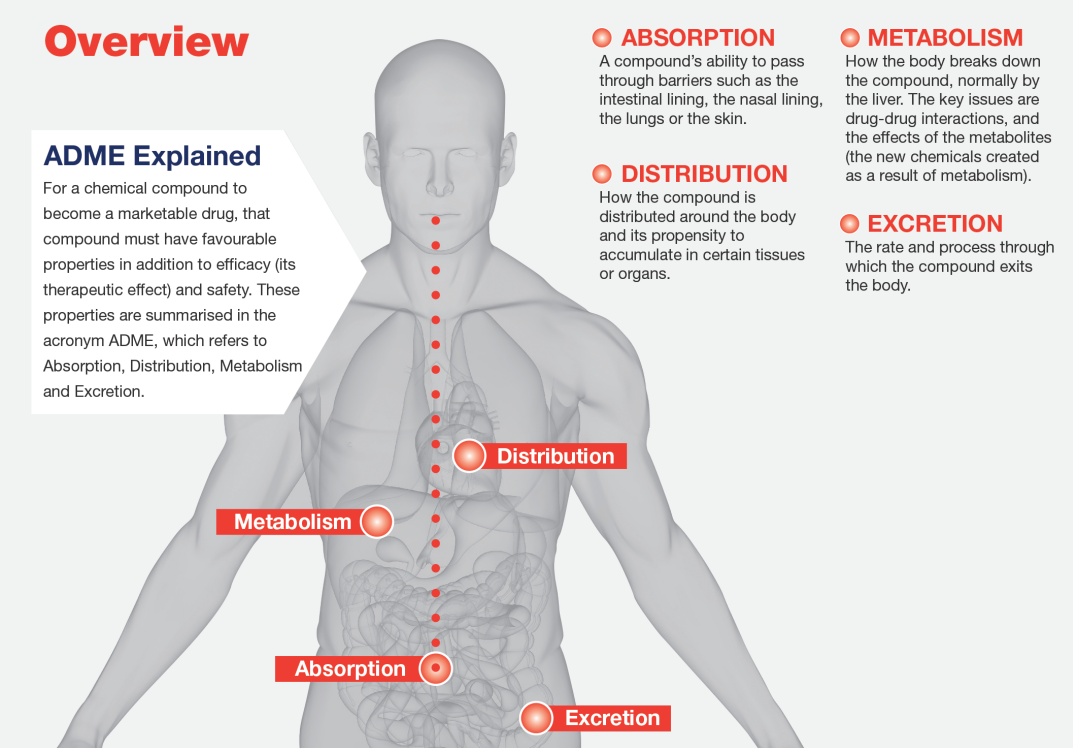
Chapter 1: Introduction

1.1. The Definition of ADME and Pharmacokinetics (PK)

ADME is an acronym for Absorption, Distribution, Metabolism and Excretion (Figure 1.1). These distinct processes determine blood and tissue levels of all molecules including pharmaceutical medicines (drugs) in the body and therefore can influence the efficacy and toxicity profile of these molecules.

Pharmacokinetics (PK) is defined as the study of the concentration of drug in the body over time, and is related to the absorption, distribution, metabolism and excretion of a drug. Pharmacokinetics is often described as 'what the body does to the drug'.

Figure 1.1: Overview of ADME



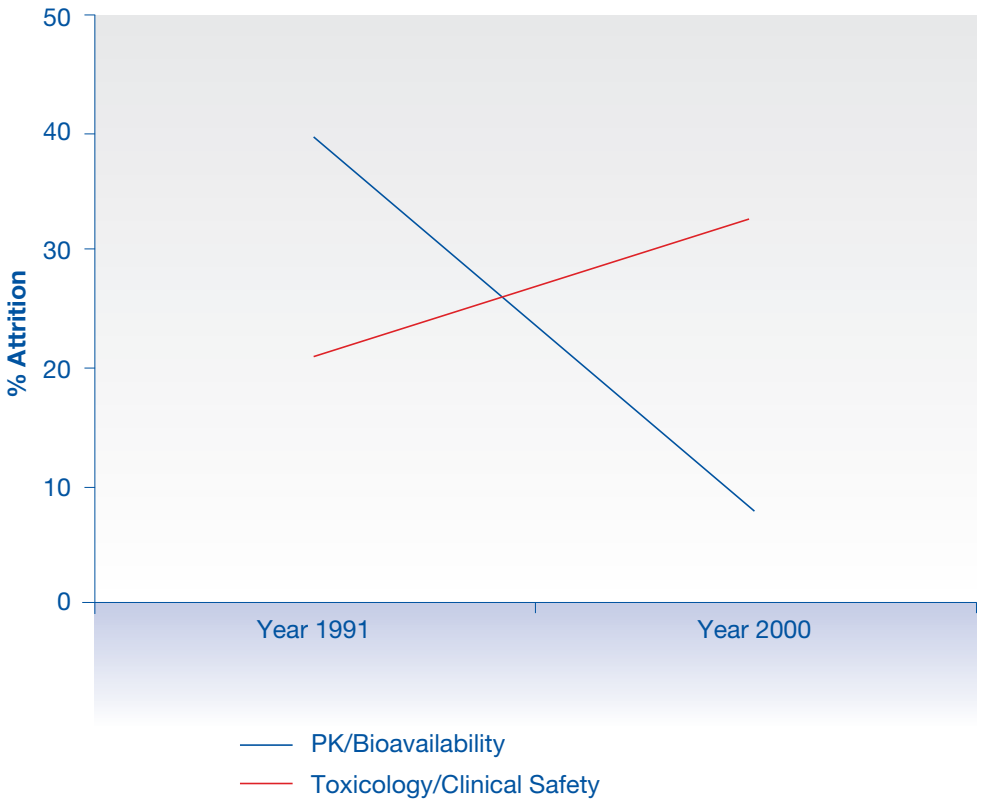
1.2. The Importance of ADME and Pharmacokinetics

Rising R&D (research and development) costs coupled with late clinical stage drug failure rates are a major concern to the pharmaceutical industry. Back in the early 1990's, poor pharmacokinetics accounted for approximately 40% of late stage attrition. The integration of early ADME into the drug discovery process appears to have had a significant impact and helped to reduce attrition rates due to poor pharmacokinetics and bioavailability to less than 10% in the year 2000 (Figure 1.2). The root causes of these problems are now being understood, and solutions are emerging for identifying and eradicating these issues before they surface in the clinic. However, an increase in attrition has been observed for areas such as toxicology and clinical safety which is driving a push for better and more standardised *in vitro* toxicity testing methods.

In the past many of the ADME related failures were a consequence of drug-drug interactions. One of the most well cited examples of this is the drug terfenadine (Seldane®) which when co-administered with CYP3A4 inhibitors caused QT prolongation and potentially fatal torsades de pointes. As a consequence, terfenadine was withdrawn from the US market in 1998. Mibefradil (Posicor®), astemizole (Hismanal®) and cisapride (Propulsid®) were also withdrawn in subsequent years as a consequence of drug-drug interactions. Due to our aging population and the increasing use of polypharmacy, the importance of identifying the potential for drug interactions at an early stage is becoming even greater. Obviously drug interactions are not the only cause of drug failure in terms of ADME/pharmacokinetics, for example, species differences in drug metabolism or other ADME parameters can also cause misleading data in preclinical toxicity studies. Inadequate efficacy can also be a consequence of poor ADME properties.

The implementation of regulatory guidelines by the FDA[1,2],the EMA[3] and the Japanese PMDA[4] have helped to standardise many of the experimental approaches for identifying potential ADME or pharmacokinetic issues.

Figure 1.2: Reasons for Attrition (1991-2000)[5].



1.3. The Link between ‘Drug-Likeness’, Physicochemical Properties and ADME

Modern drug discovery typically requires the efficient generation of high-quality compound libraries of ‘drug-like’ molecules. ‘Drug-like’ properties become increasingly more important as a project progresses from lead discovery to lead optimisation. Such properties may predict a drug candidate’s probability of surviving development.

A compound is defined as ‘drug-like’ when it:

- has the desired pharmacological effect
- has the ability to reach the target site of action
- resides in the body and target organ long enough to have a clinically meaningful effect
- is sufficiently target-selective so that the intended pharmacological action is the only physiological effect, and
- has the right pharmaceutical properties for synthesis, formulation, distribution, handling and dosing in a practical manner.

As well as determining the efficacy and safety of a molecule, these ‘drug-like’ characteristics are highly interlinked with its ADME properties. For instance, an orally administered drug may only reach its target if it is well absorbed, distributed to the target site, and not cleared too rapidly from the body.

The ‘drug-likeness’ of a molecule (and in turn its ADME properties) are dependent on its physicochemical and structural properties. One of the most heavily cited examples of where this has been applied is the ‘Lipinski Rule of Five’ which originated from the enormous amount of data gathered by the industry on properties that maximise an oral drug candidate’s probability of surviving development[6]. The Lipinski Rule of Five was received warmly by the industry because it provided a simple framework for defining the chemistry space for oral bioavailability, and these rules are still commonly applied to the design and selection of compounds for lead discovery.

Paradoxically, despite the introduction of the Lipinski Rules, there has been a trend towards generating high molecular weight and highly lipophilic compounds (commonly termed as ‘molecular obesity’). Although these molecules are typically highly efficacious, they often have an increased likelihood of failure due to other suboptimal properties (often poor ADME and toxicity characteristics). This has led to a realisation that balanced *in vitro* potency, ADME, toxicity and physicochemical properties are important for successful drug development and new approaches are now starting to emerge which lead to better candidate-quality compounds of which many comply with Lipinski Rules. Lipinski’s Rule of Five has inspired numerous refinements and investigations into optimal drug properties which have provided revised frameworks for predicting ‘drug-like’ chemical space. These are discussed further in Chapter 2.

1.4. ADME in Drug Discovery

Prior to the mid 1990s, a serial approach to drug discovery was employed which involved optimising potency prior to the optimisation of ADME and finally engineering out any toxicity liability at the end (Figure 1.3A)[7]. This sequential approach was time consuming and could lead to ADME-Tox issues only being uncovered at a late stage in the drug discovery or development process[8].

After the mid 1990s, a parallel approach to drug discovery was adopted where optimisation of potency, selectivity and ADME-Tox was performed simultaneously (Figure 1.3B)[7]. This approach has led to better quality leads with a balance of safe, active compounds with good ADME properties. For example, a less potent compound might be the best choice if it has a longer half-life than more potent analogues. The impact of this approach is shorter timelines and a higher success rate.

Figure 1.3A: Serial Approach to Drug Discovery (prior to the mid 1990s)[7]

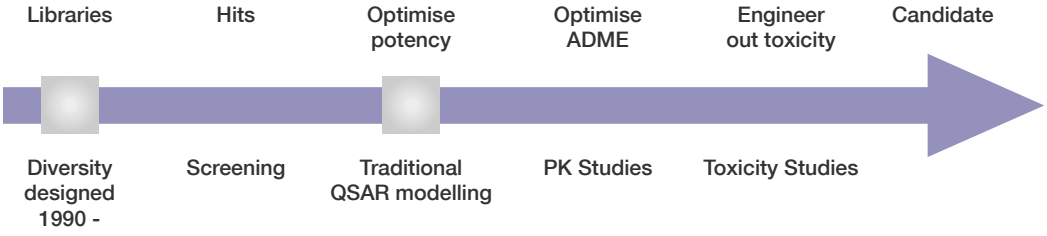
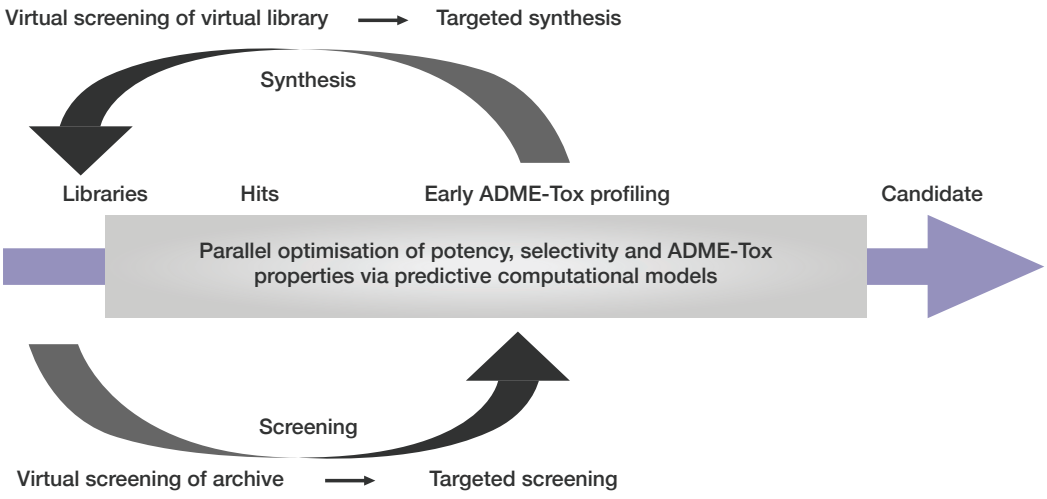


Figure 1.3B: Parallel Approach to Drug Discovery (post the mid 1990s)[7]



Reprinted from *Drug Discov Today* **6(21)** Manly CJ *et al.* The impact of informatics and computational chemistry on synthesis and screening, 1101-1110. Copyright 2001 with permission from Elsevier[7].

A number of early stage screens, many of which are routinely performed in 96 or 384 well format, are now available to characterise ADME properties early in drug discovery. These include, amongst others, Caco-2 permeability, microsomal/hepatocyte stability, cytochrome P450 inhibition, nuclear receptor screening for cytochrome P450 induction and plasma protein binding. The screening strategy which each company employs is dependent on their particular project and is based on the route of administration, therapeutic area, the cost of the individual screens and specific liabilities identified in the project.

1.5. ADME in Drug Development

During the candidate selection stage, the candidate drug and typically one or two follow-up molecules will be characterised in more detail. In terms of ADME, this may involve more in-depth drug metabolism and transporter studies and pharmacokinetic studies in the species selected for assessing preclinical toxicity. These data, along with data from preclinical toxicology studies, are included in regulatory submissions prior to clinical trials as they are helpful in guiding potential safety and efficacy of the molecule as well as defining human clinical dose.

It is important that the potential for drug-drug interactions is fully evaluated prior to Phase II and III clinical trials. This involves characterising (using *in vitro* methods) which enzymes or proteins are involved in the molecule's drug metabolism or drug transport, reversible and time dependent inhibition and induction of key drug metabolising enzymes and inhibition of relevant transporter proteins. Regulatory guidelines are available to guide the design and interpretation of these studies[1,2,3,4]. The data are important in determining if a clinical human drug-drug interaction study is required prior to the drug reaching the market.

1.6. Integrating ADME and Physicochemical Data with Physiological Modelling (Physiologically Based Pharmacokinetic (PBPK) Modelling)

There is a common tendency to oversimplify ADME data interpretation. *In vitro* assay results are often considered independently of one another (even though they are interdependent properties), and artificial 'cut-off' values are assigned to each *in vitro* parameter.

Data on various ADME properties are sometimes tabulated and each property given red or green traffic light colours according to whether the *in vitro* result for each property fitted previously defined acceptance limits. This so-called 'traffic light' system is illogical because the ADME properties act interdependently and interact with physiological processes to drive pharmacokinetics. The danger with this empirical approach is that it will result in otherwise good compounds being eliminated. Moreover, the time and money spent in screening compounds will not have improved the quality of compounds in the pipeline.

Evaluating the data as a whole can be achieved using PBPK (physiologically-based pharmacokinetic) modelling approaches where all the key *in vitro* ADME data are integrated along with physicochemical data. It has been shown that using *in vitro* data combined with a generic PBPK model is more accurate and less biased than allometric scaling from interspecies *in vivo* PK data[9]. The added advantage of using the PBPK approach is that it helps identify which ADME properties most influence plasma levels, thus allowing the prioritisation of properties for optimisation.

1.7. Purpose of the ADME Guide

This guide has been prepared to provide an easy to read summary of the main *in vitro* ADME assays performed during the drug discovery and development process for small molecule therapeutic agents. The screening strategy devised during drug discovery often differs between different projects depending on the therapeutic area and key issues which have been identified and need to be addressed during the lead optimisation process. Generating high quality ADME data using optimal protocol design is important even at an early stage. Misleading inaccurate data can either lead to a poor quality compound being taken forward or a potential blockbuster being discarded. Therefore factors such as solubility, compound instability or non-specific binding should be important considerations before embarking on the characterisation of the ADME properties.

The guide focuses predominantly on small molecule drug discovery and development. The approaches for determining the ADME properties of biologics are significantly different to those for small molecules with less *in vitro* tools being available. For this reason the therapeutic property optimisation of biologics is not covered in detail in this guide.

1.8. References

1 US FDA Guidance for Industry – In Vitro Drug Interaction Studies - Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions (Jan 2020)

2 US FDA Draft Guidance for Industry – Drug Interaction Studies - Study Design, Data Analysis, Implications for Dosing, and Labelling Recommendations (Feb 2012)

3 European Medicines Agency (EMA) Guideline on the Investigation of Drug Interactions (Adopted 2012)

4 Japanese Pharmaceuticals and Medical Devices Agency (PMDA) Drug Interactions Guidelines (July 2018)

5 Kola I and Landis J (2004) Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov* **3**; 711-715

6 Lipinski CA *et al.*, (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliver Rev* **23(1-3)**; 3-25

7 Manly CJ *et al.*, (2001) The impact of informatics and computational chemistry on synthesis and screening. *Drug Discov Today* **6(21)**; 1101-1110

8 Baxter AD and Lockey PM (2001) 'Hit' to 'lead' and 'lead' to 'candidate' optimisation using multi-parametric principles. *Drug Disc World* **2**; 9-15

9 Brightman FA *et al.*, (2006) Application of a generic physiologically-based pharmacokinetic model to the estimation of xenobiotic levels in rat plasma. *Drug Metab Dispos* **34**; 84-93

Chapter 2: Physicochemical Drivers

Aqueous Solubility

2.1. The Importance of Aqueous Solubility

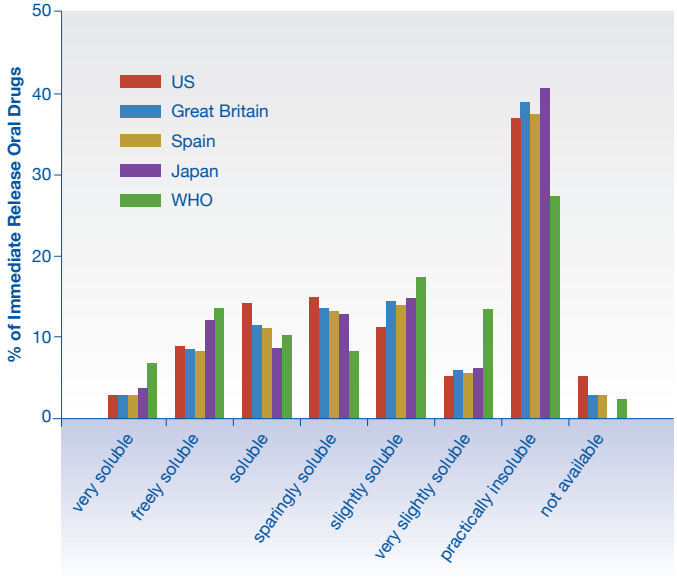
Solubility is the maximum dissolved concentration of a solute in a particular solvent at a given temperature.

Poor solubility:

- can limit the absorption of compounds from the gastrointestinal (GI) tract, resulting in reduced oral bioavailability.
- may necessitate novel formulation strategies and hence increase cost and delays in drug development.
- can lead to misleading data in *in vitro* assays.

It is reported that approximately 40% of currently marketed drugs and up to 75% of compounds currently under development are poorly water soluble[1]. Understanding the aqueous solubility of a compound is, therefore, a priority and needs to be established prior to *in vitro* testing. It is advisable to address solubility issues early in drug discovery by way of structural modifications, as proceeding with a highly insoluble compound can be expensive and challenging to deal with as a compound moves through the drug discovery and development process. Solubility data allow medicinal chemists to assess the effect of functional groups on the solubility of a compound series.

Figure 2.1: A Comparison of the Solubility Categories for The Top 200 Oral Drug Products in the United States (US), Great Britain, Spain and Japan and from the World Health Organisation (WHO) Essential Drug List[2].



Solubility Classification	mg/mL
Very soluble	>1000
Freely soluble	100 - 1000
Soluble	33 - 100
Sparingly soluble	10 - 33
Slightly soluble	1 - 10
Very slightly soluble	0.1 - 1
Practically insoluble	<0.1

Reprinted (adapted) with permission from Takagi T *et al.*, A Provisional Biopharmaceutical Classification of the Top 200 Oral Drug Products in the United States, Great Britain, Spain, and Japan. *Mol Pharm* **3(6)**; 631-643. Copyright 2006 American Chemical Society[2].

2.2. Factors affecting Solubility

There are many factors which can affect solubility (and dissolution) including:

- the structural and physicochemical nature of the compound
    - *Molecular weight* – As a general rule solubility decreases as molecular weight increases.
    - *Lipophilicity* – Hydrophilic compounds tend to be very soluble in water and other polar solvents, whereas highly lipophilic compounds tend to be insoluble in water yet soluble in nonpolar solvents such as benzene or cyclohexane.
    - pK_a – The charged form of a compound is more soluble than the neutral form and this differs depending on the pH of the solvent for acidic or basic compounds.
    - *Particle size* – If the particle size is reduced then the surface area of the solid is increased relative to size enabling faster dissolution.
    - *Solid form of the compound* – Amorphous solids consist of a non-crystalline structure in which the atoms and molecules are not organised in a lattice pattern whereas crystalline solids have ordered three dimensional arrangement of particles. Because no crystalline lattice exists with amorphous solids, dissolution is generally faster than with crystalline solids and the lack of order means solubility of amorphous solids can vary between different preparations.
    - *Salts:* Choosing different salt forms can help to increase dissolution rate and solubility.
- the solvent chosen
    - *pH* – For ionisable compounds, the solubility may be affected by the pH of the solution. Acidic compounds tend to be more soluble at high pH values, and basic compounds tend to be more soluble at low pH values.
    - *Composition* – The ionic strength of the solvent or the addition of lipids, proteins or surfactants can all influence the solubility of a compound.
- the conditions selected
    - *Thermodynamic or kinetic measurement* – Kinetic solubility generally tends to be higher than thermodynamic solubility due to the fact the initial stock is already prepared in an organic solvent such as DMSO and the insoluble material which forms consists of more soluble higher energy metastable crystal forms[3].
    - *% Cosolvent* – In kinetic measurements the % of the stock organic solvent can influence the solubility. For this reason the % organic solvent is typically kept at 1% or below of the aqueous solvent to minimise this effect and to more closely mimic the *in vitro* assays.
    - *Temperature* – For the majority of solids increasing the temperature increases the solubility of a compound in a solvent.

- *Equilibration time* – This is most important in the case of thermodynamic measurements where equilibrium is typically achieved within 24 hr.

- *Separation techniques* – For thermodynamic measurements, the excess solid material is usually separated by either filtration or centrifugation. Filtration has the limitation that the solute may bind to the filter which may lead to error for poorly soluble compounds. However this may be reduced by pre-rinsing with a saturated solution of the solute to saturate the binding sites. For the centrifugation methods often ultracentrifugation is performed to ensure sufficient pelleting of the insoluble material.

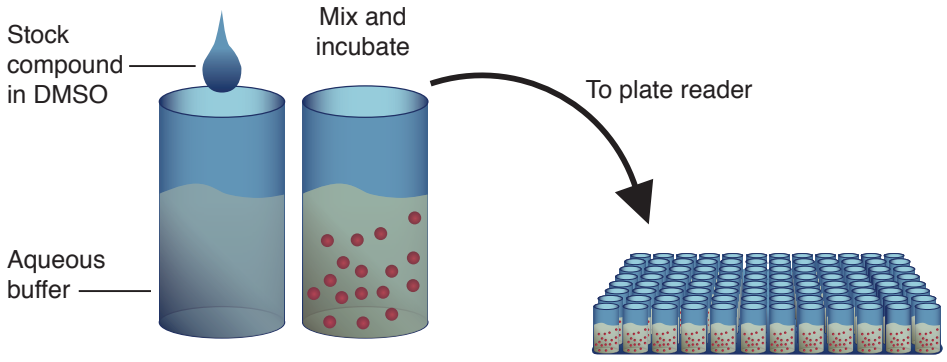
2.3. Methods used to Determine Solubility

There are generally two methods used to evaluate solubility:

2.3.1. Kinetic Solubility

Kinetic solubility measurements are determined from a pre-dissolved solution of the compound in organic solvent (often a stock solution of DMSO) which is diluted into an aqueous buffer. No equilibrium is reached and the measurement is routinely used to determine the concentration at which the compound precipitates. This assay is often run as a turbidimetric plate-based assay where the endpoint assesses formation of precipitate through either nephelometry (light scattering) or absorption measurements above background levels. The final results are typically presented as an estimated precipitation range (i.e., lower bound, upper bound and calculated mid-range). It is assumed that at some point within this upper and lower bound range, the compound precipitates. The kinetic solubility assay mimics the conditions used in many *in vitro* assays where solutions are typically prepared in DMSO stocks and then diluted in aqueous assay media. Therefore kinetic solubility is a valuable initial screen prior to starting efficacy, ADME or *in vitro* toxicity screens in order to identify potential issues or to determine appropriate concentration ranges.

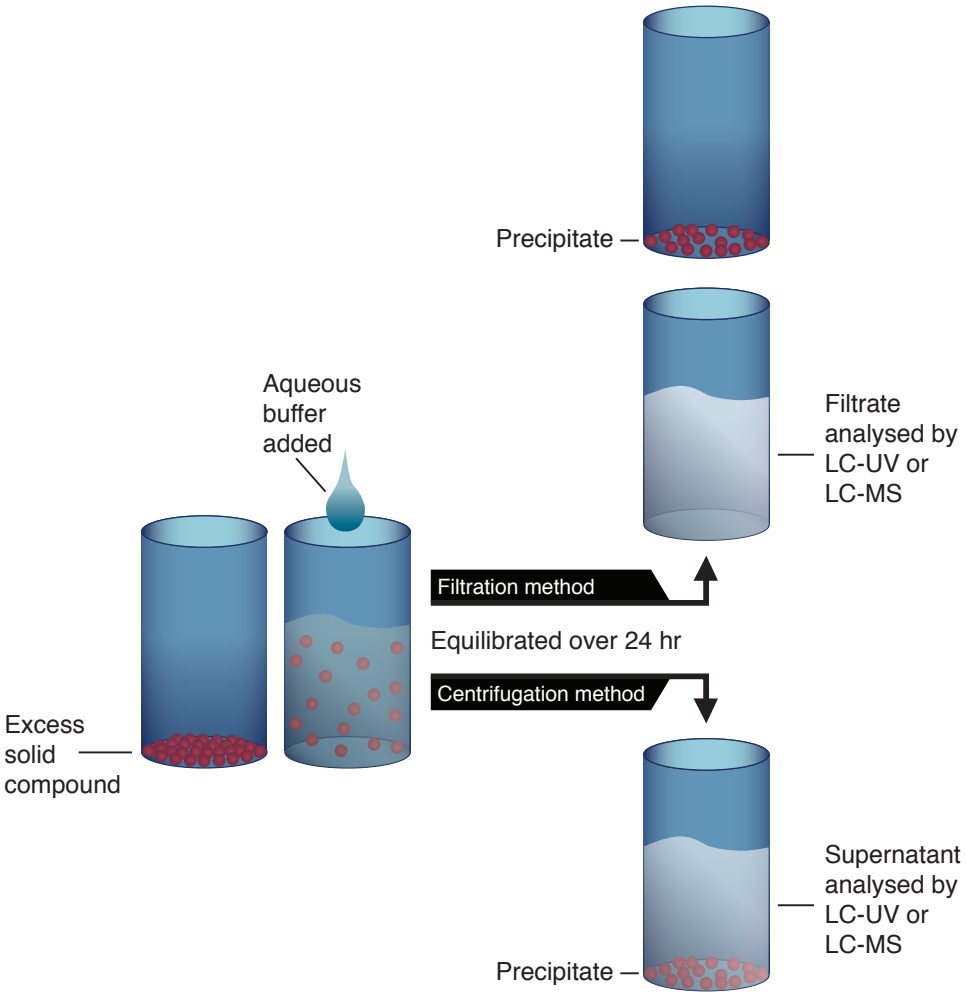
Figure 2.2: Schematic of Kinetic Solubility Method



2.3.2. Thermodynamic Solubility

Thermodynamic solubility is determined by the addition of aqueous buffer to an excess of solid compound. The solubility is the concentration of compound in the saturated solution in the presence of the excess solid, and the solution and solid are mixed, typically over 24 hours, to ensure equilibrium. The excess solid compound is then removed by either filtration or centrifugation (or ultracentrifugation). The filtrate (in the case of filtration) or the supernatant (in the case of centrifugation) are then analysed by LC-UV or LC-MS. The thermodynamic measurement will vary depending on the solid form of the compound. For example, amorphous forms tend to have a higher solubility than crystalline solids. Thermodynamic solubility is typically assessed in late discovery or early development where the solid form has been well characterised.

Figure 2.3: Schematic of Thermodynamic Solubility Method



2.4. The Difference between Dissolution and Solubility

Dissolution is a kinetic process and is measured as a rate. Dissolution testing tends to be performed during drug development for solid oral dosage forms (e.g., tablets and capsules) to provide quality control information (e.g., lot to lot consistency), to guide new formulation development or to predict *in vivo* release profiles and bioavailability. Dissolution methods typically follow standardised methods published by the United States Pharmacopeia (USP).

Solubility is the maximum dissolved concentration of a solute in a particular solvent at a given temperature, and is a measure of when the rate of precipitation equals the rate of dissolution in equilibrium. This is typically performed at an earlier stage than dissolution testing and can be used to establish if solubility is likely to be an issue during the drug discovery and development process in terms of insolubility in the *in vitro* assays, reduced absorption in the GI tract or formulation challenges.

Both dissolution and solubility are important parameters, either of which can be limiting. For example, after oral administration the rate of dissolution must significantly exceed the intestinal transit rate for optimal absorption. If the solubility is acceptable but the dissolution rate is low then the solubility limit may not be reached during the transit time. Furthermore, in the case of low equilibrium solubility there may not be enough compound in solution to allow high levels of flux across the intestine even if the compound has a rapid dissolution rate[1].

2.5. The Biopharmaceutics Classification System (BCS)

The Biopharmaceutics Classification System (BCS), originally proposed by Amidon *et al.*, (1995)[4], is useful for categorising the potential bioavailability of oral drugs. It divides drugs into 4 classifications based on their solubility and permeability.

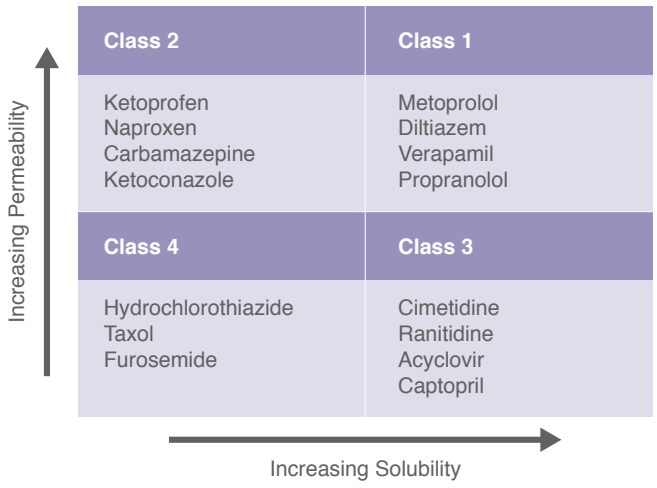
Class 1: High Permeability, High Solubility

Class 2: High Permeability, Low Solubility

Class 3: Low Permeability, High Solubility

Class 4: Low Permeability, Low Solubility

Figure 2.4: Categorisation of Marketed Drugs[5] using the Biopharmaceutic Classification System (BCS)



Class 1 drugs have optimal solubility and permeability properties for good oral bioavailability. The properties of Class 2 drugs can potentially be improved by developing suitable formulation strategies. Most Class 3 and Class 4 drugs need improvement through structural modification in the lead optimisation process.

Over recent years, there has been a move in drug discovery programs away from lipophilic, insoluble but highly potent compounds (BCS Class 2), to a more BCS Class 3-like chemical space. These compounds have much better solubility, are generally less bound to plasma proteins, but are likely to be less passively permeable, and more likely to be subject to transporter effects[6].

BCS has been adopted by the regulatory authorities (e.g., FDA[7]) for biowaiver studies (i.e., waiver of *in vivo* bioavailability and/or bioequivalence studies for immediate release solid oral dosage forms). This typically applies to subsequent formulations after the initial bioavailability of the immediate release form has been established. For these studies 3 factors are considered;

- **Solubility:** A drug substance is considered HIGHLY SOLUBLE when the highest dose strength is soluble in 250 mL or less of aqueous media over a pH range of 1 to 6.8 at 37±1°C.
- **Permeability:** A drug substance is considered HIGHLY PERMEABLE when the extent of absorption in humans is determined to be ≥85% of an administered dose, based on mass-balance or in comparison to an intravenous reference dose. Either *in vivo* approaches or *in vitro* models such as the Caco-2 cell monolayer permeability model are permitted.
- **Dissolution:** A drug product is considered to be RAPIDLY DISSOLVING when ≥85% of the labelled amount of drug substance dissolves within 30 minutes using USP apparatus I or II in a volume of ≤500 mL buffer solutions.

2.6. Compendial and Biorelevant Media

2.6.1. Compendial Media

Compendial buffers such as simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) are often used to assess solubility to mimic the different pH values in the stomach and the small intestine respectively. These buffers can be used with or without the gastric and intestinal enzymes, pepsin for the stomach and pancreatin for the intestine. Despite their use in evaluating pH-dependent effects, these aqueous buffers cannot mimic factors such as osmolality, ion strength, viscosity, surface tension or the impact of food in the GI tract which are important for solubilising BCS Class 2 and 4 compounds.

2.6.2. Biorelevant Media

More complex biorelevant media have been developed to simulate the conditions in the stomach and intestine before and after meals. Different compositions of gastrointestinal content can influence solubility depending on the physicochemistry of the drug. For example, fat and bile salts are important for the solubility of lipophilic drugs and buffering capacity and pH are important factors for the solubility of ionisable drugs[8].

The biorelevant media include:

- **FaSSGF (fasted state simulated gastric fluid)** mimics the fasted state gastric conditions. It contains pepsin and low amounts of bile salts (sodium taurocholate) and phospholipids (lecithin).
- **FaSSIF (fasted state simulated intestinal fluid)** mimics fasting conditions in the proximal small intestine. It contains bile salts (sodium taurocholate) and phospholipids (lecithin).
- **FeSSGF (fed state simulated gastric fluid)** is difficult to mimic using a standardised biorelevant media. Heat treated cow's milk and Ensure® Plus have both been proposed for this purpose.
- **FeSSIF (fed state simulated intestinal fluid)** mimics the fed state conditions in the proximal small intestine. It contains higher concentrations of bile salts (sodium taurocholate) and phospholipids (lecithin) than FaSSIF to reflect the biliary response to food intake.

Both the compendial and biorelevant media are commonly used for dissolution testing and Caco-2 permeability assays whereas for solubility assessment, compendial media are more commonly used.

Lipophilicity and pK_a

2.7. Definition of Lipophilicity and pK_a

Lipophilicity is the affinity of a molecule for a lipophilic (non-polar) environment.

The pK_a of a singly-ionising compound is the pH at which the molecule is 50% protonated and it provides an indicator of the extent of ionisation potential of the compound. Most drugs are either weak acids or weak bases. Acids are most highly ionised at a high pH (i.e., in an alkaline environment). Bases are most highly ionised in an acidic environment (low pH).

2.8. The Importance of Lipophilicity and pK_a in Drug Discovery

Lipophilicity and pK_a are two important physicochemical properties which determine the pharmacokinetic, pharmacodynamic and toxicological behaviour of drugs. These properties can influence distribution across the lipid bilayer of cells and into tissues, absorption and the binding characteristics of a drug as well as being important factors in determining the solubility of a compound. Many of the prediction technologies available today rely heavily on the physicochemical properties of a molecule.

2.9. Lipinski's Rule of Five

A very simple set of rules based on the structural and physicochemical properties of a molecule were published by Lipinski in 1997[9]. Despite their simplicity, these rules have been extremely effective at assisting with the design of orally administered 'drug-like' molecules which are more likely to succeed in the clinic. The 'Rule of Five' is based on a distribution of calculated properties among several thousand drugs. As with most rules there are exceptions and these include compound classes that are substrates for biological transporters.

The rule states that poor absorption or permeation is more likely when:

- There are more than 5 H-bond donors (expressed as the sum of OHs and NHs);
- The molecular weight is over 500;
- The logP is over 5
- There are more than 10 H-bond acceptors (expressed as the sum of Ns and Os)

2.10. QED (Quantitative Estimate of Drug-Likeness) Approach

More recently, based on a set of 771 oral drugs approved by the US FDA, Hopkins and co-workers (Bickerton *et al.*, 2012)[10] developed a quantitative estimate of drug-likeness (termed QED) as an alternative approach to Lipinski's Rule of Five.

The approach uses a set of eight commonly used molecular properties including molecular weight, octanol-water partition co-efficient (logP), number of hydrogen bond donors, number of hydrogen bond acceptors, polar surface area, number of rotatable bonds, number of aromatic rings and the number of structural alerts which are weighted based on their contribution to drug-likeness. Rather than being a binary classification as is the case with Lipinski's Rule of Five, QED ranks and quantifies compounds on a continuous scale of drug-likeness between zero (all properties unfavourable) and one (all properties favourable).

2.11. Summary of Other 'Rule' Based Approaches

Many other metrics involving logP/logD have recently been discussed in the literature, including the Pfizer 3/75 rule[11] (suggesting that clogP >3 and total polar surface area <75Å correlates with an increased risk of *in vivo* toxicity findings) and the GlaxoSmithKline 4/400 rule[12] (suggesting that compounds with clogP >4 and molecular mass >400 Da have a less favourable ADME and toxicity profile). Hann[13] has also suggested a sweet-spot of drug discovery space, based on molecular weight and logP.

2.12. Methods used to Determine Lipophilicity

2.12.1. Shake Flask Method

The standard approach for determining lipophilicity is the 'shake flask' method which measures the ability of a molecule to partition between immiscible non-polar and polar liquid phases. Often octanol is chosen as the non-polar phase and aqueous buffer as the polar phase. Octanol does, however, have several limitations as it supports hydrogen bonding and also contains approximately 4% v/v water at equilibrium. These properties differ from the properties of the inner hydrocarbon core of cell membranes, and for compounds which can form hydrogen bonds, octanol can over-represent the actual membrane-crossing ability[14]. It has been proposed that a quartet of systems may be useful in the screening strategy including octanol (amphiprotic), alkane (inert), chloroform (a proton donor) and propylene glycol dipelargonate (PGDP; largely a proton acceptor) in order to fully cover a range of partition properties[14]. However, this recommendation does not seem to have taken hold throughout the industry and octanol remains the most popular solvent for these studies.

The partitioning values which can be measured are logP or logD.

- **LogP** is the partition coefficient of the compound between an organic phase and an aqueous phase at a pH where all of the compound molecules are in the neutral form.

- **LogD** is the distribution coefficient of the compound between an organic phase and an aqueous phase at a specified pH. It can be used to understand the pH dependent lipophilicity of a drug.

A common approach for assessing logD using the shake flask method is detailed below:

Following pre-saturation of the octanol with the buffer (and buffer with octanol), the test compound is equilibrated in octanol:buffer. The ratio of octanol:buffer can be varied in order to cover a wider range of lipophilicity values. After equilibration, the two phases are separated and the test compound is analysed (typically by LC-MS or LC-MS/MS) in the aqueous and octanol phases. The logD is calculated from the following equation:

$$\text{LogD} = \text{Log}\left(\frac{C_{oct}}{C_{aq}}\right)$$

Where:

C_{oct} = Concentration in octanol sample (corrected for dilution)
C_{aq} = Concentration in aqueous sample (corrected for dilution)

2.12.2. Lipid Water Partitioning

The cell membrane consists of lipids and proteins therefore partitioning between lipid membranes and water is likely to be a better representation of the *in vivo* situation than a solvent based system. Indeed, liposomes, which are vesicles containing lipid bilayers with water inside the lipid core, provide relevant data for predicting membrane uptake and absorption. Unlike octanol, the liposomes do not form distinct immiscible layers with water and so typically separation is performed by ultracentrifugation or equilibrium dialysis.

Sovicell have developed an alternative method for lipid water partitioning. Their TRANSIL™ technology involves the lipid bilayer being non-covalently attached to a bead[15]. The lipid orientation and fluidity is similar to liposomes, and data between TRANSIL™ and liposome partition coefficients are comparable[16]. However, the fact that the TRANSIL™ beads are more easily separated from the unbound compound ensures the assay is more amenable to high throughput screening.

2.12.3. Chromatographic Methods

Determination of the chromatographic hydrophobicity index (CHI) is based on a reversed phase HPLC method with calibration performed using a set of standards for which the CHI value has been previously determined[17]. CHI provides a rapid screen for determination of lipophilicity. It is a sensitive technique which is not affected by impurities and is not dependent on quantification of concentration.

Immobilised artificial membranes are another chromatographic approach. Originally developed by Pidgeon and Venkataram (1989)[18], these consist of phospholipids covalently attached to a solid phase HPLC support. Although column life is relatively short, the throughput is relatively high and the method correlates well with passive Caco-2 permeability (for molecular weights between 200 and 800)[19].

2.13. The Relationship between Lipophilicity and the ADME Properties of a Compound

Lipophilicity measurements such as $logD_{7.4}$ can be used to provide a very early indication of the likely ADME properties of a molecule (see Table 2.1). As a general rule, a $logD_{7.4}$ of between 1 and 3 is considered optimal for the drug design of orally bioavailable compounds.

Table 2.1: General $LogD_{7.4}$ Characterisation of ADME Parameters[3,15]

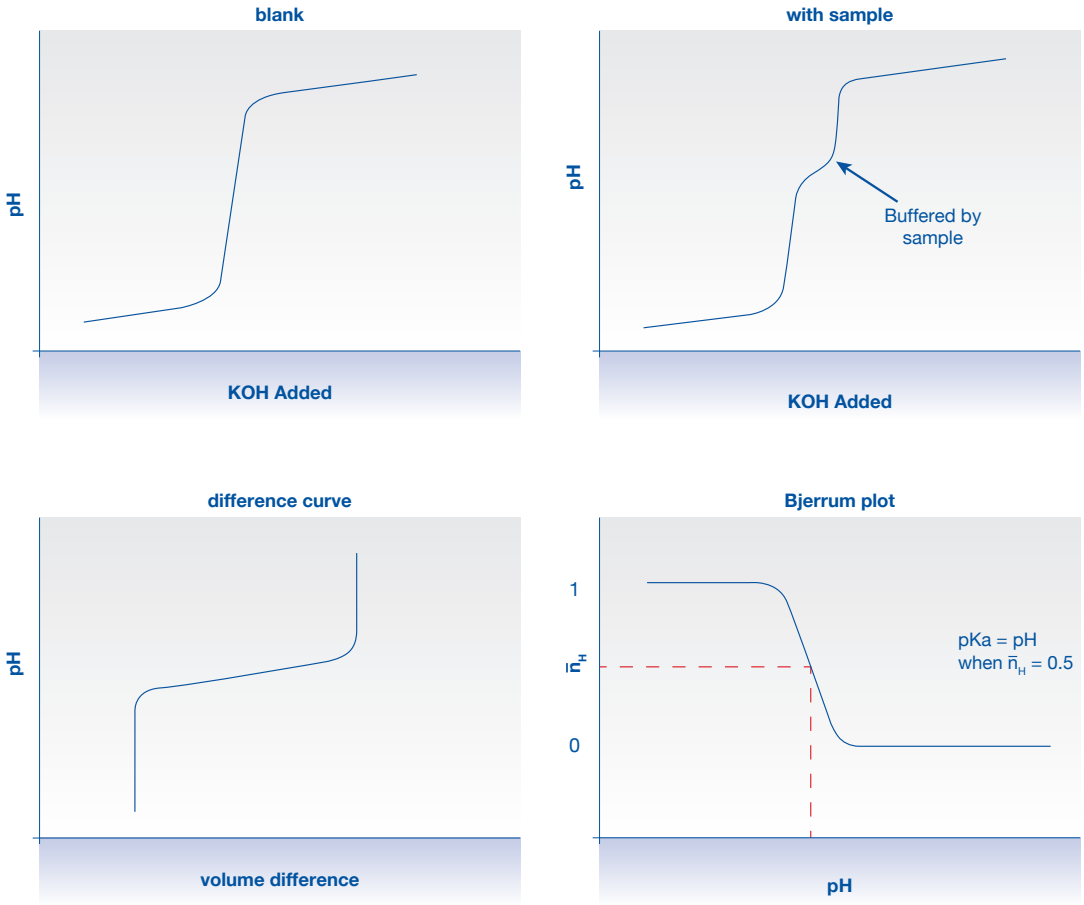
$LogD_{7.4}$	Effect on ADME Properties
< 1	• Permeability issues (Intestinal and CNS) • Susceptibility to renal clearance • Low metabolic liability
1 - 3	• Optimal range for CNS and non-CNS orally dosed drugs • Low metabolic liability
3 - 5	• Lower solubility • Increased metabolic liability
> 5	• Low solubility • Poor oral availability • High metabolic liability

2.14. Methods used to Determine pK_a

2.14.1. Potentiometric Titration (pH-Metric) Method

One of the most popular instruments for determining pK_a by potentiometric titration is currently the SiriusT3 instrument developed by Sirius Analytical Instruments. This system compares a blank acid-base titration to a titration in the presence of the compound. A difference curve is produced from the volume of potassium hydroxide required to reach a given pH with and without the compound. The difference curve is converted to a Bjerrum plot. The axes are reversed and the volume difference is converted to units of $\bar{n}_H$ (average number of bound protons per molecule of compound). For a singly-ionising compound, the pK_a is the pH at which the molecule is 50% protonated. The pH-metric method will measure all pK_as between 2 and 12, provided the sample is in solution throughout the experiment.

Figure 2.5: Graphs showing the Titration Curves with and without Sample, the Difference curve and the Bjerrum Plot.



For compounds which ionise, logP can also be determined using this instrument by performing the titrations in a two-phase water-octanol system. From the measured pK_a and logP values, the logD value can be calculated as a function of pH.

2.14.2. pK_a by UV Spectroscopy

In cases of poor solubility or small sample amounts, calculation of the pK_a from UV measurement is the best approach for test articles which contain a chromophore. Approximately 70% of samples have UV-active pK_as. The method relies on the change in UV spectra at different pH values. The SiriusT3 developed by Sirius Analytical is one of the most widely used instruments for UV-metric pK_a determination. This instrument can offer a rapid UV method which measures absorbance at 250 wavelengths and 54 pH values in a buffered solution in approximately 5 min. The slower UV-metric method in unbuffered solution extends the pH range below 1 and above 13. Measurements of logP, logD and pH-solubility profiles can also be determined using this instrument.

2.14.3. Capillary Electrophoresis

pK_a values can be measured by capillary electrophoresis. This technique is based on the different electrophoretic mobility of a compound in the neutral and ionised form. Ionised molecules move through the mobile phase faster as mobility by capillary electrophoresis is proportional to charge. pK_a is determined by the inflection point of the effective mobility versus mobile phase pH[3].

2.15. The Relationship between pK_a and the ADME Properties of a Compound

Most drugs are weak acids or weak bases and exist in solution in equilibrium between unionised and ionised forms. The ionisation potential of a compound affects the distribution of the chemical in solution and affects the availability of the compound to enter into physical, chemical and biological processes. According to the pH partition hypothesis, only unionised non-polar drugs penetrate the cell membrane, and at equilibrium, the concentrations of the unionised species are equal on both sides. The pK_a of a compound influences properties such as logD and solubility as well as the absorption, distribution, metabolism, elimination and potency of a compound.

Table 2.2: General Characterisation of ADME Parameters based on pK_a[20,21,22]

ADME Property	Effect of Ionisation Potential
Solubility	Acidic compounds tend to be more soluble at high pH values, and basic compounds tend to be more soluble at low pH values.
Permeability	Acidic compounds tend to be less permeable at high pH and basic compounds tend to be less permeable at low pH.
Metabolism	Electrostatic interactions are determined by the pK_a of a compound. These interactions can affect binding of the compound to the active sites of enzymes. For example, nitrogen containing bases where the basic nitrogen is 5-7Å from the site of metabolism have been shown to be important in the metabolism of compounds by CYP2D6.
Protein Binding	Binding of drugs to plasma proteins tends to be by hydrophobic and electrostatic interactions. Typically, acidic compounds with moderate lipophilicity are more likely to bind to serum albumin whereas basic compounds with moderate lipophilicity are more likely to bind to α_1-acid glycoprotein.
Excretion	Urinary pH is an important factor in the excretion of a drug. For example, acidic drugs are ionised at alkaline urinary pH and basic drugs are ionised at acidic urinary pH. Only unionised compounds in the tubular fluid will be reabsorbed by passive diffusion.

2.16. Prediction of Physiochemical Properties

There are many commercial suppliers of software for predicting pK_a, logP and logD from chemical structure (for example, Daylight Chemical Information, Advanced Chemistry Development (ACD) and CompuDrug). These software tools are developed using large databases of previously generated experimental values. The accuracy of the software prediction is based on the diversity of the training set used for developing the model as well as the quality of the experimental data. Although there are exceptions, predictions for physicochemical properties are generally considered to be some of the more reliable *in silico* predictions.

As well as general classification bands for predicting expected biological properties, lipophilicity and pK_a values are widely used in drug discovery for QSAR (quantitative structure activity relationship) and QSPR (quantitative structure property relationship) modelling.

2.17. References

1 Williams HD *et al.*, (2013) Strategies to address low drug solubility in discovery and development. *Pharmacol Rev* **65(1)**; 315-499

2 Takagi T *et al.*, (2006) A provisional biopharmaceutical classification of the top 200 oral drug products in the United States, Great Britain, Spain, and Japan. *Mol Pharm* **3(6)**; 631-643

3 Kerns EH and Di L (2008) Drug-like properties: concepts, structure design and methods: from ADME to toxicity optimization. ISBN 0123695201 Academic Press

4 Amidon GL *et al.*, (1995) A theoretical basis for a biopharmaceutic drug classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability. *Pharm Res* **12(3)**; 413-420

5 Basanta Kumar Reddy B and Karunakar A (Feb 2011) Biopharmaceutics Classification System: A regulatory approach. *Dissolution Technologies*; 31-37

6 Smith D (2013) Evolution of ADME science: where else can modeling and simulation contribute? *Mol Pharmaceutics* **10(4)**; 1162-1170

7 FDA Guidance for Industry. Waiver of *in vivo* bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a Biopharmaceutics Classification System (Dec 2017)

8 Klein S (2010) The use of biorelevant dissolution media to forecast the *in vivo* performance of a drug. *AAPS J* **12(3)**; 397-406

9 Lipinski CA *et al.*, (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliver Rev* **23(1-3)**; 3-25

10 Bickerton GR *et al.*, (2012) Quantifying the chemical beauty of drugs. *Nat Chem* **4**; 90-98

11 Hughes JD *et al.*, (2008) Physicochemical drug properties associated with *in vivo* toxicological outcomes. *Bioorg Med Chem Lett* **18(17)**; 4872-4875

12 Gleeson MP (2008) Generation of a set of simple, interpretable ADMET rules of thumb. *J Med Chem* **51**; 817–834

13 Hann MM *et al.*, (2012) Finding the sweet spot: the role of nature and nurture in medicinal chemistry. *Nat Rev Drug Discov* **11**; 355-365

14 Smith DA *et al.*, (2012) Pharmacokinetics and metabolism in drug design. Volume 51 of *Methods and Principles in Medicinal Chemistry.* John Wiley & Sons

15 Hartmann T and Schmitt J (2004) Lipophilicity – beyond octanol/water: a short comparison of modern technologies. *Drug Discov Today* **1(4)**: 431-439

16 Loidl-Stahlhofen A *et al*., (2001) Multilamellar liposomes and solid-supported lipid membranes (TRANSIL): screening of lipid-water partitioning toward a high-throughput scale. *Pharm Res* **18**; 1782-1788

17 Valko K *et al*., (1997) Chromatographic hydrophobicity index by fast gradient RP-HPLC: a high throughput alternative to logP/logD. *Anal Chem* **69**; 2022-2029

18 Pidgeon C and Venkataram UV (1989) Immobilised artificial membrane chromatography: supports composed of membrane lipids. *Anal Biochem* **176(1)**; 36-47

19 Chan EC *et al.*, (2005) Modeling Caco-2 permeability of drugs using immobilised artificial membrane chromatography and physicochemical descriptors. *J Chromatogr A* **1072(2)**; 159-168

20 Lewis DF (2000) On the recognition of mammalian microsomal cytochrome P450 substrates and their characteristics towards the prediction of human P450 substrate specificity and metabolism. *Biochem Pharmacol* **60(3)**; 293-306

21 Urien S *et al.* (2001) The significance of plasma-protein binding in drug research. In *Pharmacokinetic optimisation in drug research* Ed. Testa *et al.* 189-197

22 Timbrell JA (1991) In *Principles of Biochemical Toxicology* 65-67

Chapter 3: Drug Metabolism

3.1. Background to Drug Metabolism

Drug metabolism is the process where drugs are enzymatically altered, typically to more water soluble metabolites, to aid excretion from the body. This is often termed a 'biotransformation' of the drug. It is reported that approximately three quarters of the top 200 prescribed drugs in the United States in 2002 were cleared by drug metabolism[1].

Drug metabolism reactions can be generally divided into Phase I and Phase II reactions:

- **Phase I reactions:** involve oxidation, reduction and hydrolysis. The major Phase I enzyme families include the cytochrome P450 (CYP) superfamily, the flavin-containing mono-oxygenases (FMO), the monoamine oxidases, alcohol or aldehyde dehydrogenases, reductases, esterases, amidases and epoxide hydrolases.

- **Phase II reactions:** involve additions (or conjugations) of highly polar groups to the molecule. These can be sequential to Phase I reactions, for example a compound may first be hydroxylated (Phase I reaction) and then the same hydroxyl group may be conjugated to glucuronic acid (Phase II reaction). However, Phase I reactions do not always precede Phase II reactions and occasionally, direct Phase II reactions occur if susceptible functional groups are present on the molecule. Common Phase II reactions include glucuronidation, sulphation, methylation, N-acetylation and glutathione conjugation.

Many organs in the body contain drug metabolising enzymes, however, it is the liver which is the main site of drug metabolism in the body for the majority of drugs.

3.2. The Importance of Drug Metabolism

The rate of drug metabolism impacts on pharmacokinetic parameters such as oral bioavailability, clearance and half-life. This in turn can affect the efficacy and toxicology of the drug by influencing the concentrations of the drug within the plasma and tissues of the body. For example, a drug that is rapidly metabolised may require multiple daily dosing or higher doses to maintain a concentration in the bloodstream or target organ that is sufficient to elicit a therapeutic effect. However, very slowly metabolised drugs may also cause issues if they remain in the body for long periods, causing accumulation of the drug and potential toxicity.

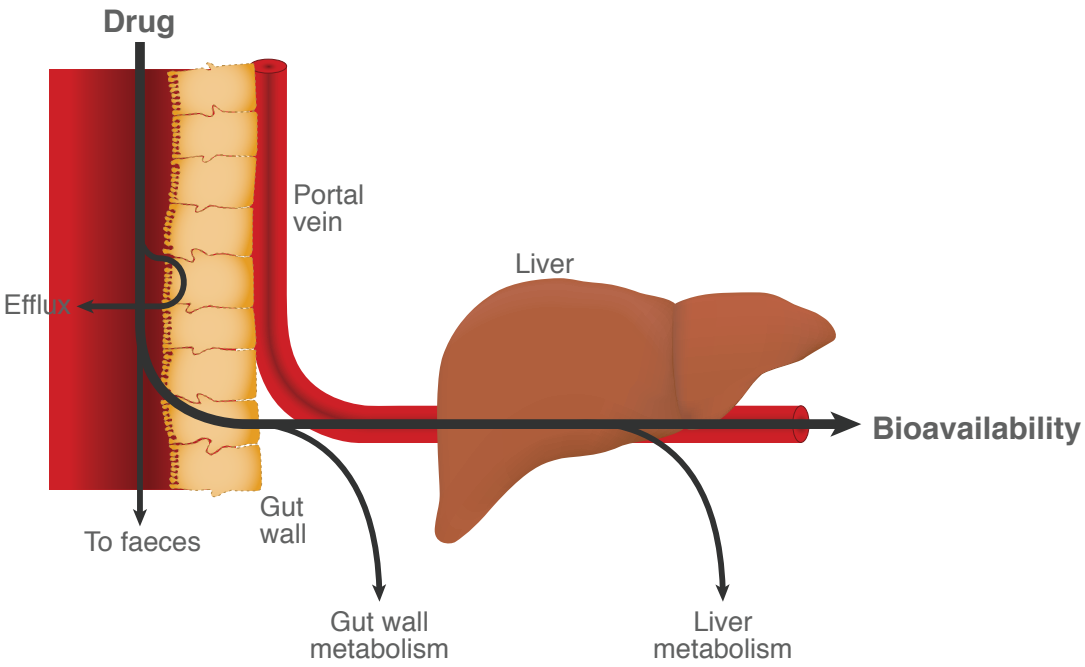
The ideal scenario is that a drug has an optimal half-life for once-a-day dosing to aid patient compliance with no toxic effects from drug accumulation. The half-life is the time taken for the drug plasma concentration to decrease by 50%, which is dependent on the clearance and rate and extent of metabolism of the drug.

In some instances rapid drug metabolism can be beneficial. This applies to prodrugs, which are administered in an inactive form and then converted in the body into the active drug. This conversion occurs either by enzymatic or chemical transformation.

3.3. First Pass Metabolism

First pass metabolism is the term used to explain metabolism which occurs before a drug reaches the systemic circulation. Typically this is used to refer to orally administered drugs which undergo metabolism either in the gut and/or in the liver before reaching the systemic circulation. Along with the solubility and permeability of a drug, first pass metabolism is a major determinant of the bioavailability of a drug. The schematic below (Figure 3.1) illustrates the various barriers to the drug reaching the systemic circulation caused by first pass metabolism.

Figure 3.1: Schematic illustrating First Pass Metabolism by the Gut Wall and the Liver prior to Reaching the Systemic Circulation.



3.4. CYP and non-CYP mediated Metabolism

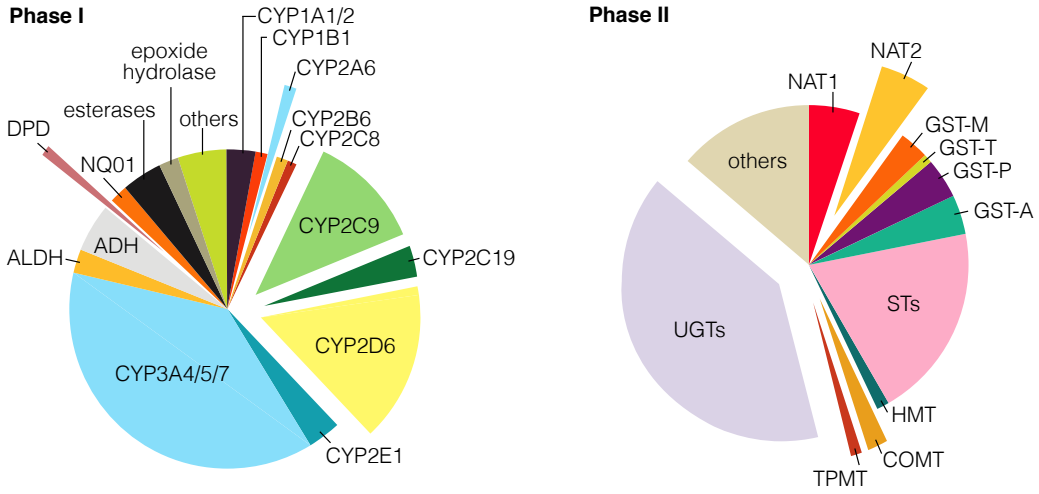
The cytochrome P450s (CYPs) are a family of enzymes primarily involved in the Phase I oxidative metabolism of many drugs. Two thirds of drugs cleared by metabolism are metabolised at least in part by the cytochrome P450 enzymes with CYP3A4 accounting for almost 50% of CYP activity[1].

It is important to consider, however, that approximately one third of the top 200 prescribed drugs which undergo drug metabolism are substrates for metabolic clearance mediated by enzymes other than CYPs. The most prevalent are UDP-glucuronosyl transferases (UGTs) and esterases accounting for approximately 8% and 5% of the metabolised drugs respectively[1,2].

Numerous other enzymes exist including, amongst others, flavin-containing monooxygenases (FMO), monoamine oxidases (MAO), aldehyde oxidases (AOX), aldehyde dehydrogenases (ALDH), aldo-keto reductases (AKR), alcohol dehydrogenases (ADH), hydroxysteroid dehydrogenases (HSD), sulphotransferases (SULT), N-acetyltransferases (NAT) and glutathione S-transferases (GST).

The interest in non-CYP mediated metabolism is growing due to its involvement in the metabolism of drugs as well as its relevance in drug-drug interactions. The EMA[3], FDA[4] and Japanese PMDA[5] guidance on drug interactions suggest that both CYP and non-CYP pathways should be elucidated if the pathways are thought to contribute a significant amount to drug elimination.

Figure 3.2: The Percentage of Phase I and II Metabolism of Drugs that Each Enzyme Contributes Correlates with the Relative Size of the Corresponding Pie Chart[6].



ADH = alcohol dehydrogenase; ALDH = aldehyde dehydrogenase; CYP = cytochrome P450; DPD = dihydropyrimidine dehydrogenase; NQO1= NADPH: quinone oxidoreductase; COMT= catechol O-methytransferase; GST= glutathione S-transferase; HMT= histamine methyltransferase; NAT = N-acetyltransferase; STs = sulphotransferases; TPMT= thiopurine methyltransferase; UGTs= UDP-glucuronosyl transferases

From Evans and Relling (1999)[6] Pharmacogenomics: Translating functional genomics into rational therapeutics. *Science*, **286**, 487-491. Reprinted with permission from AAAS.

3.5. The Relationship between Drug Metabolism and Toxicity

Drug toxicity can either result from the parent drug itself, or from potentially reactive or toxic metabolites which are formed. There may be several reasons for the toxicity.

- **Drug-drug interactions** – Drug-drug interactions (often shortened to DDI) can arise when two or more different drugs are co-administered and interfere with either the metabolism or the transport of the other drug. In terms of drug metabolism, these effects can lead to either a decrease in the metabolism of the parent drug leading to elevated plasma levels (e.g., in the case of enzyme inhibition), increased metabolism of the parent drug leading to a potential reduction in efficacy or an increased formation of a toxic metabolite (e.g., in the case of enzyme induction). Drug-drug interactions involving drug metabolising enzymes will be covered as a separate topic in Chapter 5 within this guide.

- **Genetic polymorphisms** – Large variations in drug metabolising activity can be explained in some instances by genetic variation (genetic polymorphisms) resulting in the following four phenotypes; poor metabolisers (PMs), intermediate metabolisers (IMs), extensive metabolisers (EMs) and ultrarapid metabolisers (UMs). These genetic polymorphisms may be more common in specific ethnic groups. For example, there is a higher incidence of CYP2D6 PMs in the Caucasian population compared to Asians[7]. Conversely, there is a high incidence of CYP2C19 PMs in the Asian population compared to the Caucasian population[7]. PM status can lead to elevated plasma levels of the parent drug which may lead to exaggerated pharmacological effect or toxicity. There are a small number of cases where the UMs are at an increased risk and this is especially relevant where a prodrug is being administered or if a toxic metabolite is formed.

- **Species differences** – Large variation can be observed between different species in terms of drug metabolism. This can be problematic if disproportionately higher levels of metabolite are formed in humans than those present in the animals chosen for preclinical safety testing. The regulatory authorities now request that *in vitro* cross-species drug metabolism and metabolite profiling studies are performed prior to initiation of clinical trials using either liver microsomes, liver slices or hepatocytes. If disproportionate levels of metabolite are formed in humans then it may be necessary to perform non-clinical safety studies on the metabolite as well as the parent compound.

- **Age** – Geriatric and pediatric patients often exhibit differences in binding proteins, drug metabolising enzymes and/or drug transporters and renal filtration/secretion which can impact on the pharmacokinetics of a drug.

- **Impaired hepatic function** – As the liver is the primary site of drug metabolism, any impaired hepatic function can significantly affect the drug metabolism and pharmacokinetics of a drug or its metabolites. Under these circumstances, a dose reduction for hepatically-cleared therapies may be necessary.

- **Impaired renal function** – Chronic renal failure alters intestinal, renal and hepatic drug metabolism, transport and plasma protein binding, affecting plasma levels of drugs and leading to the risk of adverse effects[8].

- **Reactive metabolites** – Certain drugs are able to undergo bioactivation to a reactive metabolite which, if not adequately detoxified, can covalently bind to biological macromolecules (e.g., protein or DNA) and subsequently cause drug-induced toxicity.

3.6. Metabolic Stability

3.6.1. *In vitro* Systems to Evaluate Metabolic Stability

As mentioned earlier the liver is the major drug metabolising organ for the large majority of pharmaceutical drugs. For this reason, the *in vitro* models used to investigate drug metabolism often focus on hepatocytes or subcellular fractions of the liver such as microsomes, cytosol, S9 or mitochondria where concentrations of particular enzymes are high.

- **Liver microsomes**

  The most popular subcellular fraction used during drug discovery tends to be microsomes as these are easy to prepare and store, are amenable to high throughput screening, and are a relatively low cost option. Microsomes contain Phase I oxidative enzymes including the CYP enzymes but do not have an intact cell membrane. Moreover, microsomes require the addition of relevant co-factor(s) to the incubation.

  Microsomes tend to underpredict intrinsic clearance for those compounds which undergo metabolism by cytosolic or Phase II enzymes. UDP-glucuronosyl transferase (UGT) is one of the most common Phase II enzymes present in microsomes, however, in contrast to CYPs and the flavin-containing monooxygenases, the active site of the UGTs resides in the lumen of the endoplasmic reticulum (ER), and the ER membrane provides a diffusional barrier for substrates, cofactors, and products[9]. Disruption of this barrier is required to remove the latency of UGTs in microsomal incubations. Typically, alamethicin has been used for this purpose[10]. Alamethicin forms pores in the membrane and allows access to the enzyme without affecting gross membrane structure or general intrinsic enzyme catalytic activity. By supplementing the microsomal incubations with alamethicin and the cofactors, NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) and UDPGA (uridine diphosphoglucuronic acid), both CYP mediated and UGT mediated metabolism can be evaluated.

- **Liver S9**

  S9 fraction is the post-mitochondrial supernatant fraction. Although S9 fraction is easy to use and contains both cytosolic and microsomal enzymes, the activity tends to be lower than microsomes and as a consequence higher protein concentrations are often required. As with microsomes, S9 often requires the addition of a co-factor. Extrapolating from *in vitro* metabolic stability data to *in vivo* clearance data is less common for S9, and data are often used for qualitative purposes to identify if cytosolic enzymes are responsible for the formation of a metabolite.

- **Hepatocytes**

  Hepatocytes are more representative of the *in vivo* situation because they contain a cell membrane and do not require additional co-factors. Hepatocytes have the advantage that they contain the full complement of enzymes for both Phase I and Phase II metabolism. Cryopreservation of hepatocytes enables the cells to be stored for long periods of time and ensures no supply problems or delays in screening. With advances in cryopreservation techniques, cell viability and activity have improved dramatically, and cryopreserved cells now provide a viable alternative to freshly isolated cells.

3.6.2. *In vitro* Metabolic Stability Assessment

For all the *in vitro* models, a pool of human donors is typically assessed to reduce the influence of inter-individual variability in catalytic activity. Typically the metabolising system is incubated with the substrate (parent compound) over time at 37°C and the disappearance of the substrate is monitored at the individual time points by LC-MS/MS. From a plot of ln peak area ratio (parent compound peak area/internal standard peak area) against time, the gradient of the line is determined. Subsequently, half-life and intrinsic clearance are calculated using the equations below:

$$\text{Elimination rate constant (k)} = (-\text{ gradient})$$

$$\text{Half life } (t_{1/2}) \text{ (min)} = \frac{0.693}{k}$$

$$\text{Intrinsic clearance } (CL_{int}) = \frac{V \times 0.693}{t_{1/2}}$$

where V = Incubation volume (µL)/ protein (mg) for microsomal and S9 stability
or V = Incubation volume (µL)/Number of cells for hepatocyte stability.

The units for CL_{int} are µL/min/mg protein for microsomal and S9 stability or µL/min/million cells for hepatocyte stability.

In drug discovery, knowledge of metabolic pathways is limited and, therefore, substrate depletion over a number of time points is an accepted approach to calculate intrinsic clearance. A substrate concentration below the K_m is typically chosen to reduce the possibility of saturation of the enzymes.

3.6.3. Predicting *in vivo* Total Clearance from Microsomal and Hepatocyte Intrinsic Clearance

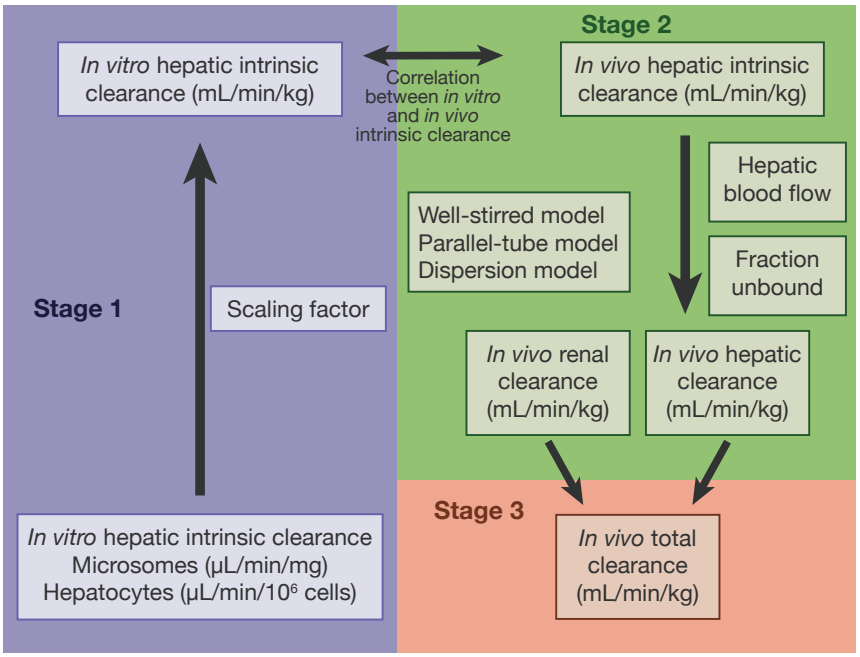
Figure 3.3 illustrates the scheme by which the *in vitro* intrinsic clearance can be related to *in vivo* total clearance, and the approach is described in more detail below.

- **Stage 1:** In order to compare the *in vitro* intrinsic clearance to the *in vivo* intrinsic clearance it is necessary to convert the units of the *in vitro* intrinsic clearance from either µL/min/mg protein (microsomes) or µL/min/10^6 cells (hepatocytes) to mL/min/standard weight of the species or mL/min/kg. The scaling factors used are based on protein recovery during microsome preparation (mg protein/g liver) and hepatocellularity (cells/g liver). Values for these scaling factors can be found in the literature[11,12].

- **Stage 2:** Intrinsic clearance is a pure measure of enzyme activity towards a drug and is not influenced by other physiological determinants of liver clearance such as hepatic blood flow or drug binding within the blood matrix. Therefore, conversion from intrinsic clearance to hepatic clearance requires knowledge of these parameters, as well as the relationship between circulating drug concentrations and the drug concentration at the enzyme site. For the latter, several mathematical models have been developed as it is not possible to determine practically how these two drug concentrations relate. The most frequently used models are the well-stirred model, the parallel-tube model and the dispersion model. For drugs with a low clearance, the differences between the models are minimal, however, larger differences between the models exist if the drug is highly cleared[13].

- **Stage 3:** Conversion from hepatic metabolic clearance to total clearance is relatively simple and assumes that total clearance is the sum of the hepatic and renal clearance, and that no other organs play a significant role in the metabolism and excretion of the compound. This assumption is correct for the majority of compounds.

Figure 3.3: Approach used for Prediction of *in vivo* Total Clearance from Microsomal and Hepatocyte *in vitro* Clearance



3.6.4. Correlation between *in vitro* Intrinsic Clearance from Microsomal and Hepatocyte Studies and *in vivo* Intrinsic Clearance

The determination of the *in vivo* hepatic clearance rates from *in vitro* data is problematic and can be error-prone due to

- the complexity of metabolism
- inter-individual variability
- the poor understanding of certain processes that are not yet modelled in current *in vitro* systems.

Figures 3.4 and 3.5 show the correlation between *in vitro* and *in vivo* clearance for a set of known drugs in both microsomes and hepatocytes, respectively[11]. For all the compounds selected for this study, the metabolism was well understood. The data suggest *in vitro* metabolising systems can be used to predict *in vivo* clearance for this particular set of compounds. It is clear, however, that microsomal systems tend to underestimate the clearance for rapidly metabolised compounds. This discrepancy is likely to be linked to the lack of active Phase II metabolising enzymes in the microsomal preparations. For series of compounds which are metabolised extensively by Phase II enzymes, undergo product inhibition or are extensively bound non-specifically to microsomal protein, the predicted clearance may be a poor representation of the observed clearance *in vivo*. Accordingly the best way to use the *in vitro* data is to classify compounds into high, medium and low categories, rather than providing a precise estimate of *in vivo* clearance.

Figure 3.4: Correlation between *in vitro* Microsomal Intrinsic Clearance and *in vivo* Hepatic Intrinsic Clearance.
High, medium and low classification bands, scaled from Table 3.1, are displayed on the graph.

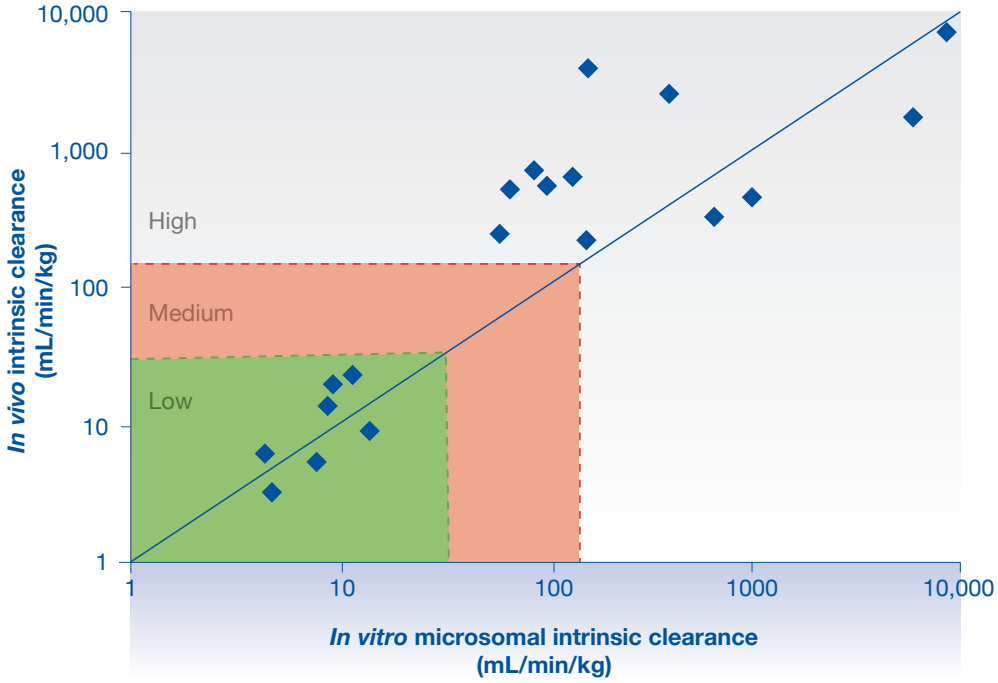
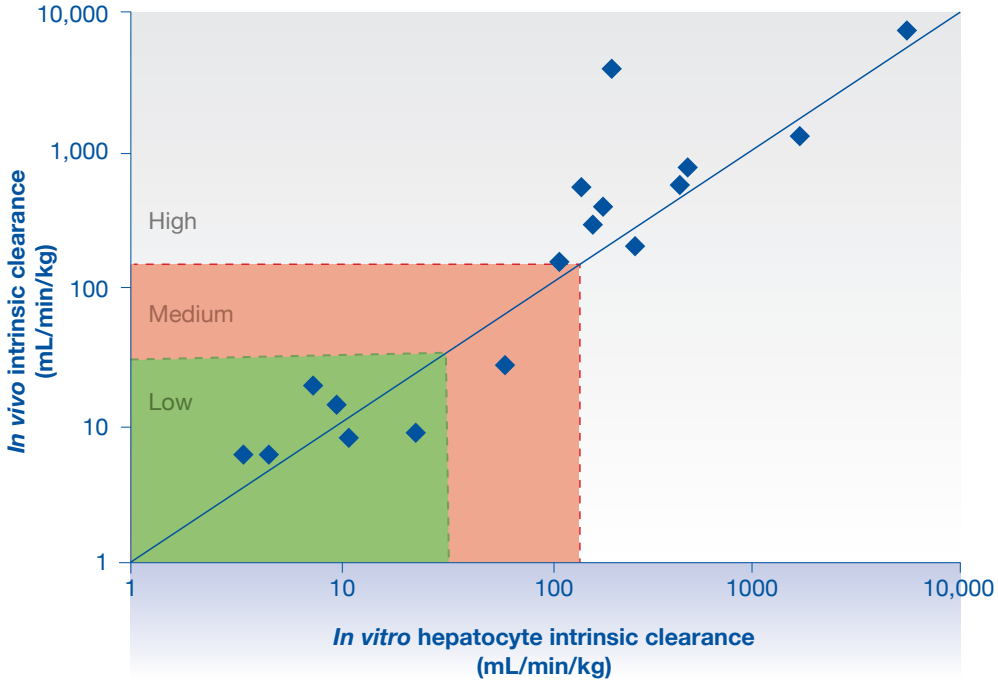


Figure 3.5: Correlation between *in vitro* Hepatocyte Intrinsic Clearance and *in vivo* Hepatic Intrinsic Clearance.
High, medium and low classification bands, scaled from Table 3.1, are displayed on the graph.



3.6.5. Reasons for Disparity between the Intrinsic Clearance calculated from *in vitro* Metabolising Systems and the Observed *in vivo* Intrinsic Clearance

Estimating *in vivo* intrinsic clearance from *in vitro* intrinsic clearance has its potential pitfalls, and several assumptions are made during the conversion which may not be reliable in certain circumstances. These are discussed in reviews by Iwatsubo *et al.* (1997)[13] and Houston (1994)[11] and summarised below.

- **Metabolism in extrahepatic tissues** – *In vitro* hepatic metabolising systems only address clearance in a single organ. In reality, although the liver is the major site of drug metabolism, many other tissues are capable of metabolising drugs. If the extra-hepatic metabolism is significant, the *in vitro* intrinsic clearance is likely to under-predict the *in vivo* intrinsic clearance.

- **Equilibrium between blood and hepatocytes** – It is assumed that there is rapid equilibrium of drugs between the blood and hepatocytes. When the intrinsic clearance in hepatocytes is much greater than the efflux clearance from hepatocytes to blood, the *in vivo* intrinsic clearance is rate-limited by the influx process from blood to hepatocytes. Such an incorrect assumption of rapid equilibrium can be one of the reasons why *in vivo* intrinsic clearance is less than *in vitro* intrinsic clearance.

- **Active transport through the sinusoidal membrane** – If the drug is undergoing active transport through the sinusoidal membrane, the unbound concentration of drug in the hepatocytes will be higher in the case of influx, or lower in the case of efflux, than the unbound concentration in the blood, resulting in a corresponding overestimation or underestimation of *in vivo* intrinsic clearance, respectively.

- **Inter-individual variability** – Inter-individual variability can exist both *in vivo* and in the *in vitro* metabolising systems. The *in vivo* clearance can be influenced dramatically by CYP induction (e.g., smoking can induce CYP1A2), polymorphisms in metabolism, gender, age and physiological conditions such as stress. For *in vitro* studies, pooled microsomes or hepatocytes can reduce the problems associated with inter-individual variability.

3.6.6. Interpretation of Metabolic Stability Data

- **Compound ranking** – Classification bands can be used to categorise compounds into low, medium or high clearance. The classification bands shown in Table 3.1 have been calculated by Cyprotex using scaling factors from the literature and assuming a fraction unbound value of 1 and an extraction ratio (the fraction of drug that is eliminated from the blood by an organ) of 0.3 and 0.7 for the low and high boundaries, respectively, as defined in Wilkinson and Shand (1975)[14].

Table 3.1: Classification Bands Typically used for Categorising Compounds into Low, Medium or High Clearance.

Clearance Category	*In vitro* microsomal intrinsic clearance (µL/min/mg protein)		*In vitro* hepatocyte intrinsic clearance (µL/min/10^6 cells)	
	Human	Rat	Human	Rat
Low	≤ 8.6	≤ 13	≤ 3.5	≤ 5.1
Medium	8.6 - 47	13 - 72	3.5-19	5.1-28
High	≥ 47	≥ 72	≥ 19	≥ 28

With the exception of pro-drugs, compounds in the high clearance category are generally considered to be unfavourable. The prediction is that they will be rapidly cleared *in vivo* and have a short duration of action. The therapeutic area and likelihood of co-administration with other compounds is an important consideration because these may affect the overall plasma concentration of the drug and therapeutic margins.

- **Reaction phenotyping**

  Reaction phenotyping (or enzyme mapping) is used to determine which enzymes are involved in the metabolism of a specific test article. The data from these studies can be important in identifying potential drug interactions in the clinic with common co-medications or for anticipating possible variable pharmacokinetics caused by genetic polymorphisms in certain enzymes.

  Recombinantly expressed enzymes are the most popular way of assessing reaction phenotyping. Another approach uses correlation analysis where banks of human liver microsomes prepared separately from at least 10 donors and previously characterised for activity towards individual probe substrates are used to correlate with the activity towards the test compound. Inhibition of the metabolism of the test compound in human liver microsomes using selective chemical inhibitors or antibodies is another technique for reaction phenotyping.

  Using recombinant enzymes, a number of scaling approaches exist, for example, the RAF (relative activity factor) method[15] or the ISEF (intersystem extrapolation factor) method[16] which help to understand relative contribution of the individual enzymes.

  Understanding which enzymes (CYP and non-CYP) are involved in the formation of the major metabolites is essential during drug development to understand potential drug-drug interactions. *In vivo* mass balance studies are often performed to understand the main elimination pathways and systemic metabolite exposure, and this information is combined with the *in vitro* metabolism and reaction phenotyping data to corroborate metabolic pathways and the enzymes responsible.

- **Species-specific differences**

  Large differences in drug metabolism can exist between species for certain compounds, and therefore it is important to assess compounds in multiple species to understand these differences.

  The multi-species metabolic stability data may be used to

  - identify the appropriate species for pre-clinical development
  - determine if preclinical safety testing is required for metabolites
  - predict human pharmacokinetics using scaling approaches often in combination with preclinical *in vivo* data

- **Predicting *in vivo* pharmacokinetics in conjunction with other assays and physiologically based PK models**
  Predicting *in vivo* clearance rates using only microsomal stability data can be inaccurate. However, considering other physicochemical and ADME data in conjunction with knowledge of physiological processes important in clearance, can substantially alleviate this situation. The major physiological aspects that will impact clearance include organ tissue composition, distribution rates into all major organs, renal and biliary clearance and active transport mechanisms, all of which can be modelled mathematically[17].

3.7. Enzyme Inhibition

The mechanism of enzyme inhibition can be reversible, quasi-irreversible or irreversible.

- **Reversible inhibition** is caused by non-covalent binding of the inhibitor to a site on the enzyme. Reversible inhibition can be categorised into one of four types;

  - *Competitive:* where the substrate and inhibitor bind to the same site which is usually the active site of the enzyme. The presence of the inhibitor increases the apparent K_m but the V_{max} remains unchanged for the substrate.

  - *Non-competitive:* where the inhibitor binds to a site on the enzyme which is not the active site yet reduces the enzyme activity without affecting binding of the substrate. The presence of the inhibitor decreases the V_{max} but the K_m remains the same for the substrate.

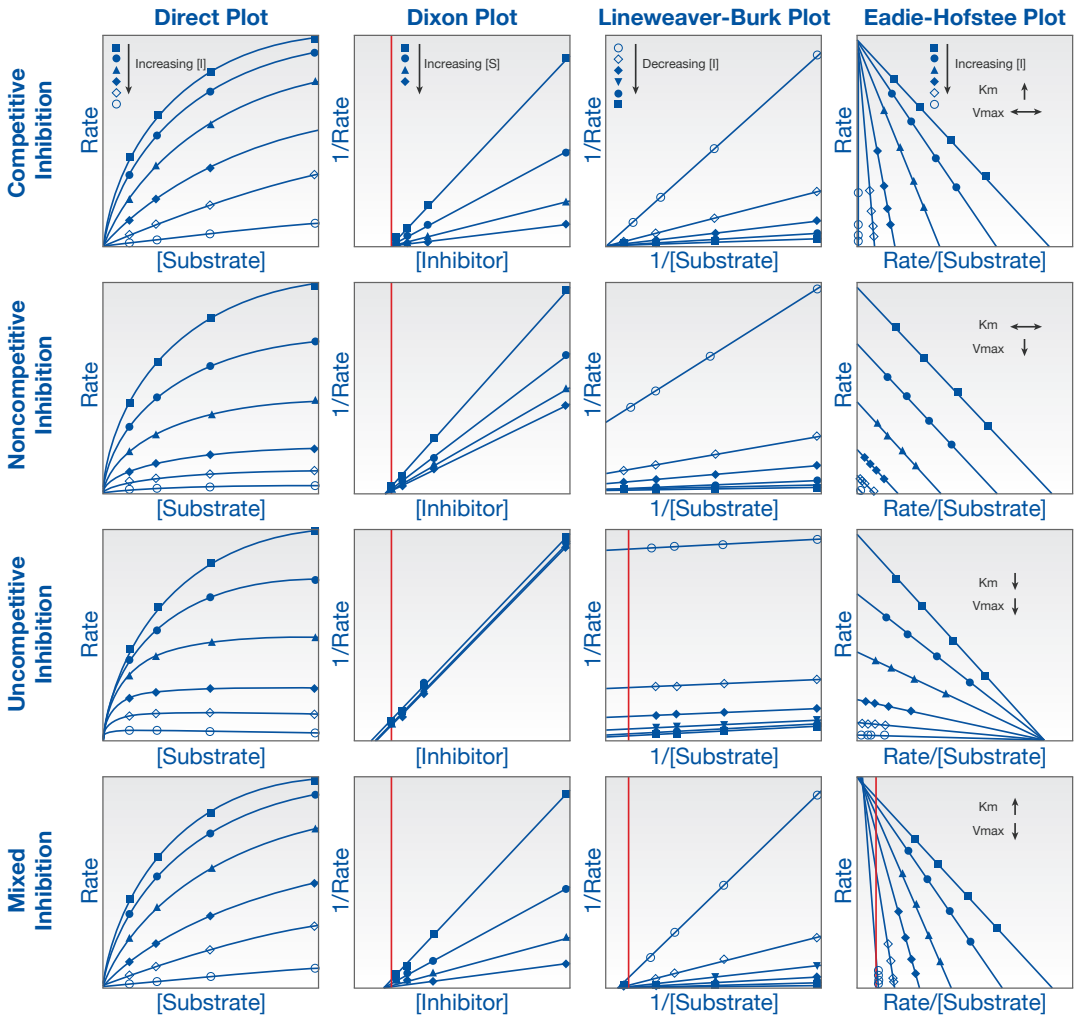
  - *Uncompetitive:* where the inhibitor binds to the enzyme-substrate complex only and the binding site can be the same or different from the active site. The presence of the inhibitor reduces both the V_{max} and the K_m of the substrate.

  - *Mixed:* where the inhibitor may bind to the free enzyme or the enzyme substrate complex. In the presence of the inhibitor V_{max} is reduced but K_m may be increased or decreased for the substrate.

    Transformations of the Michaelis-Menten equation are used for graphical depiction of the type of inhibition as well as calculation of the K_i (inhibition constant). These are illustrated in Figure 3.6[18].

Figure 3.6: Theoretical Graphical Representation of the Different Mechanisms of Reversible Enzyme Inhibition using Direct Plots and Various Transformations such as the Dixon Plot, Lineweaver-Burk Plot and the Eadie-Hofstee Plot.

The Eadie Hofstee plot is one of the preferred plots for distinguishing between the different types of inhibition[18]. It is important to note that for mixed inhibition the K_m can either increase or decrease. For the purposes of the illustration, the mixed inhibition shows an example of where the K_m increases in response to the inhibitor.



- **Irreversible inhibition**, as the name suggests, is a non-reversible interaction which typically occurs through covalent bond formation. The consequences of irreversible inhibition are considered to be more serious than reversible inhibition because the enzyme must be re-synthesised before activity is restored[19]. The irreversible interaction can be a consequence of the formation of a reactive metabolite which forms a covalent bond with the enzyme. This can lead to hapten formation and in some cases triggers an autoimmune response[20]. Two types of irreversible interaction are often referred to: time dependent inhibition and mechanism based inhibition. Although these terms are often used interchangeably there are distinct differences scientifically. Time dependent inhibition is defined as an interaction where there is enhanced inhibition if the test compound is pre-incubated with the metabolising system prior to addition of the substrate[19]. Mechanism-based inhibition specifically refers to a subset of time dependent inhibition which defines inactivation of the enzyme by a chemically reactive metabolite[19].

- **Quasi-irreversible inhibition** occurs when the test compound is metabolised to form a stable complex with the ferrous iron of the heme of the CYP enzyme. Like irreversible inhibition, quasi-irreversible inhibition also requires synthesis of new enzyme before normal CYP activity is restored. Although the interaction is extremely strong it is not a covalent bond and the bond can be broken under extreme conditions, for example in the presence of potassium ferricyanide[19,21].

3.7.1. Reversible Inhibition

3.7.1.1. Determination of Reversible Inhibition

Typically, the seven main CYP isoforms (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4) with two substrates recommended for CYP3A4 are investigated. Evaluation of UGT inhibition may also be performed if direct glucuronidation is one of the major elimination pathways of the investigational drug. Isoform-specific substrates are incubated individually with a range of test compound concentrations in the presence of human liver microsomes (or expressed enzyme) and cofactor.

The incubations should be performed under linear conditions with respect to time and protein concentration and use a substrate concentration at, or below, the K_m. Ideally, the probe substrate selected should be predominantly metabolised by a single enzyme and should have a simple metabolic scheme preferably with no sequential metabolism. Generally the amount of the probe substrate consumed should be no more than 10%[22]. At the end of the incubation, the formation of metabolite is monitored by LC-MS/MS (liquid chromatography with triple quadrupole mass spectrometry) or fluorescence, at each of the test compound (potential inhibitor) concentrations.

Two main methods are employed.

- The use of microsomes with individual isoform-specific substrates to indicate which of the enzymes are inhibited by the test compound.

- The use of recombinant individually expressed enzymes.

Each method has advantages and disadvantages.

- **Human liver microsomes inhibition method – Main features**
    - Amenable to high-throughput format
    - Closer to the *in vivo* situation than recombinant systems as all the enzymes are present in the correct ratios.
    - Inter-individual differences in expression levels of enzymes can be taken into account by pooling microsomes from different donors.
    - Majority of reactions use LC-MS/MS so the analysis is sensitive and specific.
    - Marker probe substrates are used[3] – high specificity and well documented in the literature.
    - The use of human liver microsomes is the preferred option for regulatory studies.
- **Recombinant enzyme inhibition method – Main features**
    - Very high-throughput systems with fast turnaround permitted by the option of a fluorescent end-point.
    - No inter-individual differences in enzyme expression levels.
    - The enzyme is not present in its native environment (no other enzymes present) and is often over-expressed – so is not representative of the *in vivo* situation.
    - Extrapolation of data to the 'patient in clinic' situation is not as robust.
    - Need for specificity of probe substrates is decreased.
    - Analysis may be either by LC-MS/MS or fluorescence.

3.7.1.2. Interpretation of Reversible CYP Inhibition Data

- **Percentage inhibition** – a single concentration of test compound (potential inhibitor) at a single time point. Useful for ranking compounds in a series at an early stage in drug discovery.
- **IC_{50}** – the test compound (potential inhibitor) concentration required to inhibit activity by 50% (obtained using a single concentration of substrate, single time point and multiple inhibitor concentrations). Useful for ranking compounds, and setting conditions for potential K_i evaluation.
- **K_i** – inhibition constant (obtained using multiple substrate concentrations, single time point and multiple inhibitor concentrations). Useful for predicting clinical drug-drug inhibition potential and also for identifying the type of inhibition (e.g., if competitive, non-competitive, uncompetitive or mixed inhibition). The relevance of the K_i should be determined by considering clinical data such as the maximum dose and C_{max} levels. Details of the calculations used are included in the EMA[3], FDA[4] and Japanese PMDA[5] drug interaction guidelines. It is important to note that the FDA guidelines[4] indicate that half the IC_{50} can be used as an estimate of K_i if

the substrate concentration used is at K_m. The data are used to determine if a clinical drug-drug interaction study is required. More information on the relevance of the K_i data in the prediction of clinical drug-drug interactions is included in Chapter 5.

3.7.2. Time Dependent Inhibition of CYPs

3.7.2.1. Determination of Time Dependent Inhibition

All time dependent inhibition studies include some form of pre-incubation step where the test compound is incubated with a metabolising system (typically human liver microsomes) with and without cofactor, followed by a second incubation in the presence of the marker substrate. The seven main CYP isoforms (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4) are typically investigated.

Three standard methods exist for assessing time dependent inhibition including a single point assay, an IC_{50} shift assay or a more in-depth k_{inact}/K_I study.

- **Single point time dependent inhibition:** The single point assay is typically performed at a single concentration of test compound (potential inhibitor), a single substrate concentration and a single pre-incubation time in the presence and absence of cofactor.

- **IC_{50} shift:** The IC_{50} shift assay evaluates a range of inhibitor concentrations, a single probe substrate concentration with and without a single pre-incubation time in the presence and absence of cofactor.

- **k_{inact}/K_I:** The k_{inact}/K_I assay investigates a range of inhibitor concentrations, a single substrate concentration and a range of pre-incubation times in the presence of cofactor.

For these standard time dependent inhibition methods, both irreversible and quasi-irreversible inhibition are detected, but cannot be distinguished between. However, follow-up studies can be performed to understand the nature of the binding. For example, potassium ferricyanide can be used to dissociate quasi-irreversible metabolite intermediate complexes to restore the enzymatic activity of the CYP, a process which is not possible if true irreversible binding occurs[19]. Alternative approaches include the use of dialysis or repeated microsomal washing[19].

3.7.2.2. Interpretation of Time Dependent Inhibition Data

The initial screening results (single point and IC_{50} shift assays) provide a flag for potential issues with time dependent inhibition, and furthermore is indicative of reactive metabolite formation. A more detailed assessment can be performed to determine k_{inact} and K_I which can be used to establish the potential for clinically relevant drug interactions using the equations recommended in the regulatory guidelines[3,4,5]. Further details on the relevance of time dependent inhibition to clinical drug-drug interactions is included in Chapter 5.

3.8. Cytochrome P450 (CYP) Induction

3.8.1. Mechanism of CYP Induction

There are two main mechanisms by which induction of CYP enzymes may occur.

- **Nuclear receptor-mediated induction.** The most common mechanism of CYP enzyme induction is transcriptional gene activation. Nuclear receptors, such as the aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR), mediate drug-induced changes in the expression of Phase I and Phase II enzymes and transporters. Induction of CYP1A2, CYP2B6 and CYP3A4 gene expression can serve as sensitive representative endpoints for activation of AhR, CAR and PXR respectively[23].

- **Stabilisation of the mRNA or enzyme.** This is a less common mechanism but includes examples such as troleandomycin which induces rat CYP3A by decreasing the rate of CYP3A protein degradation with no increase in the rate of protein synthesis[24].

3.8.2. Methods to assess CYP Induction

For early screening methods, nuclear receptor activation assays provide valuable surrogates for determining CYP induction. Most nuclear receptors reside in the cytoplasm and then translocate to the nucleus on ligand binding. In the nucleus PXR or CAR heterodimerise with retinoid X receptor α (RXRα). AhR heterodimerises with the AhR nuclear translocator. The heterodimers then interact with response elements of the respective target genes and cause transcriptional activation[25]. A number of techniques have been employed to evaluate this process, the main ones being ligand binding assays and cell-based transactivation assay. The transactivation assay uses a cell line which has been stably or transiently transfected with the nuclear receptor and reporter gene vectors.

For regulatory assessment, the preferred way to assess CYP induction is to use cultures of human hepatocytes (fresh or cryopreserved) from at least 3 donors because the resulting data is the most clinically relevant. Human hepatocytes are incubated with the test compound typically over a 48-72 hr period. Induction of the CYP enzymes can then be investigated by assessing the catalytic activity of an isoform specific probe substrate or through analysing mRNA levels of test wells compared to vehicle control wells. Catalytic activity has the advantage that it can detect induction due to protein stabilisation, however, if the test compound is a mechanism-based inhibitor then the induction effects can be masked using this approach. For the latter reason, assessing increases in mRNA levels is considered to be a more reliable approach for CYP induction unless protein stabilisation is anticipated.

3.8.3. Interpretation of CYP Induction Data

Several methods have been proposed for analysing data from CYP induction studies. These include determining a concentration-dependent fold increase and assessing if this is greater than a laboratory-specific predetermined threshold, a basic model incorporating the E_{max} and EC_{50}, or the use of correlation methods.

The correlation methods[4] involve either calculating a Relative Induction Score (RIS) or determining $[I_{max,u}]/ EC_{50}$. The clinical relevance of the induction effect is determined by extrapolating from a curve of induction magnitude (*in vivo* change in AUC of

an enzyme probe drug e.g., midazolam) versus RIS or [$I_{max,u}$]/ EC_{50} that has been established previously in the same cell population with known inducers[26].

3.9. References

1 Williams JA *et al.*, (2004) Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUC_i/AUC) ratios. *Drug Metab Dispos* **32(11)**; 1201-1208

2 Beaumont K *et al.*, (2010) ADMET for the medicinal chemist. In *RCS Drug Discovery Series No. 1: Metabolism, Pharmacokinetics, and Toxicity of Functional Groups: Impact of Chemical Building Blocks on ADMET*. Edited by Smith DA; 61-98

3 The European Medicines Agency (EMA) Guideline on the Investigation of Drug Interactions (Adopted 2012)

4 US FDA Guidance for Industry – In Vitro Drug Interaction Studies - Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions (Jan 2020)

5 Japanese Pharmaceuticals and Medical Devices Agency (PMDA) Drug Interactions Guidelines (July 2018)

6 Evans WE and Relling MV (1999) Pharmacogenomics: Translating functional genomics into rational therapeutics. *Science* **286**; 487-491

7 Bertilsson L (1995) Geographical/interracial differences in polymorphic drug oxidation. Current state of knowledge of cytochromes P450 (CYP) 2D6 and 2C19. *Clin Pharmacokinet* **29(3)**; 192-209.

8 Dreisbach AW & Leotora JJL (2008) The effect of chronic renal failure on drug metabolism and transport. *Expert Opin Drug Metab Toxicol* **4(8)**; 1065-1074

9 Meech R and Mackenzie PI (1997) Structure and function of uridine diphosphate glucuronosyltransferases. *Clin Exp Pharmacol Physiol* **24(12)**; 907-915.

10 Fisher MB *et al.*, (2000) *In vitro* glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. *Drug Metab Dispos* **28(5)**; 560-566.

11 Houston JB (1994) Utility of *in vitro* drug metabolism data in predicting *in vivo* metabolic clearance. *Biochem Pharmacol* **47**; 1469-79

12 Carlile DJ *et al.*, (1999) Microsomal prediction of *in vivo* clearance of CYP2C9 substrates in humans. *Br J Clin Pharmacol* **47**; 625-35

13 Iwatsubo T *et al.*, (1997) Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacol Ther* **73**; 147-171

14 Wilkinson GR and Shand DG (1975) Commentary: a physiological approach to hepatic drug clearance. *Clin Pharmacol Ther* **18**; 377-390

[15] Crespi CL (1995) Xenobiotic-metabolizing human cells as tools for pharmacological and toxicological research. *Adv Drug Res* **26**; 179-235

[16] Chen Y *et al.*, (2011) Utility of intersystem extrapolation factors in early reaction phenotyping and the quantitative extrapolation of human liver microsomal intrinsic clearance using recombinant cytochrome P450. *Drug Metab Dispos* **39(3)**; 373-382

[17] Brightman FA *et al.*, (2006) Application of a generic physiologically-based pharmacokinetic model to the estimation of xenobiotic levels in rat plasma. *Drug Metab Dispos* **34**; 1-10

[18] Madan A *et al.*, (2001) *In vitro* approaches for studying the inhibition of drug-metabolizing enzymes and identifying the drug-metabolising enzymes responsible for the metabolism of drugs. In *Drugs and The Pharmaceutical Sciences: Drug-Drug Interactions* (Rodrigues AD Ed) Informa Healthcare, New York; 217–294

[19] Grimm SW *et al.*, (2009) The conduct of *in vitro* studies to address time-dependent inhibition of drug-metabolizing enzymes: a perspective of the pharmaceutical research and manufacturers of America. *Drug Metab Dispos* **37(7)**; 1355-1370

[20] Fontana E *et al.*, (2005) Cytochrome P450 enzymes mechanism based inhibitors: common sub-structures and reactivity. *Curr Drug Metab* **6(5)**; 413-454

[21] Rock D *et al.*, (2009) Experimental characterization of cytochrome P450 mechanism based inhibition. In *Drugs and the Pharmaceutical Sciences. Handbook of Drug Metabolism*. Second Edition (Pearson PG and Wienkers LC Ed) Informa Healthcare USA Inc New York; 541-569

[22] Fowler S and Zhang H (2008) *In vitro* evaluation of reversible and irreversible cytochrome P450 inhibition: current status on methodologies and their utility for predicting drug-drug interactions. *AAPS J* **10(2)**; 410-424

[23] Hewitt NJ *et al.* (2007) Induction of hepatic cytochrome P450 enzymes: methods, mechanisms, recommendations, and *in vitro–in vivo* correlations. *Xenobiotica* **37**; 1196-1224

[24] Watkins PB *et al.* (1986) Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P450p in rat hepatocytes *in vivo* and in primary monolayer culture. *J Biol Chem* **261**; 6264-6271

[25] Pinne M and Raucy JL (2014) Advantages of cell-based high-volume screening assays to assess nuclear receptor activation during drug discovery. *Expert Opin Drug Discov* **9(6)**; 1-18

[26] Fahmi OA and Ripp SL (2010) Evaluation of models for predicting drug-drug interactions due to induction. *Expert Opin Drug Metab Toxicol* **6(11)**; 1399-1416

Chapter 4: Permeability and Drug Transporters

4.1. Permeability and its Importance

Drug permeability is the rate at which the drug crosses a biological membrane barrier. This is important as it influences the following parameters;

- Intestinal absorption and oral bioavailability
- Blood brain barrier permeability
- Penetration into cells and so can affect efficacy or toxicity within the cell
- Elimination by the kidney and the liver

4.2. Common Approaches to Improve Permeability

A balance between the different physicochemical properties is required for optimal permeability. Structural modification to address permeability issues is a common approach used in lead optimisation[1].

- **Lipophilicity** is one of the main determinants of permeability with highly lipophilic compounds readily passing through cell membranes via passive diffusion. Therefore increasing logP is a common approach used to increase permeability.
- **Ionisation and polarity** – According to the pH partition hypothesis, only unionised non-polar drugs penetrate the cell membrane, therefore removing ionisable groups and reducing polarity are strategies used to improve passive permeability.
- **Hydrogen bonding** – Hydrogen bonding can have a detrimental effect on permeability therefore reducing hydrogen bond donors or acceptors is often used to enhance permeability.
- **Size** – Larger drugs with a higher molecular weight do not cross the lipid bilayer of the cell membrane as easily as smaller molecules.
- **Prodrugs** – Improving permeability through the use of prodrugs can be achieved in a couple of different ways. Firstly, increasing the lipophilicity of the molecule by masking polar functional groups and hydrogen bonds with ester or amide linkers is a common approach to address poor passive permeability[1]. An example of this type of prodrug is oseltamivir which is an ethyl ester prodrug and undergoes rapid conversion by carboxylesterase to the active drug, oseltamivir carboxylate[2]. Secondly, prodrugs can be substrates of uptake transporters. Enalapril is an example of an ester prodrug which improves the bioavailability from 3% (active drug, enalaprilat) to 40%. The ethyl ester moiety increases lipophilicity and is also a substrate of the PEPT1 transporter[1].

4.3. Main Modes of Movement across a Cell Membrane

Cell membranes consist of phospholipids (amphipathic molecules which consist of two hydrophobic fatty acid chains linked to a polar phosphate containing head) and proteins. The phospholipids form bilayers in aqueous conditions whereby the hydrophobic tails are embedded in the internal region of the membrane and the polar head is exposed on the outside.

The proteins in the membrane have specialised functions to allow transport of molecules across the membrane, to act as receptors for transmitting external signals to the cell or to enable electron transport and oxidative phosphorylation.

The composition of the cell membrane differs depending on the type of membrane. For example plasma membranes consist of approximately 50% lipid and 50% protein whereas the inner mitochondrial membrane has a higher percentage of proteins (approximately 75%)[3].

- **Paracellular permeability** *(between cells)* typically occurs via passive diffusion (process driven by a concentration gradient) and involves the solute moving through an intracellular space between cells. This route of transport is favoured by small hydrophilic polar solutes.
- **Transcellular permeability** *(through cells)* is the most common mechanism by which drugs cross membranes. It can occur via passive diffusion, facilitated or active processes. This route of transport is favoured by more lipophilic solutes.
  - *Transcellular passive diffusion* – involves movement of solutes via a diffusion gradient moving from a high concentration to a lower concentration. Charged, hydrophilic or zwitterions tend not to cross via this mechanism due to the hydrophobic environment of the cell membrane.
  - *Facilitated diffusion (or Facilitated transport)* – is a form of passive diffusion across a biological membrane in which a carrier facilitates the movement of an otherwise membrane-impermeable molecule or ion down its concentration or electrochemical gradient. Unlike active transport, the process does not require energy. There are two types of facilitated diffusion carriers;
    - *Channel proteins* which transport only water or certain ions. They tend to be selective and transport can be very rapid. Some of these channels are gated and are closed unless certain signals are present.
    - *Uniporters* which normally transport organic molecules such as amino acids or sugars.
  - *Active transport* – an energy driven process mediated by membrane-bound transport proteins. These processes move a substrate against its concentration gradient. There are primary active transporters that generate energy themselves (e.g., through hydrolysis of ATP), and secondary active transporters that utilise energy stored in voltage and ion gradients generated by a primary active transporter such as sodium/potassium ATPase. Secondary active transporters can be symporters (co-transporters) or antiporters (exchangers). Active transport can lead to efflux or uptake into the cells, and several of these transporters are known to play a role in clinically relevant drug-drug interactions.

4.4. Methods for Evaluating Permeability

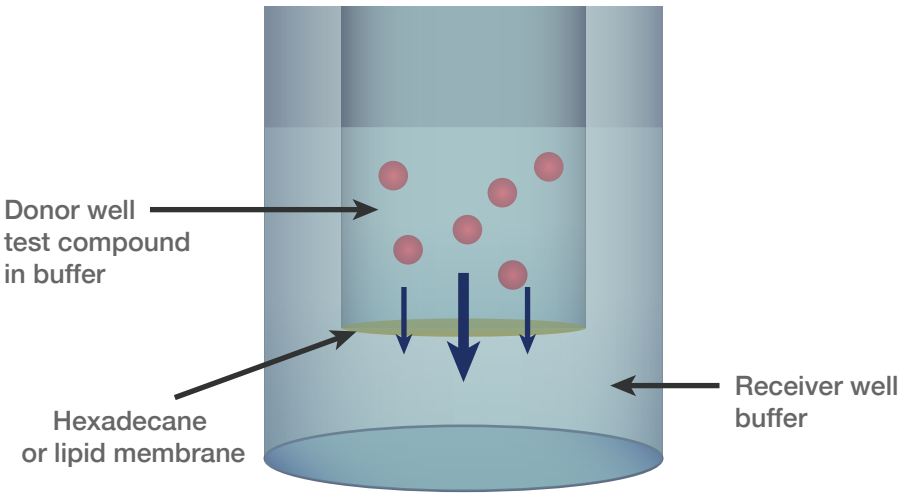
4.4.1. PAMPA

- **Background**
  The parallel artificial membrane permeability assay (or PAMPA) was first introduced by Kansy *et al.*, in 1998[4]. Along with the use of physicochemical properties, PAMPA is another early stage approach for estimating passive permeability. However, the absence of a cellular barrier containing relevant transporter proteins limits the predictive capability of PAMPA and reduces its widespread use.

- **Assay overview**
  An artificial hexadecane or lipid membrane is prepared in specially constructed 96-well plates (see Figure 4.1). Different lipid membranes can be employed to represent different organs e.g., brain lipid extracts can be used to represent passive permeability across the blood brain barrier. A typical protocol would involve the test compound being added to the donor compartment and, following an incubation at room temperature, the permeation of the compound across the membrane is quantified by a method such as LC-MS/MS or UV absorbance.

- **Data interpretation**
  PAMPA derives an apparent permeability co-efficient (P_{app}) for each compound which can be used to classify the permeability into high or low permeability categories. Lucifer yellow is often included to check membrane integrity. As lucifer yellow is a paracellular marker it should not pass through the PAMPA membrane.

Figure 4.1: PAMPA Concept



4.4.2. Caco-2 Permeability

- **Background**

  One of the most popular *in vitro* models for evaluating intestinal permeability is the Caco-2 (human colorectal adenocarcinoma cell line) assay. This assay is commonly employed during early discovery, especially in lead optimisation. Due to the expression and functional activity of several intestinal transporters such as P-gp and BCRP, the model is also often used for more in-depth later stage regulatory transporter studies[5,6,7,8].

- **Assay overview**

  The Caco-2 assay (see Figure 4.2) is typically performed in 96-well plates, whereby Caco-2 cells form a confluent polarised monolayer over a period of up to 21 days. Once confluent, the test compound is added to the apical compartment (representative of intestinal lumen) and the flux of the compound across the monolayer is monitored. Flux of the compound from the basolateral compartment (representative of the blood) to the apical compartment can also be assessed to determine whether the compound is subject to efflux. Moreover, inhibitors of efflux transporters, such as verapamil or fumitremorgin C, can be included in the incubation to determine whether a compound is a P-gp or BCRP substrate, respectively.

  When the incubation period has concluded the amount of compound that has permeated across the cells is measured by LC-MS/MS and P_{app} values are calculated. The permeability of lucifer yellow (paracellular transport), and transepithelial electrical resistance (TEER) measurements are used as markers of confluent monolayer integrity.

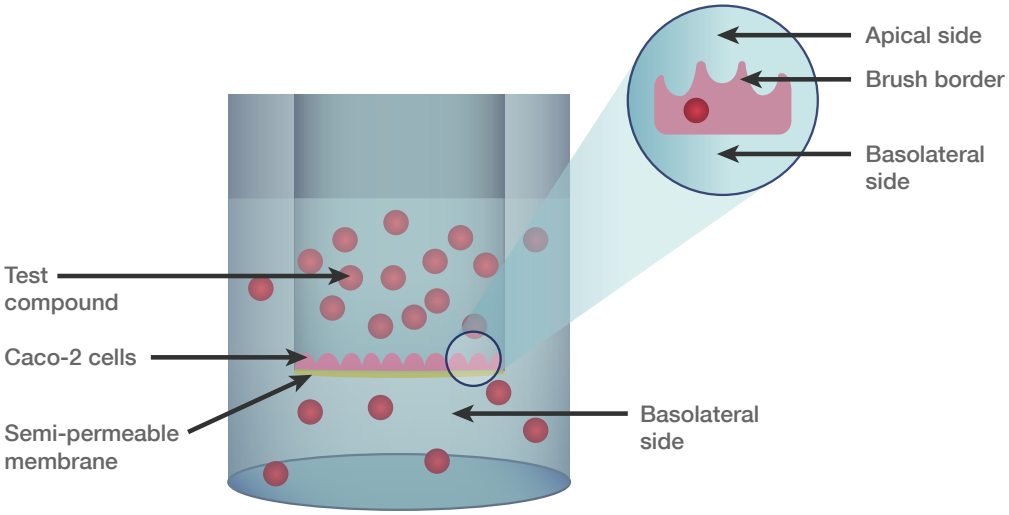
- **Data interpretation**

  Compounds can be ranked in terms of their Caco-2 P_{app} values and their potential human intestinal absorption. The relationship between Caco-2 permeability and human intestinal absorption is shown in Figure 4.3. Reference compounds can be screened alongside the test compounds and can be used as markers for ranking the test compounds by comparison of their P_{app} values. For example, atenolol (paracellular transport) and propranolol (passive transcellular transport) have known human intestinal absorption of 50% and 90% respectively[9,10]. If a compound has a P_{app} higher than atenolol yet lower than propranolol in the Caco-2 assay then the human intestinal absorption can be estimated to be between 50% and 90%.

  If a bidirectional assay is performed, Caco-2 permeability data can also be reported as an efflux ratio, i.e., P_{app} (basolateral–apical)/P_{app} (apical–basolateral). If the efflux ratio is greater than two, this indicates drug efflux is occurring, which can be confirmed using specific inhibitors. A known P-gp substrate, such as talinolol, is screened as a control compound to confirm that the cells are expressing functional P-gp efflux proteins. Efflux may indicate poor *in vivo* absorption.

  Recovery of the compound at the end of the incubation is a useful parameter for assessing quality of the data. Low recovery may indicate poor solubility, metabolism of the compound by the cells or issues with non-specific binding or retention of the compound in the monolayer.

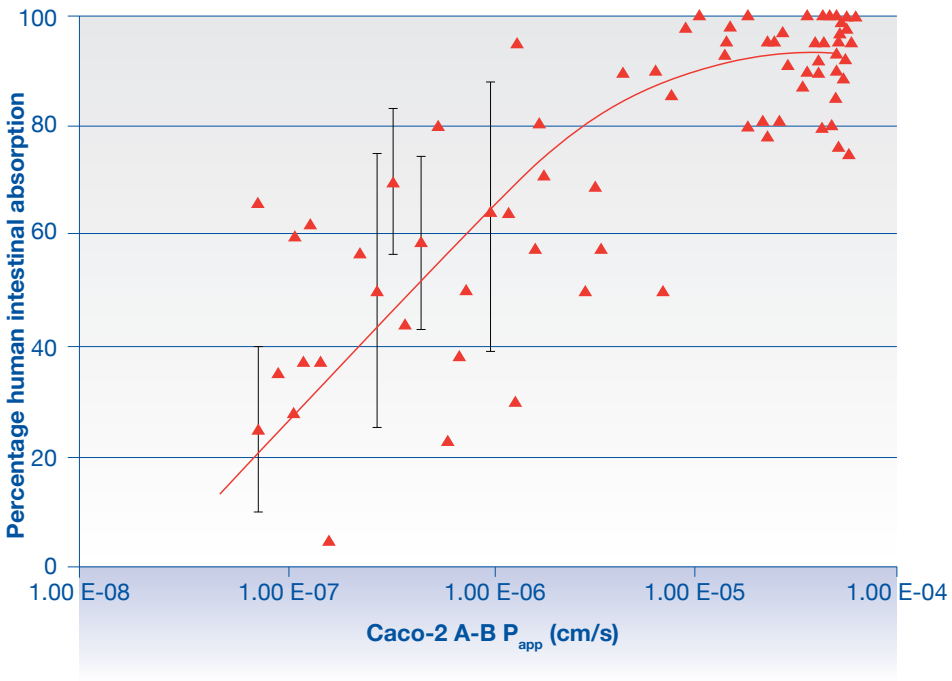
Figure 4.2: Schematic of the Caco-2 Permeability Assay



- **Relationship between Caco-2 permeability and human intestinal absorption**
  The relationship between Caco-2 permeability and human intestinal absorption is displayed in Figure 4.3.

Figure 4.3: Relationship between Caco-2 Permeability and Percentage Human Intestinal Absorption

Caco-2 data generated by Cyprotex. Human intestinal absorption data taken from Zhao *et al.* (2001)[9].



Owing to differing protocols and inter-individual differences, there is considerable variability in the observed clinical intestinal absorption values shown in Figure 4.3. Several compounds are also known to exhibit dose-dependent absorption (shown by the error bars on the graph). The relationship is not linear, but can be used to accurately group compounds into high, medium and low absorption potential categories for prioritisation.

4.4.3. Unstirred Water Layer and its Effect on Permeability

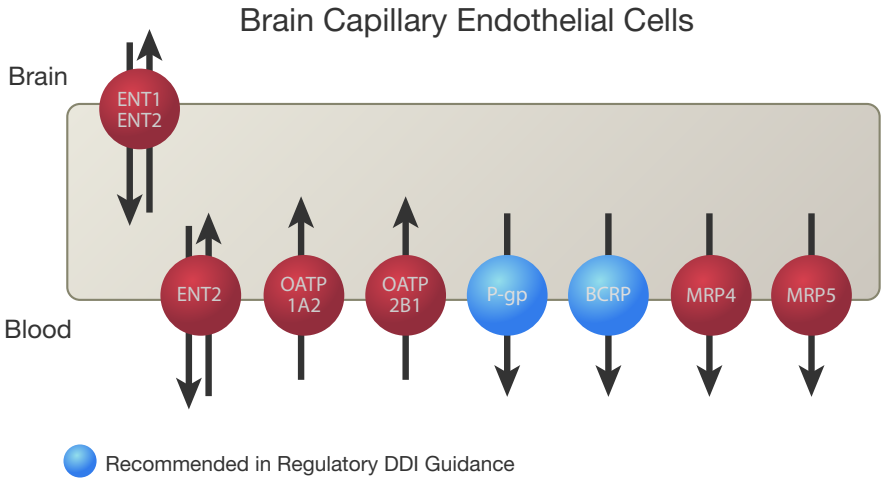
The unstirred water layer is an aqueous diffusion layer adjacent to the intestinal membrane which separates the brush border (apical) membrane of enterocytes from the fluid in the small intestine lumen. This layer may affect the permeability of some lipophilic molecules which are insoluble in aqueous media and diffusion may be impaired. The unstirred water layer is also present in cell based models such as the Caco-2 permeability model, and it has been shown that stirring increases the permeability of lipophilic drugs such as testosterone[11]. There is considerable debate on whether stirring should be adopted as a standard approach for *in vitro* permeability models. Despite this, the cost of implementing this adaptation and the impact on automation and throughput, as well as the fact that many of the lipophilic compounds affected would still be classed as highly permeable, has led to the majority of companies not adopting the stirring approach.

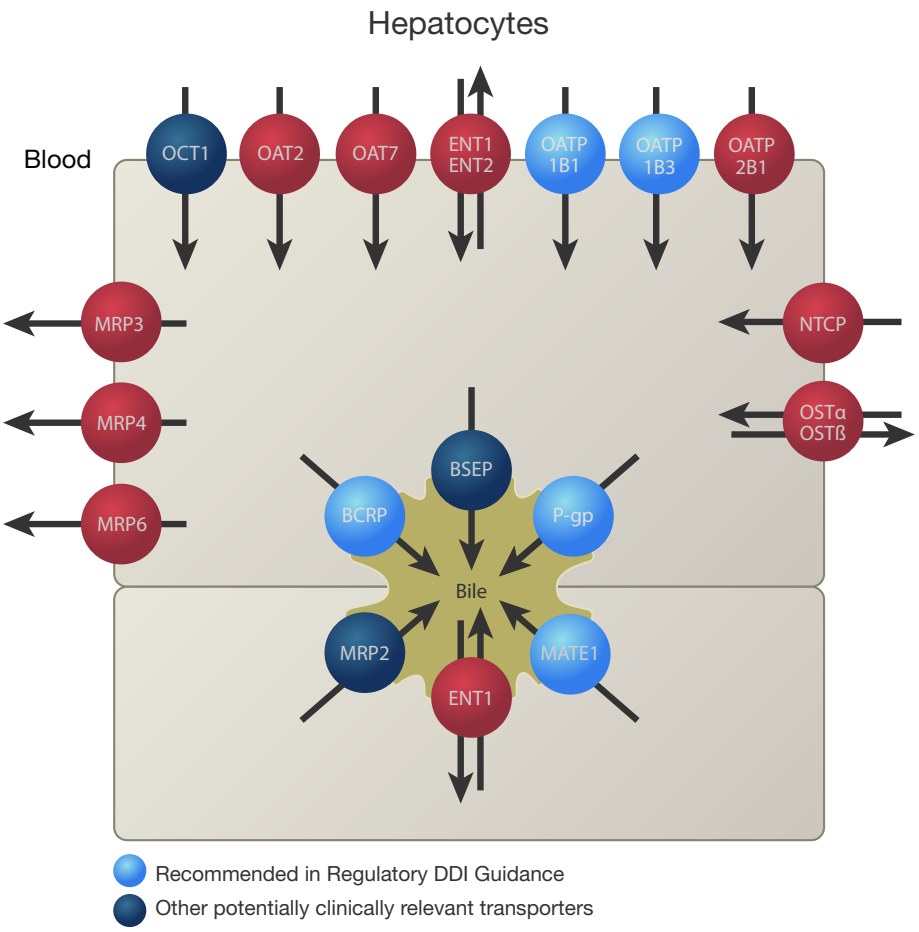
4.5. Drug Transporters

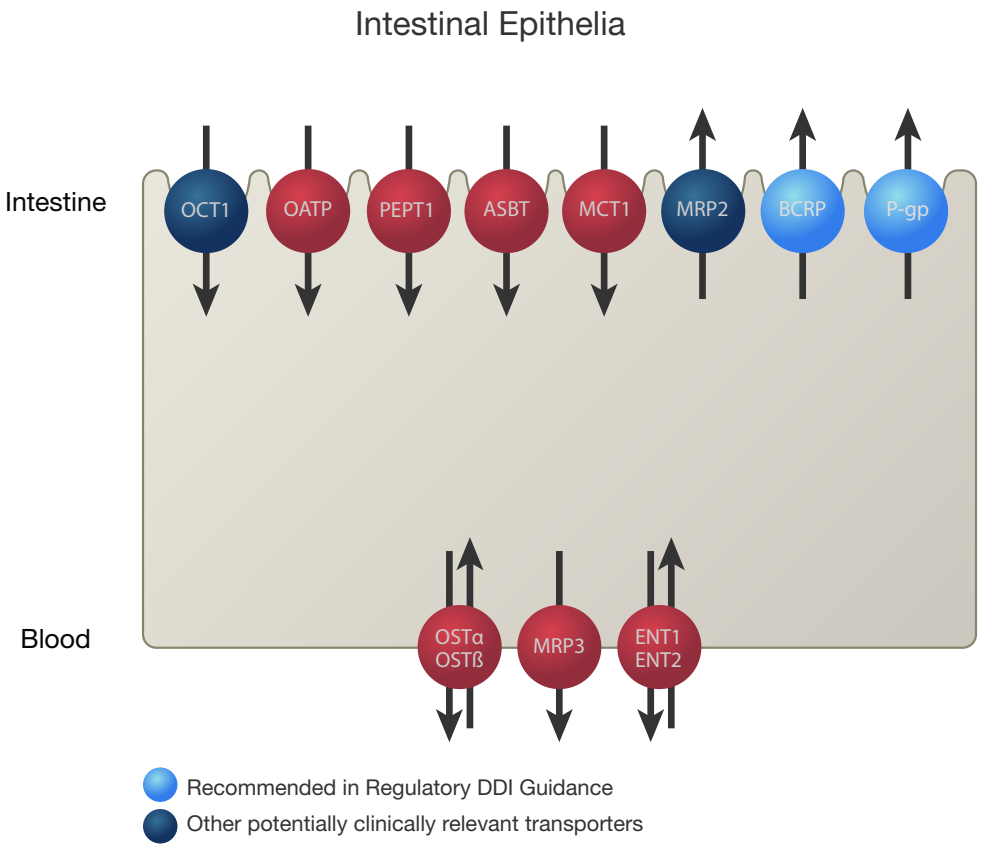
Many efflux and uptake drug transporters exist which can influence the permeability of compounds into specific cells within particular organs or tissues. These transporters exist in many tissues including, but not limited to, intestinal epithelia, hepatocytes and bile canaliculi, kidney proximal tubules and brain capillary endothelial cells.

The main drug transporters in these tissues are illustrated below:

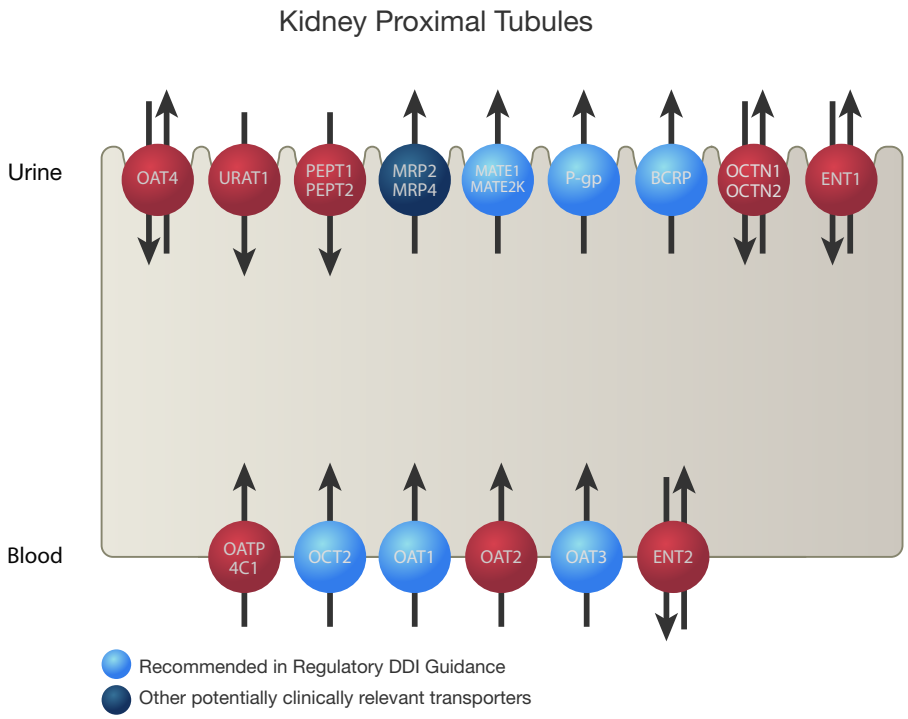
Figure 4.4: Key Drug Transporters in Brain Capillary Endothelial Cells, Hepatocytes, Intestinal Epithelia and Kidney Proximal Tubules.







cyprotex AN EVOTEC COMPANY



The industry is now becoming increasingly aware of the role of these transporters in clinically relevant drug interactions. The drug interaction guidelines from the FDA[6], EMA[7] and Japanese PMDA[8] provide recommendations on which transporters should be assessed for transporter substrate identification and inhibition studies. These include P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1 and MATE2-K which are the key clinically relevant transporters, all of which should be investigated for transporter inhibition studies[6,7,8]. For substrate studies, the decision on which transporters to evaluate is dependent on the extent of hepatic elimination (total hepatic metabolism + biliary secretion) and renal secretion. Additional transporters such as BSEP and OCT1 are also becoming increasingly important.

In addition to these broadly recommended transporter studies, there are a number of other potentially clinically relevant transporters which may be important for particular drug discovery programmes. These are discussed in ITC review papers published in March 2010[13] and July 2013[12].

4.5.1. Methods for Evaluating Drug Transport

4.5.1.1. Caco-2 Cell Line

More details on the Caco-2 permeability assay are provided in section 4.4.2.

- **Background**

  The efflux transporters, P-gp and BCRP, are functionally active in the Caco-2 cell line, and so this model is appropriate for determining P-gp and BCRP substrates and inhibitors.

- **Assay overview**

  Permeability is assessed across a polarised cell monolayer either from the apical to basolateral direction or vice versa, with efflux determined from the bidirectional assay.

- **Data interpretation**

  For substrate studies, if an efflux ratio is ≥2 then the transporter responsible for the efflux is identified by including an inhibitor of that transporter in the test compound permeability assay (e.g., verapamil as an inhibitor for P-gp or fumitremorgin C as an inhibitor for BCRP). The specific inhibitor should reduce the efflux ratio if the test compound is effluxed by that transporter.

  Transporter inhibition studies can also be performed using Caco-2 cells. For these studies, known probe substrates (e.g., loperamide or digoxin for P-gp, and estrone 3-sulphate for BCRP) are incubated at a single concentration (at or below the K_m) typically with a range of test compound (or potential inhibitor) concentrations to calculate an IC_{50} (inhibitor concentration which produces 50% inhibition). The relevance of the IC_{50} should eventually be determined by considering clinical data such as the dose and C_{max} levels and the concentration of inhibitor at the interaction site (intestine, hepatic inlet or plasma). Details of the calculations used are included in the EMA[7], the FDA[6] and the Japanese PMDA[8] drug interaction guidelines, and are discussed further in Chapter 5.

4.5.1.2. Transfected Cell Lines Overexpressing Transporter(s)

- **Background**

  Cell lines transfected with the transporter(s) of interest are popular tools for evaluating transporter interactions. These exist for all the main transporters recommended by the regulatory authorities. For example, MDR1-MDCK cells are a common choice for evaluating P-gp substrates and inhibitors. It must be noted that in the case of BCRP, polarised cell lines such as MDCKII-BCRP have relatively high levels of functional endogenous transport which need to be corrected for and it is generally considered that Caco-2 may be more suitable in this case[14].

- **Assay overview**

  The methods for the efflux transporter cell lines typically follow a similar protocol to the Caco-2 permeability assay where permeability is assessed across a polarised cell monolayer either from the apical to basolateral direction or vice versa, with efflux determined from the bidirectional assay. The wild type cell line can be used to identify any potential interference from endogenous non-human transporters (e.g., canine P-gp in the MDCK cells). However, it has been reported that, for MDR1-MDCK cells, the wild type, non-transfected parental cell line (MDCK) may not be a true background for the endogenous transporter activity in the transfected cell line due to reduced expression of canine P-gp in the MDR1-MDCK cells relative to that expressed in the MDCK wild type cells[15].

  For the SLC transporter assays, the transfected cell lines are grown in a monolayer on the base of wells in a plate and incubated with the test compound. At the end of the incubation, the amount of compound in the cells is quantified. Control cells (parental incorporated with empty vector) are used to correct the data for passive permeability or endogenous transporter processes. For substrate studies, data are presented as pmol/mg protein giving an uptake ratio for different incubation times, and transporter specific accumulation is confirmed using a known inhibitor.

  Both the efflux and SLC transporter cell lines can also be used for investigating transporter inhibition. For these studies, known probe substrates are incubated at a single concentration (below the K_m) typically with a range of test compound (or potential inhibitor) concentrations to calculate an IC_{50} (inhibitor concentration which produces 50% inhibition of the vehicle control transport activity). Some known OATP1B1/3 inhibitors demonstrate time dependent inhibition, therefore, pre-incubation with the investigational drug is advised[6].

- **Data interpretation**

  Generally, for efflux transporters (e.g., P-gp and BCRP), the test compound is considered to be a substrate for a transporter if the net flux ratio is ≥2 and is inhibitable by a known inhibitor.

  For the SLC transporters, the test compound is considered a substrate for a transporter if the uptake ratio is ≥2 and is inhibitable by a known inhibitor.

For transporter inhibition studies, an IC_{50} is typically calculated. The relevance of the IC_{50} should eventually be determined by considering clinical data such as the dose and C_{max} levels and the concentrations of the inhibitor at the interaction site (intestinal, hepatic inlet or plasma). Details of the calculations used are included in the EMA[7], FDA[6] and Japanese PMDA[8] drug interaction guidelines and described further in Chapter 5.

4.5.1.3. Inside-out Vesicles

- **Background**

  Inside-out membrane vesicles have the ATP binding and substrate binding site of the transporter facing the buffer on the outside of the vesicle. Often the vesicles are prepared from transfected cell lines which overexpress a single transporter. The vesicles are useful in identifying substrates or inhibitors of efflux transporters. Transport occurs in an ATP-dependent manner.

- **Assay overview**

  The uptake is initiated by the addition of ATP. A parallel incubation is performed in the presence of AMP or buffer alone (in place of ATP) to correct for passive permeability/non-specific binding. Ice cold buffer is used to stop the transport and filtration is used to separate the vesicles from the incubation solution. Vesicles are solubilised to release the compound trapped inside. The amount of compound released is quantified.

- **Data interpretation**

  ATP-dependent uptake activity is reported as 'Uptake activity in the presence of ATP' minus 'Uptake activity in the presence of AMP or buffer alone'. The uptake ratio is calculated as shown below;

$$\text{Uptake ratio} = \frac{\text{Uptake activity in transporter vesicles (+ATP)}}{\text{Uptake activity in transporter vesicles (+AMP or buffer alone)}}$$

For substrate studies, the test compound is considered a substrate for the transporter if the uptake ratio is ≥2 and is inhibitable by a known inhibitor.

For inhibition studies, the percentage of remaining vehicle control transport activity is reported as the 'Uptake activity in the presence of inhibitor' divided by the 'Uptake activity in the absence of inhibitor'. From this, an IC_{50} can be determined.

Table 4.1: Comparison of the Advantages and Disadvantages of the Different *in vitro* Models for Assessing Permeability and Drug Transporters

In Vitro Model	Advantage	Disadvantage
PAMPA	• High throughput, low cost option • Useful as front line screen for ranking passive permeability • Broad range of pH values can be studied	• Poorly predictive of human intestinal absorption • Ineffective at predicting transporter-based effects
Caco-2 permeability	• Human cell line • Both passive and active transport assessed • Predicts human intestinal absorption and BCS classification • Used in regulatory DDI studies	• Inter-laboratory variation in data • Long lead time for cell monolayer formation
Cell lines overexpressing transporters	• Typically express a single transporter which reduces need for specific substrates and inhibitors	• Need to assess control cells due to potential interference with endogenous transporters • Typically overexpression of single transporter so not representative of transporter interactions in the body • Poorly permeable compounds may not be able to cross basolateral membrane to gain access to efflux transporter in MDCK cells
Inside out vesicles (prepared from transfected systems)	• High throughput • High transporter expression can be achieved • Allows evaluation of "true" kinetic parameters as compounds have direct access to transport protein	• Difficult to determine transporter substrates with medium-to-high passive permeability • Interplay of multiple transporters cannot be assessed • Only applicable to efflux transporters

4.6. BCS (Biopharmaceutics Classification System)

Caco-2 permeability is one of the *in vitro* methods recommended for categorising drugs according to the BCS for determining the potential bioavailability of oral drugs. Originally proposed by Amidon *et al.*, (1995)[16], BCS divides drugs into 4 classifications based on their solubility and permeability.

BCS Categories

Class 1: High Permeability, High Solubility,

Class 2: High Permeability, Low Solubility

Class 3: Low Permeability, High Solubility

Class 4: Low Permeability, Low Solubility

The regulatory authorities have adopted BCS for biowaiver studies (waiver of *in vivo* bioavailability and/or bioequivalence studies for immediate release solid oral dosage forms)[17]. More information on BCS can be found in Chapter 2.

4.7. BDDCS (Biopharmaceutics Drug Disposition Classification System)

Originally proposed by Wu and Benet (2005)[18], the BDDCS is complimentary to the BCS scheme, and assists in identifying the importance of drug transporters in predicting drug disposition routes as well as potential drug-drug interactions following oral dosing. It is based on the assumption that highly permeable BCS Class 1 and 2 drugs are predominantly eliminated by metabolism in humans. Conversely, poorly permeable BCS Class 3 and 4 drugs are predominantly eliminated by renal and biliary excretion of unchanged drug. Furthermore, CYP3A4 metabolism is mainly observed for BCS Class 1 and 2 drugs rather than BCS Class 3 and 4 drugs.

By considering permeability rate, metabolism and solubility, transporter effects can then be predicted using the BDDCS scheme, and this is illustrated below in Figure 4.5. High metabolism assumes ≥70% metabolism, and low metabolism assumes <70% metabolism.

Figure 4.5: Overview of the BDDCS Classification System

	High Solubility	Low Solubility
High Permeability Rate/ High Metabolism	**Class 1** Transporter effects minimal in gut and liver	**Class 2** Efflux transporter effects predominate in gut, but both uptake and efflux transporters can affect liver
Low Permeability Rate/ Low Metabolism	**Class 3** Uptake transporter effects predominate (but can be modulated by efflux transporters)	**Class 4** Uptake and efflux transporter effects could be important

4.8. References

1 Kerns EH and Di L (2008) Drug-like properties: concepts, structure design and methods: from ADME to toxicity optimization. ISBN 0123695201 Academic Press.

2 Huttunen KM *et al.*, (2011) Prodrugs—from serendipity to rational design. *Pharmacol Rev* **63(3)**; 750-771

3 Cooper GM (2000) The cell: A molecular approach. 2nd Edition, Sinauer Associates

4 Kansy M *et al.*, (1998) Physicochemical high throughput screening: parallel artificial membrane permeability assay in the description of passive absorption processes. *J Med Chem* **41**; 1007-1010

[5] Elsby R *et al.*, (2008) Validation and application of Caco-2 assays for the *in vitro* evaluation of development candidate drugs as substrates or inhibitors of P-glycoprotein to support regulatory submissions. *Xenobiotica* **38(7-8)**; 1140-1164

[6] US FDA Guidance for Industry – In Vitro Drug Interaction Studies - Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions (Jan 2020)

[7] European Medicines Agency (EMA) Guideline on the Investigation of Drug Interactions (Adopted 2012)

[8] Japanese Pharmaceuticals and Medical Devices Agency (PMDA) Drug Interactions Guidelines (July 2018)

[9] Zhao YH *et al.*, (2001) Evaluation of human intestinal absorption data and subsequent derivation of a quantitative structure-activity relationship (QSAR) with the Abraham descriptors. *J Pharmaceut Sci* **90**; 749-784

[10] Yazdanian M *et al.*, (1998). Correlating partitioning and Caco-2 permeability of structurally diverse small molecular weight compounds. *Pharmaceut Res* **15**; 1490-1494

[11] Karlsson J and Artursson P (1991) A method for the determination of cellular permeability coefficients and aqueous boundary layer thickness in monolayers of intestinal epithelial (Caco-2) cells grown in permeable filter chambers. *Int J Pharmaceut* **71(1-2)**; 55-64

[12] Hillgren KM *et al.*, (2013) Emerging transporters of clinical importance: an update from the International Transporter Consortium. *Clin Pharmacol Ther* **94(1)**; 52-63

[13] The International Transporter Consortium (2010) Membrane transporters in drug development. *Nat Rev Drug Disc* **9**; 215–236

[14] Elsby R *et al.*, (2011) Validation of membrane vesicle-based breast cancer resistance protein and multidrug resistance protein 2 assays to assess drug transport and the potential for drug-drug interaction to support regulatory submissions. *Xenobiotica* **41(9)**; 764-783

[15] Li J *et al.*, (2013) Kinetic analysis of human and canine P-glycoprotein-mediated drug transport in MDR1–MDCK cell model: Approaches to reduce false-negative substrate classification. *J Pharm Sci* **102**; 3436-3446

[16] Amidon GL *et al.*, (1995) A theoretical basis for a biopharmaceutic drug classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability. *Pharm Res* **12(3)**; 413-420

[17] FDA Guidance for Industry. Waiver of *in vivo* bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a Biopharmaceutics Classification System (Dec 2017)

[18] Wu C-Y and Benet LZ (2005) Predicting drug disposition via application of BCS: transport/absorption/elimination interplay and development of a Biopharmaceutics Drug Disposition Classification System. *Pharm Res* **22**; 11-23

Chapter 5: Drug–Drug Interactions

5.1. Drug-Drug Interactions and their Consequences

Drug-drug interactions (commonly abbreviated to DDI) occur when one drug affects the pharmacokinetics or pharmacodynamics of a co-administered drug. Pharmacokinetic DDI are a significant safety concern as substantial changes in blood and tissue concentrations of the drug or metabolite can occur. These fluctuating exposure levels can alter the safety and efficacy profile of a drug and or its metabolites, especially for drugs with a narrow therapeutic index.

DDI are becoming increasingly important due to the aging population and the practice of polypharmacy within this population and also the administration of combination therapies in other conditions such as HIV and cancer. It is reported that DDIs from polypharmacy are responsible for approximately 26% of adverse events leading to hospitalisation[1].

DDI are a major regulatory hurdle which can lead to early termination of development, refusal of approval, dosage adjustments, prescribing restrictions or withdrawal of drugs from the market. The majority of the clinically relevant drug interactions occur through interactions with drug metabolising enzymes or drug transporters, and this chapter focuses predominantly on these interactions. However, it is important to note that other interactions can occur and include those related to plasma protein binding, food effects, pH effects on solubility, the effects of drugs on gastric emptying or intestinal motility and pharmacodynamic interactions.

One of the most well recognised examples of serious DDIs is that of the antihistamine, terfenadine (Seldane®) which was issued with a black box warning from the FDA in 1992 and was subsequently withdrawn from the US market in 1998. If terfenadine is administered with a CYP3A4 inhibitor (e.g., ketoconazole or erythromycin) or if the patient has liver disease, QT interval prolongation can occur which may result in the potentially fatal torsades de pointes[2]. Mibefradil (Posicor®), astemizole (Hismanal®) and cisapride (Propulsid®) were also withdrawn in subsequent years as a consequence of drug-drug interactions.

Table 5.1: Examples of Drugs Withdrawn from the US Market due to Serious DDI[3]

Drug	Date Withdrawn from US Market	Reason for Withdrawal
Terfenadine (Seldane®)	February 1998	QT interval prolongation when co-administered with CYP3A4 inhibitors
Mibefradil (Posicor®)	June 1998	Potent inhibitor of CYP3A4 and P-gp
Astemizole (Hismanal®)	July 1999	QT interval prolongation when co-administered with CYP3A4 inhibitors
Cisapride (Propulsid®)	January 2000	QT interval prolongation when co-administered with CYP3A4 inhibitors

Statins are also attracting considerable interest with respect to DDI due to their widespread use and the fact they are commonly co-administered with other drugs. Skeletal muscle abnormalities such as myalgia and rhabdomyolysis can occur with statins, which have been linked to both transporter-based and drug metabolism-based clinical drug interactions[4,5].

It was the realisation that DDI were a serious and potentially life-threatening issue that led to the first regulatory guidance documents introduced in the US and Europe in 1997. These original guidance documents focused predominantly on drug metabolism but subsequent versions included consideration of drug transporter interactions and other factors such as the role of genetic polymorphisms.

The latest versions of these guidelines[6,7], along with the Japanese PMDA guidelines[8], detail a number of *in vitro* assays which are designed to detect potential drug-drug interactions and identify if additional clinical studies are required prior to launch of the drug to market.

In terms of DDI, drugs are often described as 'victim' or 'perpetrators'. Perpetrators are drugs that inhibit or induce drug-metabolising enzymes and/or drug-transporting proteins. Victim drugs are those which are metabolised by drug metabolising enzymes and/or transported by drug-transporter proteins.

5.2. Drug-Drug Interactions associated with Drug Metabolism

When interactions occur, the activity of the drug metabolising enzymes can be either inhibited or induced. In the case of enzyme inhibition, the plasma levels of co-administered drugs may be increased leading to potentially exaggerated pharmacological effect or drug toxicity. In the case of enzyme induction, the metabolism of the drug itself or a co-administered therapy may be increased, leading to decreased plasma levels and the potential for reduced efficacy or the increased formation of a toxic metabolite.

The regulatory authorities suggest evaluating the potential for interactions for both cytochrome P450 (CYP) and non-CYP enzymes. The CYP enzymes play a major role in the metabolism of drugs and, because of the high specificity and low capacity of many of these enzymes, they are most likely to be involved in clinically relevant DDI. Other non-CYP pathways should also be characterised if they play a significant role in the metabolism of an investigational new drug. It is important to note that major metabolites should also be investigated for drug interaction potential.

5.2.1. Reaction Phenotyping

If a drug is metabolised, reaction phenotyping studies identify which enzyme is responsible for this metabolism. The methods used to study metabolic stability and reaction phenotyping are covered in Chapter 3.

Understanding which enzyme is involved in the metabolism of a drug is important in:

- identifying potential DDIs with concomitant medications which may be inhibitors or inducers of the same enzymes.
- establishing if the drug is metabolised by an enzyme which exhibits genetic polymorphisms as this may result in significant inter-individual variability. Examples of clinically important genetic polymorphisms in drug metabolism are illustrated in Table 5.2.
- determining if pharmacologically active metabolites are formed.
- identifying the extent of drug metabolism and the main metabolites formed.

Table 5.2: Clinically Important Genetic Polymorphisms of Drug Metabolism that Influence Drug Response[9].

Enzyme	Frequency of Polymorphism	Drug	Drug Effect
CYP2C9	14-28% (heterozygotes) 0.2-1% (homozygotes)	Warfarin	Haemorrhage
		Tolbutamide	Hypoglycaemia
		Phenytoin	Phenytoin toxicity
		Glipizide	Hypoglycaemia
		Losartan	Decreased antihypertensive effect
CYP2D6	5-10% (poor metabolisers) 1-10% (ultra-rapid metabolisers)	Antiarrhythmics	Proarrhythmic and other toxic effects
		Antidepressants	Toxicity in poor metabolisers, inefficacy in ultrarapid metabolisers
		Antipsychotics	Tardive dyskinesia
		Opioids	Inefficacy of codeine as analgesic, narcotic side-effects, dependence
		ß-adrenoceptor antagonists	Increased ß-blockade
CYP2C19	3-6% (whites) 8-23% (Asians)	Omeprazole	Higher cure rates when given with clarithromycin
		Diazepam	Prolonged sedation
Dihydropyrimidine dehydrogenase	0.1%	Fluorouracil	Neurotoxicity, myelotoxicity
Plasma pseudo-cholinesterase	1.5%	Succinylcholine	Prolonged apnoea
N-acetyltransferase	40-70% (whites) 10-20% (Asians)	Sulphonamides	Hypersensitivity
		Amonafide	Myelotoxicity (rapid acetylators)
		Procainamide, Hydralazine, Isoniazid	Drug-induced lupus erythematosus
Thiopurine methyltransferase	0.3%	Mercaptopurine, Thioguanine, Azothioprine	Myelotoxicity
UDP-glucuronosyl-transferase	10-15%	Irinotecan	Diarrhoea, myelosuppression

Reprinted from *The Lancet*, **356**, Meyer UA. Pharmacogenetics and adverse drug reactions, 1667-1671. Copyright 2000 with permission from Elsevier[9].

Initially, *in vitro* metabolism studies are usually performed prior to Phase I trials to understand the main metabolites formed. Defining differences between metabolites formed in preclinical toxicity species and humans can be important in defining preclinical safety testing of metabolites. In addition to toxicological considerations, identifying any pharmacological effects of major metabolites is also essential.

Understanding which enzymes are involved in the formation of the major metabolites is established through *in vitro* reaction phenotyping studies. These are described in more detail in Chapter 3. Human radiolabelled mass balance studies are often performed prior to phase III trials to understand the main elimination pathways and systemic metabolite exposure, and this information is combined with the *in vitro* data to corroborate metabolic pathways and the enzymes responsible.

Typically enzymes (CYP and non-CYP) involved in metabolic pathways estimated to contribute to ≥25% of drug elimination should be identified and, if possible, their *in vivo* contribution confirmed and quantified through either an interaction study with a potent selective inhibitor of the enzyme or through pharmacogenetic studies if a polymorphic enzyme is involved in the metabolism. If multiple enzymes are responsible for ≥25 % of its systemic clearance then the potential of complex DDI should be investigated.

Minor metabolic routes may be important in specific populations. For example, if the patient is hepatically or renally impaired, if the drug is metabolised by a polymorphic enzyme, or if the patient is taking medication that is a strong inducer of the minor pathway.

5.2.2. Enzyme Inhibition or Induction

Enzyme inhibition and induction studies identify the potential of an investigational drug (or its major metabolites) to inhibit or induce clinically relevant drug metabolism of other co-administered drugs, respectively. Further background information on enzyme inhibition and induction including an overview of the methods used is included in Chapter 3.

For enzyme inhibition and induction data, quantitative assessment of the *in vitro* data in conjunction with clinical pharmacokinetic data is used to determine if an *in vivo* clinical DDI study is required. The analysis can use a number of different models including basic, mechanistic static or dynamic (e.g., physiologically based pharmacokinetic – PBPK) models. The basic and mechanistic static models are derived from Bjornsson *et al.*, 2003[10] and Fahmi *et al.*, 2009[11].

5.2.2.1. Basic Models used to Quantify Potential Enzyme Inhibition or Induction

The basic models are practically the simplest to use but they are considered to represent the worst case scenario in terms of the risk of interaction and so further evaluation using a mechanistic static or dynamic model may be used if a positive result is obtained using the basic model. For the basic model, an R value cut-off is used to determine the likelihood of an interaction. The R value is the ratio of the intrinsic clearance of the probe substrate in the absence and presence of the interacting drug. The basic models are illustrated in Table 5.3.

Table 5.3: Basic Models for Reversible Inhibition, Time Dependent Inhibition and Induction.

Type of Interaction	FDA Guidance 2020	EMA Guidance 2012	Japanese PMDA Guidance 2018
Reversible Inhibition	Investigational drug likely to be a reversible inhibitor if: R_1 value ≥ 1.02 (or for CYP3A4 inhibitors given orally, R_1 value ≥ 11) $R_1 = 1 + \frac{[I]}{K_{I,u}}$ Where: $K_{I,u}$ is the *in vitro* unbound reversible inhibition constant. $[I] = [I_{max,u}]$ is the maximal unbound* systemic inhibitor concentration in plasma at steady state. Or for CYP3A4 inhibitors dosed orally, [I] is $[I]_{gut}$ = Molar Dose/250mL.	Investigational drug likely to be a reversible inhibitor if: $\frac{[I]}{K_i} \geq 0.02$ Where: [I] is the unbound* mean C_{max} obtained during treatment with the highest recommended dose. K_i is the *in vitro* reversible inhibition constant. Or for CYP3A4 inhibitors dosed orally, $\frac{[I]}{K_i} \geq 10$ Where: [I] is the maximum dose taken at one occasion/250mL.	Investigational drug likely to be a reversible inhibitor if: R_1 value ≥ 1.02 (or for CYP3A4 inhibitors given orally, R_1 value ≥ 11) $R_1 = 1 + \frac{[I]}{K_i}$ Where: K_i is the *in vitro* unbound reversible inhibition constant. [I] is the maximal unbound* systemic inhibitor concentration in plasma at the highest dose. Or for CYP3A4 inhibitors dosed orally, [I] is $[I]_{gut}$ = Molar Dose/250mL.
Time Dependent Inhibition	Investigational drug likely to be a time dependent inhibitor if: R_2 value ≥ 1.25 $R_2 = \frac{(k_{obs} + k_{deg})}{k_{deg}}$ AND $k_{obs} = \frac{(k_{inact} \times 50 \times [I_{max,u}])}{(K_{I,u} + 50 \times [I_{max,u}])}$ Where: k_{deg} is the apparent first order degradation rate constant of the affected enzyme. k_{obs} is the observed (apparent first order) inactivation rate of the affected enzyme. k_{inact} is the maximal inactivation rate constant. $K_{I,u}$ is the unbound inhibitor concentration which yields 50% of the maximum inactivation rate. $[I_{max,u}]$ is the maximal unbound* plasma inhibitor concentration at steady state.	Investigational drug likely to be a time dependent inhibitor if: R value ≥ 1.25 $R = \frac{(k_{obs} + k_{deg})}{k_{deg}}$ AND $k_{obs} = k_{inact} \times \frac{[I]}{(K_I + [I])}$ Where: k_{deg} is the apparent first order degradation rate constant of the affected enzyme. k_{obs} is the apparent inactivation rate constant. k_{inact} is the maximal inactivation rate constant. K_I is the inhibitor concentration which yields 50% of the maximum inactivation rate. [I] is the unbound* mean C_{max} obtained during treatment with the highest recommended dose. Or for CYP3A4 inhibitors dosed orally, [I] is the maximum dose taken at one occasion/250mL.	Investigational drug likely to be a time dependent inhibitor if: R_2 value ≥ 1.25 $R_2 = \frac{(k_{obs} + k_{deg})}{k_{deg}}$ AND *For systemic enzymes:* $k_{obs} = \frac{(k_{inact} \times 50 \times [I])}{(K_I + 50 \times [I])}$ *For CYP3A4 inhibitors dosed orally:* $k_{obs} = \frac{(k_{inact} \times 0.1 \times [I_{gut}])}{(K_I + 0.1 \times [I_{gut}])}$ Where: k_{deg} is the apparent first order degradation rate constant of the affected enzyme. k_{obs} is the apparent inactivation rate constant. k_{inact} is the maximal inactivation rate constant. K_I is the inhibitor concentration which yields 50% of the maximum inactivation rate. [I] is the maximal unbound* systemic inhibitor concentration in plasma at the highest dose. $[I]_{gut}$ = Molar Dose/250mL.
Induction	Investigational drug is a likely CYP inducer if: Fold increase in mRNA is ≥ 2. The increase in mRNA is greater than 20% of the response of the positive control. OR R_3 value ≤ 0.8 $R_3 = \frac{1}{[1+dx((E_{max} \times 10xI_{max,u})/(EC_{50}+(10xI_{max,u})))]}$ Where: $[I_{max,u}]$ is the maximal unbound* systemic inhibitor concentration in plasma at steady state. d is a scaling factor which is assumed as 1 for the basic model. EC_{50} is the concentration causing half maximal effect. E_{max} is the maximum induction effect. Correlation methods such as RIS (relative induction score) and $[I_{max,u}]/EC_{50}$ can also be used.	Investigational drug is a likely CYP inducer if: The drug gives rise to more than a 100% increase in mRNA which is concentration dependent. The increase in mRNA is greater than 20% of the response of the positive control. RIS (relative induction score) correlation method can also be used.	Investigational drug is a likely CYP inducer if: The drug gives rise to more than 100% increase in mRNA which is concentration dependent. The increase in mRNA is greater than 20% of the response of the positive control. OR R_3 value ≤ 0.8 $R_3 = \frac{1}{(1+dxE_{max} \times 10\ x[I]/(EC_{50}+10x[I]))}$ Where: [I] is the maximal unbound* systemic inhibitor concentration in plasma. d is a scaling factor which is assumed as 1 for the basic model. EC_{50} is the concentration causing half maximal effect. E_{max} is the maximum induction effect. Correlation methods such as RIS (relative induction score) and $[I_{max,u}]/EC_{50}$ can also be used.

*For calculation of the unbound C_{max}, the fraction unbound value should be set to 1% if the fraction unbound value is less than 1%.

5.2.2.2. Mechanistic Static Model

The mechanistic static model combines the net effects of enzyme inhibition (both reversible and time-dependent) and induction, and includes more detailed information such as bioavailability and fractional metabolism data. The equation is shown below:

$$AUC_R = \left(\frac{1}{[A_h \times B_h \times C_h] \times f_m + (1 - f_m)} \right) \times \left(\frac{1}{[A_g \times B_g \times C_g] \times (1 - F_g) + F_g} \right)$$

Where:

Type of Interaction	Gut	Liver
Reversible Inhibition	$A_g = \frac{1}{1 + \frac{[I]_g}{K_i}}$	$A_h = \frac{1}{1 + \frac{[I]_h}{K_i}}$
Time Dependent Inhibition	$B_g = \frac{k_{deg,g}}{k_{deg,g} + \frac{[I]_g \times k_{inact}}{[I]_g + K_i}}$	$B_h = \frac{k_{deg,h}}{k_{deg,h} + \frac{[I]_h \times k_{inact}}{[I]_h + K_i}}$
Induction	$C_g = 1 + \frac{d \times E_{max} \times [I]_g}{[I]_g + EC_{50}}$	$C_h = 1 + \frac{d \times E_{max} \times [I]_h}{[I]_h + EC_{50}}$

The individual parameters in the equations are determined in a similar manner to the basic equations with the exception of:

F_g is the fraction available after intestinal metabolism.

f_m is the fraction of systemic clearance of the substrate mediated by the enzyme that is subject to inhibition/induction

$[I]_h$ is the maximal unbound inhibitor/inducer concentration in portal vein ($[I]_u$, inlet, max).

$[I]_h = f_{up} \times ([I]_{max,p} + (((F_a \times F_g \times k_a \times Dose)/Q_h)/R_B))$ in FDA guidance or,
$[I]_h = f_{ub} \times ([I]_{max,b} + ((F_a \times F_g \times k_a \times Dose)/Q_h))$ in EMA and Japanese guidance taken from Ito *et al.*, 2002[12].

$[I]_g$ is the concentration of inhibitor in the enterocyte.

$[I]_g = F_a \times k_a \times Dose/Q_{en}$ taken from Rostami-Hodjegan and Tucker, 2004[13].

f_{ub} is the unbound fraction in blood; f_{up} is the unbound fraction in plasma

$[I]_{max,b}$ is the maximal total (free and bound) inhibitor concentration in the blood at steady state

$[I]_{max,p}$ is the maximal total (free and bound) inhibitor concentration in the plasma at steady state

F_a is the fraction absorbed after oral administration (a value of 1 can be used if data are not available)

k_a is the first order absorption rate constant *in vivo* and a value of 0.1 min^{-1} can be used if data are not available[14].

Q_{en} is blood flow through the enterocytes (e.g., 18L/hr/70kg taken from Yang *et al.*, 2007a[15])

Q_h is hepatic blood flow (e.g., 97L/hr/70kg taken from Yang *et al.*, 2007b[16])

R_B is the blood to plasma concentration ratio

If the AUC_R is >1.25 then this suggests inhibition or if the AUC_R is <0.8 then this indicates induction. If the AUC_R falls outside the range of between 0.8 and 1.25 then a dynamic model (e.g., PBPK) may be used as an alternative approach.

5.2.2.3. Dynamic Model (e.g., PBPK Model)

Physiologically based pharmacokinetic (PBPK) modelling is a method of dynamic modelling which can be used to quantitatively predict the magnitude of the drug-drug interactions and can provide an alternative to dedicated clinical trials. The model technique is also useful in improving the design of drug-drug interaction studies including dedicated trials and population PK studies.

The PBPK models integrate physiological information and drug-dependent parameters providing a more accurate and dynamic determination of the interactions to reflect the effect of the interacting drug on the entire pharmacokinetic profile of the substrate, and can be useful in understanding complex drug-drug interactions where a combination of mechanisms exist. When *in vivo* clinical pharmacokinetic data become available the model can be refined. PBPK models are built for the substrate and interacting drug and then linked together to simulate drug-drug interaction potential. PBPK modelling can also be valuable in determining the drug interaction potential of metabolites especially if the metabolites are major.

When using these models for the purpose of predicting potential clinical DDI, it is important to provide comprehensive details on model assumptions, physiological and biochemical plausibility, variability and uncertainty, to illustrate validity of the model.

According to the EMA[7], providing the model is suitably validated and the simulation shows <20% inhibition of the probe substrate with appropriate sensitivity analyses then it is assumed that the investigational drug does not cause significant interaction and no *in vivo* interaction study is required.

5.3. Drug-Drug Interactions associated with Drug Transporters

The focus on transporter-based DDI has increased dramatically over the past decade. Clinically relevant interactions have been demonstrated in the case of P-gp, BCRP, OATP, OAT, OCT and MATE transporters. Studying the DDI potential of these transporters using *in vitro* methods is therefore recommended to establish if clinical DDI studies are required. Other transporters, such as the MRP transporters, also may need to be evaluated if related drugs in the same class are known to be substrates or inhibitors. It is important to note that major metabolites of the investigational drug should also be evaluated for transporter DDI potential.

Table 5.4: Examples of Clinically Relevant Transporter Based Drug-Drug Interactions

Transporter	Victim	Perpetrator	Reference
P-gp	Digoxin	Ritonavir	Ding R *et al.*, (2004)[17]
BCRP	Topotecan Rosuvastatin	Elacridar Cyclosporine	Kruijtzer CM *et al.*, (2002)[18] Simonson SG *et al.*, (2004)[19]
OATP1B	Rosuvastatin	Cyclosporine	Simonson SG *et al.*, (2004)[19]
OAT	Furosemide	Probenecid	Li M *et al.*, (2006)[20]
OCT2	Metformin	Cimetidine	Somogyi A and Muirhead M (1987)[21]
MATE	Metformin Procainamide	Cimetidine Cimetidine	Somogyi A and Muirhead M (1987)[21] Somogyi A *et al.*, (1983)[22]

As with drug metabolising enzymes, genetic polymorphisms exist with the key transporters and have been implicated in several clinically relevant differences in exposure. For example polymorphisms in OATP1B1 and BCRP have been used to explain ethnic differences for statin exposure between Asian and Caucasian subjects[23]. If transporter polymorphisms are present and investigational drug levels are elevated, individuals may have an altered sensitivity to potential DDI via other pathways.

5.3.1. Transporter Substrate Identification Studies

These studies identify the potential of an investigational drug (or its main metabolites) to be a substrate of a clinically relevant transporter. Further background information on drug transporters including an overview of the methods used is included in Chapter 4.

The choice of transporters to assess is dependent on the renal and/or hepatic elimination of the drug. For example investigational drugs estimated to have ≥25% hepatic elimination (clearance by hepatic metabolism and biliary secretion totalling ≥25% of total clearance) should be evaluated *in vitro* to identify if they are potential substrates for OATP1B1 and OATP1B3. Furthermore, if renal active secretion is ≥25% of total clearance for the investigational drug then it is important to identify if the drug is a potential substrate for OAT1, OAT3, OCT2, MATE1 and MATE2-K. All investigational drugs should be assessed to determine if they are substrates for P-gp and BCRP. However, if the investigational drug is categorised as BCS (Biopharmaceutics Classification System) Class 1 (i.e., highly permeable and highly soluble) where the intestinal absorption is not rate-limiting then it may be acceptable to exempt such a drug from *in vitro* P-gp or BCRP (and potentially other transporter) substrate studies.

Currently, Caco-2 cells (for efflux transporters) or cell lines over-expressing the transporter of interest (both efflux and uptake transporters) tend to be the most common systems used.

For efflux transporters such as P-gp and BCRP, if the net flux ratio is greater than or equal to 2, and is inhibitable by a P-gp or BCRP inhibitor (i.e., reduction of flux ratio of >50% or to unity) then it can be assumed that the investigational drug is a P-gp or BCRP substrate, respectively.

For SLC (solute carrier) transporters such as OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1 and MATE2-K, *in vitro* substrate identification studies are performed to evaluate uptake into transporter-overexpressing cells compared to empty vector control cells. If the uptake of the investigational drugs into the transporter expressing cells is ≥2 compared to the control (empty vector) cells and is inhibitable by a known inhibitor (i.e., reduction of uptake by >50% to unity) then the investigational drug is assumed to be a substrate.

OATP1B1 and OATP1B3 substrate studies may be exempt if there is evidence for low active hepatic uptake of the drug. Active hepatic uptake is more likely if the investigational drug has low passive membrane permeability, has high hepatic concentrations in relation to other tissues, or if it is an organic anion and charged at physiological pH.

If the transporter has been identified and interactions are likely to be clinically relevant then an *in vivo* study with a potent (selective, if available) inhibitor is recommended. If the transporter is subject to genetic polymorphisms then a clinical pharmacogenetics study may be an alternative approach.

5.3.2. Transporter Inhibition Studies

Transporter inhibition studies identify the potential of an investigational drug (or its major metabolites) to inhibit clinically relevant transport of other co-administered drugs. All the guidelines recommend evaluating the inhibition potential of investigational drugs on P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3 and OCT2. More recently, MATE1, MATE2-K and OCT1 have also been routinely requested by the regulatory authorities. Inhibition of BSEP is also recommended if elevated bile acids levels are observed in the clinic. This is because the BSEP transporter is involved in biliary clearance of bile acids, and inhibition of this process may lead to cholestasis and potential hepatotoxicity. Further background information on drug transporters including an overview of the methods used is included in Chapter 4.

Caco-2 cells (for efflux transporters) or cell lines over-expressing the transporter of interest (for both efflux and uptake transporters) tend to be the most popular models, although vesicles are often used for some transporter (e.g., BSEP) inhibition studies.

Table 5.5 illustrates how the data from the transporter inhibition studies are interpreted. If the investigational drug is shown to be a likely inhibitor of the transporter then a clinical DDI study is recommended with a sensitive substrate.

Table 5.5: Interpreting Data from the Transporter Inhibition Studies

Transporter	FDA Guidance 2020	EMA Guidance 2012	Japanese PMDA Guidance 2018
P-gp and BCRP or any intestinal transporter	Investigational drug likely to be an inhibitor if: *Oral route* $\frac{[I]_{gut}}{IC_{50}\ (or\ K_i)} \geq 10$ *Metabolite or parenteral route* $\frac{[I]_1}{IC_{50}\ (or\ K_i)} \geq 0.1$ Where: $[I]_{gut}$ = dose of inhibitor /250mL. $[I]_1$ = C_{max} of metabolite or inhibitor	Investigational drug likely to be an inhibitor if: K_i value ≤ 0.1 fold the maximum dose on one occasion / 250mL (or maximum possible concentration at the pH range of the GI tract)	Investigational drug likely to be an inhibitor if: *Intestinal transporters* $\frac{[I]}{IC_{50}} \geq 10$ Where: [I] = dose of inhibitor /250mL.
Hepatic transporters (e.g., OATP1B1 or OATP1B3)	Investigational drug likely to be an inhibitor if: $1 + \frac{f_{u,p} \times [I_{in,max}]}{IC_{50}} \geq 1.1$ (R value*)	Investigational drug likely to be an inhibitor if: *For hepatic uptake (oral administration)* K_i ≤ 25-fold the unbound* maximum hepatic inlet blood concentration *For renal uptake or efflux, hepatic efflux and hepatic uptake (after iv administration)* K_i ≤ 50-fold unbound* C_{max}	Investigational drug likely to be an inhibitor if: $1 + \frac{f_{u,b} \times [I_{in,max}]}{K_i} \geq 1.1$ (R value*)
Renal transporters (e.g., OAT1, OAT3, OCT2, MATE1 or MATE2-K)	Investigational drug likely to be an inhibitor if: *OAT1, OAT3, OCT2, MATE1 and MATE2-K** $\frac{[I_{max,u}]}{IC_{50}} \geq 0.1$		Investigational drug likely to be an inhibitor if: *OAT1, OAT3 and OCT2* $1 + \frac{[I_{max,u}]}{K_i} \geq 1.1$ Investigational drug likely to be an inhibitor if: *MATE1 and MATE2-K* $1 + \frac{[I_{max,u}]}{K_i} \geq 1.02$

*For calculating the unbound values, the fraction unbound value should be set to 1% if the fraction unbound value is less than 1%.

$[I_{in,max}]$ (FDA; estimated maximum plasma inhibitor concentration at inlet to the liver) = $[I_{max}] + (((k_a \times Dose \times F_a \times F_g)/Q_h)/R_B)$

$[I_{in,max}]$ (PMDA; estimated maximum blood inhibitor concentration at inlet to the liver) = $[I_{max}] + ((k_a \times Dose \times F_a \times F_g)/Q_h)$

where,

$[I_{max}]$ is the maximum systemic plasma concentration of inhibitor

$[I_{max}]$ (PMDA for hepatic inlet calculation only) is the maximum systemic blood concentration of inhibitor

Dose = dose of inhibitor

F_a is the fraction absorbed (if unknown, use 1)

F_g is the intestinal availability (if unknown, use 1)

k_a is the absorption rate constant of the inhibitor (if unknown, use 0.1 min^{-1})

Q_h is the hepatic blood flow

R_B is the blood to plasma concentration ratio (if unknown, use 1)

5.3.3. Transporter Induction

There is a lack of validated *in vitro* models for testing transporter induction and as such regulatory authorities (e.g., FDA) do not currently recommend *in vitro* evaluation of investigational drugs as transporter inducers. However, CYP3A4 induction may be used to inform P-gp induction studies as both are induced by a similar mechanism (i.e., PXR activation).

5.4. Other Factors for Consideration in DDI Studies

- **Non-specific binding** – Only free (unbound) drug can interact with drug metabolising enzymes in the microsomal incubations. Indeed, it has been demonstrated that non-specific microsomal binding may result in an underestimation of inhibitor potency (i.e., overestimation of IC_{50} or K_i values) when dealing with lipophilic basic drugs[24,25] with the potential implication being an underestimation of risk from drug-drug interactions. This risk may be greater when dealing with mechanism-based inhibitors, due to the relatively high microsomal concentrations that are typically used during pre-incubation of the inhibitor in these experiments. Furthermore binding to the vessel is another factor to consider in understanding the unbound concentration of the inhibitor in drug metabolism and transporter based assays. It is recommended that the fraction unbound value for non specific binding is used to correct the IC_{50} or K_i value.

- **Plasma protein binding** – Plasma protein binding can affect the extent of free drug concentration which may influence binding to the enzyme and the drug's subsequent inhibitory potential. To relate the *in vitro* data to the clinical situation it is recommended that plasma protein binding is taken into consideration. In the guidelines[6,7,8] it also suggests that a fraction unbound less than 1% should not be used in the calculations due the uncertainty in determining accurate fraction unbound values below 1%. Under these circumstances the value should arbitrarily be set to 1%.

- **Cytotoxicity** – Understanding the potential for cytotoxicity may be important prior to cell-based assays. This is particularly important for assays such as CYP induction where incubation times extend over several days, and is less critical in assays performed over shorter incubation times such as the SLC transporter assays (typically minutes).

- **Aqueous solubility** – Establishing the solubility limit prior to the assays is recommended to prevent misleading data being generated and to assist with choosing the concentration range to assess.

- **Drug metabolism** – For CYP induction, the EMA and FDA guidelines[6,7] suggest measuring the concentration of parent drug in the medium at several time points on the last day of the incubation to determine the concentrations remaining in the incubations.

5.5. References

[1] McDonnell PJ and Jacobs MR (2002) Hospital admissions resulting from preventable adverse drug reactions. *Ann Pharmacother* **36(9)**; 1331-1336

[2] Rangno R (1997) Terfenadine therapy: Can we justify the risks? *Can Med Assoc* **157(1)**; 37-38

[3] Baxter K *et al.*, (2008) Drug-drug interactions. In *Drug Benefits and Risks: International Textbook of Clinical Pharmacology*. (Van Boxtel CJ *et al.*, Eds.) IOS Press; 247-262

[4] Kusuhara H *et al.*, (2013) *In vivo* characterisation of interactions on transporters. In *Transporters in drug development – discovery, optimization, clinical study and regulation.* (Sugiyama Y and Steffansen B, Eds) Springer Science and Business Media, New York; 67-98

[5] Elsby R *et al.*, (2012) Understanding the critical disposition pathways of statins to assess drug-drug interaction risk during drug development: It's not just about OATP1B1. *Clin Pharm Ther* **92**; 584-598

[6] US FDA Guidance for Industry – In Vitro Drug Interaction Studies - Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions (Jan 2020)

[7] European Medicines Agency (EMA) Guideline on the Investigation of Drug Interactions (Adopted 2012)

[8] Japanese Pharmaceuticals and Medical Devices Agency (PMDA) Drug Interactions Guidelines (July 2018)

[9] Meyer UA (2000) Pharmacogenetics and adverse drug reactions. *Lancet* **356**; 1667-1671

[10] Bjornsson TD *et al.*, (2003) The conduct of *in vitro* and *in vivo* drug-drug interaction studies: A Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. *Drug Metab Dispos* **31(7)**; 815-832

[11] Fahmi OA *et al.*, (2009) Comparison of different algorithms for predicting clinical drug-drug interactions, based on the use of CYP3A4 *in vitro* data: predictions of compounds as precipitants of interaction. *Drug Metab Dispos* **37(8)**; 1658-1666

[12] Ito K *et al.*, (2002) Which concentration of the inhibitor should be used to predict *in vivo* drug interactions from *in vitro* data? *AAPS PharmSci* **4(4)**; 53-60

[13] Rostami-Hodjegan A and Tucker GT (2004) *In silico* simulations to assess the *in vivo* consequences of *in vitro* metabolic drug-drug interactions. *Drug Discov Today Technol* **1**; 441-448

[14] Ito K *et al.*, (1998) Prediction of pharmacokinetic alterations caused by drug-drug interactions: metabolic interaction in the liver. *Pharmacol Rev* **50(3)**; 387-412

[15] Yang J(a) *et al.*, (2007) Misuse of the well-stirred model of hepatic drug clearance. *Drug Metab Dispos* **35(3)**; 501-502

[16] Yang J(b) *et al.*, (2007) Prediction of intestinal first pass drug metabolism. *Curr Drug Metab* **8(7)**; 676-684

[17] Ding R *et al.*, (2004) Substantial pharmacokinetic interaction between digoxin and ritonavir in healthy volunteers. *Clin Pharm Ther* **76**; 73-84

[18] Kruijtzer CM *et al.*, (2002) Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. *J Clin Oncol* **20**; 2943-2950

[19] Simonson SG *et al.*, (2004) Rosuvastatin pharmacokinetics in heart transplant recipients administered an antirejection regimen including cyclosporine. *Clin Pharmacol Ther* **76**; 167-177

[20] Li M *et al.*, (2006) Drug-drug interactions involving membrane transporters in the human kidney. *Expert Opin Drug Metab Toxicol* **2**; 505-532

[21] Somogyi A and Muirhead M (1987) Pharmacokinetic interactions of cimetidine. *Clin Pharmacokinet* **12**; 321-366

[22] Somogyi A *et al.*, (1983) Cimetidine-procainamide pharmacokinetic interaction in man: evidence of competition for tubular secretion of basic drugs. *Eur J Clin Pharmacol* **25**; 339-345

[23] Birmingham BK *et al.*, (2015) Impact of ABCG2 and SLCO1B1 polymorphisms on pharmacokinetics of rosuvastatin, atorvastatin and simvastatin acid in Caucasian and Asian subjects: a class effect? *Eur J Clin Pharmacol* **71**; 341-355

[24] Tran TH *et al.* (2002) Microsomal protein concentration modifies the apparent inhibitory potency of CYP3A inhibitors. *Drug Metab Dispos* **30(12)**; 1441-1445

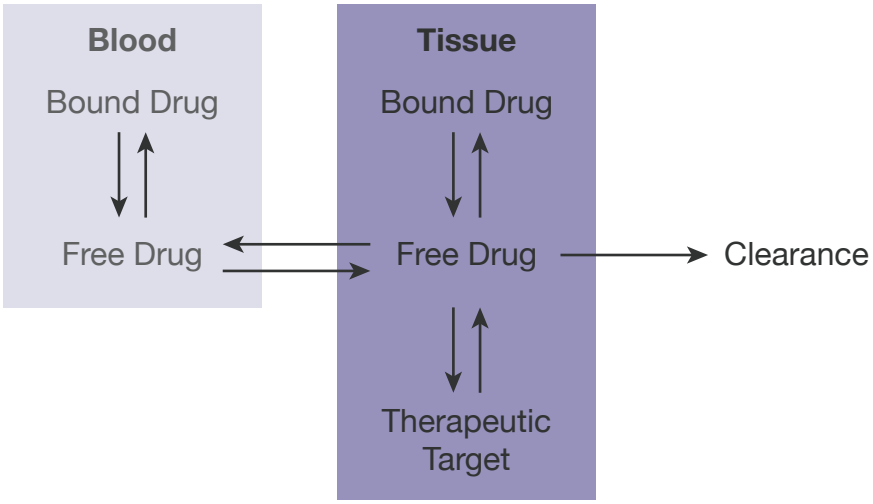
[25] Margolis JM and Obach RS (2003) Impact of nonspecific binding to microsomes and phospholipid on the inhibition of cytochrome P450 2D6: Implications for relating *in vitro* inhibition data to *in vivo* drug interactions. *Drug Metab Dispos* **31(5)**; 606-611

Chapter 6: Plasma Protein Binding

6.1. The Importance of Plasma Protein Binding

In many instances, it is only unbound drug which is available for passive diffusion to extravascular or tissue sites, only unbound drug which can bind to the therapeutic target and only unbound drug which can undergo excretory processes.

Figure 6.1: Simplified Schematic of Free Drug Hypothesis



The free drug hypothesis[1] assumes that only free (unbound) drug passes through membranes, that at steady state the free drug concentration is the same on both sides of any biomembrane, and that at the target site of action it is the free drug which exerts its pharmacological effect. The main exceptions include if the drug undergoes active transport, has poor passive permeability or has poor blood flow to the tissue.

Plasma protein binding can influence the therapeutic action and pharmacokinetics of a drug by retaining the drug in the plasma compartment, and can[2]:

- Affect the volume of distribution of the drug and influence distribution into tissues.
- Reduce the metabolic clearance by the liver and, in turn, increase the half-life.
- Limit the blood brain barrier permeability.
- Impact on the drug reaching the therapeutic target, thus reducing efficacy.
- Require changes to dosing regimens in terms of higher loading doses yet lower maintenance doses.

There is considerable debate on the value of optimising series of compounds based on plasma protein binding data in a discovery setting to improve the pharmacokinetics and efficacy of a molecule. It is generally thought that it is the free drug concentration at the target which is important for efficacy. As living organisms are dynamic systems with multiple physiological processes (e.g., drug transport and membrane permeability, tissue binding, drug metabolism and other clearance mechanisms) occurring simultaneously, *in vitro* methods may not reflect this complexity and may be misleading in terms of extrapolation from fraction unbound (Fu) measured *in vitro* to *in vivo* free drug concentration[1].

Despite this, plasma protein binding is still a widely used technique for:

- **Scaling for predicting *in vivo* clearance** – It is generally accepted that only unbound drug undergoes clearance mechanisms such as metabolism and renal excretion. The effect of plasma protein binding on clearance is dependent on the major route of clearance of the drug, and if it is hepatically cleared then also on the liver extraction ratio. For a drug with a high extraction ratio, metabolic clearance is less influenced by plasma protein binding whereas the metabolic clearance of a drug with a low extraction ratio could be significantly affected by plasma protein binding. With respect to renal clearance, most renally excreted drugs are hydrophilic and therefore have low plasma protein binding, so plasma protein binding may have limited impact on their rate of clearance[3].

- **Establishing human dose** – Fraction unbound is important for accurately predicting a starting dose for first in human studies based on the required unbound exposure or Css, unbound[3].

- **Determining cross species comparisons** – Species differences in plasma protein binding are important when predicting human pharmacokinetics from preclinical species data. This is especially important when differences are significant, and may be crucial in explaining differences in efficacy or potential toxicity. Table 6.1 illustrates species differences in plasma protein binding for 4 compounds. In the case of cefoperazone the fraction unbound differs by more than 13 fold between dog and monkey[4].

Table 6.1: Interspecies Differences in Plasma Protein Binding.
Data taken from Berry LM *et al.,* (2011)[4].

Drug	Rat Fu	Dog Fu	Monkey Fu	Human Fu
Cefazolin	0.13	0.43	0.058	0.091
Cefoperazone	0.36	0.38	0.029	0.066
Valproic acid	0.37	0.22	0.080	0.052
Topotecan	0.21	0.17	0.88	0.72

- **Understanding drug-drug interaction risk** – It is generally considered that the risk of clinically relevant interactions via displacement from plasma protein binding sites is low[5,6]. However, the EMA drug interaction guidelines[6] still recommend evaluating the potential for displacement interactions of drugs known to be markedly protein bound. This is particularly important for drugs which have a fraction unbound less than 1%, a narrow therapeutic window, a high hepatic extraction ratio (if administered IV) or a high renal extraction ratio. The fraction unbound value is also used to convert the total C_{max} to the unbound C_{max} in order to understand the relevance of *in vitro* drug interaction data for cytochrome P450 or transporter based interactions[6].

- **Establishing the effect of disease states or conditions** – Under certain conditions or disease states, levels (or binding capacity) of plasma proteins can alter dramatically. For example, in patients with severe inflammation (e.g., cancer patients), α_1-acid glycoprotein levels increase whereas human serum albumin levels decrease. In the case of the anti-cancer drug, etoposide, which is >95% bound to plasma proteins, a high degree of inter-patient variability in the free drug concentration has been reported[3].

- **Understanding the pharmacokinetics and efficacy of CNS therapies** – Determining a brain to plasma ratio value (K_p) in a PK study as a measure for predicting efficacy can be misleading as it does not reflect free drug concentrations in the brain[1,7]. The brain to plasma ratio (K_p) can be converted to the unbound K_p ($K_{p,uu}$) using the fraction unbound in plasma and brain tissue as described below[7]:

$$K_{p,uu} = K_p \times \left(\frac{fu_{brain}}{fu_{plasma}} \right)$$

$K_{p,uu}$ is more useful than K_p as it determines the extent of distribution equilibrium between the unbound fraction in brain and plasma. If the $K_{p,uu}$ is close to unity then this indicates passive diffusion across the blood brain barrier (or equal rates of efflux and influx). $K_{p,uu}$ <1 indicates efflux at the blood brain barrier and $K_{p,uu}$ >1 indicates uptake at the blood brain barrier[7].

- **Non linear pharmacokinetics** – If non-linear concentration dependent binding is observed over the range of therapeutic doses then it is difficult to relate total drug concentration to pharmacologically active and safe free concentrations. This often occurs when a drug has a high affinity for a specific binding site and levels of drug are high, leading to saturation of the binding site at increased doses, and limiting the concentration dependent increase in binding. This can be a particular challenge for drugs with a narrow therapeutic index. Examples of drugs exhibiting non-linear plasma protein binding include diflunisal,cephalosporin and trandolaprilat[3].

- **PBPK modelling approaches** – Plasma protein binding data can be used in conjunction with other *in vitro* parameters (including permeability, lipophilicity, metabolic clearance and solubility) to predict pharmacokinetics[8].

6.2. Major Drug Binding Plasma Proteins

The two major proteins to which drugs bind in blood are albumin (in humans this is called human serum albumin or HSA) and α_1-acid glycoprotein. Some of the more lipophilic drugs also bind to lipoproteins (γ-globulin) although these interactions are less common[2].

- **Human serum albumin** – HSA accounts for approximately 60% of the total plasma protein concentration and is present at concentrations of 500-750μM[2] in blood serum. It has a number of different binding sites and primarily binds strongly to acids (binding sites I, II and III). Basic and neutral drugs are also capable of binding to HSA binding sites IV, V and VI[2].

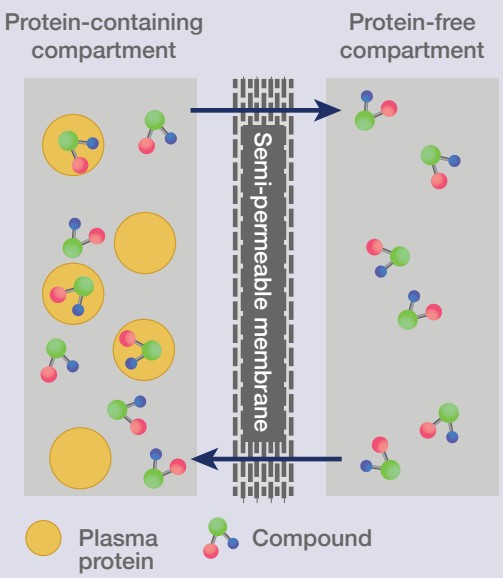
- **α_1-Acid glycoprotein (AAG)** – In healthy individuals, AAG is present in the blood at a concentration of between 12-30 μM[3], however in certain disease states this can increase over three-fold[2]. As well as levels of AAG fluctuating, binding to AAG can also be affected by factors such as gender, age, obesity, pregnancy, ethnicity, disease state and diurnal changes. AAG is a high affinity, low capacity plasma protein which typically binds basic drugs (e.g., amines) and one of its primary functions is to carry steroids around the body.

6.3. Methods used to Determine Plasma Protein Binding

Protein binding may be assessed by a number of methods including equilibrium dialysis, ultrafiltration, ultracentrifugation, gel filtration, and binding to HSA and AAG immobilised beads. The most popular methods are described in more detail below:

- **Equilibrium dialysis** – Equilibrium dialysis is generally considered to be the gold standard method for determining protein binding, and is one of the most commonly used. The method consists of two compartments, a protein free compartment typically containing buffer and a protein containing compartment typically containing plasma, or solutions of HSA or AAG. The compartments are separated by a semi-permeable membrane with a molecular weight cut-off to restrict the passage of proteins but allow the diffusion of drug molecules. The system is allowed to equilibrate over time at 37°C before measuring the concentration of drug in the individual compartments, and calculating the fraction unbound. This method has been shown to be accurate and reliable, and plate-based dialysis systems are now available which are compatible with high-throughput automated systems.

Figure 6.2: Schematic of Equilibrium Dialysis Method

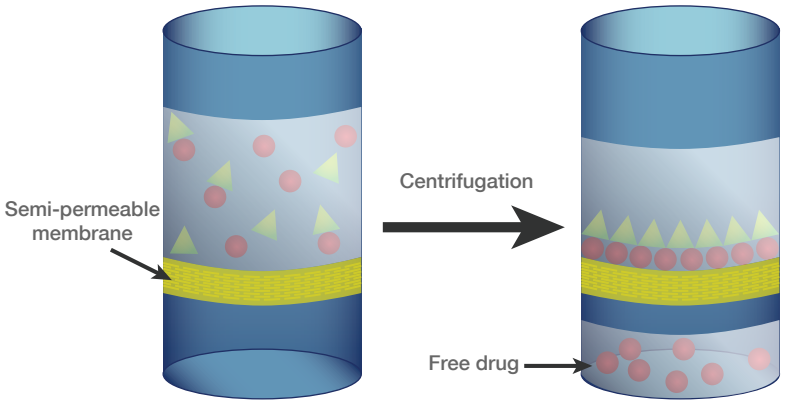


Method features:

- Constant physiological temperature (37°C)
- Non-specific binding is less of an issue with equilibrium dialysis methods as compared with filtration methods

- **Ultrafiltration** – Ultrafiltration devices incorporate membranes capable of separating free from plasma bound drug by centrifugal force. This technique is faster than equilibrium dialysis but is often seen as less reliable. The volume of the filtrate should not exceed 10% of the total sample volume to reduce the possibility of changes in binding caused by increases in concentration of the drug in the unfiltered material, and to maintain initial equilibrium conditions. Drawbacks of the method include membrane leakage and non-specific binding to the apparatus[3]. However in cases of poorly soluble compounds the presence of plasma in the initial solution may be beneficial in circumventing this problem.

Figure 6.3: Schematic of Ultrafiltration Method



Method features:

- Not temperature controlled
- Rapid (<1 hour)
- High throughput

- **Ultracentrifugation** – This approach has no membrane and involves centrifuging a drug-protein solution at high speeds (e.g., 500,000g) for long time periods to sediment the protein at the bottom of the tube. The supernatant is then analysed for drug free concentrations. Although issues such as non-specific binding are reduced, for higher molecular weight compounds sedimentation of the drug may occur. Contamination issues may also occur due to a floating lipid layer on the surface following ultracentrifugation[3].

6.4. Tissue Binding and Red Blood Cell Binding

- **Tissue binding:** Due to the higher protein content in tissue compared with plasma, tissue binding is thought to significantly influence the volume of distribution at steady state and the half-life of drugs. It is therefore important to understand tissue binding in order to determine the potential pharmacological and toxicological effects[3]. Equilibrium dialysis is typically used to evaluate the binding of drugs to tissue homogenate.

- **Red blood cell binding:** During preclinical and clinical studies, typically plasma is analysed for drug concentrations to understand the pharmacokinetics of the molecule. If the drug binds to red blood cells then the plasma levels will not be representative of those in the blood compartment and so the final pharmacokinetic parameters may be misleading. At blood to plasma ratios of greater than 1 (usually as a consequence of the drug distributing into the erythrocyte), the plasma clearance significantly overestimates blood clearance and could exceed hepatic blood flow. Blood to plasma ratio measurements can be performed to understand the partitioning between the red blood cell and plasma compartments. This involves incubating the blood with the test article, centrifuging at the end of the incubation to separate red blood cells from plasma and determining test compound concentration in each fraction. The ratio of the test article in the red blood cell fraction and the plasma is used to calculate the blood to plasma ratio. Alternatively whole blood protein binding determined using equilibrium dialysis can be compared with plasma protein binding data to understand if a difference in the fraction unbound is observed.

6.5. References

1 Smith DA *et al.*, (2010) The effect of plasma protein binding on *in vivo* efficacy: misconceptions in drug discovery. *Nat Rev Drug Disc* **9**; 929-939

2 Kerns EH and Di L (2008) Drug-like properties: Concepts, structure design and methods: from ADME to toxicity optimization. ISBN 0123695201. Academic Press

3 Bohnert T and Gan L-S (2013) Plasma protein binding: from discovery to development. *J Pharmaceut Sci* **102(9)**; 2953-2994

4 Berry LM, *et al.*, (2011) Species differences in distribution and prediction of human V_{ss} from preclinical data. *Drug Metab Dispos* **39(11)**; 2103-2116

5 Benet LZ and Hoener BA (2002) Changes in plasma protein binding have little clinical relevance. *Clin Pharmacol Ther* **71**; 115-21

6 The European Medicines Agency (EMA) Guideline on the Investigation of Drug Interactions (Adopted 2012)

7 Reichel A (2009) Addressing central nervous system (CNS) penetration in drug discovery: basics and implications of the evolving new concept. *Chem Biodiv* **6**; 2030-2049

8 Brightman FA *et al.*, (2006) Application of a generic physiologically-based pharmacokinetic model to the estimation of xenobiotic levels in rat plasma. *Drug Metab Dispos* **34**; 84-93

Chapter 7: Challenges and Future Outlook

7.1. Current Challenges in the Industry

ADME is a critical part of the drug discovery and development process, and focusing on optimising these properties at an early stage has helped to reduce late stage attrition associated with poor pharmacokinetics. Regulatory guidelines for areas such as drug-drug interactions and metabolite profiling have helped to standardise many of the *in vitro* protocols to allow clear guidance on how to interpret data. Despite this, challenges still exist in the extrapolation of *in vitro* data to the clinical setting.

Physiologically based pharmacokinetic models are now commonly used for integrating the *in vitro* and physicochemical data with the human physiology. Although these work well for a wide range of prototypical molecules, these predictions often fail where more complex pharmacokinetics or interactions occur. One area which is still proving challenging is the integration of drug transporter data within these models.

The field of drug transporters is evolving rapidly. These transporters play an important role in the disposition of drugs within the body which, in turn, determines drug concentrations in particular organs, tissues or cells within the body. Knowledge of these localised concentrations is critical for understanding efficacious effect at drug targets and/or organ specific toxicity. However, predicting these concentrations is difficult due to the vast number of transporters within the body and the fact our awareness of their presence and function is still developing.

Inter-individual variability associated with genetic polymorphisms, age, underlying disease or conditions, or polypharmacy often complicate the pharmacokinetics within the clinic and may play a role in unexpected lack of efficacy or idiosyncratic toxicity not seen within the general population. These factors add to the complexity and may restrict the ability to accurately predict *in vivo* pharmacokinetics in all sub-populations.

7.2. Future Outlook

There is an increasing trend towards the use of biologics including monoclonal antibodies, vaccines, gene therapies and recombinant proteins. Of the top 10-selling pharmaceuticals in 2014, 7 were biologics and 3 were small molecules[1].

Table 7.1: List of the Top Selling Drugs of 2014[1]

Drug	Indication	Sponsor	2014 Sales (USD)	Type
Humira (Adalimumab)	Autoimmune disease	AbbVie	$12.5 billion	Monoclonal antibody
Sovaldi (Sofosbuvir)	Antiviral	Gilead Sciences	$10.3 billion	Nucleotide analogue/ Small molecule
Remicade (Infliximab)	Autoimmune disease	Johnson & Johnson and Merck & Co	$9.24 billion	Monoclonal antibody
Rituxan (Rituximab)	Haematological cancers and autoimmune disease	Roche (Genentech) and Biogen Idec	$8.68 billion	Monoclonal antibody
Enbrel (Etanercept)	Autoimmune disease	Amgen and Pfizer	$8.54 billion	Recombinant fusion protein
Lantus (Insulin glargine)	Diabetes	Sanofi	$7.28 billion	Recombinant protein
Avastin (Bevacizumab)	Cancer	Roche	$6.96 billion	Monoclonal antibody
Herceptin (Trastuzumab)	Cancer	Roche	$6.79 billion	Monoclonal antibody
Adavir (Fluticasone and Salmeterol)	Respiratory disease	GlaxoSmithKline	$6.43 billion	Small molecules
Crestor (Rosuvastatin)	Hyperlipidemia	AstraZeneca and Shionogi	$5.87 billion	Small molecule

Typically, ADME of biologics is only considered after candidate selection during the development stage, and is primarily limited to *in vivo* pharmacokinetic evaluations[2] – a situation which resembles where we were with small molecules over 20 years ago. Poor cross-species extrapolation and off-target interactions are common issues identified with monoclonal antibody therapies at this stage. Drug-drug interactions have also been associated with therapeutic proteins, specifically cytokines, cytokine modulators and peptide hormones, due to their ability to potentially alter CYP and transporter mediated activity. Furthermore, delivery of these large molecules can prove problematic, and novel drug delivery techniques are now becoming more popular including the use of nanoparticles for the delivery of more challenging drugs.

The ADME properties of biologics and nanoparticles differ considerably to small molecules. Increasing our knowledge of the mechanisms involved in the ADME processes of large molecules/nanoparticles, and expanding the number of tools available for studying these processes will enable appropriate *in vitro* assays to be implemented at an earlier stage reducing the possibility of any later stage issues.

In terms of small molecules, new improved *in vitro* tools continue to be introduced. Enhancing clearance prediction especially in the case of low clearance compounds is likely to occur with the advent of new cell types (e.g., stem cells) and models (e.g., 3D cell culture) which may allow for longer term cultures where enzymatic activity is preserved. Flow systems where cell culture chambers are interconnected may also have advantages. In these systems, multiple tissues can be assessed simultaneously and exposed to both parent and formed metabolites (assuming a hepatic chamber is incorporated with metabolising capability). Integrating ADME with *in vitro* toxicity will assist in explaining metabolism-mediated toxicity, species related toxicity and exposure of drugs (and ultimately safety) in various situations or populations.

LC-MS/MS is advancing rapidly and the sensitivity and speed of analysing small molecules is constantly increasing. This technology is also now emerging as a valuable tool for qualitative and quantitative analysis of large molecules. Currently this process is fairly time consuming as sample preparation may require immunocapture or enzyme digestion, and sensitivity can be limited[2], however, as demand increases more automated processes are expected to be introduced to enable more rapid analysis.

PBPK (physiologically based pharmacokinetic) modelling approaches are likely to play an increasing role in extrapolating the *in vitro* data to the clinical situation, and the importance of these models is expected to grow as they become more sophisticated and advanced.

The introduction of early stage standardised *in vitro* ADME assays coupled with new advanced technologies in the field of mass spectrometry and *in silico* modelling have been highly successful in driving a reduction in a late stage attrition, ensuring safer and more efficacious drugs reach the market. Clearly the landscape is changing and new challenges lie ahead. Biologics are starting to take centre stage in the drug discovery and development process, and many of the traditional *in vitro* ADME assays will need to adapt to address this market. An increased focus on research and development in this field is required to establish translation of the *in vitro* data to the clinical data, which should lead to new industry guidance and established methods being introduced.

7.3. References

1 Philippidis A. The top 25 best-selling drugs of 2014. *Genetic Engineeering News* Feb 2015

2 Prueksaritanont T and Tang C. (2012) ADME of biologics – what have we learned from small molecules? *AAPS J* **14(3)**; 410-419

Cyprotex Europe
Tel (UK): +44 (0) 1625 505 100
No. 24 Mereside Alderley Park
Macclesfield Cheshire SK10 4TG UK

Cyprotex North America
Tel (USA): +1 888 297 7683
200 Staples Drive, 1st Floor
Framingham MA 01702 USA

Email: eduguide@cyprotex.com
Website: www.cyprotex.com

Read online: