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Methodologies to study human intestinal absorption. A review.

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Abstract

This review is the first report of the project V/630030 entitled: "Development and evaluation of *in vitro* methodologies to assess the internal exposure of man to ingested contaminants". This study is intended to give an overview on *in vitro*, *in situ* and *in vivo* methodologies most commonly used to study the intestinal absorption of ingested compounds. What information can be derived from each method and how this relates to human intestinal absorption is reviewed. This information is useful for researchers to choose an experimental set-up for studying intestinal absorption. This report is also aimed at risk assessors on pharmaceuticals and toxic compounds and the applicability of the methodologies for studying intestinal absorption to risk assessment practice of orally ingested toxicants is discussed.

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Samenvatting

In de risicobeoordeling worden blootstellingsniveaus en normen uitgedrukt in een externe blootstelling (inname) terwijl de inwendige blootstelling (opname) bepaalt of er toxische effecten optreden. Van oraal ingenomen stoffen wordt vaak maar een fractie van de externe dosis opgenomen in het lichaam waardoor de opname lager is dan de inname. Bij het Laboratorium voor Blootstellingonderzoek en Milieu-Epidemiologie is in 1999 een project gestart met als doel de opname van oraal ingenomen stoffen in de darm bij de mens te kunnen bepalen aan de hand van *in vitro* methodieken. Dit rapport geeft een overzicht van de methodieken die veelal gebruikt worden om de absorptie van stoffen in de darm te bestuderen. In dit review ligt de nadruk op de bruikbaarheid van deze methodieken voor (ad hoc) risicoschatting. Daartoe worden de volgende eisen aan een methodiek gesteld: de methode moet snel, flexibel en relatief goedkoop zijn en moet een accurate schatting van de humane absorptie van oraal ingenomen stoffen in de darm kunnen maken.

Twee belangrijke factoren liggen ten grondslag aan de opname van stoffen in de darm. Ten eerste moet een stof in het maagdarmkanaal vrijkomen uit zijn matrix (bijv. voeding, speelgoed, grond). De fractie van de stof die vrijkomt uit de matrix wordt de bioaccessibility van een stof genoemd. In de wetenschappelijke literatuur is weinig eenduidige informatie over methodes die de extractie simuleren van stoffen uit hun matrix in het maagdarmkanaal bij de mens. Het dynamisch digestiemodel, beschreven door Minekus et al., maakt gebruik van geavanceerde laboratorium apparatuur. Dit model is vooral geschikt om het vrijkomen van de stof in het maagdarmkanaal in detail te bestuderen en is minder geschikt voor screeningsdoeleinden. Voor dit doel zijn de statische digestiemodellen waarschijnlijk beter geschikt. In Laboratorium voor Blootstellingonderzoek en Milieu-Epidemiologie bij het RIVM is een statisch digestiemodel ontwikkeld met als doel de bioaccessibility van zware metalen en hydrofobe organische stoffen uit ingeslikte grond te bepalen. Dit digestiemodel is representatief voor humane fysiologie en een eenvoudige, makkelijk hanteerbare en flexibele methode. Dit digestiemodel kan als een uitgangspunt genomen worden voor een testprocedure om bioaccessibility van stoffen uit een willekeurige matrix te bepalen.

De tweede factor, die opname van stoffen in het maagdarmkanaal beïnvloedt, is de passage van de stof over het darmepitheel (transport van maagdarmkanaal naar bloed). In de literatuur zijn veel *in vitro*, *in situ* en *in vivo* methodieken beschreven waarmee het transport van stoffen over het darmepitheel bestudeerd wordt. Op basis van de gereviewde data kunnen het *in vitro* Caco-2 cellijn model, de *in vitro* mucosale darmplakjes en de *in situ* en humane *in vivo* perfusie methodes gebruikt worden om de opname van stoffen in de darm van de mens te schatten. De *in situ* en in vooral de *in vivo* perfusie studies zijn meer kostbaar en tijdrovend dan de *in vitro* absorptiemodellen. Indien absorptie op een specifieke plaats in de darm of interspecies vergelijkingen belangrijk zijn, zijn de mucosale darmplakjes een bruikbare methode. Een groot nadeel van deze methode is dat vers geïsoleerde darm gebruikt moet worden omdat de functionaliteit van het weefsel snel achteruitgaat. Het is moeilijk om humaan weefsel te verkrijgen dat nog vers en functioneel is, en bovendien kunnen de experimentele condities niet makkelijk veranderd worden. Deze nadelen zijn niet van toepassing voor het *in vitro* Caco-2 cellijn transport model dat in vergelijking met de andere methodes makkelijk te hanteren en te manipuleren is. Dit model is representatief voor humane absorptie en is bovendien reproduceerbaar, snel, relatief goedkoop en kan het eenvoudig in groot aantallen uitgevoerd worden.

Tot dusverre worden de bioaccessibility en het transport over de darmwand van stoffen in afzonderlijke experimenten bepaald en slechts recent zijn in enkele gevallen deze twee methodieken gecombineerd. Dit is een veel belovende benadering en het verdient aanbeveling om dit verder te onderzoeken en te valideren. De *in vitro* methodes voor bioaccessibility en transport over de darmwand kunnen een waardevolle bijdrage leveren wanneer een snelle indicatie gewenst is van de humane absorptie van een stof uit een willekeurig product (bijv. geneesmiddelen, voeding, speelgoed, grond, etc). De informatie die met deze methodes verkregen wordt, kan geïmplementeerd worden in humane blootstellingmodellen zoals CONSEXPO en kan bijdragen tot een betere risicoschatting.

Summary

Concepts in risk assessment practice are expressed as an external exposure while the internal exposure determines whether toxic effects will occur. Often only a fraction of the ingested external exposure is absorbed resulting in a lower internal exposure. In 1999 a project has been started at the Laboratory of Exposure Assessment and Environmental Epidemiology on development and evaluation of an *in vitro* methodology to determine the human intestinal absorption of ingested compounds. This report gives an overview on the methodologies most commonly used to study the intestinal absorption of ingested compounds. The use of these methodologies in (ad hoc) health risk assessment practice is of particular interest in this review. Therefore, the methodologies are reviewed on their potential to provide a fast and relative inexpensive, accurate estimation of the human intestinal absorption of compounds.

Two major factors can be distinguished determining intestinal absorption of compounds. First, the release of the compound from its matrix (e.g. food, toy, soil) in the gastrointestinal tract, the so-called bioaccessibility of the compound, is considered. Not much conclusive information is available on methods that simulate the extraction of compounds from their matrix in the human gastrointestinal tract. The dynamic digestion model, described by Minekus et al., requires advanced laboratory equipment, and is especially suitable for a detailed study on the release of the compound from its matrix rather than for use of screening purposes. For this purpose the static digestion models are probably more useful. The static model, developed in our laboratory to study the bioaccessibility of heavy metals and hydrophobic organic compounds from ingested soil, may provide a good starting point for a test procedure combining a simple, easy to manipulate model and a model representative for human physiology.

The second factor affecting intestinal absorption of compounds, is the passage across the intestinal epithelium by the compound (i.e. transport from the gastrointestinal tract to blood). Many *in vitro*, *in situ* and *in vivo* methodologies address the passage of the intestinal epithelium by the compounds. Based on the reviewed literature, the *in vitro* Caco-2 cell culture model, the *in vitro* mucosal sheets method, and the *in situ* and the human *in vivo* perfusion methods can be used to estimate the passage of the intestinal epithelium in humans of ingested compounds. The *in situ* and especially *in vivo* perfusion studies are more expensive and more time consuming than the *in vitro* absorption models. If site-specific absorption or interspecies comparison is an important issue, intestinal mucosal sheets will provide a useful method. A major disadvantage of this method is that fresh intestinal material should be used because of the rapid decrease in viability of the tissue. Therefore, it is difficult to obtain truly fresh human material, and the conditions can not be manipulated as easily because of the fragile tissue viability. Overall, the Caco-2 cell culture model is by far the easiest to manipulate, very reproducible, fast, relative inexpensive, can be performed in large numbers and is representative for human absorption.

Up to now the determination of bioaccessibility and permeability of compounds is rarely determined in one single experiment but the few cases that they are combined are promising and need to be further explored and validated. These methods can provide a powerful tool to obtain a rapid indication of the human absorption of a compound from an arbitrary product e.g. pharmaceuticals, contaminants in food, toys, soil etc. The information obtained with the *in vitro* methods can for example be implemented in human exposure models such as

CONSEXPO and can contribute to a more rational and optimised health risk assessment practice.

1. Introduction

In health risk assessment of orally ingested toxic compounds, concepts and standard settings are expressed as an external exposure whereas the internal exposure (is amount of contaminant present in blood, organs and tissues) determines whether toxic effects will occur. The external exposure is the amount of contaminant ingested by a person. Often only a fraction of the ingested contaminant reaches the blood, organs and tissues. Thus, the internal exposure is often lower than the external exposure. The fraction of the external exposure reaching the systemic circulation is called the oral bioavailability of the compound. Figure 1.1 shows the three processes that are important for the oral bioavailability of a compound. First, the bioaccessibility of the compound: the matrix (e.g. food, consumer product, soil) affects the bioavailability of a compound since the compound should be released from its matrix before the compound is accessible for intestinal absorption. This means that the amount of contaminant in the form of matrix A may lead to a different internal exposure of the contaminant than the same amount of the contaminant in the form of matrix B. The oral bioavailability of a compound should, therefore, always be considered in relation to its matrix. The second process affecting the oral bioavailability of a compound is the ability of the compound to pass the intestinal epithelium and to reach the portal vein unmetabolised (intestinal permeability). The third process is the susceptibility of the compound for metabolism in the liver (Figure 1.1).

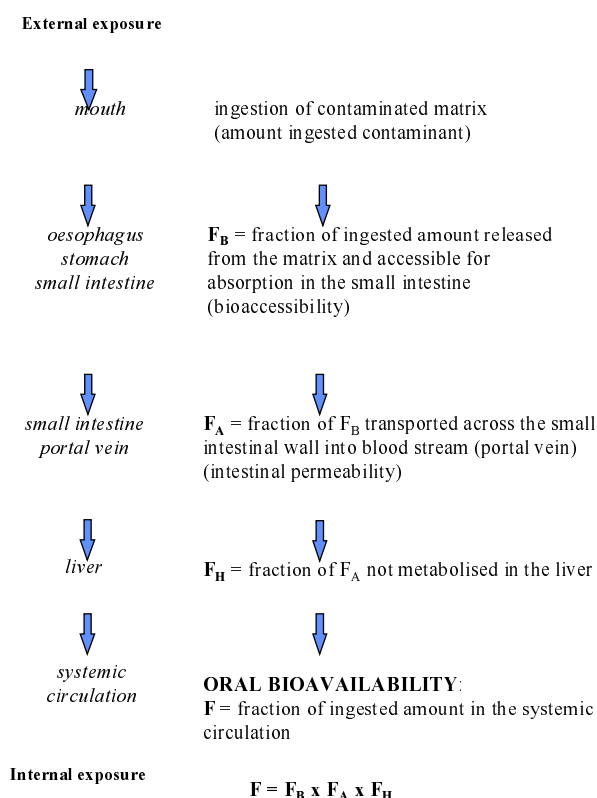


Figure 1.1. Schematic representation of processes determining oral bioavailability.

In current risk assessment practice standard setting of a toxic compound is based on *in vivo* studies in laboratory animals and occasionally based on acute toxicity accidents in man. The matrix the toxicant is applied in can influence the internal exposure by affecting the intestinal

absorption of the toxicant. This effect of the matrix is often not considered in the risk assessment of the toxicant when the toxicant is applied in another matrix than was used in the toxicity study. This could be accounted for by performing another toxicity study with the toxicant applied in the matrix of interest. However, *in vivo* toxicity studies are expensive and time-consuming, therefore, methods that could give an estimation of the human oral bioavailability of a compound in a short span of time would be a valuable tool for health risk assessment. Since as well as the variety of compounds as the variety of matrices can be great, the method(s) should be capable of handling many different conditions, should be reproducible, fast and relatively cheap. Considering the use of these methods for ad hoc risk assessment makes special demands on the swiftness of the methods. The methods should then be fast (days to weeks).

This report will focus on the passage of compounds across the intestinal epithelium. As release of the compound from the matrix in the gastrointestinal tract is a prerequisite to absorption of a compound, methods to determine the release of a compound from the matrix are shortly discussed. Being the first contact site of all orally ingested compounds, the epithelium of the gastrointestinal tract has an important absorptive function for nutrients but it also needs to present a barrier to absorption of potentially harmful compounds. It is this barrier that not only may limit the absorption of harmful compounds but also limits the absorption of many orally administered pharmaceuticals. The last decade considerable attention has been paid especially by the pharmaceutical industry in finding comprehensive methods capable of predicting human oral absorption. Implementation of such methods in risk assessment of orally ingested toxicants would provide information, which could speed up and contribute to a more rational and optimised health risk assessment. Besides the use for risk assessment practice, these methods could also be used to gain insight into the absorption of pharmaceutical drugs or active components in food.

Outline of report.

This report gives an overview of *in vitro*, *in situ* and *in vivo* methodologies commonly used to evaluate intestinal absorption of orally ingested compounds. What information can be derived from each method and how this is related to intestinal absorption or bioavailability is discussed as well as the applicability of the methodology. In chapters 2-3, the histology of the small intestine, some of the physicochemical factors influencing oral bioavailability, the mechanisms of transport of drugs across the intestinal epithelium and the role of metabolism are considered. Chapter 4 describes shortly some methods to determine the release of a compound from the matrix. The technique of the methodologies, used to evaluate intestinal absorption, is described in chapters 5-7, divided in *in vitro*, *in situ* and *in vivo* methodologies. Chapter 8 summarises the information collected from the methodologies and the potential of the methods as a tool for (ad hoc) health risk assessment is discussed.

2. Histology of the small intestine

The major functions of the gastrointestinal tract are to digest food, to absorb major nutrients such as sugars and aminoacids, but also to serve as a barrier to digestive enzymes, ingested (potentially harmful) compounds, bacteria, and finally to remove undigested and unabsorbed (food) compounds into the large intestine. Absorption of compounds in the gastrointestinal tract occurs primarily in the three sections of the small intestine (duodenum, jejunum, ileum) and to much lesser extent in colon, stomach and oral cavity. Each of these segments has unique anatomical, biochemical and physiological properties (Table 2.1 and Figure 2.1).

Table 2.1. Biological and physical parameters of the human intestinal tract [Daugherty and Mrsny, 1999].

Gastrointestinal segment	Surface area	Segment length	Residence time	PH of segment	Prominent catabolic activities
Oral cavity	100 cm ²		seconds to minutes	6.5	Polysaccharides
Esophagus	200 cm ²	0.25 m	seconds		
Stomach (fed)	3.5 m ² (variable)	2.5 cm (variable)	8-15 min* (0.5-3 h*)	1-2 (2-5)	Protease, lipases
Duodenum	1.9 m ²	0.35 m	0.5-0.75 h	4-5.5	Polysaccharides, oligosaccharides, peptidases, lipases, nucleases
Jejunum	184 m ²	2.8 m	1.5-2 h	5.5-7.0	Oligosaccharides, peptidases, lipases
Ileum	276 m ²	4.2 m	5-7 h	7.0-7.5	Oligosaccharides, peptidases, nucleases, nucleotidases
Colon and rectum	1.3 m ²	1.5 m	1-60 h (35 h)	7.0-7.5	Broad spectrum of bacterial enzymes

* Half-emptying time.

The small intestinal mucosa is composed of a monolayer of epithelial cells, the lamina propria, which is a connective tissue, and the muscularis mucosa, which is a thin layer of smooth muscle (Figure 2.1). The deeper connective tissue layer underlying the mucosa is called submucosa. The lamina propria is a structural support for the confluent monolayer of epithelial cells and is highly folded to form villi. The large number of villi (20-40 per square millimeter) increases the surface area (approximately 10-fold) of the epithelium available for absorption and digestion. At the base of the villi reside the crypts of Lieberkühn, from which the epithelial cells originate and differentiate. The epithelial cells are a heterogeneous population of cells which include: enterocytes or absorptive cells; goblet cells, which secrete mucin; endocrine cells, which produce hormones and peptides; Paneth cells, which secrete large amounts of protein rich materials; M cells, which transport antigens present in the intestinal lumen. The crypt cells are undifferentiated cells, which start to differentiate into the above mentioned cells as the cells move up the crypt to the base of the villi.

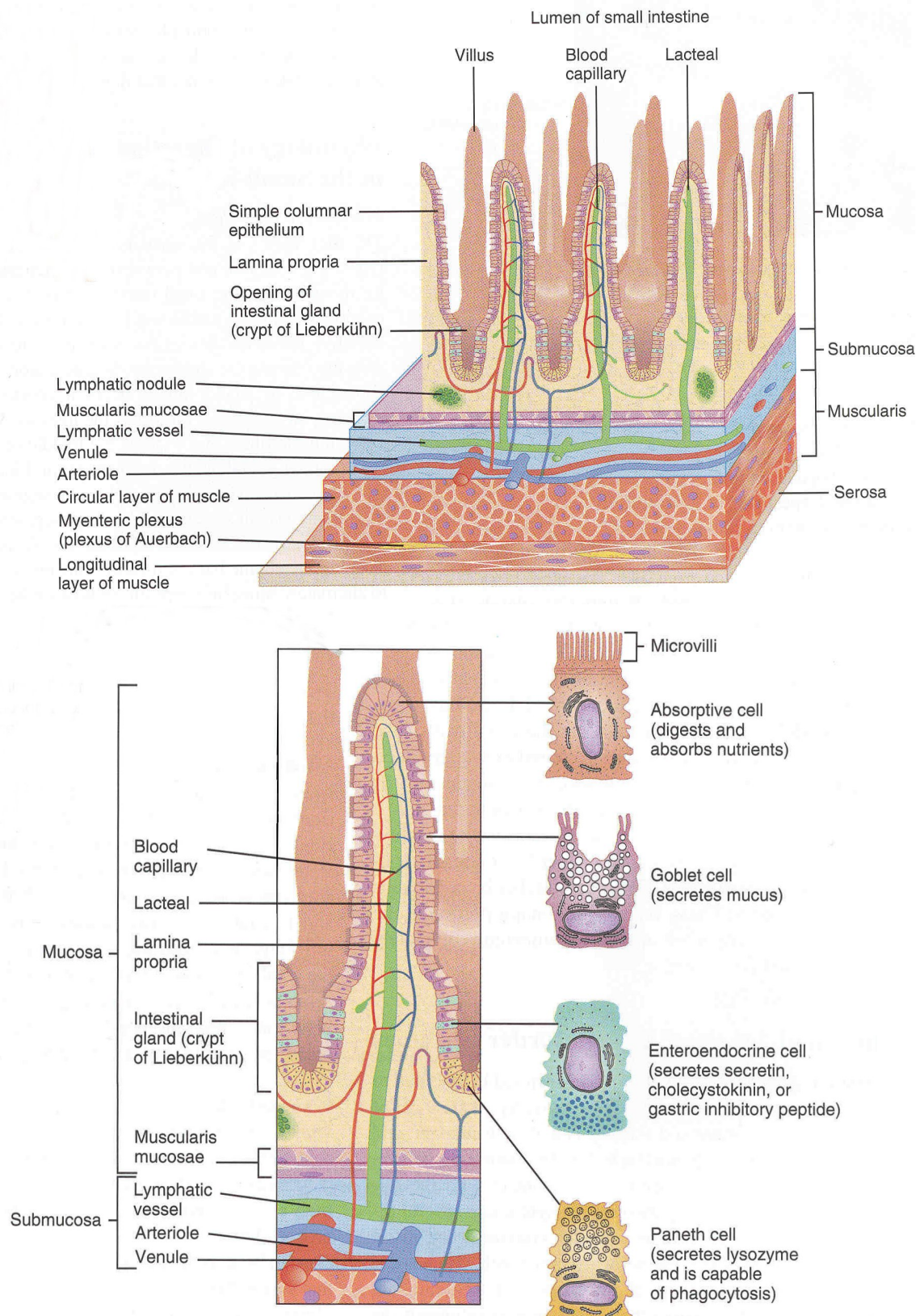


Figure 2.1. Histology of the small intestine. Above: Three dimensional view of layers of the small intestine showing villi. Under: Enlarged villus showing lacteal, capillaries, and intestinal gland [Tortora and Garbowski, 1996].

The most common epithelial cell is the enterocyte. This cell type is responsible for the majority of the digestion and absorption of both nutrients and drugs in the small intestine. The cells are highly polarised with distinct apical (lumen) and basolateral (serosa) membranes. The apical membrane has microvilli (approximately 1 μm in height, surface area increment 20-fold, total surface area 200 m^2) and here reside the digestive enzymes such as disaccharides and peptidases. The apical membrane also expresses transport systems for the absorption of monosaccharides, peptides, amino acids, fatty acids and ions. In contrast to the apical membrane, the basolateral membrane has smooth contours, no digestive enzymes and expresses other transport systems including Na-K ATPase, glycosyltransferases, and adenylylase.

Molecules that have passed the monolayer of epithelial cells are transported by the blood and lymphatic vessels located in the lamina propria (Figure 2.1). By contracting, the muscularis mucosa may modulate the thickness of the unstirred water layer at the surface of the mucosa, affecting the absorption of lipophilic molecules and helping to empty the luminal contents of the crypts. The villus epithelium is renewed every 2 to 4 days. This means that every day approximately 30 g of cell material is drained off the intestinal lumen. The height of the villi is related to rate of renewal but under pathological conditions (antibiotics, chronic diarrhoea, cytostatics) the villi can virtually disappear.

3. Absorption of ingested compounds

3.1 Factors influencing absorption

The fate of an ingested compound, as soon as it enters the small intestine, is shown in figure 3.1. The compound, if not degraded in the intestinal lumen by enzymes or bacteria, is absorbed by the intestinal epithelium (gut wall) and is transported by the blood stream (Portal Vein) to the liver. Here the compound can be metabolised and the compound and its metabolites can be cleared into the systemic blood circulation or into the bile. The compound (and metabolites) cleared in the bile re-enters the intestinal lumen and can be absorbed again. This is called the enterohepatic circulation. The fraction of the compound that is not absorbed in the intestine is excreted in the faeces. Compounds (and metabolites) that have entered the systemic circulation can be excreted either by the kidneys in the urine or in the faeces in the large intestine.

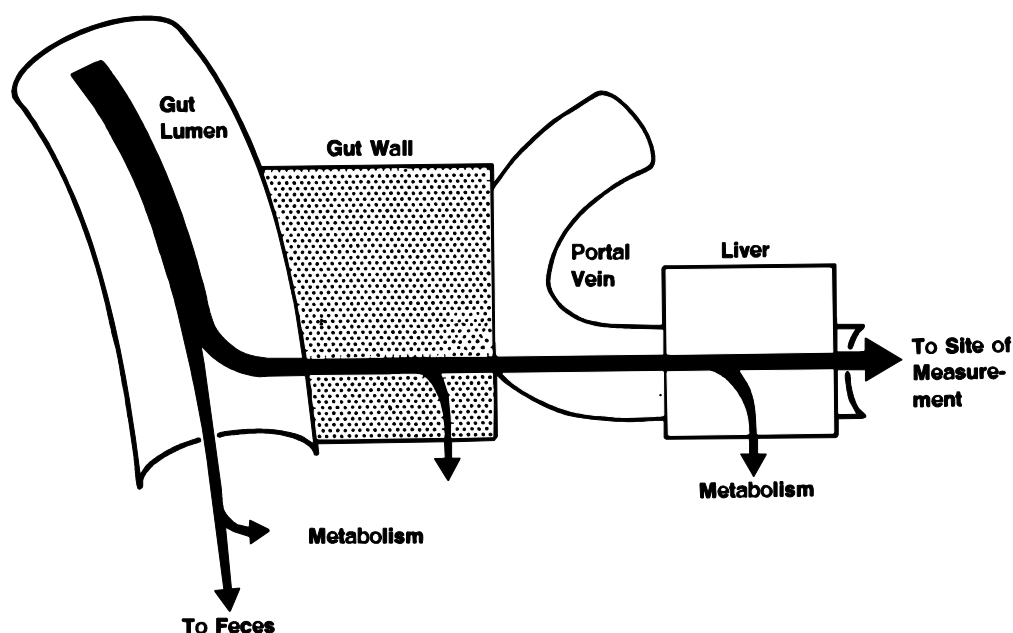


Figure 3.1. Steps involved in the bioavailability of an ingested compound. The first step is the release of the compound from its matrix into the gut lumen. Second, the compound has to be absorbed by the intestinal cells and transported into the portal vein. Third, the compound can be degraded in the liver before it enters the body. [Rowland and Tozer, 1989].

For the oral route, the extent of absorption of a compound in the gastrointestinal tract depends on both physiological factors and on the physicochemical properties of the compound. Physiological factors that will determine the extent of absorption of a compound include the gastrointestinal pH, gastric emptying, gastrointestinal transit, the composition of the intestinal lumen (e.g., pH, enzymes, food), the intestinal epithelium and the liver. The physical properties (e.g. molecular weight, molecular size, lipophilicity, hydrogen bonding potential, pKa), chemical properties (e.g. chemical and enzymatic stability, drug-drug or drug-food interactions) but also solubility, and hence the importance of the matrix, influence the bioavailability of a compound.

Dissolution of a compound is crucial, as whatever the absorption route across the intestinal epithelium may be, a compound has to be dissolved in order to be accessible for absorption.

Both physical properties of the compound as well as the matrix affect the bioaccessibility of the compound (chapter 4). The surface area exposed to the solvent is proportional to the rate of dissolution. Reducing the size of solid particles will thus improve the rate of dissolution. When the bioaccessibility of a compound is high and release from the matrix is much faster than the passage of the intestinal epithelium, most of the compound has dissolved before an appreciable amount has been absorbed (Figure 3.2A). In this case, permeability studies as described in chapters 5-7 may provide a good estimation of the *in vivo* intestinal absorption of the compound. When bioaccessibility proceeds relatively slowly, absorption of the compound cannot be faster than the rate at which the compound is released from its matrix. In this dissolution rate-limited absorption, the concentration of the compound in solution at the absorption site is low because the released compound rapidly enters the body (Figure 3.2B). In this case, a good correlation between *in vitro* bioaccessibility (chapter 4) with *in vivo* absorption of the compound can be achieved. The dissolution profile will determine the concentration profile along the intestine for a much greater time and absorption will occur over an extended period of time and along the complete intestine duodenum till ileum (colon). If the absorption window of a compound is restricted to the first part of the small intestine (duodenum-jejunum), the rate of release is likely to influence the extent of absorption.

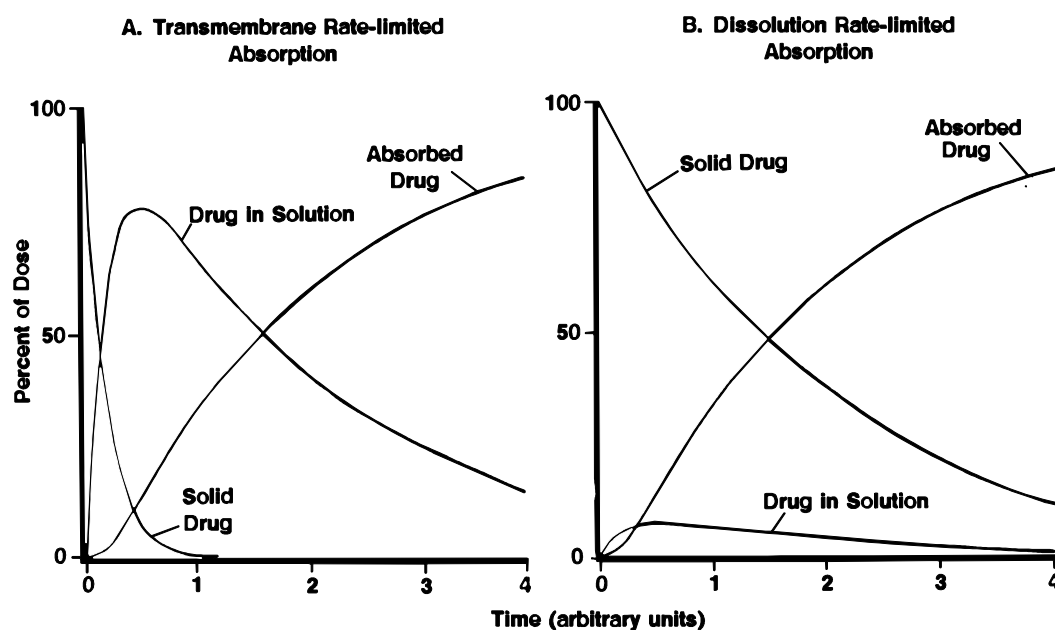


Figure 3.2. When dissolution of a compound is fast, permeability across the intestinal cells is rate-limiting (A). In contrast, dissolution of the compound determines the intestinal absorption rate in case B. [Rowland and Tozer, 1989].

As many compounds are either weak acids or weak bases, and assumed that only the non-ionised molecule is sufficiently lipophilic to cross the biological membranes, the intestinal pH may influence the intestinal absorption by affecting the ionisation of weak acids and bases (Figure 3.3) [Palm et al., 1999]. Bases dissolve more rapidly in an acid solution, whereas for analogous reasons, acids dissolve more rapidly in an alkaline solution. The pH of the gastrointestinal fluids vary between pH 1.0 and 7.5. The fraction unionised only changes dramatically for acids with pKa values between 3.0 and 7.5, and for these compounds a change in the rate of transport with pH is expected when permeability is rate limiting. However, even more than the gastrointestinal pH, the absorption of acids and bases can be markedly improved by using a salt. For example, sodium tolbutamide is absorbed much more

rapid than the free acid, which has a very low aqueous solubility (<10 mg/L). There are other physicochemical interactions, which may also play a role in limiting bioavailability, for example interaction of a compound with the mucus. The mucus layer coating the intestinal epithelium surface contains mucin glycoproteins, enzymes and electrolytes and can reduce the absorption (rate) of compounds by creating hydrogen or ionic bonds. These factors greatly influence the intestinal absorption of (therapeutic) peptides.

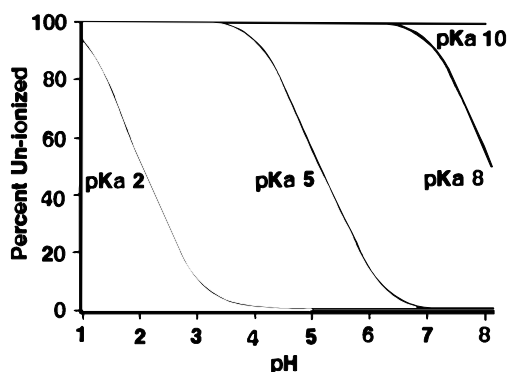


Figure 3.3. Profound changes in the fraction un-ionised occur with pH for compounds with a pKa value between 2 and 8. Very weak acids, $pK_a > 8$, are predominantly un-ionised at all pH values between 1 and 8. For strong acids, $pK_a < 2$, the fraction un-ionised is high at stomach pH values but low at pH values of the small intestine. [Rowland and Tozer, 1989].

Another general factor affecting absorption is the time a compound is in contact with the intestinal surface. Gastric emptying and intestinal transit will in this way affect the absorption (rate) of a compound. Factors known to accelerate gastric emptying time include hunger, moderate physical exercise, prone position and drugs like metoclopramide. Other factors decrease the gastric emptying for example food, emotion, pain, intensive physical exercise, or certain drugs as anticholinergics, amphetamines, and morphinics. Furthermore, drug-drug and food-drug interactions can alter the absorption (rate) of a compound by forming a non-absorbable complex (e.g. iron with tannates, phosphates [Hallberg, 1981], by competition for the same transport system (e.g. zinc and lead affect each others absorption [Conrad and Barton, 1978]), or by reduction in metabolic clearance (e.g. grapefruit juice increases the bioavailability of felodipine [Dresser et al., 2000]). Moreover, degradation of compounds (for example therapeutic peptides) in the intestinal lumen by peptidases, lipases and amylases limits the intestinal absorption of these compounds. Despite all these variables which may affect the bioavailability of an ingested compound, as long as the enzymatic stability is satisfactory, the solubility and passage of the intestinal epithelium is likely to be the critical factor in the absorption of a compound.

3.2 Mechanisms of the passage of compounds across the intestinal epithelium

When a compound is bioaccessible for intestinal absorption, the rate of absorption, i.e. the passage of the intestinal epithelium, is the determining step in intestinal absorption of the compound. Various absorption mechanisms can be distinguished (Figure 3.4). Basically, a compound can be absorbed either by transport through the epithelial cell (transcellular pathway) or by transport along the cells (paracellular pathway). Since the transcellular pathway occupies more than 99.9% of the total surface area (absorptive cells with microvilli),

most compounds are absorbed by the transcellular route. The different pathways of intestinal absorption are shown in Figure 3.4 and are discussed below.

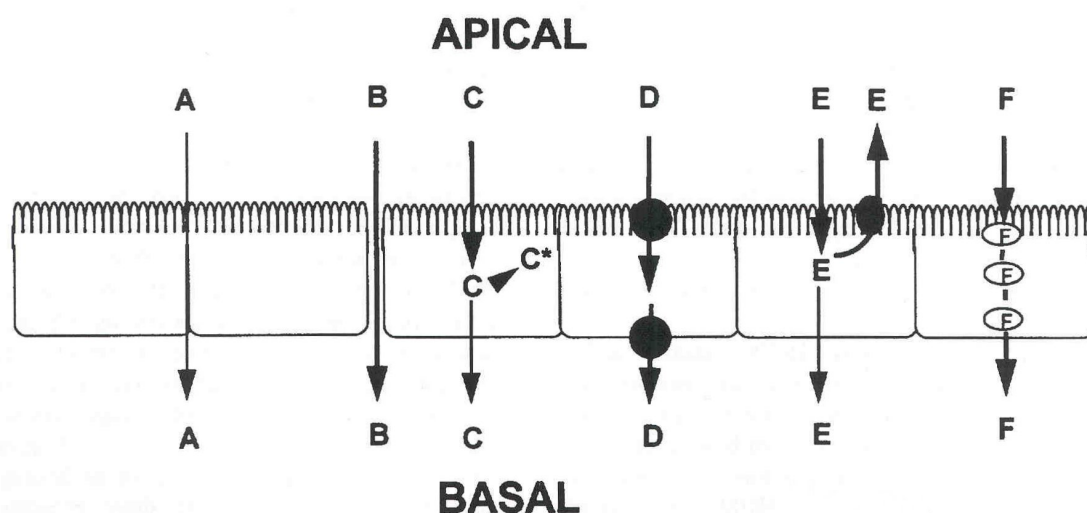


Figure 3.4: Pathways of intestinal absorption. (A) paracellular diffusion; (B) paracellular diffusion enhanced by a modulator of tight junctions; (C) transcellular passive diffusion, (C*, intracellular metabolism); (D) carrier mediated transcellular transport; (E) transcellular diffusion modified by an apically polarised efflux mechanism; (F) transcellular vesicular transcytosis [Hunter and Hirst, 1997].

1. **Transcellular passive transport:** The transport of compounds that are absorbed by passive diffusion is along the concentration gradient and shows linear absorption kinetics. A molecule to cross the apical and basolateral membrane by passive diffusion (pathway C) must have the appropriate physicochemical properties. Single physicochemical properties of compounds such as lipophilicity are roughly correlated to passive drug transport across cell membranes (Figure 3.5). Un-ionised lipophilic compounds ($\log D_{\text{oct},7.4} > 0$) are rapidly absorbed due to their solubility in the lipid bilayers [Artursson and Karlsson, 1991, Wils et al., 1994]. Very hydrophilic molecules ($\log D_{\text{oct},7.4} < -1$) do not solve in the lipid bilayer and can not pass the cell membrane. For very lipophilic compounds ($\log D_{\text{oct},7.4} > 4$), low solubility in the unstirred water layer may decrease the permeability [Wils et al., 1994]. Permeability of such compounds may greatly affected by inclusion of bile salts [Dulfer et al., 1996, Mithani et al., 1996]. In general, poor transcellular permeation of a compound is more likely when the compound follows “the rule of 5”: more than 5 H-bond donors, more than 10 H-bond acceptors (sum of N’s and O’s), the molecular weight > 500D and the $\log D_{\text{oct},7.4} > 4.15$ [Lipinski et al., 1997]. Substrates for transporters and natural products are exceptions. The passive transcellular diffusion of a compound is depended on the net effect of these factors. Therefore, a good correlation was found for fraction absorbed of a compound and its calculated polar surface area. These calculations are based on molecular properties, including polarity and size, to assess the three dimensional shape, and an excellent correlation was obtained between the dynamic polar van der Waals’

surface area of a homologues series of β -adrenoreceptor agonists and their permeability rates in Caco-2 cells and excised rat intestinal segments (see chapter 5) [Palm et al., 1996]. In Figure 3.5 is shown that the correlation for the calculated dynamic polar van der Waals' surface areas was stronger than those between $\log D_{\text{oct},7.4}$ and permeability. Again substrates for transporters are excluded from such a correlation.

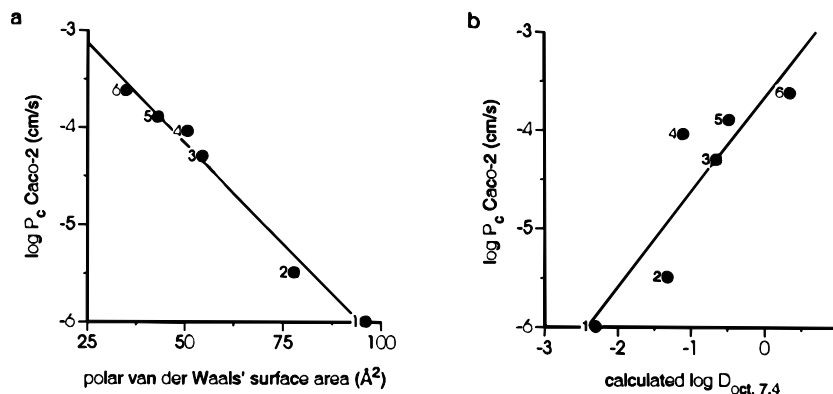


Figure 3.5. Linear correlation between $\log$ cellular permeability (P_c), determined from apparent permeabilities in the Caco-2 model, and the structural descriptors: (a) dynamic polar van der Waals' surface area ($r^2=0.99$) and (b) calculated $\log D_{\text{oct},7.4}$ values ($r^2=0.80$). The numbers refer to 1, atenolol; 2, practolol; 3, pindolol; 4, metoprolol; 5, oxprenolol; and 6, alprenolol [Palm et al., 1996].

2. *Transcellular protein-mediated transport*: Hydrophilic compounds ($\log D_{\text{oct},7.4} < 0$) can not readily pass the lipid bilayers but their transcellular absorption can be facilitated by substrate specific carriers or by active transport proteins (pathway D in Figure 3.4). This transport route is important for the absorption of nutrients such as monosaccharides, amino acids and di/tripeptides, but is also important for the absorption of electrolytes and bile salts and for specific classes of drugs. For example, L-dopa and D-cycloserine are absorbed by intestinal amino acid transport systems, while orally available cephalosporins and renin antagonists are substrates for the intestinal oligopeptide transporter [Tsuji and Tamai, 1996]. Both carrier and active transporter mediated absorption of compounds can be saturated (K_m and V_{max}), is characterised by non-linear absorption kinetics. Whereas carrier-mediated absorption is always along a concentration gradient, active transport is possible against a concentration gradient. Active transport requires an energy supply: either by hydrolysis of ATP (primary energy source) or by a coupled transport of usually Na^+ or H^+ (secondary energy source).
3. *Paracellular transport*: The tight junctions form an aqueous pore between the absorptive cells. Since the surface area in between the tight junction is only 0.1% of the total surface area, drug transport across the intestinal epithelium by this route is minimal (pathway A in figure 3.4). The epithelial junctions become progressively tighter from small intestine to the colon, which mainly decreases the permeability of polar compounds along the intestinal tract [Rouge et al., 1996]. Small hydrophilic molecules (ions) are allowed to pass between cells through the tight junctional opening but the tight junctions form a transport barrier for the absorption of larger molecules ($>400\text{D}$) and are impermeable for macromolecules. This is shown in Figure 3.6 for the oral bioavailability of PEG oligomers in rat, dog and man. In rat and man, oral bioavailability of PEG oligomers $>600\text{D}$ is poor whereas in dog the decrease in bioavailability occurs at higher molecular weight and even at 1000D a low but substantial part of PEG oligomers is still absorbed in

dog. Tight junctional transport is not inversely proportional to the molecular weight of the compound, but depends more on the molecular size. Furthermore, the tight junctional permeability is charge (cations) selective, but the influence of the size is dominant over the charge of a molecule. Transport through the tight junctions is a diffusion-controlled process and shows linear absorption kinetics.

4. The tight junctional barrier function is physiologically regulated, for example, by serosal peptide hormones [Hochman and Artursson, 1994], but it can also be hampered in pathophysiological conditions [Hollander 1988, Malin et al., 1996, Schulzke et al., 1998]. Furthermore, the tight junctions can be opened by a variety of nutritional and xenobiotic compounds or by using absorption enhancers (e.g. salicylates, EDTA, surface active agents) [Hochman and Artursson, 1994]. The latter compounds may open the tight junctions or change the permeability of the intestinal lipid bilayer. The disadvantage of the use of absorption enhancers is the a-specific nature. The intestinal barrier becomes permeable not only for the intended compound, but it can also become more permeable for toxic xenobiotics or even antigens. Another approach to increase the absorption of a hydrophilic compound is to alter the physico-chemical properties of the compound itself. Prodrugs (often esters) can be created to increase the lipophilicity of the compound, and thus increase the membrane permeability, or target the prodrug to a carrier-mediated transport system (prodrug of AZT is a substrate for the di/tri-peptide carrier). The prodrug is then hydrolysed in the enterocytes or in blood to release the active drug.

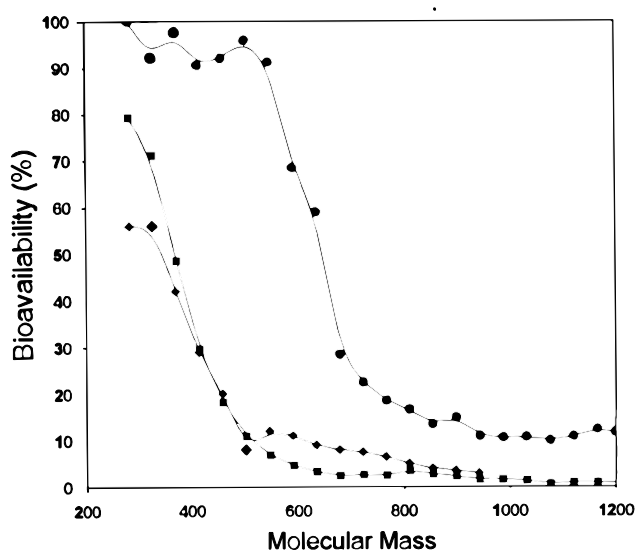


Figure 3.6. Oral bioavailability of PEG oligomers in rat (♦), dog (●), and human (■) against molecular mass. [He et al., 1998]. PEG oligomers are transported via paracellular transport.

5. *Endocytosis*: The tight junctions are normally impermeable for macromolecules, and macromolecules such as peptides and proteins can be transported via specific receptor-mediated endocytosis or via non-specific endocytosis (pathway F in Figure 3.4). Specialised, so-called M cells in the Peyer's patches have less microvilli and show high endocytic activity but the absorptive cells show also endocytic activity [Neutra, 1998]. Endocytosis of compounds is minimal in adult small intestine and is not a quantitatively significant mechanism for drug absorption in the intestine. However, the intimate contact with lymphocytes suggests that endocytosis is probably an important absorption route for potentially antigenic macromolecules [Mayer, 1998, Rouge et al., 1996]. After

endocytosis, the vesicles coalesce in the cell with the lysosomes, leading to digestion of the contents. Therefore, the absorption kinetics is not linear.

6. *Efflux transporters*: Compounds that are adapted for passive transcellular absorption may be substrates for enterocytic intracellular metabolism (pathway C* in Figure 3.4) and for apically polarised efflux mechanisms (pathway E in Figure 3.4). In this way, by metabolism and secretion, the intestinal epithelium plays an important role in protecting the body against the absorption of ingested xenobiotics. No single efflux system explains the diversity of drugs secreted by the intestine but the role of P-glycoprotein in intestinal secretion has received great attention [Hunter and Hirst, 1997].

The role of P-glycoprotein, product of the MDR1 gene, as a drug transport protein is first discovered in cancer cells. P-glycoprotein is a membrane glycoprotein, member of the ABC-transport family, and transports a variety of hydrophobic anti-cancer agents out of tumour cells. In humans two *mdr* genes have been detected, but only the *MDR1* gene encodes for the multidrug-related P-glycoprotein efflux pump. The second MDR gene product in humans, MDR3, has a discrete substrate specificity and transports cholesterol and phosphatidylcholine into the bile [Smit et al., 1993].

In normal tissue MDR1 P-glycoprotein is expressed in many secretory organs including liver, kidney and intestinal tract epithelia. In the intestinal tract P-glycoprotein is localised in the brush border of the mature enterocyte [van der Valk et al., 1990, Meyers et al., 1991]. The localisation of P-glycoprotein in the intestine is in agreement with a secretory function of P-glycoprotein, and P-glycoprotein has been shown to limit the oral bioavailability for various pharmaceuticals [see references in Hunter and Hirst, 1997]. However, more insight in the function of P-glycoprotein in the intestine and in the P-glycoprotein mediated pharmacokinetics of compounds has been gained from knockout mice.

Rodents have 3 *mdr* genes from which the *mdr1a* and *mdr1b* P-glycoproteins show similar characteristics as the human MDR1 P-glycoprotein. Absence of *mdr1*-type P-glycoprotein in mice has a profound effect on the tissue distribution and on the hepatic and intestinal clearance of hydrophobic cationic drugs [Smit et al., 1998]. It was shown in these knockout mice that P-glycoprotein in the blood-brain barrier is very important in preventing the accumulation (up to a factor 20) of a variety of drugs in the brain [Schinkel et al., 1994, 1998, Jonker et al., 1999]. Moreover, intestinal P-glycoprotein has a prominent role in the extrusion of several drugs from the blood into the intestinal lumen, and in preventing drugs in the intestinal lumen from (re-entering) the bloodstream. Other efflux systems such as organic cation and anion transporters (OAT, OATPs, MRPs) are now known to be expressed in the small intestine and in the liver and kidneys. What their contribution to the intestinal absorption or elimination of compound is, is yet to be discovered.

7. *Metabolism (and efflux transporters)*: It has recently become apparent that the role of P-glycoprotein in intestinal absorption of compounds can not be studied without considering the role of the metabolising P450-3A family [Soldner et al., 1999, Watkins 1997]. P450-3A4 is present, like P-glycoprotein, in the villus tip enterocyte. Furthermore, the substrate specificity of P-glycoprotein and P450-3A4 show a significant overlap [Wacher et al., 1995]. Thus, P-glycoprotein together with P450-3A4 may form a co-ordinated intestinal barrier resulting in a limited oral availability [Hall et al., 1999, Watkins et al., 1997]. Bioavailability of a compound could be maximised by inhibition of increasing drug absorption (inhibition P-glycoprotein) and reducing drug biotransformation (inhibition P450-3A) as was shown for example by co-administration of verapamil and digoxin [Pedersen et al., 1983]. To what extent P-glycoprotein or P450-3A4 contribute to the absorption is difficult to determine because of the significant

overlap in substrate specificity. Selective inhibition of the intestinal P450-3A4 activity (no effect on liver P450-3A4 activity) by grapefruit juice increased the oral bioavailability of many P450-3A4 substrates (e.g. felodipine, nifedipine, saquinavir). Grapefruit juice, however, appears to enhance the P-glycoprotein-mediated drug transport [Soldner et al., 1999], and this may, therefore, potentially counteract the P450-3A4 inhibitory effect. Seville orange juice has been reported to inhibit intestinal P450-3A4 without affecting P-glycoprotein mediated transport of cyclosporin A in man [Edwards et al., 1999] and Seville orange juice may therefore be a candidate to study the contribution of intestinal P450-3A4 to the bioavailability of a compound.

P450s occur at highest concentrations in the duodenum, near the pylorus, and at decreasing concentrations distally, being the lowest in the ileum. Highest concentrations occur from midvillus to villous tips, with little or none occurring in the crypts of Lieberkuehn. The P450-3A5 protein and CYP1A1 have been detected in enterocytes but the contribution to the first pass metabolism has not been established. Furthermore, microsomal P450-2C8-10, and 2D6 forms have also been identified in human small intestine. P450-3A is known to be a multigene family both in rats and in humans [de Waziers et al., 1990, and references therein]. However, several compounds that are supported by P450-3A4 in humans are supported by P450-2B1/B2 in rats. In humans, this P450 isoenzyme is not usually expressed. Expression of phase II enzymes have also been found in enterocytes of the small intestine. The human small intestine contains the acid (pi) and the basic (alpha) forms of glutathione-S-transferase [Peters, 1988, de Waziers et al., 1990]. Among extrahepatic sites, the small intestine has the highest level of sulphotransferase activity and the acetyltransferase activity is one-half of that in the liver [Prueksaritanont et al., 1996]. Thus other interactions between efflux and metabolism than P-glycoprotein and P450-3A4 are likely to exist in the small intestine, for example the interaction between organic anion transporter proteins (OATP and MRPs, located in the basolateral and in the apical membrane) and glutathione S-transferases. A better understanding of the interactions between intestinal secretory efflux systems and cellular metabolism pathways will allow optimisation of oral drug absorption.

In **summary**, compounds that are well absorbed in the intestine must meet various conditions. The “rule of 5”, small ($M_r < 500$ D), lipophilic ($0 < \log D_{\text{oct},7.4} < 4.15$), and without too many H-donor and H-acceptor sites, will indicate whether a compound is likely to be transported via the passive transcellular route. When a compound is too hydrophilic (or too large or too many H-donor and H-acceptor sites) to be transported via the transcellular route and a high and fast absorption is required, such as is the case for nutrients, the compound should be transported across the intestinal epithelium by specific carrier-mediated mechanisms. For compounds that are transported via the transcellular route, the amount of compound reaching the portal vein can be reduced by efflux transporters or by metabolism in the intestinal cells. To access the diffusive paracellular pathway the compound must be small ($M_r < 400$ D) and hydrophilic. In general the absorption of paracellularly transported compounds is slow.

4. In vitro systems for studying bioaccessibility

As was discussed in chapter 3.1, release of a compound from its matrix is a prerequisite to intestinal absorption. Therefore bioaccessibility of a compound should be determined in the small intestine, where absorption takes place. Release of a compound from its matrix starts in the mouth. It is continued in the stomach and the small and large intestines. Absorption of compounds occurs mainly in the small intestine. The number of *in vitro* studies on bioaccessibility is growing, but is still a developing area of investigation. To obtain reliable bioaccessibility data, the *in vitro* system should mimic the *in vivo* situation in the gastrointestinal tract. Although the release of a compound in the gastrointestinal tract is a dynamic process of continuous changes in physiological parameters, most methods consist for practical reasons of a series of static steps. The test material is exposed to an enzyme solution at a fixed pH and temperature during a fixed period of time simulating a particular step of digestion. In general, the methods described involve a single, two- or three-step procedure simulating stomach, stomach/small intestine and stomach/small intestine/large intestine or mouth/stomach/small intestine.

The bioaccessibility tests performed by the pharmaceutical industry, to select during the development phase of an orally administered drug those drugs with a high intestinal absorption, follow a single step procedure simulating only one part of the gastrointestinal tract at a time. The most frequently used *in vitro* systems to study drug release from orally administered solid formulations are the rotating disk apparatus and the rotating basket apparatus (Figure 4.1). The solid drug is placed in a vessel and the release in the medium, maintained at 37°C, is measured in time. Mostly simple aqueous dissolution media at pH 7 are being used. For compounds that are quickly dissolved (>85% dissolved in 30 min [USP23]) such a simple solution may be adequate.

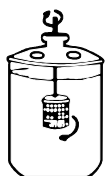


Figure 4.1. Rotating basket apparatus used for dissolution studies.

In figure 4.2A is shown for a water-soluble compound (acetaminophen) that the dissolution rate in water and in more physiologically based solutions including bile salts was similar. However, using milk as solution greatly affected the release of this compound, which was due to interaction of the tablet with milk components. For compounds with a slow dissolution profile, dissolution at different pH and with inclusion of bile salts in the dissolution media greatly affected the dissolution profile (Figure 4.2 B+C) [Galia et al. 1998, Dressman et al.1998]. In the case of weak base, dissolution in the stomach (low pH) will be of great importance as was shown for ketoconazole in Figure 4.2B. On the other hand for a weak acid dissolution under intestinal conditions (a higher pH than the stomach pH) will be important. In the case of a neutral compound, the presence of solubilising components such as bile salts and phospholipids is of the determinant of solubility and hence dissolution profile (Figure 4.2C). Thus, media for *in vitro* dissolution tests based on physiological relevance are affecting and probably better mimicking the *in vivo* dissolution especially of compounds with a slow dissolution profile. However, physiologically relevant compounds such as bile salts

and phospholipids are too expensive to use for screening purposes and are, therefore, not commonly used for dissolution tests in the pharmaceutical industry.

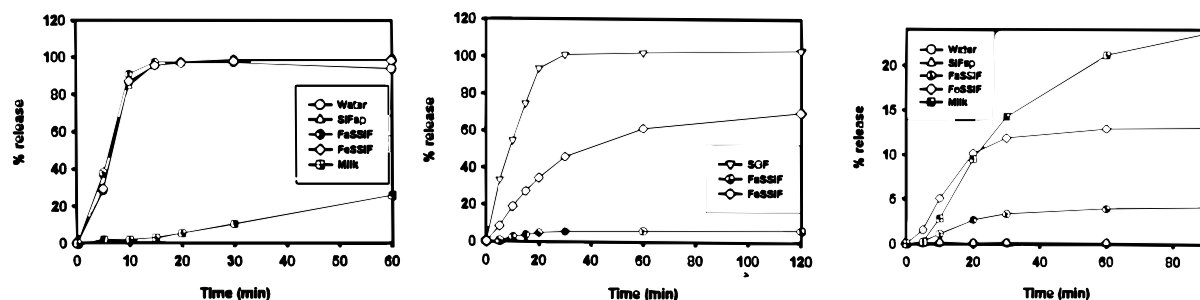


Figure 4.2. Dissolution profiles of compounds, which are (A) good aqueous soluble (acetaminophen), (B) a weak base (ketoconazole), (C) poor aqueous soluble (danazol), in different dissolution media: water (open circle), low pH (triangle), 3 mM taurocholate (diamond), 15 mM taurocholate (filled circle), milk (filled square). [Galía et al., 1998].

More physiologically based methods consisting of a two- or three steps procedure have been described for the bioaccessibility of compounds from soil and food [Ruby et al., 1992, Rotard et al., 1995, Minekus et al. 1995, Glahn et al., 1996, Sips et al., 1998]. These methods simulate subsequent stomach/small intestine, or stomach/small intestine/large intestine, or mouth/stomach/small intestine by using enzyme solutions, pH and residence time periods typical for each compartment. In our laboratory a modified version of the model of Rotard et al. [1995] was developed [Sips et al., 1998] based on the physiology of children to study the bioaccessibility of heavy metals and hydrophobic organic compounds from soil [Oomen et al., 2000]. The digestion process is schematically presented in Figure 4.3.

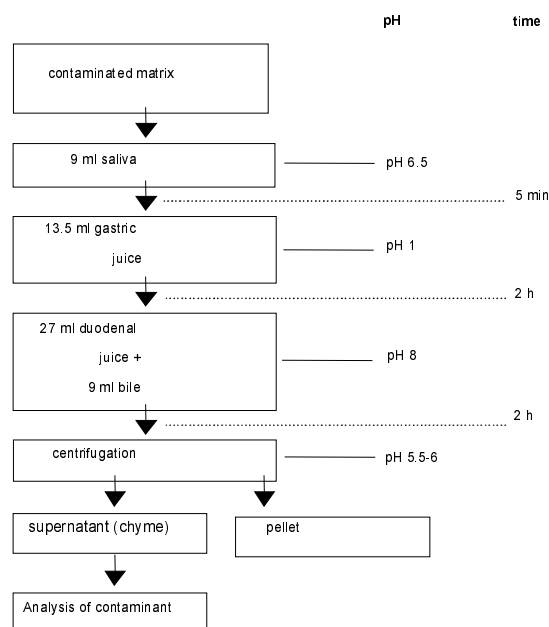


Figure 4.3. Schematic representation of the procedure of an artificial digestion method to determine the bioaccessibility of contaminants in soil. [Sips et al., 1998].

Enzymes and bile isolated from animals are used to prepare artificial saliva, gastric juice, duodenal juice and bile solutions. Saliva is added to the matrix and rotated for 5 min at 37°C.

Subsequently, gastric juice is added, and the mixture is rotated for 2h at 37°C. In the last digestion step duodenal juice and bile are added to the mixture, which is rotated for another 2h. Finally, the suspension is centrifugated for 5 min at 3000g, yielding a pellet of not dissolved matrix material and supernatant (i.e. artificial chyme) containing the contaminant released from its matrix. The volume and the ratio of digestion juices are based on the daily production of man, physiological transit times are applied and the gastric pH is set to 1 to imitate the fasting conditions of a child. Another, more sophisticated, dynamic model was introduced by Minekus et al. [1995]. This latter model is also a multi-compartmental model including the stomach and small intestinal parts jejunum and ileum (if required also colon) but not the mouth. Peristaltic movement is simulated in this model and stomach and duodenal pH and water absorption in the small intestine are continuously monitored and controlled. Compounds that are released from the matrix into the chyme are removed from the chyme by dialysis with hollow-fibre devices. In this model, the bioaccessibility of a compound is not determined as the fraction that is released from the matrix but by the fraction that is subsequently dialysed. The bioaccessibility of compounds that form complexes with ingredients of the intestinal fluids such as bile salt complexes, but can still be absorbed, may be underestimated because these complexes will not be dialysed. The advanced equipment used in this model restricts the particle size of the matrix. The size of tablets or small pieces of toys is too large to be handled by the model.

In **summary**, various *in vitro* models and test procedures have been described in literature to estimate dissolution and bioaccessibility of compounds. The dynamic model described by Minekus et al. is simulating the *in vivo* situation as close as possible. Not only the gastrointestinal juices and transit times are simulated, as is the case with many static models, but also the dynamic conditions that occur in the lumen of the gastrointestinal tract are simulated. However, this model requires advanced laboratory equipment, and is less suitable to apply on a random matrix or to use for screening purposes. For these reasons the model might be less useful as a tool for (ad hoc) health risk assessment. The static models, as described by Rotard et al. and Sips et al., provide a good starting point for a test procedure combining a simple, easy to manipulate model and a model representative for human physiology. A disadvantage of most *in vitro* systems is that validation of the system to the human *in vivo* situation has not been described in literature.

5. *In vitro* models for studying intestinal permeability

The success of *in vitro* models to determine the intestinal absorptive potential of a compound and to study the mechanism of absorption will depend on how closely the model mimics the characteristics of the *in vivo* situation. Although it is difficult to reproduce *in vitro* all of the characteristics of the intestinal mucosa, various systems have been developed which, to varying degrees, mimic the characteristics of the intestinal mucosa. Various *in vitro* models utilising excised tissues, isolated cells and membrane preparations, and cultured cells are reviewed. Principle guidance for this chapter are the recent reviews by Barthe et al. [1999] and Hillgren et al. [1995] supplemented with the conclusions of each review meeting on the *in vitro* model topics.

5.1 Ussing chamber / Mucosal sheets

Ussing chambers and their variants are a popular *in vitro* system, where small sections of intestinal mucosa are clamped between two chambers containing buffer, and hence the transport of molecules across the tissue and between the chambers can be measured (Figure 5.1). The diffusion chambers used are generally designed after the Ussing chamber, which were developed by Ussing and Zerahn in 1951 to measure ion fluxes across biological tissues.

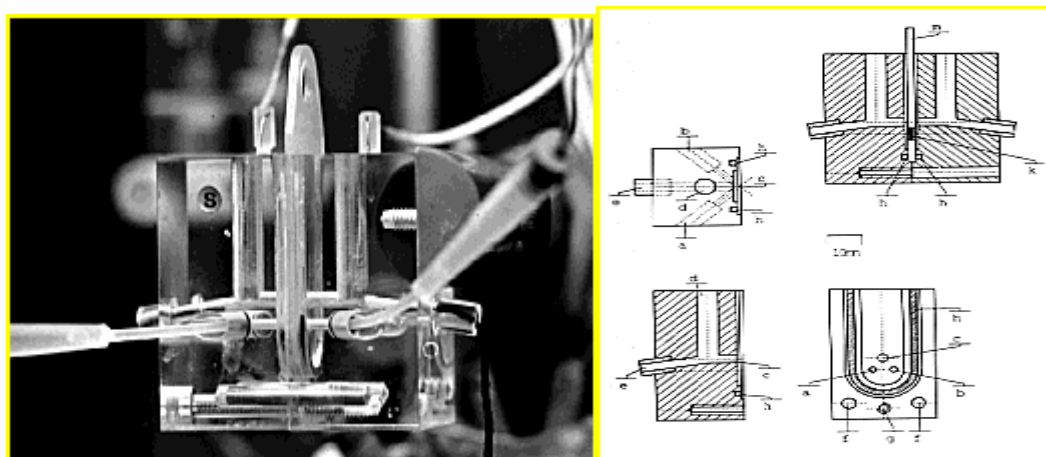


Figure 5.1. Picture and schematic drawing of a transport-Ussing chamber device. Intestinal mucosal sheet is mounted in between the two identical half-chambers. Trans epithelial electrical resistance can be monitored.

<http://www.bio.uva.nl/onderzoek/KDekker/pict02.htm?21,14> (University of Amsterdam)

To prepare the tissue, the intestine should be isolated under anesthesia or immediately after sacrifice of the animal. Subsequently, intestinal parts of interest are rinsed with buffer to remove the intestinal content. To improve the oxygenation of the tissue and, thus, the viability and integrity, the underlying musculature is commonly removed, so called "stripped", to yield mucosal sheets. For smaller animals (mice, rat, goldfish), the musculature is stripped of the mucosa according to the "goldfish" method [Bakker et al., 1993]. In short, the intestine is carefully placed over a wetted, small plastic rod with a rounded top (lumen in contact with the rod). The outer layer of the intestine is carefully incised longitudinal (not through the complete tissue) on both sides of the mesenterium and the outer muscularis can be pulled off in the length direction. Then, flat sheets of mucosal tissue

can be cut and placed in the side-by-side diffusion chambers. For larger animals (dog, pig, chicken etc.) the mucosal sheets can be prepared as follows: the intestine is cut open in the longitudinal direction and placed with the mucosa on top on a flat underground. Subsequently, the mucosa layer is scraped of the musculature by gently pulling with a flat object (razor blade, plastic card etc.). A steel punch can be used to obtain mucosal sheets.

The feature of Ussing-like chambers is that they allow measurement of the electrical parameters of the mucosal sheet during the course of the experiment. The short circuit current may indicate the viability of the tissue during the experiment. It has been noted that the short circuit current increased after removal of the musculature (refs in Hillgren et al., 1995). It was not determined whether the ion flux was decreased due to poor oxygenation of the serosal side with the muscle layer intact or whether the musculature forms a barrier to the ion flux. The stripped mucosa sheets have been used primarily to study ion fluxes and the effect of various drugs and neurotransmitters on them (refs in Hillgren et al., 1995). However, to measure the electrical parameters simple salt buffers are used as incubation medium. The viability of the tissue for time incubations longer than 1 hour is very uncertain in such media (refs in Barthe et al., 1999). In addition, the intestinal mucosa is metabolically very active, and it is, therefore, important that there is an adequate supply of energy sources such as glucose or glutamine.

To improve the viability of the tissue, cell culture media are being used in transport studies, which means that the electrical parameters can not be measured during the course of the experiment. The transport barrier integrity is monitored by determination of the transport of a poorly or non-absorbable compound. For transport studies, the compound of interest is added to the donor compartment and its accumulation in the receiver compartment is measured as a function of time. The flux across the tissue is defined as the rate of accumulation normalised for tissue surface area. Passive diffusion will be characterised by equal rates of transport from serosal-to-mucosal and mucosal-to-serosal. On the other hand carrier-mediated processes are characterised by a non-equal ratio between these two fluxes and the transport can be saturated. Thus, mechanisms by which a compound is transported across the intestinal epithelium (carrier- and transporter-mediated transport, passive transcellular and paracellular transport, endocytosis) can be studied by using mucosal sheets. The conditions to which the tissue is exposed are easily manipulated and the samples obtained are “clean” buffer samples, which can be easily analysed by HPLC. The sensitivity of the analysis of a compound may be a problem since the surface area of the mucosal sheets is often restricted to 0.2 to 1 cm². Provided sensitive drug-metabolite assays are available, metabolism can be evaluated and transport of metabolites can be determined.

Permeability of compounds may be position dependent due to the different morphology and mucosal cell differentiation down the intestine e.g. amino acid and di/tripeptide transporters are expressed in jejunum and ileum but not in colon. This *in vitro* model is a powerful tool for studying transport at specific position of the intestine (duodenum, jejunum, ileum, large intestine) [Jezyk et al., 1992]. Furthermore, an interspecies comparison can be made under the same experimental conditions [Jezyk et al., 1992]. Diet and pathological conditions can affect the intestinal transport barrier that can easily be investigated by using this *in vitro* model [van der Klis and Versantvoort, 1999, Malin et al., 1996].

As with all *in vitro* methods, there are limitations to the Ussing chamber technique, mainly arising from the absence of an intact mesenteric blood supply or lymph drain. Although the bathing solutions can be and should be oxygenated, oxygenation of the intestinal tissue is

poor compared to the oxygenation by blood. The integrity of the intestinal transport barrier, as was discussed above, only allows measuring the transport of compounds for utmost a few hours. This is mainly a problem to reliably measure compounds that are very slowly transported. The absence of an intact mesenteric blood supply may also form a problem in those situations where rapid removal of transported drug from the acceptor side is essential to maintain sink conditions. This is for instance the case when rapid diffusion occurs and the rate limiting step is located at the deeper tissue levels. The absence of an intact lymph drain may result in accumulation of very lipophilic compounds in the tissue. Like most other *in vitro* (*in situ* and *in vivo*) models, one of the pitfalls of this methodology is that dissolution and solubility of a compound are not taken into account. Furthermore, the mucosal sheets are incubated with “clean” buffer instead of the intestinal lumen content. This may greatly affect the absorption rate of certain compounds.

In summary: The technique lends itself to fast and reproducible intestinal absorption studies. It can be applied to study the mechanism of the transport, and is very useful for interspecies comparison and position specific (e.g. jejunum, ileum) transport. A major drawback of the technique is that truly fresh intestines should be used because of the rapid decrease in viability of the tissue, implying that it is difficult to use human material. However, determining the transport of a non-absorbable compound simultaneously can monitor the integrity of the transport barrier of the intestinal tissue. The technique is rather simple, commercially available, and if electrical parameters are not to be measured the equipment is not too expensive.

5.2 Everted sacs

The everted gut sac technique was first described by Wilson and Wiseman [1954] to study the transport of sugar and amino acids. An intestinal sac is prepared by inverting a segment of the intestine over a glass rod. Thus, the mucosal epithelium with the villi is turned inside out, while the muscular membrane forms the inner surface of the everted sac. The inner compartment is filled with buffer, both ends are tied off. The sac is then placed in a beaker of well-oxygenated solution containing the compound of interest and transport into the sac is determined (Figure 5.2).

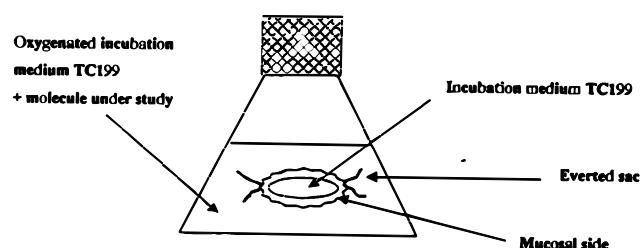


Figure 5.2. Everted sac: the intestinal sac is incubated in oxygenated tissue culture medium while shaking. Each sac is removed at the required time point. Analysis of the compound is determined inside the sac (serosal space) and in the tissue.

Handling and preparation of the tissue are important. Immediately after killing the animal, the entire intestine is quickly excised, flushed through several times with saline solution at room temperature and placed immediately into oxygenated tissue culture medium at 37°C. Then the intestine is gently everted over a glass rod (diameter 3 mm for rat intestine), filled with fresh oxygenated tissue culture medium and divided into sacs approximately 2-4 cm in length. In simple buffer solutions the viability of the sacs was poor, but by using oxygenated

tissue culture medium tissue viability could be maintained for up to 120 min. This was demonstrated by oxygen consumption, glucose and amino acid uptake, as well as by histology at light and electron microscope level [Bridges et al., 1978, Barthe et al., 1998]. To check sac viability and integrity, glucose concentration can be monitored inside and outside the everted sac at the end of the incubation. As glucose is actively transported, the concentration inside the sac should be higher than in the incubation solution. At the end of the incubation, the compound of interest should be analysed in both the solution inside the sac and in the tissue. The tissue is dissolved in 1M NaOH and the protein content is measured to enable normalisation of the transport of the compound for each sac.

The mechanism of uptake of compounds can be studied and differentiated with this technique. To obtain the kinetics of uptake and transport, test compounds are incubated with sacs for various time periods. One sac is required for each time point, since the serosal compartment is a closed compartment. Since the underlying musculature is not removed, the musculature may provide a transport barrier and underestimate the actual transport that will take place *in vivo*. By using mucosal sheets, the ratio of mucosal to serosal vs. serosal to mucosal transport provides a first indication for passive or active and carrier-mediated transport. Such a simple evaluation can not be made by using the everted sacs but identification of the mechanisms can be made based on non saturable/saturable transport characteristics (passive/protein mediated) and on the effect of modifiers (P-glycoprotein, paracellular transport), metabolic inhibitors (active transport) or competition with structural similar compounds (carrier-mediated).

In **summary**: The everted sac system offers a simple and inexpensive technique without specialised equipment. It can provide information on the mechanism of absorption and affords to investigate differences in absorption of compounds along the length of the gastrointestinal tract and an interspecies comparison. Similarly to the use of mucosal sheets, truly fresh intestines should be used because of the rapid decrease in viability of the tissue, implying that it is difficult to use human material. Another major disadvantage is that the serosal compartment is a closed compartment with a small volume. Therefore, the concentration of compounds will rapidly rise inside the sac and back-flux of the compound will occur. This will distort the kinetics of rapidly absorbed compounds. Furthermore, many sacs are needed to obtain information on the kinetics of compounds since for each time point a sac is required.

5.3 Isolated epithelial cells and membrane vesicles

5.3.1 Isolated intestinal enterocytes

To study the transport of compounds across the intestinal epithelium, the ideal system would be a monolayer of enterocytes. The uptake and transport of compounds could then be studied without the complications of the musculature and the submucosa. Isolation of intestinal enterocytes can be accomplished using proteolytic enzymes (trypsin and hyaluronidase), chelating agents (EDTA and citrate-EDTA), or mechanical dissociation (scraping and vibration). The intestinal segment is removed from the animal and filled with buffer containing the enzymes or chelating agents or filled with a simple physiological buffer solution when mechanical dissociation is used. The absorptive cells will be released into the buffer solution whereas the crypt cells are still connected with the tissue. The best technique

seems to vary with the species being used [Kimmich, 1990]. Proteolytic enzymes may damage proteins in the membrane or decrease viability [Eade et al., 1984]. Chelating agents can increase the permeability of the membrane to ion fluxes [Kimmich 1990]. Mechanical dissociation can also damage external proteins and weaken the cell membrane [Kimmich 1990]. Furthermore, the isolation procedures tend to lack reproducibility, with large variability in viability, number and characteristics of cells obtained.

Isolated enterocytes allow uptake experiments to be done in a controlled environment and for several compounds or conditions to be done with one animal. Enterocytes can be isolated from different regions of the intestine to explore the region specific uptake of a compound. However, enterocytes generally have a very short lifetime once isolated [Kimmich, 1990] and do not attach to filter inserts implying that no monolayers can be obtained. Thus, experiments must be done rapidly using freshly isolated enterocytes in suspension. This allows for only uptake, not transport, to be studied. Moreover the polarity of the uptake, whether it is taking place from the apical or basolateral surface cannot be distinguished. If a receptor or a carrier at the basolateral surface of the enterocytes mediates uptake of a compound, the conclusion may be that this compound is absorbed when, in fact, it would not be absorbed *in vivo* (absorption takes place at the apical surface of the enterocyte). Also it is not possible to determine if the compound is transported through the enterocyte or sequestered inside the cell. Nowadays, this method is not widely applied any more for studying intestinal absorption.

In **summary:** Till a solution can be found to allow freshly isolated enterocytes to grow as a monolayer on permeable filters, this method is not widely applied for study of intestinal absorption of compounds.

5.3.2 Membrane vesicles

Over the past three decades membrane vesicles have been prepared to study epithelial transport processes. Membrane vesicles allow further simplification of the system. They not only isolate the absorptive cells from the underlying musculature and submucosa, but also allow one to look at the interaction of a substrate with only the brush border or basolateral membrane of the enterocyte without the interference of intracellular metabolism. In 1973, Hopfer et al. were the first to demonstrate that the glucose uptake into brush border membranes isolated from rat intestine was a sodium-dependent process as it is *in vivo*. Since then, brush border membrane vesicles have been isolated from numerous species, including man, and have been used to study the transport of both nutrient and drugs.

The most common method of preparation of brush border membrane vesicles for transport studies involves divalent cation precipitation with either calcium or magnesium. The reasons for the popularity of this method are the ease and speed (within 3h of animal sacrifice) of the procedure and the need for little specialised equipment. The divalent ion (generally 10 mM) is added after homogenisation and aggregates the basolateral membranes to intracellular organelles. It is hypothesised that the brush border is protected from the divalent cation precipitation by the ability of the glycocalyx to compensate the charge so that the brush border membranes do not aggregate. A spin at low speed precipitates the basolateral membranes and intracellular organelles. The supernatant contains the brush border membranes and can be isolated by ultracentrifugation. To increase vesiculation, the membrane preparation is normally homogenised in a glass teflon homogeniser or run through a gauge needle. The orientation of the brush border membrane vesicles is normally for > 90%

right side out; i.e., the vesicular membranes are oriented in the same fashion as the brush border *in vivo*. The vesicles are free of intracellular organelles other than for some skeletal microfilaments (actin, calmodulin, villin), which can be removed by extraction with high concentrations of thiocyanate [Hopfer et al., 1983]. This has been shown to result in increased enrichment of purity and in enhancement of measured transport rates [Hopfer et al. 1983].

Different procedures have been developed for the isolation of basolateral membrane vesicles but the most widely accepted method is that of Scalera and co-workers [1980]. The method is based on high-speed differential centrifugation followed by separation on a percoll density gradient. The gradients are then fractionated and tested for Na⁺-K⁺-ATPase activity, which is a marker for the basolateral membrane, and for a marker of the brush border, such as alkaline phosphatase, sucrase, or aminopeptidase. The basolateral membrane vesicles are approximately 60% inside out as tested by using an enzyme marker and comparing the activity in presence and absence of a membrane permeabiliser, such as Triton X-100.

Transport of compounds into isolated membrane vesicles can be studied by utilising rapid filtration techniques. The vesicles are incubated with a (radiolabeled or fluorescent) substrate. The uptake reaction is terminated by dilution of the vesicular mixture with an ice-cold buffer solution, and the vesicles are immediately collected by filtration. Non-specific bound radiolabeled substrate to the filter is washed off with aliquots of ice-cold buffer solution. Alternatively, efflux experiments can be carried out by preloading the vesicles with radiolabeled (fluorescent) substrate, then transferring the vesicles into a medium without substrate. Efflux of the substrate from the vesicles is followed in time.

Membrane vesicles have been and are being used to characterise various transport systems, including determining their kinetic parameters and studying the effect of various physiological parameters on the regulation of the membrane transport [glucose, di/tripeptide]. A major advantage of the use of membrane vesicles over the use of other biological preparations is that the plasma membranes from the luminal and basolateral sides are studied separately and that the preparation is free of interference from metabolic enzymes. The mechanism of carrier-mediated transport can, thus, be studied in detail. The paracellular pathway and endocytosis do not contribute to the transport system studied. Another disadvantage is that the vesicles are often contaminated with small amounts of other membranes so the purity of the vesicles should be assessed periodically. Due to the small volume of the vesicles, initial velocities of uptake deviate very early from linearity. Furthermore, the detection method has to be very sensitive; in practice only radiolabeled or fluorescent substrates are being used. This implies that the membrane vesicles are not suitable to use as a screening tool to study membrane transport.

In summary: Membrane vesicles can be prepared from intestinal tissue. Little specialised equipment is needed for this technique. Position specific transport (jejunum, ileum etc.) and interspecies comparison can easily be studied. Truly fresh and viability of the tissue are less strict for the preparation of membrane vesicles compared to the use of mucosal sheets and intestinal sacs. Therefore, it is somewhat easier to obtain human material. Carrier-mediated transport processes can be studied in detail by using membrane vesicles, since the apical and basolateral membranes are separated from each other. But the consequences are that the use of membrane vesicles is restricted to carrier-mediated transport processes only and that this technique is not suitable to estimate the intestinal absorption of compounds *in vivo*.

5.4 Cell cultures

As discussed above, several *in vitro* methods have been developed to study the intestinal absorptive and barrier functions. These models are less suitable to use as a screening tool to investigate human intestinal absorption of compounds because of difficulties in obtaining viable human tissues. Nowadays most studies are being performed with human intestinal cells grown on permeable filter supports (Figure 5.3). The popularity of cell cultures, predominantly Caco-2 cells, in studies of intestinal drug transport and metabolism can probably be explained by the ease with which new information can be obtained from these simple *in vitro* models. Drug transport experiments using cell cultures are easy to perform in large numbers and under controlled conditions, resulting in the generation of a wealth of information. This information can often easily be translated into fundamental principles since the cell culture models lack many of the obscuring variables found in more complex (and complete) drug absorption models. For instance, much of our more recent knowledge on intestinal active drug transport mechanisms has been derived from cell culture studies as new information on passive transcellular and paracellular drug transport mechanisms [Hochman and Artursson, 1994, Adson et al., 1995, Pade and Stavchansky, 1997]. The good correlation between passive drug transport through the cell monolayers and that seen *in vivo* has made it possible to use the cell cultures for studies on structure-absorption relationships.

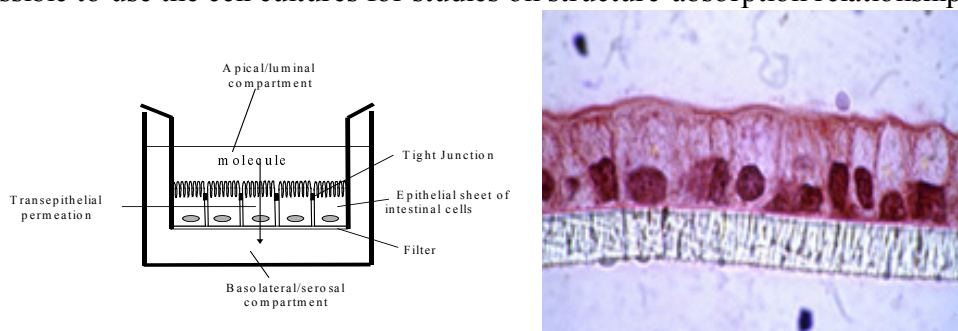


Figure 5.3. Two compartment system to study transport of compounds across a monolayer of intestinal cells. Right: Stained cross-section of Caco-2 cells grown on permeable filter supports.

Caco-2 cells, derived from a human colon carcinoma, spontaneously differentiate after reaching confluency, forming a polarised monolayer with well-defined brush border on the apical surface and tight junctions [Pinto et al., 1983]. The brush borders express digestive enzymes (e.g. sucrase isomaltase, lactase, alkaline phosphatase) and many intestinal carrier systems (e.g. amino acids, di/tripeptides, nucleosides, bile acids and vitamin B12) [Quaroni and Hochman 1996, Hidalgo and Li 1996]. Furthermore, Caco-2 cells express efflux systems (P-glycoprotein, MRP1-3), phase I metabolic enzymes (cytochrome P450s) and phase II conjugating enzymes (sulfotransferase, UDP-glucuronyltransferase, glutathione S-transferase) and thus represent an intestinal-like biochemical barrier [Hunter and Hirst, 1997, Prueksaritanont et al., 1996]. When cultured for 2-4 weeks on permeable filter supports (Figure 5.3), Caco-2 monolayers become more polarised than on plastic and thus provide a good *in vitro* model for conducting intestinal drug transport studies.

Transport studies are performed with cells grown on permeable filter inserts, which are placed in a well of a normal cell culture plate (Figure 5.3). When cells are grown to confluence, the cell layer forms a barrier between the two compartments (apical/mucosal and basolateral/serosal). The compound of interest is added to one compartment and the appearance in the other compartment is measured. To improve the homogeneity of the solutions (reduction of the unstirred water layer on top of the cells), the cell culture plate

should be placed on a shaker during the transport experiments. The transport is usually expressed as the apparent permeability coefficient (P_{app} in cm/s) determined as:

$$P_{app} = dQ/(dT \times A \times C_0)$$

Where dQ/dT is the transport rate of the compound across the cell layer ($\mu\text{g/s}$ or mol/s), C_0 is the initial concentration of the compound ($\mu\text{g/ml}$ or mol/ml) in the donor compartment, and A is the surface area of the filter (cm^2). The permeability coefficient of a compound across the Caco-2 monolayer cells was correlated to the human *in vivo* absorption of compounds [Artursson and Karlsson 1991]. As can be seen in Figure 5.4, an S-shape curve was obtained between permeability coefficient of several compounds across the Caco-2 monolayer and % absorption in human. Well absorbed compounds had a $P_{app} > 1 \times 10^{-6}$ cm/sec, whereas compounds with a low absorption (<10%) had a $P_{app} < 1 \times 10^{-7}$ cm/sec [Artursson and Karlsson 1991]. The absolute P_{app} values may vary among different laboratories [Artursson and Karlsson, 1991, Wils et al., 1994, Stewart et al., 1995], however, compounds, which are moderately absorbed such as mannitol, are at a similar position in the S-shape. Therefore, the permeation coefficient of an unknown compound should be compared with the permeation coefficient of a reference compound such as mannitol in order to estimate the absorption of a compound. Many pharmaceutical companies are now using Caco-2 monolayers to screen for potential absorption problems in drug discovery programs during the high-throughput screening. The Caco-2 monolayers have also been used in preformulation studies of the cellular effects of co-solvents and absorption enhancers [Muranishi, 1990, Hochman and Artursson, 1994]. As a result, the FDA is currently evaluating the role of Caco-2 monolayers in the standard procedures of pharmaceutical drug development.

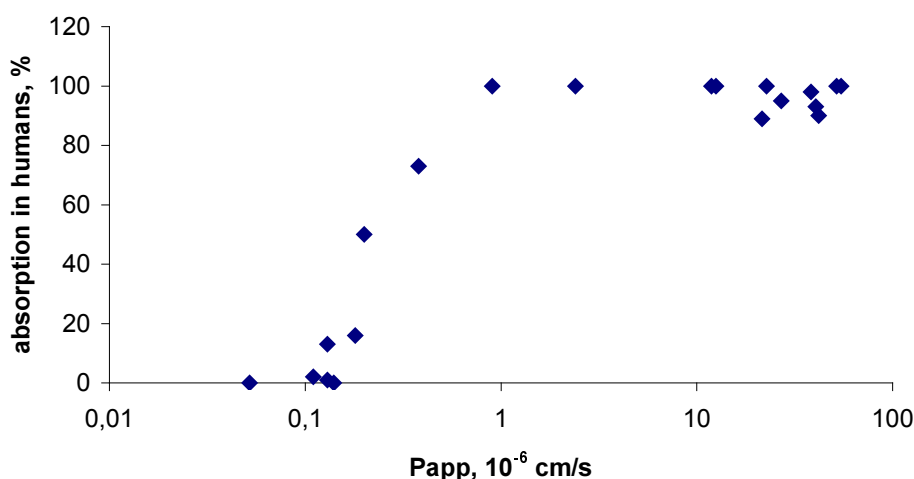


Figure 5.4. Correlation between absorbed fraction in humans after oral administration (expressed as % of the administered dose) and permeability in Caco-2 monolayers [Karlsson and Artursson, 1991].

Then, can the Caco-2 monolayers be used to predict human intestinal absorption of any compound regardless of its transport mechanisms? Although the Caco-2 cells (and other cell culture models) provide a powerful tool, these models are not perfect and refinements are needed to better predict human intestinal absorption. For instance, other contributing factors

affecting the intestinal absorption of compounds such as bioaccessibility and solubility of compounds are often neglected since transport of the compound in solution is determined in culture medium without physiologically relevant compounds. It has been shown for very lipophilic compounds (e.g. PCBs and cholesterol) that the uptake and transport through the Caco-2 cells was greatly enhanced in presence of bile salts and phospholipids [Dulfer et al., 1996, Homan et al., 1998]. Recently Glahn et al. [1996] has developed a model to assess iron availability from foods that combines simulated peptic and intestinal digestion with measurement of iron uptake by Caco-2 monolayers. Not only the bioaccessibility of iron but also the uptake of iron in Caco-2 cells was dependent on the food source (beef, chicken, fish or casein). Thus, the two most important factors determining intestinal absorption, release of compounds from the matrix and intestinal epithelium transport, can be measured in one single experiment.

Most of the known drug transporters and carrier systems of the small intestine are expressed in Caco-2 cells (Table 5.1). However, the expression level and substrate specificity of the carriers and efflux systems found in Caco-2 monolayers tend to be variable in comparison to the *in vivo* situation and, therefore, prediction of the *in vivo* intestinal absorption should always be made with precaution. Expression of transporters and tight junctions is normally ideal between certain days in culture, i.e. stage of differentiation, which may vary from transporter to transporter. For example, maximal expression of the biotin transporter was 10 days, but maximal expression of the bile acid transporter was 25 days post-seeding [Ng and Borchardt, 1993, Hidalgo and Borchardt, 1990]. Similarly the expression of intestinal metabolising enzymes is clearly different in intestinal cell cultures and the *in vivo* situation [Prueksaritanont et al., 1996]. A strategy to express relevant intestinal carriers and drug metabolising enzymes in Caco-2 cells and other cell lines is by using molecular cloning techniques. A major disadvantage of this strategy is that the natural up- and down-regulation of gene expression cannot be controlled in these systems. An alternative approach is to induce the expression of under-expressed drug metabolising enzymes such as CYP3A4 by conventional (natural) biological methods [Schmiedlin-Ren et al., 1997].

Table 5.1 Comparison of half-maximal transport concentrations (Kt) of carrier systems in Caco-2 cells and the small intestine. [Hidalgo, Li, 1996]

Carrier system	Substrate	Caco-2 cells	Small intestine
Large neutral amino acids	Phenylalanine	0.6 mM	8.5 mM ^{r, mucosa}
Acidic amino acids	Aspartic acid	56 µM	91 µM ^{h, BBMV}
Bile acids	Taurocholate	50 µM	37 µM ^{h, BBMV}
Biotin	Biotin	1.5 µM	5.8 µM ^{h, BBMV}
Folic acid	Folic acid	0.7 µM (pH 6.0)	0.6 µM ^{r, BBMV}
	Folic acid	14 µM (pH 7.1)	13 µM ^{r, BBMV}
Di/Tri-peptide	Cephalexin	2.9 mM	2 mM ^{r, R, mucosa}
Nucleosides	Thymidine	45 µM	45 µM ^{m, mucosa}

M, mouse; r, rat; R, rabbit; h, human; BBMV, brush border membrane vesicles; mucosa, mucosal tissue

Other imperfections of the Caco-2 cell cultures include the very “tight” junctions resulting in lower paracellular permeability, and the absence of other intestinal absorption barriers, such as the mucus layer. The tight-junctions of Caco-2 resemble fetal colon cells rather than small intestinal cells in this respect. The lower paracellular permeability requires very sensitive analytical methodology to study poorly permeating drugs, such as peptides. With regard to

the absence of a mucus layer, mucin is produced by goblet cells and not by the enterocytes represented by Caco-2 cells. Co-cultures of Caco-2 cells and goblet cells HT29-MTX have been established [Hilgendorf et al., 2000]. The paracellular permeability was enhanced in this co-culture compared to Caco-2 cells, however, carrier-mediated and secretion processes were reduced compared to Caco-2 cells alone.

Despite the described limitations, the use of Caco-2 cell cultures to study human intestinal absorption of compounds offers many advantages. Experiments are easy to perform (once the method has been established in the laboratory), relatively cheap and rapid, and very reproducible compared to other absorption models. Not only uptake in the cells but also transport through the cells can be monitored. Cell monolayers make it easy to manipulate transport media and experimental conditions. The ability to manipulate these factors makes the use of cell lines a very good model for study the mechanisms of transport of compounds.

In **summary**: Intestinal epithelial cell cultures, especially Caco-2 monolayers, have proven their value in various types of transport studies of compounds. Once the method has been established in a cell culture laboratory, experiments are easy to perform, relatively cheap and rapid, and very reproducible compared to other absorption models. Much of our knowledge of intestinal transport of compounds at the cellular level has been derived from Caco-2 cell culture studies. A pitfall may be that the expression level and substrate specificity of the carriers and efflux systems and metabolising enzymes found in Caco-2 monolayers tend to be variable in comparison to the *in vivo* situation. The combination of *in vitro* digestion methods with Caco-2 cell permeability is a very promising development for the assessment of compounds from foods and other matrices.

6. *In situ* perfusion

The *in situ* perfusion model is the nearest to the *in vivo* situation as the blood supply, innervation and clearance capabilities of the animal remain intact. *In situ* perfusion experiments for studying intestinal drug uptake were first introduced in the late 1960s. Under anaesthesia, the abdominal cavity is exposed and an intestinal loop is cannulated and perfused with an isotonic solution containing the compound of interest. The amount of compound in the perfusate is analysed (Figure 6.1), thus disappearance from the perfusate rather than intestinal absorption is measured. A consequence of measurement of the disappearance of the compound from the perfusion medium is that compounds with a low absorption can not be determined accurately. Recovery of non-absorbable markers is often 90%-95%, therefore, compounds with a decrease in concentration of less than 20% can not be determined accurately by perfusion techniques. It is possible to isolate more than one intestinal loop per animal and the method can be used for regional absorption studies. Perfusion experiments give no information about events at the cellular or membrane level but can be used to observe gross effects of absorption enhancers [Park and Mitra, 1992]. Often *in situ* perfusion studies are combined with blood sampling to study the appearance in blood. The perfusion technique is mainly applied in small laboratory animals, but it can also be applied to larger laboratory animals. Combination with blood kinetics is not often performed in rodents since they are too small for many blood samplings.

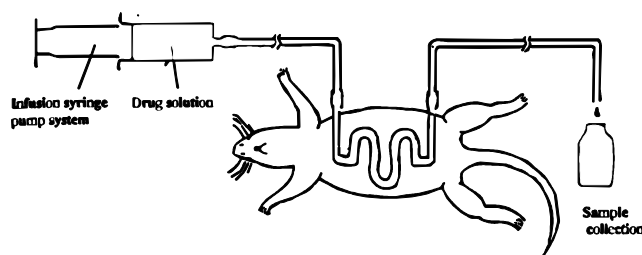


Figure 6.1. Intestinal uptake of compounds by *in situ* single-pass perfusion technique.

The basic experimental procedure is as follows: fasted animals are anaesthetised and then an intestinal segment is isolated and cannulated (Figure 6.1). This segment is rinsed with an isotonic normal saline solution before being constantly perfused with the solution containing the compound of interest. Input of the compound can be closely controlled in terms of concentration, pH, osmolality, intestinal region, and flow rate. More often than not, intestinal flow rates are higher than those known to exist *in vivo* and as a consequence, the *in situ* intestine is fully distended and the luminal hydrostatic pressure increases. This distension may affect the intestinal permeability or absorptive clearance [Chiou, 1994]. In the rat, the rate of perfusion is usually 0.2 to 0.6 mL/min and 30 to 60 minutes are allowed to attain steady-state conditions (4 times $t_{1/2}$) before the perfusate is collected for analysis. The perfusate is then collected at determined time intervals. In a single-pass perfusion experiment the amount of compound in the perfusate is measured (Figure 6.1), whereas in a recirculating perfusion the perfusate is collected in a reservoir and allowed to recirculate into the intestinal loop. In order to reliably determine the amount of compound absorbed, a significant part of the compound should be absorbed since disappearance of the compound from the perfusion medium is determined. Therefore, recirculation of the perfusate will improve the determination of slowly / poorly absorbed compounds. Since the intestine in the loop is still functioning, the volume of the perfusate (and thus the concentration of the compound) can change due to absorption or secretion of water. Therefore, a non-absorbable marker (e.g.

PEG-4000, inulin, fluorescein dextrans) should be included in the perfusion medium to monitor the volume of the perfusion medium and to indicate maintenance of viability of the intestinal tissue during the experiment. The intestinal uptake (effective permeability coefficient P_{eff}) is calculated from the disappearance of the compound from the perfusate:

$$P_{\text{eff}} = [-Q_{\text{in}} \times \ln(C_{\text{out}}/C_{\text{in}})]/2\pi rL$$

Where C_{in} and C_{out} are the inlet and outlet concentrations of the compound in the perfusate, respectively. Q_{in} is the flow rate of perfusion entering in the intestinal segment, and $2 \pi rL$ is the mass transfer surface area within the intestinal segment which is assumed to be the area of a cylinder with length L and radius r . The measurement of the rate of decrease in concentration of the compound in the perfusate does not always represents the rate of arrival of the compound in the portal vein, in particular when intracellular intestinal metabolism occurs. This problem can be overcome if this technique is applied in combination with measurements of the concentration of the compound in the portal vein. Another imperfection of the perfusion methodology is that dissolution of the compound of interest is not taken into account. The compound is dissolved in the perfusion medium (often normal saline solution is being used). Thus release from the matrix as well as effects of the digestion fluids on the absorption are not accounted for.

The *in situ* perfusion experiments are usually terminal for the animal under study and, therefore, the number of animals required is higher compared to the *in vitro* methodologies. To overcome this drawback and waste of animals, a chronically isolated intestinal loop has been developed by Poelma and Tukker [1987, 1991]. This technique consists of a long-term isolation of an intestinal segment inside the animal and allows for cross-over studies (reduction of animals) and for working without anaesthesia. Recently, it was shown that anaesthesia and surgical manipulation decreased the amount of intestinal blood flow and changed the distribution of intestinal blood flow. Both parameters are known to decrease intestinal absorption [Yuasa et al., 1993] and thus affecting the absorption of some compounds [Uhing et al., 1995]. The chronically isolated intestinal loop technique can overcome these problems.

In **summary**: The perfusion methodology is the nearest to the *in vivo* situation as the lymphatic and blood circulation systems are still intact. In perfusion studies the disappearance of the compound from the perfusate is being determined, which implies that compounds that are slowly and poorly absorbed can not be measured accurately. In combination with blood sampling and urine measurements, perfusion experiments can be used for kinetic studies. Furthermore, perfusion experiments can be used for regional absorption studies, and the effects of absorption enhancers and absorption mechanisms can be observed. The chronically isolated loop perfusion technique enables to reduce the number of animals, to study absorption in un-anaesthetised animals and allows for cross-over studies, day-night differences and to perform treatment comparison. However, this technique requires specialised personnel and very intensive animal treatment. Therefore, the perfusion methodology is not suitable for screening purposes.

7. In vivo techniques.

The ultimate information on oral bioavailability of a compound is derived from *in vivo* studies. The information that can be obtained from various *in vivo* methods is not only limited by the method itself but also by the choice of the species under investigation. A broad spectrum of methods is available for determining intestinal absorption or bioavailability of compounds in man and experimental animals. In this chapter these methods are categorised in three groups although the methods are often combined. These methods can be categorised into:

- perfusion via (multiple) lumen tubes (in man)
- mass balance studies: intake – excretion of faeces and urine (in animal and man)
- blood kinetics: following oral and intravenous administration of the compound (in animal and man)

7.1 Intestinal perfusion

Intestinal perfusion techniques were first used in man with the aim of studying the absorption and secretion mechanisms or the role of gastric emptying in drug absorption. The principle of this technique is to infuse a solution of the test compound and a non-absorbable marker such as PEG4000 into an intestinal segment and to collect perfusate samples after the solution has passed through the segment. The absorption is calculated from the disappearance rate of the compound from the perfused segment. Three main perfusion methods have been employed in the small intestine of man: A) an open system consisting of a triple lumen tube including a mixing segment system, B) a semi-open multilumen system with a proximal occluding balloon, C) and a double balloon, multilumen tube occluding a 10 cm long intestinal segment (see Figure 7.1).

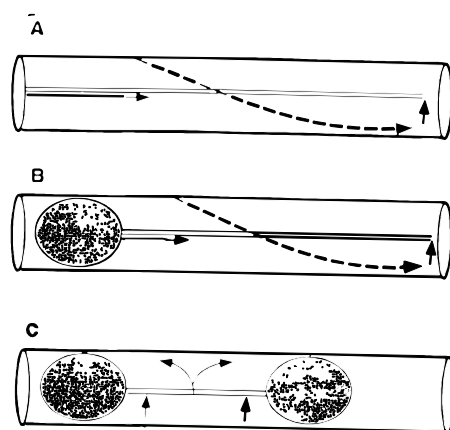


Figure 7.1 Schematic diagram of three different perfusion methodologies for human use: A, open; B, semi-open; C, double balloon. The hydrodynamic movement of the compound for the open and semi-open perfusion methodology can best be described by the parallel tube model (see dotted line for the concentration profile best describes the hydrodynamics over the intestinal length). The well-stirred model is the best hydrodynamic model for the double balloon perfusion technique. [Lennernäs, 1998].

In the triple open lumen tube method, perfusion solution and gastrointestinal fluids are mixed in a “mixing segment”, and at the distal end of the mixing segment a sample is taken which is considered to be the inlet concentration of the test segment (Figure 7.1A). The absorption is then calculated from a second outlet sample taken at the end of the test segment, which

usually is 20 to 30 cm distal to the mixing segment. Perfusion studies in an open system have several disadvantages: the perfusate can flow in either direction during physiological intestinal contractions and the composition of the perfusate will change along both mixing and test segments. This makes it difficult to define the absorption conditions and thus to determine permeability of a compound. By using an occluding balloon proximal to the test segment (Figure 7.1B), the proximal leakage is decreased and therefore the luminal conditions will be kept at equilibrium and drug permeability can be determined under better-defined conditions. Disadvantages of both methods are the high perfusion flow rates, typically between 5 and 20 ml/min compared to physiological flow rates of about 1-3 ml/min, the low and variable recovery of the non-absorbable marker, and the uncertainty of the actual length of the intestinal segment studied.

Recently, the perfusion technique was refined by developing a new intestinal perfusion instrument which consisted of a multichannel sterile tube with two inflatable balloons (Figure 7.1C), creating a 10 cm length segment and allowing single-pass perfusion of a well-defined region of jejunum [Lennernäs et al., 1992]. The tube is introduced orally after local anaesthesia and when the tube has been positioned, the balloons are inflated, creating a closed intestinal segment. An advantage of this technique is that the intestinal segment between the balloons is mixed with luminal fluids. Furthermore, the leakage out from the segment over the balloons is small, so recovery of the non-absorbable marker is more than 95%. The effective permeability P_{eff} is calculated as follows:

$$P_{\text{eff}} = [Q_{\text{in}} \times (C_{\text{in}} - C_{\text{out}})] / (C_{\text{out}} \times 2\pi Rl)$$

Where Q_{in} is the inlet perfusion rate, C_{in} and C_{out} are the concentrations of the compound in the inlet and outlet perfusate, R is the radius of human jejunum (1.75 cm), and l the length of the intestinal segment (10 cm). Combination with blood sampling allows to not only the extent of absorption but also the degree of first-pass metabolism in the gut and liver. This regional human jejunal perfusion technique has been validated, and there is a good correlation between the measured effective permeability values and the extent of absorption of drug in man determined by pharmacokinetic data [Fagerholm et al., 1996, Lennernäs, 1998]. Thus, the technique can give valuable pharmacokinetic data with human subjects. However, it is too complex to be used for routine studies. When unexpected results are obtained in *in vivo* blood and mass balance studies, *in vivo* human perfusion studies can be performed to investigate the intestinal transport processes in more detail.

7.2 Mass balance studies

The principle of mass balance studies is that the input equals the (total) output. The input is controlled by measurement of the amount of compound that is taken in. The output (excretion) of compounds is controlled by collection of urine and faeces for a certain time period. It is, therefore, a non-invasive method. In order to collect the relevant faeces, non-absorbable markers like chromium, PEG-4000 or carmine red are generally administered. In animals, mass balance studies are performed by putting them in special metabolic cages. This type of housing can induce stress, especially in rats and rabbits, and may thus affect intestinal absorption and bioavailability of compounds. Moreover, it is sometimes difficult to collect urine and faeces separately. Furthermore, in rats coprophagy may occur, which can be prevented by using an anal cup [Hara et al., 1996]. In humans, studies are performed on

metabolic wards, which are specialised in collecting faeces and urine under full mass balance control.

Mass balance studies are especially suitable for studying the bioavailability of a compound. The fraction of the compound found in the faeces that contain the non-absorbable markers, is considered not to be absorbed. The fraction of the compound recovered in the urine and in the remaining faeces represents the bioavailability of the compound (fraction=excretion/intake). The terminal elimination half-life time ($T_{1/2}$) of the compound can be determined from the excretion rate in the urine and faeces. To obtain a complete mass balance (input=output) not only the parent compound has to be determined in the urine and faeces but also its metabolites have to be detected since many compounds are being metabolised. Therefore, radiolabelled compounds are mostly used for mass balance studies. In animals, excretion in bile (instead of faeces) is generally also taken into account in pharmaceutical research programmes, in order to obtain a clear overview of all relevant excretion routes. It should be noted that no complete mass balance could be made from compounds that are excreted in the lungs. In order to determine the pharmacokinetic parameters bioavailability (F) and terminal elimination rate ($T_{1/2}$), a basic rule in this type of research is a sampling duration of 3 to 5 terminal half-lives. This means that for a compound with a terminal elimination half-life of 24h, urine and faeces have to be collected for at least 3 to 5 days. Mass balance studies are, therefore, time-consuming and these studies are very expensive in humans, due to hospitalisation of the subjects.

7.3 Blood kinetics

Blood kinetics is the most traditional way in pharmacology to determine the oral bioavailability of a compound. For this method, blood samples are collected following oral and intravenous administration of the compound. Again, the basic rule is a sampling duration of 3 to 5 terminal half-lives. For this and the limited amount of blood that can be sampled, sensitive analytical methods have to be developed. Blood kinetics is often performed in non-rodent species, since rodents are often too small for long-term blood sampling. In some cases, permanent cannulas are inserted in the jugular vein in rats, for frequent/long-term blood sampling.

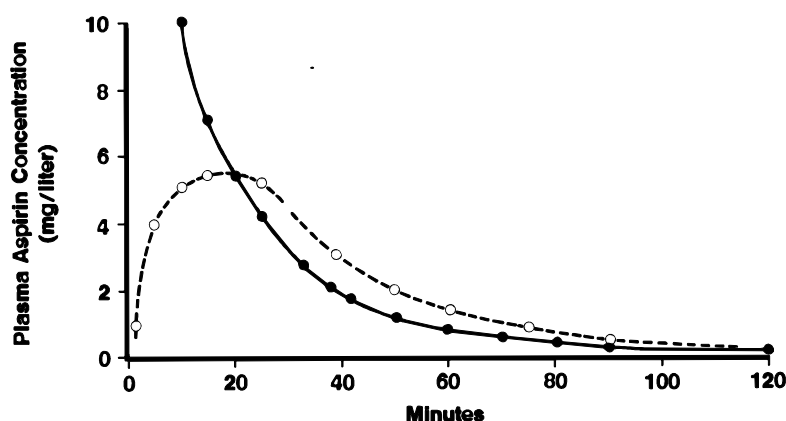


Figure 7.2. Aspirin (650 mg) was administered as an intravenous bolus (●) and as an oral solution (○) on separate occasions to the same individual. Absorption causes a delay and a lowering of the peak concentration. [Rowland and Tozer, 1989].

Blood kinetics gives information on the appearance and elimination of a compound (Figure 7.2). The blood kinetics of an intravenously administered compound reflects the distribution and elimination of the compound. For orally administered compounds, the blood concentration of the compound is always a combination of absorption, distribution and elimination. Information on the absolute oral bioavailability of the compound (F) can only be obtained following intravenous and oral administration of the compound. The area under the concentration-time curve following oral administration (AUC_{oral}) is divided by the area following an intravenous bolus (AUC_{iv}) with appropriately correcting for the dose.

Intravenous (iv) administration:

$$Dose_{iv} = Clearance \times AUC_{iv}$$

Oral administration

$$F \times Dose_{oral} = Clearance \times AUC_{oral}$$

which upon division yields:

$$F = \left(\frac{AUC_{oral}}{AUC_{iv}} \right) \times \left(\frac{Dose_{iv}}{Dose_{oral}} \right)$$

The assumption made for this calculation is that the clearance of the compound remains constant. Therefore, the doses used for intravenous and oral administration should show dose linearity. One way to determine the oral bioavailability, the oral intake and the intravenous bolus injection are administered simultaneously by using double isotopes. In this method, two isotopes of the substance of interest are used. The amounts of an orally administered isotope and an intravenously administered different isotope are measured simultaneously in blood and /or in urine and faeces. However, multiple isotopes of one compound are often not available and, therefore, most studies are performed by using different animals (humans) for each condition or by performing cross-over studies.

In case the compound is not metabolised ($F_H = 1$, Figure 1.1), the extent of absorption ($F_B \times F_A$) equals the oral bioavailability ($F = F_B \times F_A \times F_H$) and can therefore be determined by comparison of the blood concentration-time curves after oral and intravenous dosing. For compounds that are metabolised in the liver ($F_H < 1$), the intestinal absorption ($F_B \times F_A$) is higher than the oral bioavailability calculated by comparison of the blood concentration-time curves after oral and intravenous dosing. To determine the extent of intestinal absorption, the oral bioavailability should be corrected for the fraction metabolised. This can be done by measuring all metabolites formed or by using a radiolabelled or stable isotope form of the compound. In the latter situation, besides measurement of the blood *concentration* of the compound the *amount* of radiolabel or isotope is measured, which includes the compound together with its metabolites. When excretion is almost exclusively through the kidneys, the extent of absorption of a radiolabelled or stable isotope compound can also be determined by comparison of the urinary excretion of radioactivity after oral and intravenous dosing.

However, often no intravenous data are available. Costs, solubility limitations, and potential toxic compounds are major reasons for the lack of an intravenous preparation. The oral bioavailability can then be estimated based on blood kinetic studies in animals. Alternatively, a relative bioavailability can be determined by comparing different dosage forms, different routes of administration (oral, intramuscular), or different conditions (e.g., fasted, fed). For example, a compound is orally administered in solution (situation A), and orally administered

in food (situation B). The relative bioavailability (food:solution) can be calculated analogous to above described formulas for the oral administration so that,

$$\text{Relative bioavailability} = \frac{F_B}{F_A} = \left(\frac{AUC_B}{AUC_A} \right) \times \left(\frac{Dose_A}{Dose_B} \right)$$

As with the calculation of oral bioavailability, clearance is assumed to be constant and the kinetics should show dose linearity. In the example comparing the two oral conditions of a compound, a relative bioavailability of 100% means that the compound is equally bioavailable under both oral conditions although the absolute bioavailability may be poor.

In **summary**: *In vivo* studies can provide the ultimate information on intestinal absorption and bioavailability of a compound. Intestinal perfusion via multiple lumen tubes is mainly intended for research on mechanisms of absorption or for investigation whether absorption is restricted to certain locations in the intestine. As with this technique the disappearance of a compound from the intestinal lumen is measured, the compound of interest should be moderate to well absorbed. Furthermore, information on gastric emptying and dissolution in GI-tract is not included in this technique but could be obtained by comparison with data following oral administration. Mass balance and blood kinetic studies provide information on the oral bioavailability ($F = F_B \times F_A \times F_H$) of the compound but provide no direct information on the separate factors. To determine the oral bioavailability of a compound on basis of blood kinetics also an intravenous bolus dose administration is necessary, which can not be performed with potential toxic compounds in man. Mass balance studies in man require hospitalisation of the subjects, which makes these studies extra expensive and time consuming.

8. Discussion and conclusions

In this chapter the methodologies studying intestinal absorption as described in chapter 4-7 are compared. Correlation with the fraction absorbed in humans is shown for several methodologies but first what information can be derived from each methodology as well as the feasibility is discussed. Finally, the potential of these methodologies for risk assessment practice is discussed.

8.1 Comparison of intestinal absorption models

Historically, bioavailability studies for compounds are based on *in vivo* mass balance and *in vivo* blood kinetic studies in man and in laboratory animals. These studies cover the complete tract of ingestion, bioaccessibility (F_B), intestinal permeability (F_A), and metabolism (F_H) finally resulting in oral bioavailability (F). However, it is rather a black box how a compound gets from external dose to internal dose and the contribution of the three major factors to the bioavailability of the compound can not be studied easily. To investigate the usefulness of fast, reliable methodologies to study intestinal absorption, the characteristics of these *in vitro*, *in situ* and *in vivo* perfusion methodologies described in chapters 4 to 7 are summarised in Table 8.1.

Table 8.1. Summary of intestinal absorption characteristics of intestinal absorption studies.

Parameter	In vitro					In situ		In vivo		
	digestion	Sheet	sac	Vesicle	cell line	perfusion	perfusion	perfusion + blood	mass balance	blood
Bioaccessibility (F_B)	+	-	-	-	-	-	-	-	+#	+#
Permeability (F_A)	-								+#	+#
Disappearance		+	+	-	+	+	+	+	±	-
Uptake		+	+	+	+	-	-	±	-	-
Transport		+	+	-	++	-	-	+	-	-
Secretion		+	+	±	++	-	-	-	-	-
Mechanism		+	+	±	++	±	±	±	-	-
Site specific		+	+	±	-	+	+	+	-	-
Metabolism									+#	+#
Intestine	-	+	+	-	±	±	±	±	-	-
Metabolism									+#	+#
Liver (F_H)	-	-	-	-	-	-	-	±	-	-
Bioavailability (F)	-	-	-	-	-	-	-	±	+	+
Time*	++	++	+	++	++	±	-	-	--	-
Costs*	++	++	+	++	++	±	-	-	--	-
Flexibility	++	+	+	+	++	±	±	±	-	-
Screening	++	+	±	-	++	-	--	--	--	--

* + means can be studied or a favourable situation; - means can not be studied or unfavourable situation; ± means can partly be studied. #Bioaccessibility, intestinal permeability, and metabolism are all included in these studies but can not be studied in detail.

Bioaccessibility (F_B). *In vitro* digestion methods (chapter 4) can be used to estimate the bioaccessibility of a compound from a random matrix. However, the other factor contributing to intestinal absorption, the passage of the intestinal epithelium, is not considered by these methods. Release of the compound from its matrix is covered only in *in vivo* mass balance

and blood kinetic studies. With these studies it is difficult to determine the contribution of the bioaccessibility to the oral bioavailability of the compound. Relative bioavailability studies (see in chapter 7.3) may represent an *in vivo* bioaccessibility determination and such a study in human volunteers might be used to validate the *in vitro* digestion methods described in chapter 4. In all other methodologies to study the passage of the intestinal epithelium (chapters 5-7) ingestion, digestion, stomach pH, and release from the matrix are not implemented in the studies, but permeability of the compound in solution is being studied. Only recently *in vitro* bioaccessibility of iron from different food sources has been combined with intestinal uptake in Caco-2 cells [Glahn et al., 1996]. It was shown that as well as the bioaccessibility of iron was dependent on the food source as was the uptake of iron in the Caco-2 cells. Such a strategy of combination of *in vitro* digestion and *in vitro* permeability methodologies is very encouraging and needs to be further explored.

Intestinal permeability (F_A). In what way intestinal permeability is being studied is rather different among the methodologies. In perfusion studies disappearance from the perfusion medium is being measured. In an extreme situation, a compound can be adsorbed to the intestinal cells or taken up in the intestinal cells without being transported into the blood stream. In this case, the compound would disappear from the perfusion medium without being absorbed at all. Furthermore, the compound of interest should be moderate to well absorbed to obtain a reliable absorption rate based on disappearance from the perfusion medium (see also chapter 6). *In vitro* methods are easier to manipulate and disappearance from the donor solution can be measured but in general the appearance in the receiver solution, thus after passage of the intestinal cells, is being measured. Determination of appearance is more accurate than determination of disappearance: e.g. an increase in appearance from 1% to 10% is a 10-fold increase whereas the concomitant decrease from 99% to 90% is only 1.1-fold decrease. Except for membrane vesicles, which are useful to study carrier-mediated processes in detail, with the *in vitro* methods uptake in, transport through and secretion from the intestinal epithelium can be distinguished. As in the everted sac system the serosal compartment is a closed compartment with a small volume, kinetics of rapidly absorbed compounds may be distorted because of back-fluxes of the compound and many sacs are needed to study the kinetics of a compound. Thus, the mucosal sheets and the intestinal cell lines are more useful to study intestinal permeability of compounds. A major disadvantage of the use of mucosal sheets is the rapid decrease in viability. Therefore, the intestinal cell lines have great advantages to use since the cells are well defined, are easier to manipulate, the reproducibility is high, are easy to perform in large numbers, and no animals are needed.

Mechanism of transport. Over the past three decades, studies using intestinal mucosal sheets, everted gut sacs and membrane vesicles have provided information on active intestinal transport of nutrients such as sugars and amino acids. However, much of our more recent understanding on intestinal active transport mechanisms as well as information on passive transcellular and paracellular transport have been derived from cell culture studies. Drug transport experiments using cell cultures, predominantly Caco-2 cells, are easy to perform under controlled conditions, resulting in the generation of a wealth of information, which contributes to fundamental principles of intestinal drug transport at the cellular level. Rather than investigating new mechanisms, *in situ* and *in vivo* perfusion studies provide information on what the implication of the mechanisms investigated *in vitro* studies have on the transport *in vivo* or when unexpected results are obtained *in vivo*.

Site specific/Interspecies comparison. Site specific transport (jejunum, ileum etc.) can be studied with intestinal mucosal sheets, everted sacs, and perfusion studies but not using cell lines. Some regional absorption information from *in vivo* studies may be obtained from slow release formulations or from local administration of the compound. However, these studies

are no standard procedures. Intestinal mucosal sheets and everted gut sacs are very useful for interspecies comparison since transport of a compound can be compared under the same, controlled conditions lacking variables found in the more complex/complete perfusion studies or *in vivo* blood kinetic studies.

Metabolism. Expression of intestinal metabolising enzymes in intestinal cell cultures is often different from the expression found *in vivo* and therefore, intestinal mucosal sheets and everted gut sacs may provide a better model to study intestinal metabolism. Intestinal mucosal sheets and everted gut sacs are derived from laboratory animals rather than from human intestinal tissue so that extrapolation to humans is necessary. However, especially the short viability of the intestinal tissue is a point of concern. *In situ* and *in vivo* perfusion studies can only provide an indication that intestinal metabolism occurs when the metabolites formed are excreted into the intestinal lumen. The formation of metabolites can be detected *in vivo* mass balance and blood kinetic studies. Since the compounds already have passed the liver, it is with these studies not possible to discriminate between intestinal and liver metabolism.

Bioavailability (F) determinations for compounds are based on *in vivo* studies in man and laboratory animals. In order to estimate the bioavailability of a compound an intravenous bolus dose is necessary, which is often not performed because of costs, solubility limitations, and potential toxicity. *In vivo* mass balance studies can be performed to determine the bioavailability of a compound but these studies are very time consuming and costly. Of the other methodologies, only with *in situ* and *in vivo* perfusion combined with urine or blood sampling an indication of the bioavailability can be made.

Costs, time, flexibility, screening. Except for the cell lines, in all techniques animals or humans are being used. The *in vitro* methods use intestinal tissue after death of the animal (or after surgery), whereas *in situ* and *in vivo* techniques are more stressful and often invasive and especially for potential toxic compounds more a burden on the animal than the *in vitro* methods. All these techniques need the consent of an ethical committee, which needs time. Therefore, no ad hoc experiments can be carried out with these techniques. Providing the cells are continuously cultured on permeable filter supports, a transport study with Caco-2 cells can be carried out within days otherwise within several weeks. Additionally, *in vitro* methods are easier to perform in large numbers, especially the cell culture model, and to handle under controlled conditions than the *in situ* and *in vivo* methodologies.

In conclusion. For intestinal permeability tests, *in situ* and especially *in vivo* perfusion studies are more expensive and more time consuming than the *in vitro* permeability models. Perfusion studies are often performed to elucidate unexpected effects found *in vivo* blood and mass balance studies. If site specific absorption or interspecies comparison is an important issue, intestinal mucosal sheets or everted gut sacs are useful methods. A major disadvantage of these models is that fresh intestinal material should be used because of the rapid decrease in viability of the tissue. Since it is difficult to obtain truly fresh human material, animals will be used and an interspecies extrapolation is needed. Furthermore, the conditions can not be manipulated as easily because of the fragile tissue viability. Overall, the Caco-2 cell culture model is by far the easiest to manipulate, well defined, very reproducible, fast and representative for human absorption.

8.2 Correlation permeability methodologies with *in vivo* intestinal absorption

Basis for most methodologies described in chapters 5 to 7 is that the rate-limiting barrier to drug absorption across the intestinal mucosa is the single layer of intestinal epithelial cells. Thus, the transport rate across the intestinal cells of a compound may be a measure for the *in*

in vivo absorption of compounds. This correlation is shown in Figure 8.1 for Caco-2 monolayers, rat mucosal sheets, rat *in situ* perfusion and human *in vivo* perfusion. The *in vivo* absorption of compounds is measured by *in vivo* mass balance or blood kinetic studies. By these methods bioavailability (F , Figure 1.1) of a compound is determined rather than intestinal absorption ($F_B \times F_A$, Figure 1.1). For compounds that are metabolised in the liver, the bioavailability is lower than the intestinal absorption ($F = F_B \times F_A \times F_H$). The *in vivo* absorption data presented in Figure 8.1 are corrected for the fraction metabolised (F_H) so that the % absorption on the y-axis represents the true intestinal absorption of a compound. Furthermore, the compounds are mostly supplied under fasting, controlled conditions so that the matrix effects are reduced to a minimum.

Transport data of 14 to 19 structurally diverse compounds were used for this comparison and each compound was at least used in two methodologies. Furthermore, the mechanism of transport was diverse: compounds were either transported via carriers (D-glucose, L-dopa, L-leucine, L-phenyl-alanine), active (secretion) transporters (cimetidine, furosemide) or passive transcellular (metoprolol, ketoprofen, naproxen, antipyrine, salicylic acid, cortisol) or passive paracellular pathway (acyclovir, mannitol, PEG900, PEG4000, creatinine) or combination of trans- and paracellular (atenolol, terbutaline). Vesicles were not included in this comparison since only carrier-mediated transport can be studied with this technique. Because of lack in available data, everted sacs were also not included in this comparison.

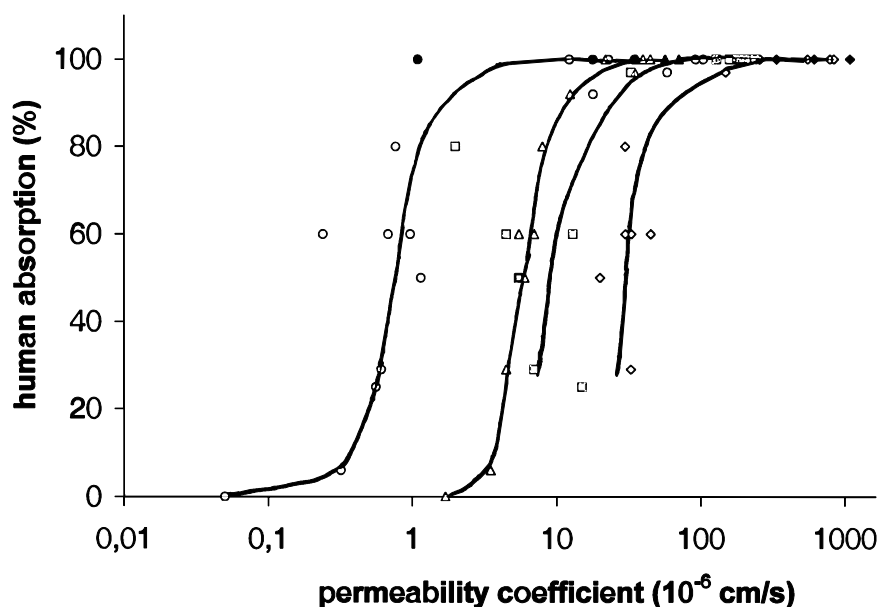


Figure 8.1. Fraction absorbed in humans after oral administration as a function of permeability coefficients in the Caco-2 model ($\circ$), rat mucosal sheets (Δ), rat *in situ* perfusion ($\square$), and human *in vivo* perfusion ($\diamond$). D-glucose, L-dopa, L-leucine, L-phenyl-alanine, all carrier-mediated transported are shown by closed symbols. [Adson et al., 1995, Artursson and Karlsson, 1991, Dowty and Dietsch, 1997, Fagerholm et al., 1996, Flanagan and Benet, 1999, Han et al., 1998, He et al., 1998, Hilgendorf et al., 2000, Karlsson et al., 1999, Lennernäs et al., 1996, Pade and Stavchansky, 1997, Palm et al., 1999, Rubas et al., 1996, Stewart et al., 1995]

An S-shaped correlation between the permeability rates of the 14 to 19 compounds and *in vivo* human absorption was found for the *in vitro* Caco-2 cell culture model and for the *in vitro* rat mucosal sheets. Compounds that are poorly absorbed *in vivo* (<10%) have a low permeability *in vitro*. With a modest increase in permeability rate (3 to 10-fold), a steep

increase in *in vivo* absorption is observed for all methodologies. These compounds are incompletely absorbed (10-80%). Because of this steep slope of the absorption-permeability curves, prediction of the *in vivo* absorption of a compound based on permeability data will provide a rough indication rather than a quantitative prediction. For compounds that are well absorbed (>80%), the curve flattens again. In this part of the curve, a higher permeability of a compound may indicate for a faster absorption rather than the absolute absorption. Such an interpretation can be made for a homologous series of compounds but should be made with care for structural and functional unrelated compounds.

Data on compounds with a low human absorption (<20%) are scarcely available in rat and human perfusion. This is likely due to the fact that in perfusion studies disappearance from the perfusion medium is being measured. Recovery of non-absorbable markers is often 90%-95%, therefore, compounds with a decrease in concentration of less than 20% can not be determined accurately by perfusion techniques. The absolute permeability rates varied between the 4 methods: Caco-2 < rat mucosal sheets < rat *in situ* perfusion < human perfusion. Especially, carrier-mediated transport is relatively slow in Caco-2 cells compared to human *in vivo* perfusion. Nevertheless, compounds, which are partly absorbed, are at a similar position in the S-shape. It should be noted that for human *in vivo* perfusion all data are measured in a single research group whereas the data for the other methods were obtained from several different laboratories. This might result in a greater variation in permeability rates for Caco-2 cells, rat mucosal sheets, and rat *in situ* perfusion than for human *in vivo* perfusion. The correlation for the rat *in situ* perfusion was less than for the other three methods.

In conclusion, based on the summarised permeability data, the *in vitro* Caco-2 cell culture model, the *in vitro* rat mucosal sheets, and the *in vivo* human perfusion can be used to estimate human intestinal absorption of ingested compounds. The other characteristics of the Caco-2 cell culture model, i.e. fast, relative inexpensive, reproducible and easy to manipulate, provide the basis for a workable method as a tool to estimate human intestinal transport of ingested compounds in a screening procedure.

8.3 Use of *in vitro* bioaccessibility and intestinal permeability models for health risk assessment

A typical experimental set-up to estimate the *in vivo* intestinal absorption of compounds from an arbitrary matrix based on *in vitro* methods would include determination of bioaccessibility of the compound and a measure of the permeability of the compound in intestinal tissue (Figure 8.2). As was discussed in chapter 8.2 for the permeability methods, the *in vitro* bioaccessibility and permeability methods can be used to provide a qualitative indication (low, medium, high absorption) of the *in vivo* intestinal absorption in humans. It is not always necessary to test both parameters in detail. When the bioaccessibility of the compound from its matrix is low, the *in vivo* absorption will be low, and it is not necessary to perform a detailed permeability study. On the other hand, when the bioaccessibility is high, intestinal membrane permeability of the compound is the rate-limiting step in absorption. In this case permeability studies should be studied in detail and may provide a good indication of the *in vivo* intestinal absorption of the compound.

Several static and dynamic physiologically based digestion models have been developed to study the bioaccessibility of compounds. For the dynamic model [Minekus et al., 1995]

advanced laboratory equipment is required and rather than using it for screening purposes is this model especially suitable for gaining detailed information on the release of the compound from its matrix. The static models [Rotard et al., 1995, Glahn et al., 1996, Sips et al., 1998] are more flexible, easier to manipulate, to apply on a random matrix, and to perform at large numbers and are, therefore, more useful for screening purposes. The *in vitro* digestion models, however, have not yet been validated to the *in vivo* bioaccessibility of compounds in humans. For permeability *in vitro* testing, the FDA is currently evaluating the implementation of Caco-2 cells in standard procedures in the development of pharmaceutical drugs and in certain circumstances such a combination of *in vitro* bioaccessibility and permeability tests of pharmaceutical drugs may replace *in vivo* bioequivalence studies. Such a standardised protocol will allow a better comparison between laboratories and will provide a wealth on information on the correlation between *in vitro* permeability and *in vivo* absorption. Furthermore, physiologically based computer models are being developed to predict human *in vivo* absorption of compounds based on combining *in vitro* permeability data, *in vitro* bioaccessibility data and physicochemical properties of the compound. This will be very useful for the interpretation of permeability data of compounds, which can not be tested *in vivo*.

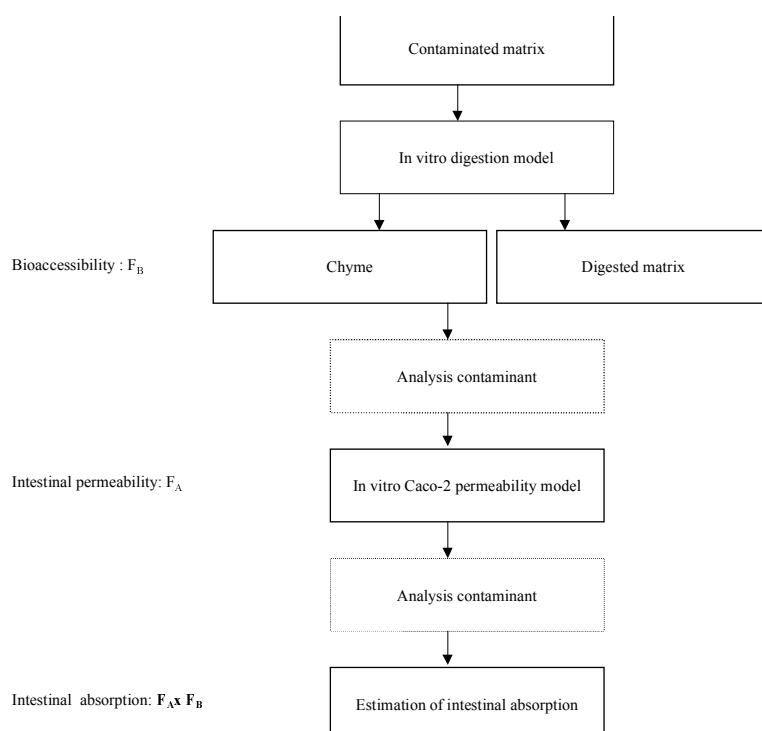


Figure 8.2. Experimental procedure to estimate *in vivo* intestinal absorption of a contaminant by *in vitro* bioaccessibility and intestinal permeability methods.

The standard setting standards for the maximal amount allowed of a toxic compound in a certain product is often based on *in vivo* toxicity studies with laboratory animals with application of the toxicant in food or drinking water and not in the product of interest. However, equal concentrations of a toxicant (e.g. lead) in food, painted toys, soil or drinking water are not likely to be equally toxic because the bioaccessibility (and thus bioavailability) of the toxicant from the various matrixes differs. In such a case, *in vivo* toxicity studies could

be performed with the toxicant in the various matrices in order to determine its toxicity. *In vivo* studies, however, are expensive and time-consuming and, therefore, it is often assumed that the toxicant is equally toxic independent of its matrix it is applied in. As a consequence the health risk will be overestimated for compounds that are poorly absorbed from a certain product resulting in a high safety standard of the compound in this product. Alternatively, release of the toxicant from its matrix could be determined by performing *in vitro* bioaccessibility measurements with different matrices. The relative bioaccessibility determined *in vitro* can be used to reflect the relative *in vivo* bioavailability of the toxicant and as a consequence reflect the toxicity, which may result in more realistic standard settings for the matrix the toxicant is applied in. As the matrix may also affect the transport across the intestinal cells [Glahn et al., 1996] an additional permeability test is recommended.

Human exposure models such as CSOIL and CONSEXPO are currently being used in risk assessment practice to estimate the internal exposure after ingestion or inhalation or dermal contact with a contaminated product. The intestinal absorption of the toxic compound is one of the main factors affecting the internal exposure after ingestion of a compound. Often the intestinal absorption of the compound is not known and the compound is assumed to be completely absorbed. As a consequence the internal exposure will be overestimated for compounds that are poorly absorbed. The information obtained with the *in vitro* bioaccessibility and permeability methods can be implemented in these exposure models and thus contribute to a better health risk assessment. Moreover, these models are useful whenever a (fast) indication is required of the human intestinal absorption of other compounds such as pharmaceuticals and active components in food.

In conclusion, these *in vitro* bioaccessibility and intestinal permeability methods provide fast (within weeks providing the analysis of the compound is not rate-limiting), accurate, and relative inexpensive tests that simulate the human intestinal absorption of ingested compounds. The strategy of combining both *in vitro* methods is very encouraging and needs to be further explored and validated. These *in vitro* methods may provide realistic information on human intestinal absorption of ingested toxicants, which can be used in human exposure models such as CSOIL and CONSEXPO, and may result in more rational exposure calculations and standard settings.

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