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In Vitro Absorption Studies and Their Relevance to Absorption from the GI Tract

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INTRODUCTION

The amount of a drug absorbed from the gastrointestinal tract into the systemic circulation is a result of many complex processes. These include physicochemical interactions with the environment in the intestinal lumen, as well as biological interactions with the physiology and anatomy of the intestinal membrane, which either interfere or aid in the transport process (Table 1.) (1,2). The absorption probability of a drug is mainly determined by the chemical structure of the molecule. The goal is to synthesize new chemical entities until a certain bioavailability is reached. Additionally, strategies concerning the development of drugs having high potency and low bioavailability using specific enhancer systems have generated increased interest.

Traditionally, the absorption process with its complexity is studied as bioavailability in vivo in different animals. However, it has become clear that there is a need for more simplified models for a better understanding of the complexity of the absorption process and the possibility of interference. In today's practical drug design and development, it is therefore crucial to have methods of drug absorption detailing the different steps involved before and during the absorption process (Fig. 1). The methods should describe the release of the drug from the dosage form (dissolution phase), and transfer of molecules through the epithelial membrane into the systemic circulation (absorption phase). They must also include different mechanisms of absorption for a wide variety of molecules as well as have a predictive value for the absorption process in man (Fig. 2). This concept is a challenge for the industry because of the discovery and development of more complex drugs, e.g., proteins and peptides (or peptidomimetics), that mimic endogenous hormones, transmitters, and enzyme inhibitors.

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To simplify the studies on factors that will affect the absorption process, they must be divided into factors affecting the effective concentration of the drug at the absorption site, and those that will affect the permeability coefficient or the diffusion probability through the membrane (Table 1) (1). In addition, it must be known if the barrier of absorption is before or during absorption through or after the actual penetration of the epithelial membrane. If a drug fails in the absorption process, new chemical entities are often synthesized. However, increased absorption can also be achieved by the use of an absorption window, e.g., by avoiding critical regions of enzymes or low permeability or by using enhancers of absorption. Systems that enhance absorption do not always contain drugs that act on the epithelial membrane (increased membrane permeability) but enhance absorption by other mechanisms such as increase in solubility, change in pH at the absorption site, and decrease in the binding to luminal material, etc. The result of the enhancement is then an increased driving force of the absorption process, i.e., the effective concentration at the absorption site.

If a new chemical entity cannot penetrate the intestinal epithelium, it will not be developed as a pharmaceutical drug. The screening of new pharmaceutical drugs will therefore be mainly focused on the diffusion probability through the epithelial membrane, and methods will be developed to study the factors governing the process.

Table 1

Physicochemical and Physiological Factors Influencing the Bioavailability of Drugs After Oral Administration (1,2)

Physicochemical	Physiological
Hydrophobicity	Surface area at the admin. site
Molecular size	Transit time and motility
Molecular conformation	pH in lumen and at surface
pKa	Intestinal secretions
Chemical stability	Enzymes
Solubility	Membrane permeability
Complexation	Food and food composition
Particle size	Disease state
Crystal form	Pharmacological effect
Aggregation	Mucus and UWL
Hydrogen bonding	Water fluxes
Polar surface area	Blood flow
	Bacteria
	Liver uptake and bile excretion

METHODS TO ASSESS DRUG ABSORPTION FROM THE GI TRACT

There are multiple nonbiological (biophysical) and biological in vitro and in vivo methods for screening the barriers of absorption mentioned in the literature (Fig. 3) (1,3,4). Each of the methods represents only one or a few steps and mechanisms in the absorption process (Figs. 1 and 2). The closer the in vivo bioavailability it describes, the more complex the method becomes. The ideal methods used for screening depend on the characteristics of the substances to be tested and their behavior in the biological system. It is clear, however, that for drug discovery and development, there is no ultimate method to use but rather a need for more than one of these screening methods. From the latest results in the literature, it is also evident that we need to get more information regarding the absorption mechanisms to be able to understand more clearly the absorption process and to overcome the different barriers.

Below, a short description is given of the main methods used today for the evaluation of the absorption of drugs. Each method also describes its advantages and disadvantages.

In Vitro Nonbiological (Biophysical) Methods

For most drugs, the absorption from the gastrointestinal tract is mediated by passive diffusion through the lipid membrane. This process is thought to be governed by physicochemical factors such as lipophilicity, solubility in water, surface charges, molecular weight, and conformational flexibility (5). The most accepted parameter to predict drug absorption is the partition coefficient reflecting partitioning into a lipid phase (e.g., octanol/water), the log P (or log D) value. This parameter is relatively easy to use for the discovery and development of pharmaceutical drugs. The optimal range in lipophilicity that would reflect a good absorption potential has been suggested to be a log P value between 0 and 3 (5) or above 3 (6). This is a general rule of thumb, because it means that very hydrophilic drugs (log $P < -3$) and very lipophilic drugs (log $P > 3$) are often associated with incomplete absorption in vivo (5,6). Drugs with log P values between -3 and 0, and a log P between 0 and 3 often have variable results (5). However, the prediction of incomplete absorption for hydrophilic and very lipophilic drugs has been argued.

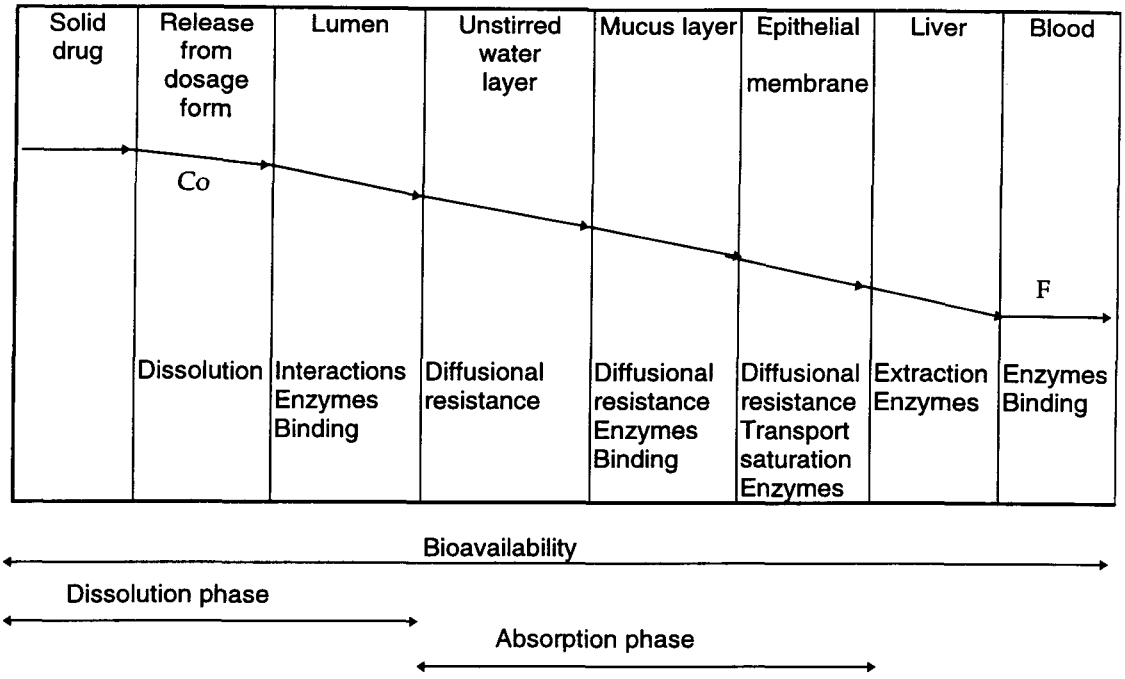

Figure 1. Schematic drawing of the different steps before, during, and after absorption of the drug from the gastrointestinal tract. *Co* represents the concentration of dissolved drug and *F* is the systemic bioavailable drug (from ref. 1).

Hydrophilic drugs like atenolol and sotalol are absorbed from the gastrointestinal tract although their partition coefficients are low (5), and very lipophilic drugs such as fluvastatin are completely absorbed (7). Today there is, therefore, a more complex view on the factors governing the partitioning into a lipid phase, e.g., the number of hydrogen bonds (8) or polar surface area (9).

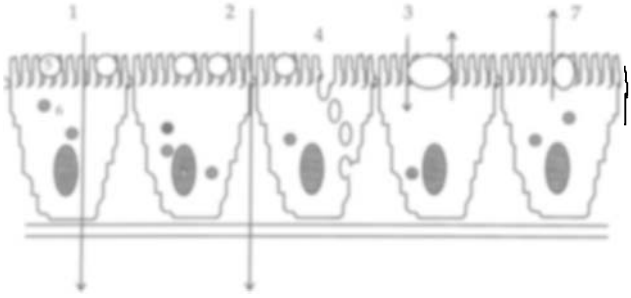

Figure 2. Schematic drawing of the mechanisms of absorption through the epithelial membrane. Numbers show: 1. passive transcellular pathway; 2. passive paracellular pathway; 3. carrier-mediated transport; 4. endocytosis; 5. brush-border enzymes; 6. cytosolic enzymes; 7. *p*-glycoprotein efflux systems; and 8. cell nucleus.

Number of Hydrogen Bonds

Calculation of the number of hydrogen bonds in a molecule has been suggested as a simple method to predict drug absorption (8). The method reflects the ability of the molecule to form hydrogen bonds with the surrounding solvent. The more bonds the molecule forms with water (luminal fluid), the less potential it has to diffuse into a lipid phase of a membrane. A good relationship between the number of hydrogen bonds of small model peptides and their permeability coefficients determined using Caco-2 cell monolayers has been established (8).

This method is simple to use, but it can be argued if the total number of hydrogen bonds of the molecule should be calculated. Fluvastatin, a very lipophilic drug (log *P* 3.8) which has a total of 8 hydrogen bonds, is completely absorbed from the gastrointestinal tract in man (7). The suggested reason for the lack of correlation to the number of hydrogen bonds for this drug is the amphilicity of the drug and its ability to form internal bonds.

van der Waals Polar Surface Area

The polar surface area has been proposed by Palm et al. (9) as an important determinant of drug absorption. The method showed dynamic molecular surface properties that were calculated using all low-energy conformations of some beta-blockers, and the water accessible surface areas that were calculated and averaged according to a Boltzmann distribution. A linear relationship was found between permeability coefficients (measured with both Caco-2 cells and excised segments from the rat intestine) and % polar surface area of beta-blockers with different lipophilicity. According to calculated values of log D_{oct} at pH 7.4, there was less good relationship with some additional impaired ranking order between the substances (calculated according to the method of Hansch and co-workers (9)).

The polar surface area has also recently been proposed by the same laboratory to explain why drugs with very high log D are not absorbed (10). The authors suggest that these very lipophilic drugs instead show a high degree of polar surface area toward the environment, and this will reduce their ability to diffuse through a lipid phase. This was shown by a bell-shaped correlation between permeability coefficients determined in HT-29 (18-C) monolayers and log D, and in contrast, by a linear relationship between permeability coefficients and calculated polar surface area (10).

The main advantage for using this method is the probable good reflection of the mechanism of transcellular transport in vivo, and that it needs no biological tissue. However, methods based on conformational analysis are very time-consuming and need further evidence for their usefulness for different types of drugs. When it comes to very flexible and large molecules, the conformational studies are also very complex to interpret.

Partition Coefficients

The measurement or calculation of log P (or log D) usually uses a determination of a drug molecule extracted into the lipid phase of an octanol/water or octanol/buffer extraction system (9,11–13). This method, which works for many molecules as a rule of thumb (see above), shows the ability of a drug to diffuse into the lipid membrane, and is therefore used to predict passive transport of drugs in vivo. The methods are less time-consuming than the biological methods and need only small amounts of drug or no drug for determination.

The predictive value of log P or log D has been questioned. Firstly, octanol does not provide the ideal lipid phase because of its own hydrogen bonding capacity. However, a delta log P, determined between two lipid phases, has been suggested, e.g., between octanol/water and iso-octane/water (14). Secondly, Leahy et al. (12) have shown that there is no simple relationship between log P and drug absorption. The curve is represented by a sigmoidal shape with a plateau (or even reported as a bell-shaped form) (6,15). The reason for this is not known, but it is suggested to be due to decreased aqueous solubility (5), uncertainty in the evaluation of the value of log P (9), or a high degree of polar surface area (10).

Chromatography

Retention time measurements through chromatography have become very popular recently. Pidgeon et al. (16) have tested fluid cell membranes, i.e., immobilized artificial membranes (IAM) columns, and found a good correlation to the determined log D values and drug absorption in mice for a group of cephalosporins. IAMs also predicted drug permeability through Caco-2 cells. The method is based on a retention of molecules on a column consisting of a solid phase of immobilized phospholipids tethered to a hydrocarbon string onto a silica column. In between the phospholipid strings are C_{10} and C_3 alkyl groups bound to the column. The mobile phase is 100% aqueous. The substance is thought to be retained on the column mainly in the ranking order of lipophilicity. Because the molecule faces the more polar head groups of the phospholipids, this would reflect the biological membrane more than the separation on a high-performance liquid chromatography (HPLC) column-like octadecyl (ODS) (16).

This method is reported to be very simple and could be used for fast screening of a large quantity of compounds. A good correlation between IAM chromatography and the membrane partition coefficient for structurally related hydrophobic drugs (but not for nonrelated compounds) have been reported (16).

The IAM method is similar to a separation on a HPLC column which has been used for screening the substances for drug absorption (16,17). The method by Merino et al. (17) is based on a fluorimetric reversed-phase HPLC method for quantification of quinolones in absorption and partition samples. The retention times of two of the quinolones correlated well with data obtained in vivo. The results from this type of separation also reflect the membrane partition coefficient of drugs and

can therefore be used when the ranking order of related compounds is evaluated. However, drugs with a very large gap between their lipophilicities will require a gradient system elution which can lead to misinterpretation. Nonrelated compounds will give different correlation lines with membrane permeability and partitioning coefficients because of the different mobile phase polarity and differences in the chemical structures (18).

The chromatographic systems (as for log *D* or log *P* calculations or measurements) need to be correlated to a biological parameter (e.g., permeability over Caco-2 cells or intestinal segments in the Ussing chamber) for a better correlation to the absorption process. However, if this can be done with a wide range of molecules as was reported for IAM chromatography (16), and before starting a large synthesis strategy, both time and effectiveness might be improved in finding a drug with good absorption potential.

In addition, the use of artificial lipoidal membrane of the polysiloxane type in the presence or absence of nonionic surfactants has been investigated. This was performed in order to design in vitro conditions and features suitable for reproducing in vivo absorption tests, as well as to validate conclusions arising from in situ gut perfusions about the effect of synthetic surfactants on drug absorption (19). The results showed good correlations to in vivo results, but for a complete evaluation, further development of the polarity of the membrane seems necessary (19).

Aqueous Solubility

Intrinsic aqueous solubility has become increasingly important as a factor influencing the absorption probability of a drug, due to a more frequent synthesis of lipophilic drugs. A microscopic mass balance approach has been developed to predict early in the development phase the fraction of suspensions of poorly soluble drugs absorbed in humans (20). The intrinsic solubility is generally measured by dissolving a weighed amount of drug into a small volume of solvent, e.g., water or a buffer solution. After an equilibrium time, the solution is filtered and the drug concentration in the solvent solution is determined analytically.

The water solubility of a drug is extremely important to understand both in the early studies of drug discovery due to the difficulty to perform biological studies, and in the development phase during in vitro dissolution tests. A drug which is not fully dissolved is not absorbed through the gastrointestinal epithelium, and efforts have been made through the years to find delivery systems containing excipients for increased solubility, e.g., surfactants (see below).

Even if drug solubility is an important factor in drug absorption from the gastrointestinal tract, it has not been extensively screened as a barrier for absorption. Drug solubility should, however, be complementary to models predicting drug permeability through the lipid membrane.

In Vitro Biological Methods

Some drugs will not perform according to physicochemical rules using the biophysical methods. These are the drugs which are susceptible to carrier-mediated processes. The transport processes for these drugs must be studied with biological methods and in addition, information is needed regarding cofactors and scaling factors to the fraction absorbed in humans. Biological methods are also used when the mechanisms of absorption (paracellular, transcellular, or carrier-mediated) and the enzymatic degradation are evaluated (Fig. 2).

Brush-Border Membrane Vesicles

The use of brush-border membrane vesicles (BBMV) in the discovery or development of drugs is usually restricted to mechanistic studies of enzyme interactions or ion transport–coupled transport processes. The method is based on a homogenization of an inverted frozen intestine to give a purified fraction of the apical cell membranes from a chosen part of the gastrointestinal tract (4,21). The BBMV are then characterized by identification of high activity of sucrase (21). The method can be used frequently and for isolated studies of the brush-border membrane transport characteristics without any basolateral membrane influence. It has been used to study the intestinal peptide carrier system (22) and to clarify the mechanism of absorption of fosfomycin (23), glucose, amino acids, and salicylate uptake (16,24).

This method represents a lipid membrane extraction process and can be used in drug absorption studies for evaluation of a biological log *D* value. The different regions of the gastrointestinal tract can be used, evaluating the influence of regional differences in lipid composition on the permeability of drugs as has been suggested (25–27). The major disadvantage of this method is that these processes represent only a fraction of the complete absorption process, i.e., into the cell. No paracellular process can be studied, nor processes which need the basolateral membrane and its function for absorption, e.g., processes coupled to the active transport

of Na^+ by the basolateral Na^+/K^+ ATP-ase (4). There might be a day-to-day variation in vesicle preparation, and a leakage of drugs from the vesicles during washing and filtration that can affect the drug concentration (24). A minor disadvantage is that due to analytical problems, radioactively labeled compounds must be used, making the method less useful during the discovery phase. However, despite its drawbacks, the method can be used for mechanistic studies of the drug absorption process, although there is no data on a direct correlation to human in vivo absorption values.

Intestinal Rings (Slices)

The use of intestinal rings or slices for drug absorption studies have been used for the kinetic analysis of carrier-mediated transport of glucose, amino acids, and peptides (4,24,28,29). The method is easy to use, whereby the intestine of the animal is cut into rings or slices of approximately 30–50 mg (2- to 5-mm width) and is put into an incubation media (which is agitated and oxygenated) for a short period of time. Samples of the incubation media and rings are then analyzed for drug content (4,24). The intestine is sometimes everted on a glass rod before cutting and different regions of the intestinal tract can be used.

The main advantage of this method is its ease of preparation. This method can be used for frequent testing of many different drugs simultaneously. However, the intestinal rings have several disadvantages. Diffusion into the tissue slices takes place on the side of the tissue (not only through the lipid membrane) as the connective tissue and muscle layers are exposed to the incubation solution. The adsorption of drug on the surface of the tissue is not corrected for and the slices do not maintain their integrity for more than 20–30 min (24). The method is also restricted by the limits of the analytical methods; i.e., low concentration of the drug within the slices needs low detection limit. The method is therefore most useful when radiolabeled compounds can be used, which means later in the drug development and for nondegradable substances. Nevertheless, good mechanistic correlation to in vivo measurements have been performed with this method for kinetic studies of carrier-mediated mechanisms of peptides (28). The method has been evaluated for prediction of in vivo absorption potential (29) which showed that under appropriate conditions, uptake into everted intestinal rings closely paralleled known in vivo bioavailability.

The Everted Intestinal Sac Method

The everted sac (everted intestine) method is based on the preparation of a 2–3-cm long tube of the gut which is tied off at the ends after evertion on a glass rod (4,30). The serosa becomes the inside of the sac and the mucosa faces the outer buffer solution. An oxygenated buffer solution is injected into the sac which is put into a flask containing the drug of interest. Samples of fluid are taken from the buffer solution in the flask. The sac is weighed before and after the experiments to compensate for fluid movement. After some modification of the method, one end of the tissue is cannulated with a polyethylene tubing (4), making it easier to withdraw samples also from the serosal side of the intestine.

An advantage with this method is its rapidness, and many drugs can be tested simultaneously, especially low permeability drugs due to the low volume of the serosal compartment. The performance is good for stirring conditions on the mucosal side; however, the oxygenation of the tissue is poor due to the unstirred and unoxygenated serosal inside of the uncannulated sac. Disadvantages are mainly the viability issue and the diffusion through the lamina propria. Histological studies have shown that structural changes already start after 5 min of incubation, and after 1 h, a total disruption of the epithelial tissue can be seen (31). As for intestinal rings, there is no correction for the binding of drug substance onto the surface of the mucosa when uncannulated sacs are used.

Cell Culture Methods

The Caco-2 cell monolayers and other cell cultures (HT-29, IEC-18) from human carcinoma as permeability methods have become increasingly popular during the past few years (32–36). They consist of a monolayer of polarized cells grown onto a filter support. The cells, when fully differentiated, express the transport characteristics of mature villus cells. Caco-2 cells (32) and HT-29(18-Cl) (35) grow into tight epithelia extremely usable for measurement of permeability coefficients of various molecules. The Caco-2 cell monolayer shows an epithelium membrane barrier function similar to the colon of man (37), but shows carrier-mediated systems similar to the small intestine (e.g., bile acid transporter, dipeptide carrier, glucose carriers, and vitamin b12, etc.) (36,38). The transport of pharmaceutical drugs is studied using both simple vials and side-by-side diffusion cells like the Ussing chamber.

Due to the huge popularity of this method, much is known about its performance in predicting the absorption of drugs in humans. A good correlation is seen especially for lipophilic high permeability drugs using the transcellular pathway and the in vivo permeability coefficients measured by perfused human jejunum (LOC-i-Gut technique) (39). The Caco-2 cell method has also been used for studying mechanisms of passive paracellular transport (37), passive transcellular transport (32,40), carrier-mediated system (peptidomimetics and antibiotics) (38), oligopeptide transporter (41), efflux systems (*p*-glycoprotein) (42), enhancers (43), and, recently, cloned Caco-2 cells with specific carrier systems (44). In addition, the importance of the unstirred water layer to the transport of very lipophilic drugs has been studied (45). The HT-29 cell lines have also been used for prediction of drug absorption and for the mechanisms of mucus as a barrier for absorption (35,46,47).

The advantages of the cell culture method are many—i.e., good performance on frequent use, both for prediction of drug absorption in humans and mechanistic studies—and have probably the best potential for use in high throughput screening (HTS) strategies. The experiments are rapid, have good precision, are less time consuming, and are less controversial than, for instance, in vivo animal studies. In addition, the method allows evaluation of drug transport under very controlled conditions, and human cells are used. The disadvantages are the tightness of the epithelium (although this can probably be regulated) showing a more colon-like system giving extremely low permeability coefficients for hydrophilic drugs, the unknown quantity and predictive value of the different carrier-mediated systems (39), unknown composition of the lipid membrane, and the lack of crypt-villus axis which is important for fluid and ion transport in vivo.

The Ussing Chamber for Excised Intestinal Segments

The Ussing chamber technique was developed by Ussing and Zerhan (48) and has been used frequently in physiological studies concerning pharmacology and physiology of ion and water fluxes. It has been used recently for drug absorption studies using rabbit, dog, rat, or monkey tissue (9,27,37,49–52) and also for human biopsies (53). The method is generally based on excised segments from the animal intestine (in some cases stripped of the serosa and the muscle layers) which are mounted between two diffusion cell compartments (51). The permeability coefficients of the compounds are calculated from the measurement of the flow rate of molecules (Papp = $dQ/dt \times 1/A \times Co$) from one side of the segment to the other (either mucosa to serosa or serosa to mucosa) (e.g., (52)), area exposed of the segment (A), and the donor concentration of the drug (Co).

The stirring of the solutions on both sides of the membrane can be achieved by either a gas lift system as originally proposed by Ussing and Zerhan (48), a more refined gas lift system as shown by Grass and Sweetana (54), or by stirring with rotors (51). The viability of the tissues is verified with the measurement of potential difference (PD), short-circuit current, and calculation of the transepithelial electrical resistance by Ohm's law (51,55,56). Sometimes extracellular marker molecules like mannitol, inulin, and PEG-4000 have been used to verify a tight epithelium (52) and to test the effects of enhancers and increased fluid absorption (57).

Although the Ussing chamber with excised segments has not been as extensively used as the Caco-2 cell monolayers, there are several advantages with this method for predicting the drug absorption in vivo in humans. Firstly, there is a good correlation with the permeability coefficients of human jejunum in vivo (58) for both passively transported low and high permeability compounds. Secondly, the technique can be used for different regions of the gastrointestinal tract, evaluating the regional absorption characteristics of drugs (27). In addition, other mucosa than the intestine can be used (i.e., buccal, nasal, esophageal, stomach, rectal, and skin), making it possible to evaluate other administration sites with the same model. The method with diffusion cells can also be used for cultured monolayers using a modified insert for the monolayer membrane.

The method is very useful for evaluating specific mechanisms of absorption, such as ionic transport processes on the transport of drug molecules due to the physiological presence of a crypt-villus axis and a heterogeneous population of cells (matured and immatured as well as cells with different functions). The method has the advantage of being available also for human tissues, slices, or biopsies from surgically removed tissues (53). One of the most challenging applications of this method is the future screening of drugs, especially for mechanistic studies and enzymatic evaluation of drugs and prodrugs where human tissue experiments are needed.

A major disadvantage with this absorption method is that the diffusion pathways for the molecules is unphysiological; i.e., the lack of vascular supply forces the molecules to diffuse through the lamina propria. This tissue part can be different for the different animals and regional segments. Some reports have also noted difficulties with the unstirred water layers, and there should be concerns regarding the stirring conditions of especially the solution in the donor compartment (58). The unstirred water layers will affect the lipophilic drugs more than the hydrophilic drugs (45). Segments from animals are often used (as for most of the absorption models) which then must be verified for human tissue. This is especially important for metabolically and for carrier-mediated transport processes. The integrity and the viability of the tissue must be verified simultaneously; otherwise, it will strongly impair the transport of the drug molecules (see below).

Intestinal Perfusion Method

The isolated perfused intestine, as well as in situ perfusions, has been reported in the literature as a technique for absorption studies (59–63). A segment of 10–30 cm of the intestine is cannulated on both ends and perfused with a buffer solution with a flow rate of 0.2 ml/min (64). The blood side is also cannulated through the mesenteric vein and artery. The difference between the in situ and in vitro is the use of the rat circulation in vivo (being a vascular perfusion in vitro (65,66)), which then allows the possibility of evaluating the influence of the hepatic clearance on the absorption of drugs. Both perfusion methods can use different evaluation systems for testing the drug absorption. An example is the difference between in and out concentrations in the perfusion solutions and/or disappearance and appearance on both sides of the membrane by analyzing the drug concentration on the blood side. The permeability, usually called the P_{eff}, is calculated from the equation, $P_{eff} = [-Q_{in} \times \ln (C_{out}/C_{in})]/2\ \pi rL$ (parallel tube model (64,67), where Q is the flow rate, C_{in} and C_{out} are the inlet and outlet concentrations of each drug, respectively, and $2\pi rL$ is the mass transfer surface area within the intestinal segment. Different length is used (10–30 cm), but for the best flow characteristics 10 cm is used (64). PEG-4000 is used for corrections to fluid flow and to verify absence of leakage in the model (64). In addition, as with the Ussing chamber and excised segments, mannitol, which is more sensitive to changes of the intestinal barrier function, is commonly used as a permeability marker molecule (63).

A major advantage with this type of absorption method is the presence of a blood supply providing the tissue with oxygen and the right flow characteristics on the serosal side of the membrane, e.g., less diffusion through the lamina propria. Secondly, different parts of the gastrointestinal tract can be used as with the Ussing chamber technique. Good stirring, i.e., flow characteristics, of the mucosal/luminal solution has been reported (64). A very good correlation has been found of perfusions to the fraction absorbed in humans and permeability of different types of drugs (64,68).

A disadvantage with the method is the anesthesia that must be used and that has been reported to affect drug absorption (69,70). To verify the integrity of the barrier, PEG-4000 is used, which can lead to misinterpretation of the integrity of the tissue due to the high molecular weight. Additionally, a less important disadvantage for the mechanistic studies is that the method is time-consuming and uses animals, which make it less usable for screening purposes.

In Vivo Biological Methods

These methods are beyond the scope of this review, but will be mentioned briefly in order to give a complete discussion of the schematic drawing shown in Fig. 3. The mainly used methods are in situ perfusions of the rat gut, bioavailability models in different animals, intestinal perfusions in man (Loc-i-gut (71) and triple lumen (72) perfusions), and bioavailability studies in man. These complex studies are usually very time-consuming and cost ineffective and are also too complex for evaluating the mechanisms of absorption in detail. In addition, only the toxicity-evaluated compounds can be used in man and the methods are therefore not used early in the developmental phase. However, some experiments must be performed early in the clinical phase in order for the complete understanding of the absorption of a certain drug, for correct information on the pharmaceutical dosage form program, and for correlation of the performance of the more simple animal models. Some examples are membrane permeability coefficient assessment, ADME studies, dose and concentration dependency, food interactions, regional absorption performance, and evaluation of enhancer systems. More mechanistic studies in humans during Phase 1 must be performed for better feedback on the pharmaceutical development and thereby for faster performance through the clinical phases. Recently, it has been suggested that for a practical drug design, a bio-

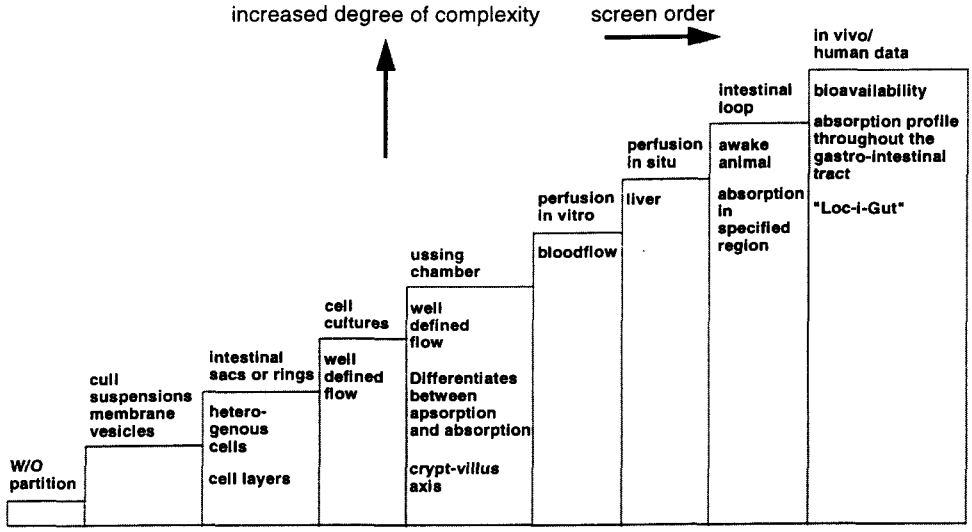

Figure 3. Methods for assessment of oral drug absorption. Some differences between the methods are written beneath the name of the method. Complexity increases from left to right (from ref. 1).

pharmaceutical classification of drug permeability coefficients and dissolution issues must be determined early in the developmental program (73).

COMPARISON BETWEEN DIFFERENT MODELS OF ABSORPTION

A schematic staircase showing the different complexity of the absorption methods is presented in Fig. 3. It illustrates the complexity of the method increasing as one moves from left to the right of the figure. Some differences among the methods are described within the figure.

All methods, regardless of what mechanisms or parts of the absorption process they represent, must be correlated to the in vivo situation and, if possible, also to the absorption in man. This is not a simple evaluation since different methods represent different parts of the whole process and the main barrier will affect the main part of the results. There are a few reports on the comparisons between some of the different biological models such as: Caco-2 cells and excised rat intestinal segments in the Ussing chamber (9,37); Caco-2 cells and perfusion of human jejunum in vivo (39); excised segments of the rat intestine in the Ussing chamber and the perfused human jejunum (58); Caco-2 cells, rat intestinal rings, and rat in situ single pass perfusion (74); and rat in situ single pass perfusion and the perfused jejunum in vivo in humans (64). In addition, comparisons among different species in the same model have also been reported, e.g., dog, monkey, and rabbit and their correlation to humans (49,50).

A good correlation has been found among Caco-2 cells, in situ perfusions of small organic molecules, a series of peptidomimetic analogs (74), model peptides (14), L- and D-methionine (75), betablockers (39), antipyrine (39), and renin inhibitors (76). A good correlation also exists between Caco-2 cells and excised segments of the rat intestine in the Ussing chamber for both the small intestine and the colon where ranking order is concerned (9,37). For most drugs, the Caco-2 cells seem to represent colon permeability characteristics (37). The Caco-2 cells and the everted intestinal rings show a weak correlation with peptidomimetics (74), while the everted rings show better correlation to the results from the perfused intestine. The permeability coefficients of some different drugs (both passive- and carrier-mediated) using excised rat jejunal segments in the Ussing chamber show a very good correlation to permeabilities in the human jejunum (58). The results also indicate discrepancies, especially for carrier-medi-

ated drugs which showed much lower permeability values in the in vitro method. This has also been found in the correlation between Caco-2 cells and the human jejunum (39).

Intestinal perfusions in the rat generally show a good correlation to fraction of dose absorbed in humans (62,66,68) and to perfused human jejunum (64) for several types of drugs. Correlations have also been made to more biophysical parameters such as partitioning coefficients. Intestinal perfusions of ileum using model peptide drugs showed a poor correlation to octanol/water partition coefficients and hydrogen-bonding numbers, but better correlation to partition coefficients determined by the difference between log P in octanol/water and iso-octane/water (14).

The BBMV have been found to correlate well with humans regarding different carrier-mediated mechanisms (22,23,77) and with intestinal rings from the rat (24). Under appropriate conditions, Leppert and Fix have shown a good correlation between everted intestinal rings from the rat and known in vivo bioavailability of 12 different compounds (29).

Using the HT-29-18-C1 cell line, permeabilities of various molecules have been compared to in vivo oral data (35). In this report, a threshold value of 2×10^{-6} cm/sec was found. Above this value, the drugs showed more than 80% absorption in vivo and below this value, the drugs were poorly absorbed. A similar threshold value can be seen also for Caco-2 cells (1×10^{-6} cm/sec) (40), excised jejunal segments of the rat in the Ussing chamber (10×10^{-6} cm/sec), perfused rat jejunum, and the perfused human jejunum in vivo (0.5×10^{-4} cm/sec) (64). These threshold values indicate a parallel shift for different methods concerning the predictive permeability versus fraction absorbed in vivo, which has been suggested recently for the following methods: in situ rat perfusion, Ussing chamber with rat jejunal segments, the perfusion of the human jejunum (58), and use of the same Caco-2 cell model (39) between different laboratories. The parallel shift for permeability coefficients between different methods and animals is expected since the lipid membrane composition can vary both with species and diet (26,27).

The values of the permeability coefficients also indicate different large experimental windows. The Caco-2 cells seem to operate roughly between 0.1 to 200×10^{-6} cm/sec, the excised segments in the Ussing chamber between 1 to 200×10^{-6} cm/sec, and the perfused rat intestine and the perfused human jejunum between 0.1 to 10×10^{-4} cm/sec. For the relevance and ability of the methods to differentiate among the different structures of molecules (although structurally related), a wider window for permeability measurements is needed as shown for the Caco-2 cell model. For the perfusion methods (both rat and humans), the ability to differentiate is less and the values show more an all-or-none mechanism.

Physiological Differences

When new chemical entities are screened for good absorption, the regional difference in the physiology must be evaluated, e.g., carriers, enzymes, luminal content, membrane surface area, membrane lipids, etc. (26,27,78). This will result in regional differences in permeability and metabolite formation for the drugs (79,80). In addition, these mechanisms are also species different (78).

Many compounds are synthesized to have systemic effects by mimicking endogenous molecules, and are therefore prone to be targets for different carrier systems, e.g., peptide, amino acid, phosphate, vitamin B, and sugar transport systems or efflux systems such as *p*-glycoprotein (42). To screen for all these factors in one simple method simultaneously will not be possible. In addition, we know too little about these mechanisms to say what should be changed in the chemical structure of a molecule to result in better absorption.

Laboratory Differences

Due to differences in the handling of animals, age, species, food, tissues, tissue media, clones of cultured cells, or different passages, laboratories will have different prediction factors of absorption (Table 2) (10,26,27,39). Data from the Caco-2 cell method show a parallel shift among four different laboratories when compared to fraction absorbed in humans (10). The ranking order among the different drugs might also vary among laboratories due to the different level of viability and integrity of the biological systems used (Table 2). This makes it difficult to compare values for absorption from several laboratories for prediction of a specific drug. Each laboratory should therefore correlate its own models to human absorption values before using them as predictive tools.

Integrity of the Tissues Used in Absorption Methods

One factor has been more or less neglected in the literature regarding the performance of the in vitro methods: the viability of the tissue during the experi-

Table 2

Examples of Important Factors Influencing the Performance of Biological Absorption Methods

Animal species
Age
Food (prior and fasting or non-fasting animals)
Method of sacrifice or anesthesia
Preparation of tissue—time
temperature
physical manipulation
Stripped or unstripped tissue
Time to equilibrium
Tissue storage (vesicles)
Region of the GI tract
Viability change during experiments
Integrity change during experiments
Absence or presence of systemic blood supply
Oxygenation
Stirring of the UWL
Cell culture clone
Passage number
Tissue culture media
Stirring

ment. This includes measurements of the potential difference, ionic current, transepithelial electrical resistance, and morphology (31,37,54). In addition, for a full understanding of the absorption process, some biochemical markers should be measured such as ATP, LDH, lactate formation, and glucose consumption (81). These substances are formed in the cells and either leakage or a high lactate production indicates membrane loss and/or ischemia. Ischemia or low oxygenation of the tissue can oxidate the membrane lipids resulting in altered membrane permeability (82). For the method of the everted sac, a histological evaluation has been performed already showing structural changes after 5 min of incubation (31). A morphology evaluation for tissues in the Ussing chamber is under investigation in our laboratory. Preliminary results show a change in effective absorptive surface area, edema, and change in cell size and number (83).

The integrity of the tissue change is time related; there is a time limit to best use the different systems (31,83). It might also be related to buffer solutions, oxygenation of the solutions, stirring conditions, preparation of the tissues, and other physical handling, as well as the temperature (Table 2). The surface exposed to the drug is different in different models and for high and low permeability drugs, as suggested by Artursson et al. (39) and Strocchi and Levitt (84). The "true" exposed surface area is the same as the serosal surface area for cultured monolayers (39), but is very variable for excised segments for the Ussing chamber or perfusions, depending on the region of the gastrointestinal tract. Due to the different handling during preparation of the tissues, the effective surface area for absorption may also be different. This will affect high and low permeability drugs differently (84). For a full understanding of the differences in results among laboratories and species, these parameters could perhaps be useful as complements to other valuable information regarding the performance of the experiments and the techniques used.

STUDIES OF SPARINGLY SOLUBLE COMPOUNDS USING VEHICLES

Many drugs with high lipophilicity also have low intrinsic solubility in water. This is of course one factor that can reduce the bioavailability of a drug from the gastrointestinal tract, due to difficulties in either dissolution or luminal precipitation. The future therapeutic dose of the drug will be an important factor in determining if it is possible to develop a medicine of a compound with low solubility (20).

The problems with the sparingly soluble compounds are evident very early in the development, when the purity and the permeability issues are studied. Most vehicles used for these studies are based on surfactant systems (Tween, Brij, chremophore, etc.) or are cosolvents (PEG-400 mixtures, ethanol, etc.) and will affect the epithelial membrane as enhancers (81,85–88). The membrane integrity will be impaired and whether the lipid fluidity or the tight junctions are affected depends on the concentration and the vehicle system used. This means that the vehicle's effect on the membrane affects compounds differently if the compounds to be tested are very lipophilic or more hydrophilic in the structure.

How do we perform these experiments with a good predictability to the human in vivo situation? An inert vehicle system for sparingly soluble compounds to be used when studying oral absorption is not known today. However, we will still be able to perform such studies by using marker molecules together with the compounds to be tested, e.g., mannitol and propranolol. If the vehicle system affects the two marker molecules, the integrity of the membrane has been impaired.

Very often, we must consider an enhancer system to develop a dosage form of a drug with low bioavail-

ability. This is because of the difficulties in performing further structural changes or because the structural changes are related to impaired potency. For enhancer studies, the methods Caco-2 cells, Ussing chambers, and perfused intestinal segments are often used (85,86). It has been found that the effects of the enhancers on the biological system are both species- and method-related. For instance, it has been found that Caco-2 cells are very sensitive to surfactants and the rat is more sensitive than the rabbit (87).

FUTURE PERSPECTIVES

The Use for Absorption Methods in High Throughput Screening (HTS)

HTS methods have recently been intensively developed in the industry for speeding up the development of drugs regarding their potency probability. For in vitro optimization, the traditional correlation of potency with structural and physical properties is increasingly supported by molecular modeling of compounds into target structures such as receptors and enzymes. For lead structure generation, the characteristics of the drugs are put into libraries, either combinatorial libraries in a few screens or individual compounds (or mixtures) in HTS.

In general, the discovery of today's pharmaceutical drugs relies on the testing of the structure/potency relationships. The compound must have its effect before testing its systemic availability. Unfortunately, it seems that there is a structure/absorption relationship, as well. This means that changes in the chemical structure will impair both potency and absorption probability. In addition, it has been reported that there is a structure-related clearance of drugs from the liver (89). If these three phenomena must be taken into account in the bioavailability assessment, how do we perform such studies for evaluating changes in the chemical structure?

Should the absorption/bioavailability probability of drugs be included early in the discovery phase? Due to the complexity of the absorption process, differences in regional performance in the gut, and species differences, this might not be a simple task to perform. Many industries today already use methods for the screening of absorption /bioavailability—e.g., culture monolayers such as Caco-2 cells, intestinal rings, and Ussing chambers—as well as for predicting oral absorption from the lipophilicity, molecular weight, and flexibility of the compound. The biological methods used today, in which the epithelial permeability coefficients are measured, require a large and high quality analytical input to analyze the minute concentrations of the compounds that are transported over the membrane as well as their probable metabolites. This sets the amount of possible screening output to a rather low value, e.g., at the most, 10 compounds a week.

If we start screening for absorption probability of drugs with HTS models, we will be forced to simplify the measurements. Permeability and aqueous solubility are today two of the main absorption characteristics of a drug and are also used for biopharmaceutical drug classification (73). In addition, methods for verifying enzymatic degradation, especially during the transport across the membrane, must be developed (51). In a HTS system, we need to reduce the amount of analytical samples and in many cases, only measure the transport of molecules undefined of its origin. Because of the uncertainty in the values of these measurements, one could argue "Is this more efficient than calculating the lipophilicity?"

Methods for Screening of Gene Products, Disease States, and Vaccines

In the near future, we will need new or modified methods specifically designed for the studies of oral delivery of vaccines, gene products, and disease states. Some methods might be well-suited for these studies, e.g., Caco-2 cells, excised segments in the Ussing chamber, and perfusions of the intestine. The new methods should be able to discriminate between systemic and local mucosal delivery of drugs. In addition, the success of the delivery of the drug is not only determined by the permeability/absorption of the drug itself, but also by the increased level of specific intracellular target molecules. The development of new nonbiological and/or biological methods for these purposes will thereby most certainly broaden the view on intestinal absorption.

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