

## Chapter 7: Biokinetics

### Introduction

The systemic toxicity of a chemical to an organism depends on: a) the external dose of the chemical; b) the exposure route; c) the absorption, distribution, metabolism and excretion (ADME) of the chemicals, which can be species-specific; and d) the intrinsic properties (biological activity/dynamics) of the chemical. These factors determine the critical concentration of a chemical at its target site(s) of action. Ideally, hazard and risk assessments should be based on the concentration–response curve(s) obtained at the target site(s), rather than the dose–response curve obtained for the whole organism.

This chapter describes the current status of non-animal methods for assessing barrier function (an important determinant of absorption and distribution) and metabolism. In addition, the use of biokinetic models is described, since these models provide a means of integrating information on ADME, to provide a means of estimating the target-tissue concentration of a chemical as a function of the external dose and time. Such information can be used in the design of *in vitro* and *in vivo* experiments for the testing of target organ and target system toxicity, thereby permitting the replacement of animal experiments, or the reduction and/or refinement of animal use. It must be emphasised that many of the biologically based parameters that need to be incorporated into biokinetic models can be obtained from *in vitro* experiments.

### Barrier Function

The following sections cover models of the skin, gastrointestinal and blood–brain barriers. Except in the case of the skin barrier, the permeability properties of the other barriers have been studied mostly in the context of pharmaceuticals rather than chemicals. However, it is important that knowledge about barrier properties obtained in the pharmaceuticals area is imported into the chemicals area.

### Current status of QSARs for membrane permeability

In the QSAR modelling of membrane penetration, it has generally been assumed that membrane penetration occurs by passive diffusion across lipid bilayers. Only recently have attempts been made to model active transport, such as the role of

P-glycoprotein in the oral absorption of drugs (1). For a molecule to pass through a biological membrane, it must have certain molecular attributes. Permeability is related to hydrophobicity, and  $\log P$  is the hydrophobicity descriptor employed in a number of QSAR models. Also, there is an upper limit on the size of the molecule that will pass through a membrane, which can be defined in terms of molecular mass, cross-sectional size, or another appropriate description of molecular bulk. The ability of a molecule to form hydrogen bonds is also known to be important, because hydrogen-bond formation slows passage through membranes (2, 3).

Most QSARs for membrane permeability have been developed in the context of drug development, to predict the likely bioavailability of candidate drugs following administration (4, 5). In principle, such QSARs are also applicable to the toxicity testing of chemicals, but they should be subjected to an independent evaluation of their mechanistic relevance and predictive performance, according to an approach similar to the one used in the validation of *in vitro* prediction models (6–8).

Table 7.1 provides a summary of the types of membrane permeability for which QSAR models are available, and some of the fundamental physico-chemical descriptors that have been found to be useful for predicting various types of permeability. Unfortunately, there are no recent reviews that encompass the whole area of QSARs for membrane permeability. Numerous studies have been aimed at the development of QSARs for skin penetration, and these have recently been reviewed by Moss *et al.* (9). An example is the following QSAR for skin penetration (10):

$$\log K_p = 0.77 \log P - 0.0103 MW - 2.3$$

(Equation 7.1)

$$n = 107, s = 0.394, r = 0.93, r^2 = 0.86$$

in which  $\log K_p$  is the logarithm of the permeability coefficient across excised human skin,  $\log P$  is the octanol–water partition coefficient, and MW is the molecular mass.

The QSAR models listed in Table 7.1 provide quantitative predictions of permeability. In certain circumstances, a qualitative prediction may be sufficient (i.e. a prediction of whether a compound is, or is not, likely to be absorbed). Lipinski *et al.* (11) proposed the so-called “rule-of-five” for identifying drugs that would have poor oral absorption. This rule states that poor absorption is likely when any two of the following conditions are satisfied: a)

**Table 7.1: An overview of QSARs for membrane permeability**

Membrane/barrier	Significant physicochemical descriptors	Reference
Cell membrane	Linear-free-energy-relationship descriptors; in particular, hydrogen-bond acidity	Platts (92)
Caco-2 cells	Polar surface area, hydrophobicity Hydrophobicity, hydrogen-bonding ability Polar surface area	Egan <i>et al.</i> (93) Österberg & Norinder (94) Stenberg <i>et al.</i> (95)
Gastrointestinal tract	Polar surface area, hydrophobicity Hydrophobicity, hydrogen-bonding ability Linear-free-energy-relationship descriptors	Egan <i>et al.</i> (93) Österberg & Norinder (94) Zhao <i>et al.</i> (96)
Skin	Hydrogen-bonding ability Hydrophobicity, molecular size	Abraham <i>et al.</i> (2) Barratt (97) Cronin <i>et al.</i> (10) Kirchner <i>et al.</i> (98)
Cornea	Hydrophobicity and molecular size	Worth & Cronin (99)
Blood-brain barrier	Hydrophobicity, hydrogen-bonding ability, molecular size Hydrophobicity, hydrogen-bonding ability, molecular size Polar surface area, hydrophobicity, hydrogen-bonding ability Hydrophobicity, hydrogen-bonding ability, molecular size Hydrophobicity, hydrogen-bonding ability	Clark & Pickett (100) Duffy <i>et al.</i> (101) Fehler <i>et al.</i> (102) Österberg & Norinder (94) van de Waterbeemd <i>et al.</i> (1)
Alveolar membrane of the mammary gland	Many, including hydrophobicity, acid dissociation constant and molecular size	Agatonovic-Kustrin <i>et al.</i> (103)
Poly(dimethylsiloxane) (Silastic) artificial membrane	Hydrogen-bonding ability	Cronin <i>et al.</i> (104)

QSAR = quantitative structure-activity relationship

molecular mass > 500; b) log P > 5.0; c) number of hydrogen-bond donors > 5; and d) number of hydrogen-bond acceptors > 10. A similar scheme has been proposed by Tice (12) for agrochemicals. Similarities have been found between the rule-of-five and the properties associated with skin permeability (10). It is therefore likely that rules similar to the rule-of-five could be defined for a larger range of membrane types and for chemicals other than drugs.

Simple decision rules, such as the rule-of-five, have been successfully used to identify candidate drugs that are likely to be poorly absorbed, and therefore to be of little therapeutic benefit. For toxicological purposes, it would be more appropriate to invert the rule-of-five. For example, a possible "inverse Lipinski" rule could state that good absorption is likely when any four of the following conditions are satisfied: a) molecular mass ≤ 500; b) log P ≤ 5.0; c) number of hydrogen-bond donors ≤ 5; and d) number of hydrogen-bond acceptors ≤ 10.

### Current status of percutaneous absorption testing

The passage of a chemical across the skin is determined by the physicochemical properties of the chemical and the permeability properties of the *stratum corneum*, the outer layer of the epidermis, which consists of non-viable keratinocytes. Penetration across the skin and into the systemic circulation may result in lethal or sublethal toxic effects, and for this reason, the assessment of percutaneous absorption for regulatory purposes is often carried out in the context of acute dermal toxicity testing. It should be noted, however, that the acute dermal toxicity test does not provide quantitative information on the amount of a chemical that crosses the skin barrier, but merely a gross indication of whether a sufficient amount crosses to cause systemic toxicity. Therefore, percutaneous absorption testing may also need to be carried out, to quantify the amount of a chemical that enters the systemic circulation.

A variety of methods are available for assessing skin penetration, including *in vivo* and *in vitro* methods, as well as QSAR models.

*In vivo* methods, which provide biokinetic information on the whole-body distribution of the chemical, have involved many animal species, although the rat has probably been used most frequently (13). The animal is killed at the end of the experiment, and the extent of percutaneous absorption is estimated from the known amount of chemical applied to the skin, and from determinations of the total amount excreted and of the amounts left on the skin and in the body. An *in vivo* method is described in draft OECD Test Guideline 427 (14).

*In vitro* methods for skin absorption (15) measure the diffusion of chemicals across excised (human or animal) skin, which may be of full or partial thickness. These methods can be based on the use of non-viable skin to measure diffusion only, or on the use of fresh, metabolically active skin to assess diffusion, taking skin metabolism into account. An *in vitro* method is described in draft OECD Test Guideline 428 (16). There is good evidence that *in vitro* data are predictive of both human and animal data (17, 18). Nevertheless, for some classes of chemicals, poor *in vitro/in vivo* correlations have been reported (for example, 19). *In vitro* methods for percutaneous absorption were reviewed by Howes *et al.* (13). A draft OECD Guidance Document for the Conduct of Skin Absorption Studies (20) describes the circumstances in which the use of the *in vitro* method would be appropriate.

Following agreement by the OECD national coordinators of the Test Guidelines Programme in May/June 2001, revised versions of OECD Test Guidelines 427 and 428, and of the technical guidance document, were adopted by an OECD Joint Meeting in May 2002.

### Current status of gastrointestinal barrier testing

The gastrointestinal barrier plays a dual role in the organism: to protect against toxic substances; and to transfer nutrients and xenobiotics (i.e. dietary products and drugs) from the lumen to the blood. Since the oral route of administration is the most common, knowledge of the absorption and metabolism of a chemical at the level of the intestinal mucosa is important, because these processes affect the bioavailability of a chemical, which is defined as the fraction of an oral dose that reaches the systemic circulation (21).

Absorption across the intestinal lining includes passive diffusion between cell junctions (paracellular transport), passive diffusion across the cell membrane and cytoplasm (transcellular transport), or uptake by means of a transporter (active transport). Uptake transporters include the sodium-

dependent bile transporter, peptide transporters, the glucose transporter, and organic anion transporters. Export transporters, including ATP-dependent export pumps (22), P-glycoprotein (P-gp) and multi-drug resistance proteins 1 and 2 (MRP1 and MRP2), represent well-characterised transporters in the apical membrane of the intestinal mucosal epithelium, which actively pump substrates back into the intestinal lumen after they have been absorbed into the intestinal epithelial cells (22, 23).

The current status of *in vitro* models of the intestinal barrier has recently been reviewed by Le Ferrec *et al.* (24). Table 7.2 provides a summary of the methods currently available for investigating the principal mechanisms of absorption, grouped as *in vivo*, *in situ* and *in vitro* methods (25–28).

The advantage of *in vivo* models is that they integrate all the factors that can influence chemical partitioning. The disadvantage is that it is very difficult to separate these variables during absorption. The advantage of *in vitro* models over *in vivo* models is that it is possible to study the mechanisms of absorption *per se*, and to by-pass stomach and liver metabolism.

ECVAM is funding a study on the development and refinement of an *in vitro* Caco-2 cell model of intestinal barrier function. This system consists of a cell monolayer across which a test chemical can be transferred. The chemical is usually applied as a solution in the donor compartment, and the amount transferred to a receptor compartment is measured. The goals of the ECVAM study are to standardise the use of Caco-2 cells for various purposes (absorption, metabolism and toxicity studies), and to improve and optimise the model with respect to the use of more-refined markers of intestinal function.

### Current status of blood–brain barrier testing

The blood–brain barrier (BBB) is formed by the brain-capillary endothelial cells and associated structures, and separates the brain parenchyma and the cerebrospinal fluid from the systemic circulation. It restricts the passage of chemicals from the circulating blood to the central nervous system. The brain capillaries are characterised by very close cell–cell contacts (tight junctions) that minimise the penetration of substances by the paracellular route. Chemicals must therefore penetrate by the transcellular route. Several transporters at the BBB play an important role in restricting the penetration of chemicals, by removing them from the brain and transferring them to the systemic circulation. Consequently, some chemicals cannot achieve concentrations in the brain high enough to exert their potential biological activities (29–31).

**Table 7.2: An overview of gastrointestinal barrier models**

<b>Models</b>	<b>Advantages</b>	<b>Limitations</b>
<b><i>In vivo</i></b>		
Rat model	Integrates all factors that can influence drug dissolution Reflects the human situation in respect to paracellular space Measures bioavailability	Animal model Differences regarding metabolism are quite common Difficult to dissect variables in the absorption process
Administration of specially designed capsules to human volunteers	Permits regional absorption studies Human model Regulatory authorities are suggesting the importance of this technique in developing sustained-release products	Not appropriate when there are limited toxicological data available on the compound
<b><i>In situ</i></b>		
Perfused rat preparations	Integrates passage and metabolism aspects All physiological factors that affect passage are present Studies of direct effects of the drug on intestinal absorption are possible Permits preliminary screening	Animal model The increase of luminal hydrostatic pressure during experiment can influence intestinal permeability Disappearance of compounds usually studied, rather than appearance of compounds
Balloon technique in humans	It is a reference technique Many physiological factors that influence passage are present Allows studies in humans Possible to study compounds secretion into the intestinal lumen	A difficult technique Expensive technique Not used in development and not used routinely Disappearance of compounds usually studied and it is less sensitive for studies on low-permeability compounds Introduce non-physiological conditions in the part of the intestine studied
<b><i>In vitro organotypic</i></b>		
Gut sac of the rat small intestine	All cell types and mucus layer are present Relatively fast and inexpensive technique Useful for mechanism of absorption and formulation studies Measures absorption at different sites in the small intestine	Animal model Not a perfused model The drug must cross the whole intestinal wall Low tissue viability (up to 2 hours)
Isolated and perfused segments	Possibility to study specific functions at the organ level Physiological cell-cell contact and native intracellular matrix are present	Short duration of experiments because of cell alterations
Ussing chambers	A human and animal model Drug absorption and passage at specific intestinal sites are possible The test drug can be added on the apical and/or the basolateral side Metabolic studies are possible Useful to study substances having local-pharmacological and transported-mediated effects Possible to study electrophysiological parameters of the intestinal barrier	Cell viability is limited Availability of human material is limited Not used for screening
<b><i>In vitro cell model</i></b>		
MDCK cells	Fast and simple method Can be used for screening testing Can be used for measurement of passive diffusion	Not an intestinal model Not of human origin Physiological factors that influence passage are not present (mucus, bile salts, cholesterol) A static model A model with only one cell type

MDCK = *Madin-Darby canine kidney*

**Table 7.2: continued**

Models	Advantages	Limitations
Caco-2 cells	Human cells Display the majority of morphological, transport and permeability features of differentiated intestinal cells Relatively fast and simple method A flexible model Permit mechanistic transport studies Drug can be added at the apical or basolateral side Permit drug screening testing Predicts passive human passage Useful for ranking drugs according to permeability; using reference compounds it is also possible to predict fraction absorbed for passively transported compounds Already used during drug discovery Well-established model Good availability of “reference” data	Physiological factors that could influence passage are not present (mucus, bile salts, cholesterol) A static model Cells have tumoural origin A model with only one cell type Low levels of CYP3A4
TC7 cells (derived from Caco-2 cell line)	Express higher levels of CYP3A4 Grow faster than Caco-2 cells	Physiological factors that could influence passage are not present (mucus, bile salts, cholesterol) A static model Cells have tumoural origin Model with only one cell type
HT29-18-C1 cells	Less transepithelial electrical resistance than Caco-2 Paracellular transport more similar to small intestine (but still lower)	Not well established
HT29-MTX/Caco-2 co-cultures	Cell model containing mucus-secreting cells	Not well established
2/4/A1 (conditionally immortalised epithelial cell line)	High permeability of the paracellular space Gives similar correlation with fraction absorbed as human jejunal permeability	Rat cell line Not well established
Primary isolated epithelial cells		Difficult to culture Limited viability Loss of <i>in vivo</i> anatomical and biochemical features

At present, there are no scientifically validated models of the BBB. The methods available can be classified into three groups — *in vivo* methods, primary cell cultures, and cell lines (Table 7.3). Several *in vitro* models have been designed to reproduce the physical and biochemical behaviour of the intact BBB; however, most of them lack some of the features of the *in vivo* barrier (32–34).

An ECVAM study on *in vitro* models for the BBB has recently been completed. The study included the characterisation and evaluation of: a) immortalised BBB-derived endothelial cell lines (the rat-derived cell line, SV-ARBEC, and the mouse-derived cell line, MBEC4); b) cell lines not derived from the BBB (MDCK, Caco-2 and ECV-C6 cells); and c) primary cells derived from the BBB (bovine brain endothelial cells and human brain endothelial cells). To evalu-

ate these models, the *in vitro* data were compared with the *in vivo* data obtained in a mouse brain uptake assay. The aim of the study was to identify the *in vitro* models which best reflect the structural and functional characteristics of the BBB. It was found that the *in vitro*–*in vivo* correlations were low for all of the models evaluated, although the data obtained with the MDCK and Caco-2 cell lines correlated well with each other. One of the conclusions was that an MDCK cell line expressing the multi-drug resistance protein 1 (MDCKmdr-1) might be capable of distinguishing between chemicals that cross the BBB by passive diffusion and those that are substrates for active efflux. A workshop to consider the implications of the report on the study, and to propose further work, is to be held in Stockholm, Sweden, during 2002.

**Table 7.3: An overview of blood–brain barrier models**

Models	Advantages	Disadvantages
<b><i>In vivo</i></b>		
Tissue distribution assay	Studies of brain uptake <i>in vivo</i> , either after single dose or steady state	Animal model Several animals needed Number dependent on number of time points used Not human
Microdialysis	Studies of brain uptake <i>in vivo</i> , either after single dose or steady state Single animal used to study kinetics (continuously sampling from one animal over time) Possible to study kinetics simultaneously in several tissues in the same animal	Animal model Low throughput Not easy to set up or use
Positron emission tomography		
<b><i>In situ</i></b>		
Perfused head		Technically demanding

### Barrier Function: Summary, Conclusions and Recommendations

Some QSARs for membrane permeability, such as those based on log P and MW, may be sufficiently predictive to identify chemicals that are likely to cross biological barriers by passive diffusion. These QSARs could be used for priority setting, but chemicals that are not predicted to undergo passive diffusion should not be neglected, since non-passive modes of membrane transport can also occur. All QSARs should eventually be validated in an independent manner, through an independent body such as ECVAM.

A variety of *in vitro* methods for percutaneous absorption are available and could be used for priority setting.

For gastrointestinal absorption, the Caco-2 culture model is considered to be sufficiently reproducible for use as a high-throughput screening system in the priority setting of chemicals. However, further work is needed to assess the predictive capacity of this system.

For assessing distribution across the BBB, a number of *in vitro* systems are under development. Further work is needed to validate these systems.

#### Short-term prospects

1. The acceptance by the OECD Council in 2002 of the draft OECD TG 428 (*in vitro* percutaneous absorption).

2. The further development of QSAR models for barrier function, including penetration across the skin, gastrointestinal barrier and BBB.
3. The further development of reconstituted human skin models for percutaneous absorption testing, to make their barrier properties similar to those found *in vivo*.
4. An evaluation of the feasibility of predicting bioavailability from *in vitro* data, and not just the fraction (percentage) absorbed, as currently performed by using the Caco-2 model.
5. Further optimisation of a test protocol for the BBB involving primary endothelial cells co-cultured with primary astrocytes.

#### Medium-term prospects

1. The validation of QSARs for barrier function, including penetration across the skin, gastrointestinal barrier and BBB, following a preliminary assessment of their goodness-of-fit and mechanistic relevance.
2. The prevalidation of *in vitro* models of the gastrointestinal barrier and the BBB.

#### Recommendations for research and development

1. Further investigations on the expression of transport/efflux proteins in cell lines derived

from the human gastrointestinal tract, and the influence of such transporters on absorption.

2. Research on the effects of anti-transport mechanisms (MDR, P-gp) and gut wall metabolism (CYP3A4) on bioavailability.
3. Further investigations on co-cultures consisting of cell lines with enterocytic markers and with mucus secretory functions, to increase understanding of the effects of mucus on the absorption rate.
4. The establishment of new cell models of the BBB that have characteristics more consistent with the *in vivo* situation, with an emphasis on the use of human cell lines.
5. The design and evaluation of a battery of *in vitro* assays, including, for example, measurements of protein binding and clearance, in addition to permeability, to predict brain distribution. Such a battery could include, for example, MDCK cells transfected with genes for transporter proteins, such as *mdr-1*.

## Recommendations

The ESAC should consider endorsing a statement on the applicability of *in vitro* methods for percutaneous absorption, assessed on the basis of a weight-of-evidence approach.

## Xenobiotic Metabolism

### The scientific background to metabolism

Metabolism is the process by which a chemical is structurally changed in the body by enzymic and/or non-enzymic reactions. Information on the metabolism of a substance is important in the evaluation of its toxic potential. For example, the determination of metabolic stability can provide information on the potential for bioaccumulation. Information on the enzymes involved in metabolism can help to establish the importance of different clearance pathways, and can be useful in the prediction of clearance rates and the saturability of clearance processes. Metabolic studies can also be used to identify the tissue and cellular targets for any effect, and to establish the extent of inter-individual differences due to genetic polymorphisms, environmental factors or pathophysiological effects. The results of metabolic studies can also be useful when undertaking interspecies comparisons of the effects of chemicals.

A chemical that is absorbed orally is transported via the portal circulation to the liver, where it may be subjected to hepatic metabolism, followed by elimination in bile or through the kidneys. There is also the possibility of extra-hepatic metabolism. A typical drug metabolism pathway is the oxidation of the parent drug (phase I oxidation), followed by conjugation of the oxidised moiety with highly polar molecules, such as glucose, sulphate, methionine, cysteine or glutathione (phase II conjugation). The key enzymes for phase I oxidation are the isoforms of the cytochrome P450 (CYP) family of enzymes (an updated list of which is available from <http://www.imm.ki.se/CYPalleles>).

The major human CYPs involved in chemical metabolism are CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP1B1, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 (35–41).

The key phase II enzymes include *N*-acetyltransferases (NAT), UDP-dependent glucuronosyl transferase (UGT), phenol sulfotransferases (PST), oestrogen sulfotransferases (EST), and glutathione S-transferases (GST). The structures and functions of phase II enzymes have been reviewed elsewhere (42–44).

### Current status of *in vitro* approaches for assessing metabolism

A number of *in vitro* systems are available for studying metabolism. These include precision-cut tissue slices, subcellular fractions such as the microsomal fraction, primary cells in suspension, primary cells in culture, continuous cell lines, immortalised primary cells, and genetically engineered cell lines.

To date, no *in vitro* methods for determining phase I or phase II biotransformation, or for evaluating metabolism-dependent toxicity, have been validated according to ECVAM's principles and procedures (Chapter 2). The current OECD Test Guideline 417 (45) for assessing the toxicokinetic effects of chemicals is based on *in vivo* studies. However, many studies provide support for the usefulness of *in vitro* methods for assessing metabolism and metabolism-dependent toxicity. From such studies, it is evident that there are enormous species differences in toxicokinetics; this is especially true of the metabolic differences between humans and rodents (38). Therefore, there is a great need for human-based *in vitro* models that would offer better predictions of potential hazard to humans than could ever be obtained from laboratory animal studies (46–49). The status of the available approaches for assessing metabolism is summarised in Table 7.4.

Recently, there has been success in relating the rate of *in vitro* metabolism of several compounds with the corresponding events *in vivo*. This repre-

**Table 7.4: An overview of *in vitro* methods for assessing metabolism**

Strategies	Test systems	Endpoints	Applicability	Formal status
First tier	<p>Microsomes from human hepatocytes or from genetically engineered cell lines expressing human genes</p> <p>Cell lines, primary monolayer cultures, genetically engineered cell lines expressing human genes</p> <p>Microsomes, human hepatocytes, genetically engineered cell lines expressing human genes</p>	<p>Identification of metabolite formation by LC/MS</p> <p>Cell morphology, viability, membrane damage, liver-specific endpoints, genotoxic endpoints</p> <p>Quantification by LC/MS, HPLC or fluorescence</p>	<p>Most important metabolic pathways</p> <p>Metabolism-mediated toxic effects</p> <p>Metabolic stability and Inhibition</p>	<p>Prevalidation studies to be initiated</p>
Second tier	<p>Short-term and long-term hepatocyte cultures (for example, human hepatocyte sandwich cultures), precision-cut liver slices and liver-derived cell lines expressing or re-expressing biotransformation enzymes, highly differentiated human cell lines</p>	<p>Assaying the activity of the biotransformation enzymes (and, if appropriate, their individual isoenzymes); quantifying the protein level by using techniques such as immunoblotting or HPLC; and quantifying the mRNA levels by using Northern blotting, the nuclease protection assay or the reverse transcriptase polymerase chain reaction</p>	<p>Induction</p>	<p>Prevalidation study to be initiated</p>
Third tier	<p>Genetically engineered cell lines expressing human genes</p>	<p>Quantification by LC/MS</p>	<p>Polymorphism</p>	<p>Prevalidation study to be initiated</p>

*HPLC = high-performance liquid chromatography; LC/MS = liquid chromatography/mass spectrometry.*

sents a major step, since the value of *in vitro* metabolism systems has usually been considered to be purely qualitative in nature. The basis of this relationship is the use of intrinsic clearance, a parameter which is a pure measure of enzyme activity toward a chemical *in vivo*, and is not influenced by other physiological determinants of clearance, such as hepatic blood flow or drug binding. The *in vitro* equivalent of this parameter is the  $V_{max}:K_m$  ratio. The utility of *in vitro* intrinsic clearance as a predictor of *in vivo* intrinsic clearance, and therefore hepatic clearance and total body clearance, has been assessed with a database of 35 drug substrates for rat CYPs (50). Other studies have demonstrated the usefulness of the intrinsic clearance approach for predicting kinetics in humans (51, 52).

### Current status of computer-based approaches for assessing metabolism

A number of computer-based expert systems for predicting metabolism and metabolism-dependent toxicity are undergoing development, and have been reviewed elsewhere (53, 54).

An example is the METEOR system, a rule-based system for the prediction of metabolism, which is being developed by LHASA (55). METEOR consists of a knowledge base of biotransformations that describe the metabolic reactions catalysed by specific enzymes, and which are related to specific substrates. The system can be linked with DEREK (Deductive Estimation of Risk from Existing Knowledge), to provide a means of assessing both metabolism and toxicity.

Two other rule-based expert systems, HazardExpert and MetabolExpert, are being developed and marketed by CompuDrug Chemistry Ltd. HazardExpert enables predictions to be made for a number of toxicological endpoints, taking into account factors such as species, dose, route and exposure duration. HazardExpert can be linked to MetabolExpert, which makes qualitative predictions of the metabolites of compounds. These predictions are made by using a rulebase consisting of molecular fragments. By linking HazardExpert with MetabolExpert, the user can obtain predictions of toxicity, not only for the parent molecule, but also for its potential metabolites.

Another approach to the prediction of metabolism (and metabolism-dependent toxicity) is provided by the Computer-Optimised Molecular Parametric Analysis of Chemical Toxicity (COMPACT) method of Lewis and coworkers (56). This method can be used to predict whether a molecule has the potential to act as a substrate for one or more of the cytochromes P450 (CYPs), or the ability to promote peroxisome proliferation. Oxidative metabolism by CYPs normally results in detoxifica-

tion, although metabolism by CYP1, for example, may result in the formation of epoxides.

The COMPACT method is based upon the premise that there are certain structural requirements of a molecule that make it susceptible to oxidative metabolism. Firstly, molecules must have planar shapes that permit them to bind to CYPs. Secondly, molecules must be susceptible to chemical oxidation. The COMPACT approach is therefore based on two physicochemical descriptors: molecular planarity and electron activation energy. Molecular planarity is a function of the cross-sectional area and molecular depth of the potential substrate, whereas the electronic activation energy is the difference between the energies of the highest occupied and lowest unoccupied molecular orbitals. A two-dimensional plot of molecular planarity and electronic activation energy for a series of molecules reveals that they can be divided into categories according to the particular CYP by which they are metabolised (57).

The prediction rate obtained with the COMPACT system was found to be improved when its predictions were considered in combination with those generated by the HazardExpert system (58), demonstrating the usefulness of a battery approach to prediction. The COMPACT approach has been extended to include the molecular (protein) modelling of the CYP enzymes themselves (59).

Another computational method for predicting potential metabolites is the META system, which is part of a suite of programs developed by Klopman and colleagues (60, 61). The rules in the META rulebase were taken from the biochemical literature, rather than being derived by a statistical approach.

### A proposed strategy for assessing metabolism

So far, no consensus has been reached on the optimal test systems to be used for obtaining the information required. However, the most widely accepted approach is to progress from general questions, involving the use of simple, inexpensive and less-specific *in vitro* models, such as human cell lines and human liver fractions, to mechanistic questions, which require technically more-demanding and more-complex models, such as cultures of human hepatocytes. In this way, the first tier acts as a preliminary screen for identifying the most important metabolic pathways, including metabolism-mediated toxic effects (62), metabolic stability and enzyme inhibition (48). Recent developments in analytical chemistry (for example, in the use of mass spectrometry), have improved the value of *in vitro* systems for determinations of metabolic pathways and of metabolic stability. Subsequent screens can then be used to study more-specific questions, such as the induction of biotransformation enzymes

(46) or the occurrence of polymorphism-related effects (49).

#### *Tier 1: Screening tests for metabolism*

To identify the most important metabolic pathways, human liver fractions or genetically engineered cell lines are incubated with the chemical, followed by quantitative analysis by techniques such as liquid chromatography (LC) and mass spectrometry (MS; 47, 63, 64).

To evaluate metabolism-mediated toxicity, *in vitro* cell culture assays can be used, which are based on primary monolayer cultures, cell lines, or genetically engineered cell lines, which express biotransformation enzymes (48, 62, 65, 66), and liver-derived cell lines re-expressing biotransformation enzymes (46). These *in vitro* tests can incorporate important toxicological endpoints, including cytotoxicity and genotoxicity (see Chapters 4 and 9, respectively). With all of these systems, it is necessary to take account of endogenous metabolism when interpreting the *in vitro* toxicity data.

To screen for metabolic stability, it is possible to use microsomes, human hepatocytes or genetically engineered cell lines expressing human biotransformation enzymes. These approaches involve the incubation of the chemical with the cells or microsomes, followed by enzyme kinetic studies based on chromatographic procedures, such as high-performance liquid chromatography (HPLC) and LC/MS (67). The exclusive use of microsomal preparations may give misleading results, because phase II enzymes, which are predominantly cytosolic enzymes and play a crucial role in the metabolic activation of chemicals, may be missing. In some cases, this problem can be solved by the exogenous addition of cofactors such as UDP-glucuronic acid (UDPGA) for glucuronidation, and 3'-phosphoadenosine-5'-phosphosulphate (PAPS) for sulphation (68).

For the assessment of inhibitory interactions with CYP enzymes, the most popular approaches involve the use of either cultures of genetically engineered cell lines containing only one specific human CYP isoform (48) or their microsomes (69). The effect on the biotransformation of the substrate is dependent on the concentrations of both the inhibitor and the substrate, determined by the inhibition constant,  $K_i$ , of the inhibitor and the Michaelis-Menten constant,  $K_m$ , of the substrate. The test compounds are incubated with the cultures or the microsomes in the presence of CYP substrates. Quantification can then be performed by appropriate fluorescence analysis, HPLC or LC/MS. In situations where more than one enzyme isoform is present, the use of complex *in vitro* models, such as human hepatocyte cultures (70), is advised, so that inhibition and metabolism can be evaluated at the same time.

In addition to enzyme inhibition by chemicals, there is also the possibility of immunoinhibition, in which polyclonal and monoclonal antibodies may competitively inhibit biotransformation enzymes (71).

#### *Tier 2: Induction of biotransformation enzymes*

Xenobiotics taken up by the human body may result in the *de novo* synthesis of enzyme molecules (including phase I and phase II biotransformation enzymes) as a result of increased transcription of the respective genes. An increase in enzyme activity may also be observed as a result of enzyme stabilisation (46).

Various *in vitro* metabolically competent models have been proposed for detecting the induction of CYPs, including precision-cut liver slices, short-term and long-term hepatocyte cultures, liver-derived cell lines expressing or re-expressing biotransformation enzymes, and highly differentiated human cell lines (46, 72). For detecting enzyme induction, these *in vitro* methods involve the use of endpoints such as: a) the activity of the biotransformation enzymes (and, if appropriate, their individual isoenzymes); b) protein levels, by using techniques such as immunoblotting or HPLC; and c) mRNA levels, by using Northern blotting (for example, through the nuclease protection assay or the reverse-transcriptase polymerase chain reaction; 46).

Although there are technical difficulties associated with the cryopreservation of hepatocytes, a number of cryopreserved hepatocyte cultures have been used for enzyme-induction studies (73).

To date, no prevalidation study has been carried out on methods for identifying the capacity of compounds to induce biotransformation enzymes. However, ECVAM has initiated a prevalidation study (46) on the use of human hepatocyte sandwich cultures (74).

#### *Tier 3: Models for evaluating polymorphic effects on metabolism*

Amino acid substitution or deletion can result in reductions in, or even loss of, the activities of phase I or phase II biotransformation enzymes. Certain individuals exhibit a severely compromised ability to metabolise chemicals that are specific substrates of these polymorphic enzymes, which can lead to serious toxic side-effects (75).

The acetyltransferases, NAT1 and NAT2, represent the best-understood polymorphic enzymes. Other phase II enzymes, such as sulfotransferase (ST), UGT, and GST, are known to contain a variety of polymorphic variants, but their functional genetic diversity is not as well understood (42–44, 76, 77).

A review by Friedberg *et al.* (78) compared insect, bacterial, yeast, and mammalian metabolism models. In general, the catalytic properties of CYPs in the various models were found to be similar.

A recent and promising innovation is the use of established cell lines, such as V79 cells, which have been genetically engineered to express selected genetic variants of human CYP enzymes, so that such polymorphic effects can be assessed (49).

### **General recommendations for the use of *in vitro* metabolism tests**

It is recommended that the *in vitro* metabolism tests used to implement the future EU Chemicals Policy should provide a simple, rapid and inexpensive means of detecting a broad array of chemically mediated metabolic effects. The models should be able to provide information on: a) the most important metabolic pathways; b) the specific human enzyme isoforms involved in the metabolism; c) the metabolic stability of the chemical; d) the structures of the metabolites; e) metabolism-dependent toxification and detoxification; and f) the regulation of biotransformation enzymes (for example, their capacity to be induced or inhibited).

A variety of high-throughput *in vitro* metabolism methods are available for use in tier 1 of the metabolism strategy (for a review, see 79). ECVAM, in consultation with experts in the field of metabolism, has plans to initiate a number of prevalidation and validation studies in the future.

### **Metabolism: Summary, Conclusions and Recommendations**

A wide variety of *in vitro* tests (referred to as tier 1 tests in this report) are available for identifying metabolic pathways, metabolism-mediated toxic effects, metabolic stability and enzyme inhibition, and these could be used immediately to obtain mechanistic information. Other tests are available for assessing enzyme induction (tier 2) and polymorphic effects (tier 3). An important question for the implementation of the EU Chemicals Policy will be whether tier 2 and tier 3 tests are necessary, and if so, at what tonnage level of production/importation.

#### **Short-term prospects**

1. The prevalidation of *in vitro* tier 1 metabolism tests.
2. The prevalidation of *in vitro* tests for induction based on human hepatocyte cultures as tier 2 systems.

3. The further improvement of techniques for the cryopreservation of human hepatocytes.
4. The further development of models for evaluating polymorphic effects on metabolism.
5. The further development of computer-based systems for predicting metabolism.

#### **Medium-term prospects**

1. The validation of *in vitro* tier 1 metabolism tests.
2. The validation of *in vitro* tests for induction based on human hepatocyte cultures as tier 2 systems.
3. The prevalidation of models for evaluating polymorphic effects on metabolism.

#### **Long-term prospects**

1. The validation of computer-based systems for predicting metabolism from chemical structure.
2. The validation of models for evaluating polymorphic effects on metabolism.

### **Biokinetic Modelling**

Biokinetic models are mathematical models based on differential equations that describe the absorption, distribution, metabolism and elimination of chemicals as a function of dose and time. Biokinetic models can be divided into two main types: a) data-based compartmental models; and b) physiologically based compartmental models, which are also known as physiologically based biokinetic (PBBK) models. PBBK models are based on the known anatomy and physiology of the organism. Compartments correspond to relevant anatomical structures, such as the liver and kidney, or to tissue types, such as fat and muscle (80, 81). Over the last decade, the development of PBBK models has been facilitated by the increasing availability of computer-based techniques that can simultaneously perform the numerical solution of the sets of differential equations that characterise biokinetic processes. The use of PBBK models is expected to contribute to the replacement, reduction and refinement of animal studies, by permitting the design of studies to be optimised through the identification of critical kinetic parameters and timeframes.

The main advantage of PBBK models over classical data-based compartmental models is their ability to estimate kinetic behaviour on the basis of a predeter-

mined model structure and independently derived model parameters, thereby avoiding the need to adjust the parameters to optimise the fit of the model. PBBK models are based on two kinds of parameters: a) species-specific anatomical and physiological parameters, such as the pulmonary ventilation rate, for which data are generally available in the literature (82); and b) compound-specific parameters, such as tissue–blood partition coefficients (PCs), and kinetic parameters, such as the Michaelis–Menten constants,  $V_{\max}$  and  $K_m$ . Compound-specific parameters have conventionally been obtained from kinetic studies performed *in vivo*. However, there is now considerable scope for deriving such parameters from *in vitro* studies (83), which should therefore lead to a reduction in the number of animal studies conducted for biokinetic modelling.

When biologically based parameters are assigned to each of the specific kinetic processes, it becomes clear that once the compound is taken up in the systemic circulation, the distribution, metabolism and excretion of a chemical are independent of the exposure route. Thus, it is possible to extrapolate from one exposure route to another by adjusting the parameters for the relevant uptake process only, since the mathematical descriptions of the distribution and elimination processes remain the same. In addition to route-to-route extrapolation, PBBK models also permit extrapolations of dose and species beyond the conditions of laboratory studies.

The distribution of a compound throughout the body is described by tissue volumes, blood flow rates, tissue–blood PCs, and, where appropriate, by the kinetic parameters of active transport processes. Tissue volumes, tissue–blood PCs and blood flow rates can be determined experimentally or may be available from the literature.

Tissue–air PCs of volatile compounds can be measured *in vitro* by incubation of the compound with a homogenate of the respective tissue in buffered saline. The equilibrium distribution of the compound is then measured by gas-chromatographic analysis of the air present in the headspace of a vial containing the homogenate/buffer mixture. Tissue–blood PCs can be calculated by dividing the tissue–air PCs by the respective blood–air PCs. This method has been applied in many studies on volatile hydrocarbons, with human and rat blood, and liver, lung, kidney, fat, muscle, and brain homogenates (84–86). This principle has been extended to determine the tissue–blood PCs of non-volatile compounds by the use of a number of *in vitro* methods (87, 88). However, these techniques are time-consuming, and often depend on the availability of a radiolabelled form of the chemical under consideration.

In addition to these *in vitro* techniques, both descriptive and mechanistic algorithms for the estimation of blood–air and tissue–blood PCs have been reported (89, 90). Such methods attempt to estimate the biological partitioning process as a func-

tion of physicochemical parameters such as the aqueous and/or lipid solubilities of the chemical.

The distribution rate of a compound to the brain can be described mathematically as a function of input into the brain from the arterial blood stream and outflow from the venous side. A venous equilibration model (81) makes the assumption that the concentration of a chemical in the venous blood leaving the brain is always in equilibrium with that in the brain tissue. This model is expected to be valid for small nonpolar, lipophilic chemicals, as well as hydrophilic ones. Thus, the perfusion rate of the brain dominates the diffusion rate through the blood–brain barrier as the rate-limiting step in brain distribution. Depending on its molecular structure, a chemical may exhibit a diffusion-limited brain uptake *in vivo*, in which case a compound-specific parameter is needed to express its diffusion rate. In the case of polar and/or ionisable compounds, an energy-dependent, saturable uptake rate may be required to describe its interaction with the BBB. *In vitro* models of the BBB are available (91), and are described in more detail above.

## Biokinetic Modelling: Summary, Conclusions and Recommendations

PBBK models describe the processes of ADME by integrating physicochemical and *in vitro* data. They can be used to determine target organ/system doses and to extrapolate between routes of exposure and between species. To date, most of the models have been developed for specific chemicals, so there is a need to develop and validate generic models, applicable to broad groups of chemicals.

### Short-term prospects

1. For the prediction of target organ/tissue distribution, a user-friendly software package is required, so that the user can enter a SMILES code or Chemical Abstracts Service (CAS) Registry number for a given chemical, then obtain a qualitative indication of the major target organs and tissues. This information could then be used to indicate the *in vitro* tests that could be performed for a chemical known to enter the body by the dermal route of exposure.
2. The further development of algorithms for predicting *in vivo* metabolic clearance from *in vitro* data.

### Medium-term prospects

1. The validation of algorithms incorporated into the biokinetic software package.

2. The validation of algorithms for predicting *in vivo* metabolic clearance.

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