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Evaluation and Prediction of Drug Permeation

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Abstract

A major challenge confronting the pharmaceutical scientist is to optimize the selective and efficient delivery of new active entities and drug candidates. Successful drug development requires not only optimization of specific and potent pharmacodynamic activity, but also efficient delivery to the target site. Following advances in rational drug design, combinatorial chemistry and high-throughput screening techniques, the number of newly discovered and promising active compounds has increased dramatically in recent years, often making delivery problems the rate-limiting step in drug research. To overcome these problems, a good knowledge of the pharmacokinetic barriers encountered by bioactive compounds is required. This review gives an overview of the properties of relevant physiological barriers and presents some important biological models for evaluation of drug permeation and transport. Physicochemical determinants in drug permeation and the relevance of quantitative and qualitative approaches to the prediction and evaluation of passive drug absorption are also discussed.

Relevant Physiological Barriers

General common features

The main physiological permeation barriers to be crossed by drugs are epithelia and endothelia. Epithelia cover the surface of the body and line all its cavities. Despite their extensive biochemical differences they all serve as highly selective permeability barriers, separating internal and external fluids. Endothelia line the capillaries and regulate the distribution of compounds between the blood and the interstitial fluids. Both barriers are relatively heterogeneous, asymmetric monolayers of epithelial or endothelial cells, respectively. Their barrier properties consist mainly of physical and biochemical components. The biochemical properties are represented by the metabolic activities of epithelial and endothelial cells. The principal physical properties of the membranes arise from their tight intercellular junctions and the lipid matrix (Conradi et al 1996).

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Current opinion is that all biological membranes have as structural framework a bilayer resulting from the orientation of amphiprotic lipids (phospholipids, glycolipids) and cholesterol in the aqueous medium. This bilayer has some of the properties of a two-dimensional fluid (fluid–mosaic membrane model) in which individual lipid molecules can diffuse rapidly in the plane of their monolayer but cannot easily pass into the other monolayer (Alberts et al 1983a). In many biological animal membranes these lipids are distributed asymmetrically. The outer half of the bilayer comprises mainly zwitterionic lipids, for example phosphatidylcholine and phosphatidylethanolamine, whereas the inner part contains negatively charged lipids, particularly phosphatidylserine. This lipid matrix provides the basic structure of the membrane in which central and peripheral proteins, performing both structural and metabolic functions, are embedded.

Plasma membranes of adjacent cells are linked by continuous strands of junctional membrane proteins called tight junctions or zonula occludens (Alberts et al 1983b). Tight junctions are dynamic structures. With the exception of the endothelial cells in the blood–brain barrier, they contain narrow gates or pores filled with water. These pores, which can

open and close, regulate paracellular transport of solutes and ions depending on shape, size and charge of the solute and on the selectivity and dimensions of the pathway (Gumbiner 1987; Audus et al 1990; Schneeberger & Lynch 1992). The number and permeability of tight junctions depend on the type of biomembrane. For the small intestine, for example, tight junctions amount to 0·1% (approx.) of the whole surface (Artursson & Karlsson 1991). Thus, the lipoid surface of a biological membrane is much greater than the aqueous one, which explains the poor ability of highly hydrophilic compounds to penetrate biological membranes passively.

Transport pathways

Drug molecules can cross cellular barriers either by moving across the cells (transcellular transport) or by passing between the cells (paracellular transport). The main physical barrier of the transcellular pathway is the lipid matrix of the membranes whereas that of the paracellular pathway is the intercellular tight junctions.

Transcellular transport. Most common drugs traverse cellular barriers by transcellular pathways. These pathways require movement of solutes across and through cells and include passive diffusion, and carrier-mediated and vesicular transport mechanisms.

For passive diffusion there are two main potential mechanisms (Burton et al 1993)—either the solute becomes distributed into the apical cell membrane and diffuses within the membrane to the basolateral side, or it diffuses across the apical cell membrane and enters the cytoplasm before exiting the cell across the basolateral membrane. It has long been assumed that synthetic drugs pass cellular barriers by passive diffusion only, which would favour lipophilic drugs. However, there is accumulating direct and indirect evidence that the carrier-mediated membrane transport mechanism is not only a pathway for endogenous molecules (i.e. amino acids, oligopeptides, monosaccharides, water-soluble vitamins, etc.) but also a transport route that can be used by xenobiotics (Tsuji & Tamai 1996). Integral membrane proteins present in the cell membrane serve as specific recognition sites for carrier-mediated transport. This transcellular pathway might be energy-consuming or not. If not, the term ''facilitated diffusion'' is generally used (Alberts et al 1983a; Camenisch et al 1996). Finally, large molecules can be transported by vesicular transport mechanisms, i.e. transcytosis. This implies receptor-mediated or adsorptive endocytosis at the apical cell membrane, followed by vesicle migration across the cell, and exocytosis at the basolateral membrane (Alberts et al 1983a; Conradi et al 1996).

Paracellular transport. Paracellular transport, the tendency of a solute to follow the aqueous extracellular route, might be the primary pathway by which hydrophilic compounds of relatively low molecular weight cross epithelial and some endothelial barriers. Transport of larger hydrophilic molecules might be enhanced by modulation of junctional pores or addition of so-called drug absorption promoters. Translocation of solutes via the paracellular route takes place primarily by passive diffusion, namely by an energy-independent process characterized by solute movement in response to a chemical potential gradient (Burton et al 1996). Because of ionizable side-chains in tight junction proteins, the junctional space has an electrostatic field with a negative net charge that might affect the paracellular flux of solutes via ionic interactions (Conradi et al 1996; Gangwar et al 1997).

Polarized efflux systems. In recent years it has been found that the barrier function of the intestinal epithelium and of the blood–brain barrier cannot be adequately described by a combination of metabolic and physical barriers alone. Another transport-limiting factor, which has only recently been appreciated, is the active efflux system (Conradi et al 1996).

Efflux systems are present in cancer cells and represent the major barrier to the uptake of a wide variety of chemotherapeutic agents. Indeed, one of the mechanisms by which cancer cells develop resistance to a cytotoxic agent is by increased expression of a 170–180 kDa membrane glycoprotein called P-glycoprotein (Gottesman & Pastan 1993; Seydel et al 1994). P-glycoprotein acts as an ATP-dependent efflux pump which reduces intracellular accumulation or transcellular flux, or both, of a wide variety of drugs. It has been found that P-glycoprotein, which is highly expressed in a variety of drug-resistant cancer cells, is also present in many normal cell types. Among the tissues with higher constitutive expression of this transporter are intestinal epithelia (Peters et al 1992) and brain capillary endothelia (Tatsuta et al 1992; Sakata et al 1994). As a result of the presence of P-glycoprotein, systemic or brain exposure to potentially harmful xenobiotics is reduced. Whereas P-glycoprotein has been the main focus of recent investigations, several such transport proteins might play similar protective roles.

In drug absorption and permeation these efflux pathways are an impediment to transport, because they return a portion of the absorbed drug back to the lumen before it is able to cross the cellular barrier. Studies performed with Caco-2 cell models or in-situ rat brain perfusion models (Augustijns et al 1993; Hunter et al 1993; Wils et al 1994a; Chikhale et al 1995; Ho et al 1995) have demonstrated that this polarized efflux system, which accelerates solute flux in the basolateral-to-apical direction, is a saturable transport system which is substrate specific and is inhibited by verapamil, a recognized inhibitor of P-glycoprotein (Chikhale et al 1995).

Nature and permeability of some physiological barriers

Because 90% (approx.) of all marketed drugs are administered orally, the principal physiological barrier drugs have to pass to enter our body is the intestinal mucosa. Alternatively, drugs can also be absorbed through the skin, the oral cavity (buccal and sublingual epithelia), the nasal mucosa or the cornea. Some of these routes have been considered as alternative paths for delivery of drugs which are inactivated by first-pass metabolism. Another important physiological barrier is the blood–brain barrier, which separates the blood from the central nervous system. Within the context of this review, we focus on three barriers, namely the intestinal mucosa, the skin, and the blood–brain barrier.

Intestinal mucosa. The mucosa of the small intestine is formed by three main layers—the intestinal epithelium, the lamina propria and the muscularis mucosae. The first faces the luminal cavity and comprises a continuous monolayer of epithelial cells, whose apical plasma membrane, also known as brush-border membrane, is further organized into a lattice of microvilli, which functionally increase the facial area. This epithelial layer exhibits continuous growth and differentiation in-vivo. The basal lamina of the intestinal epithelium is not considered a significant barrier to drug permeation and separates the underlying epithelium from the lamina propria, a connective tissue that provides structural support for the epithelium. This second layer contains the blood and lymphatic channels together with numerous cells, which play an important immunological role. The third layer, the muscularis mucosae, is a continuous sheet of smooth muscle cells (3–10 cells thick) whose role is not clearly defined. It seems that the contraction of this layer can modulate the thickness of the unstirred water layer adjacent to the epithelium (Audus et al 1990). The unstirred water layer, which includes mucus secreted by the epithelial cells, is also considered a significant barrier to the passive diffusion of lipophilic compounds. However, the presence of tight junctions together with the numerous enzymes present in the brush-border region represent the most significant barrier to intestinal drug absorption.

Skin. Skin, the major function of which is to compartmentalize and protect an animal organism from its environment, was originally considered an essentially impermeable barrier. However, sensitive techniques for blood and urine analysis showed that the skin represents a potential portal of entry by which many chemicals can gain access to the systemic circulation. Consequently, much effort is currently directed towards utilizing the skin as a non-invasive route for drug administration (Kalia et al 1998a). One of the main advantages of transdermal drug delivery over oral administration is the avoidance of variability associated with the gastrointestinal tract (effects of pH, motility, transit time and food intake) and first-pass metabolism. Although these advantages are similar to those associated with intravenous infusion, transdermal drug delivery is a safer and more convenient method of drug administration (Ridout et al 1988).

During skin absorption drugs have to pass a complex multilayer structure before reaching the blood (Flynn 1990). Structurally, the skin can be viewed as a series of layers, the three major divisions being the epidermis, dermis and subcutis or hypodermis. The subcutis lies below the dermis and functions as a fat-storage layer; being below the vascular system, this layer is not relevant to percutaneous penetration. The dermis (2 mm) is essentially an acellular collagen-based connective tissue which supports the many blood vessels, lymphatic channels and nerves of the skin. Hair follicles and sweat glands originate in this layer and open directly on to the skin surface. The avascular epidermis consists of stacked layers of cells (approx.) 0·2 mm thick (varying from 0·1 mm for the eyelids to 0·8 mm for palms and soles) (Maibach et al 1971; Ritschel & Hussain 1988). Cells which provide the epidermal tissue differ from those of all other organs in that they ascend from the proliferative layer of basal cells. They change in an ordered fashion from metabolically active and dividing cells to dense, dead and keratinized cells (Barry 1983a). The outermost layer of the epidermis, the stratum corneum, comprises 10–15 layers of flat keratin-filled cells, closely packed in a non-polar lipid matrix composed of ceramides,

cholesterol and fatty acids, but largely devoid of phospholipids (Lampe et al 1983; Ridout et al 1992; Alvarez et al 1993; Hadgraft & Pugh 1998).

The main rate-determining step in skin penetration is diffusion of the solute across the stratum corneum, which thus represents the principal penetration barrier of the skin (Houk & Guy 1988; Ritschel & Hussain 1988; Hadgraft & Pugh 1998). Under conditions of normal passive diffusion it seems that the intercellular route predominates over the transcellular route (Albery & Hadgraft 1979; Nemanic & Elias 1980; Boddé et al 1991; El Tayar et al 1991a; Alvarez et al 1993; Heisig et al 1996). Moreover, an important property of the stratum corneum is its capacity to become heavily hydrated, modifying also the permeation profile of chemicals (Ritschel & Hussain 1988; Berner et al 1989; Mouritsen & Jorgensen 1998).

The blood–brain barrier. The blood–brain barrier certainly has peculiar characteristics among the various physiological barriers. Diffusion of drugs across this endothelium separating the blood from the central nervous system (CNS) is in fact more restrictive than elsewhere. Endothelial cells provide a crucial interface between blood and tissue environments. The free diffusion of chemicals across endothelia is prevented by endothelial tight junctions, the permeability of which varies considerably depending on tissue and conditions. In peripheral tissues (intestine, kidney, salivary gland) these cell barriers have fenestrations enabling almost free exchange of water and solutes. In contrast, the endothelial barrier separating the blood from the CNS is characterized by tight junctions of severely limited permeability (which exclude molecules with a diameter larger than 20 Å), no fenestrae, and an attenuated pinocytosis (van Bree et al 1992a; Hirase et al 1997). As a common feature, both peripheral and blood–brain endothelial cells have significant metabolic activity (Brightman 1989). Moreover, and in contrast to what has long been generally accepted, the special features of the blood–brain barrier seem to be subject to endogenous regulation (van Bree et al 1992a).

Thus, the blood–brain barrier is far from being a simple barrier. Understanding its structure and function, and physicochemical and biological aspects of the control of solute transfer, is a problem of great currency in pharmaceutical research. This is true not only for the design and development of CNS-active agents, but also for drugs which must be excluded from the CNS to avoid unwanted side-effects, as exemplified by antihistamines and β-blockers.

Biological Models for the Evaluation of Drug Permeation and Transport

In recent years combinatorial chemistry and high-throughput screening techniques have yielded a large number of potential hits, lead compounds and candidates. The need to screen drug absorption and metabolism has increased as a result. An ideal screening tool should be fast and easy to use, require little material, be relatively inexpensive and yield reliable predictions. In practice, such ideal screening tools are not easy to develop. As in-vitro models increase in complexity, an increasing number of factors influencing drug absorption is uncovered. Unfortunately, the more closely the method approaches the in-vivo situation, the more it is labour-intensive and material-consuming, and the less it is suitable for screening (Palm et al 1997a).

Intestinal drug absorption

As the dominating barrier of intestinal drug absorption is the monolayer of epithelial cells lining the gastrointestinal tract, models of this barrier should be suitable for prediction of drug absorption. Several in-vitro and in-situ techniques have been developed to assess rates, extent and mechanisms of intestinal absorption (Table 1). These methods will be briefly presented in order of increasing complexity. Particular attention will be given to cultured cell systems, such as Caco-2 cells, because of their intermediate complexity, and thus potential suitability as screening models (Kuhfeld et al 1994; Stevenson et al 1995).

Artificial membranes. The main objective of the parallel artificial membrane permeation assay (PAMPA) is the classification of passively absorbed compounds, focusing on the transcellular transport route. PAMPA is now based on a 96-well microtiter plate technology (Kansy et al 1998). Using a hydrophobic microtitre filter-plate as support, the permeation of test compounds is assessed through a membrane formed by a mixture of lecithin and an inert organic solvent. This assay enables investigation of hundreds of compounds per day. Hence its greatest potential lies in the screening of chemical libraries. Unfortunately, PAMPA is a poorly characterized system which enables only a simple classification of solutes, e.g. low, intermediate and high intestinal absorption probability.

Subcellular fractions. BBMV (brush border membrane vesicles) and BLMV (basolateral membrane vesicles) are essentially used to study the mechan-

Table 1. Different in-vitro and in-situ methods for assessment of intestinal drug absorption.

	Advantages	Limitations
In-vitro methods		
Artificial membranes	Allow classification of passively transported compounds	Difficult in-vivo extrapolation
	Fast screening possible	Poorly characterized systems
Subcellular fractions	Tissue sources may be from man	Difficult in-vivo extrapolation
	Useful to obtain partitioning parameters for compounds absorbed by the transcellular route	Damage to membranes in the isolation process
Cell cultures	Good correlation with in-vivo data for passively absorbed drugs	Difficulty to compare results obtained in different laboratories
	Potential suitability as screening models	
	Insights on mechanisms of transport	
In-situ methods		
Isolated tissues	Good intermediate level of complexity	Absence of blood may compromise enzymatic activity
	Experiments performed with intact intestinal tissues	
Organ preparations	Blood and lymphatic supplies remain intact	High variability within treatments
	Physiologically and pharmacologically responsive	Low throughput

isms of transport of compounds absorbed by the transcellular route (Fan et al 1998; Koga et al 1998). These vesicles are prepared by tissue homogenization and differential sedimentation, followed by fractionation (generally by density-gradient centrifugation and differential precipitation) and subfractionation for BLMV. Finally, they are characterized by use of marker enzymes for the membrane populations (Stewart et al 1997). Tissue sources have been from man, but more frequently from such animal species as rabbits, guinea-pigs and rats.

Uptake studies in BBMV or BLMV include investigation of the transport mechanism of a solute, and determination of its location in the vesicle. The solute can either partition into the lipid membrane or it can be transported into the intravesicular space. Although subcellular fractions can be useful in assessing oral drug delivery, the extrapolation to rate and extent of absorption in-vivo is difficult. Moreover, the isolation process can damage membranes and harm their enzymatic or carrier functions.

Freshly isolated cells and cultured cells. The use of freshly isolated cells is limited to uptake studies rather than to transport studies. The main difficulty in working with mature intestinal epithelial cells, or enterocytes, is that they are highly polarized biochemically, morphologically and functionally. As mentioned above, apical and basolateral membrane domains are essentially different cellular organelles. In cell suspensions enterocytes rapidly fuse and depolarize, thus losing the integrity of separate cell-membrane populations. Unless polarity is maintained and tight junctions are formed, these cells are not representative of intestinal-barrier function (Stewart et al 1997).

Thus, an in-vitro system consisting of a monolayer of viable and polarized intestinal epithelial cells similar to that found in the small intestine is required to study drug transport and metabolism. The utility of such a system is based on the robustness of the correlation between parameters obtained in-vitro, e.g. permeability coefficients in cell monolayers, and those obtained in-vivo, e.g. fraction of dose absorbed.

Numerous attempts have been made to culture intestinal epithelial cells. Unfortunately, they have not met with great success, because intestinal epithelial cells do not retain biological or morphological characteristics when in culture (Audus et al 1990). More successful have been approaches with transformed cell lines. Several colon carcinoma cell lines from man, e.g. Caco-2 and HT-29, undergo different amounts of enterocytic differentiation in culture. Among these cells Caco-2 monolayers (Artursson & Karlsson 1991; Artursson et al 1996; Palm et al 1997a) are the most thoroughly investigated because they spontaneously achieve more differentiation than HT-29.

The development of a cell culture system that will mimic a specific biological barrier requires not only an appropriate cell line but also a microporous membrane (generally polycarbonate or nitrocellulose filters), which by itself or after treatment with

an appropriate matrix material (e.g. collagen) will support cell attachment and cell growth. Careful selection of the microporous membrane properties, such as pore size and surface area, is necessary to avoid generating artefactual data in transport experiments.

Caco-2 cell lines are now routinely cultivated as monolayers on permeable filters for studies of the transepithelial transport of drugs (Artursson 1991; Hillgren et al 1995). Most studies performed with these cells have a mechanistic or screening objective. One aim is, for example, to investigate whether a drug is actively or passively transported across the intestinal epithelium and, if the transport is active, to identify the relevant carrier. The first study attempting to correlate passive drug permeability obtained in Caco-2 cell monolayers with drug absorption after oral administration in man suggested that the cell monolayers might be used to identify drugs with potential absorption problems (Artursson & Karlsson 1991). More recent studies indicate that Caco-2 cells rank the permeability of drugs in the same order as more complex absorption models, such as excised intestinal tissues and in-situ perfusion models (Artursson et al 1996).

In summary, the results obtained to date indicate that Caco-2 cell monolayers can be used to predict the transport of drugs by different pathways across the intestinal epithelium. However, the best correlation with the absorbed fraction in-vivo is obtained for passively and rapidly absorbed drugs. Indeed, for slowly and incompletely absorbed drugs the relation between permeability coefficients obtained in Caco-2 cell monolayers and in-vivo absorption is qualitative rather than quantitative. Moreover, care needs to be taken when comparing permeability results obtained in different laboratories, because of variability in experimental conditions and in the cell lines themselves.

Isolated tissues. Isolated tissues used for permeability investigations are usually obtained from animals, but human tissues also are used sometimes. Two main experimental systems are used, ''tissue flux chambers'' and ''everted rings'' (Stewart et al 1997).

Tissue flux chambers model in-vivo absorption by measuring the transepithelial flux of drugs across intact intestinal tissue. Isolated intestinal segments are opened to form planar tissue sheets, then mounted in standard, commercially available diffusion cells filled with an appropriate buffer mimicking extracellular fluids. This versatile experimental system enables evaluation of tissue permeability, paracellular and transcellular transport, intestinal secretion, and contribution of intestinal metabolism to bioavailability.

The everted rings method (Diez-Sampedro et al 1998), which utilizes entire intestinal segments, is generally used for tissue-uptake studies. Excised intestinal segments, carefully everted and divided into small sections (rings), are incubated in oxygenated, drug-containing buffers. For analytical reasons, radiolabelled compounds are generally used.

Organ preparations. Organ perfusion studies are intermediate between in-vivo studies and experiments with isolated tissues. The organs to be perfused must be isolated from the neighbouring tissues. This isolation is not necessarily physical, because good vascularization perfusion of the investigated tissue or organ, only, will suffice (in-situ perfusion). This can be achieved by cannulating either a pair of mesenteric vessels supplying an intestinal segment, or the superior mesenteric vessel and the portal vein, achieving perfusion of almost the entire small intestine (Stewart et al 1997).

Intestinal perfusion assays base permeability calculations on the steady-state disappearance of the compound from the intestinal lumen. With these in-situ techniques neural and endocrine input, and blood and lymphatic supplies, remain intact. Input of drug can be controlled in terms of concentration, pH, osmolality, composition and flow rate. These absorption models are sensitive to physiological and pharmacological influences, which accounts for their high variability compared with rapid in-vitro systems.

Percutaneous drug absorption

Prediction of chemical transport across the skin is important both for optimization of topical and transdermal drug delivery, and for assessment of risk after dermal exposure to chemicals (Blank & McAuliffe 1985; Hostynek et al 1993). To facilitate the estimation of percutaneous absorption, a number of in-vitro model systems have been developed (Houk & Guy 1988; Ridout et al 1992). The predictive capacity of the different approaches, and their relative utility, will be discussed here; those most worthy of further study will be highlighted.

A common method for in-vitro percutaneous absorption. The most frequently used in-vitro method is a simulated membrane permeation experiment. Its design involves different membranes simulating the skin, adjustable environments, numerous protocols and the use of

diffusion cells, i.e. systems containing a donor and an acceptor compartment (Tanojo et al 1997). The donor compartment can be closed or open, depending on the type of administration protocol. The acceptor compartment can be either closed (static design) or open (flow-through design) (Bronaugh & Stewart 1985; Addicks et al 1987; Reifenrath et al 1994).

Artificial membranes. Several synthetic membranes can be used to model the transport of drugs across the skin (Houk & Guy 1988; Alvarez et al 1993). They are exemplified by cellulose acetate membranes, which have been used in various diffusion studies (Garay-Bobo et al 1969; Barry & El Eini 1976; Barry & Brace 1977). Like all dialysis membranes, those made of cellulose acetate are porous barriers which model the skin seen as a passive diffusion barrier, with very little involvement of drug partitioning into the intercellular lipid pools. Consequently, in these experimental models the rate of diffusion is determined by solute concentration and the length of the diffusion pathway. Although this mechanism is not representative of diffusion through intact skin, it might be relevant as a model for damaged skin, where the relatively aqueous dermis is the only remaining barrier (Houk & Guy 1988).

In addition to hydrophilic acetate membranes, an array of synthetic polymer membranes containing hydrophobic groups has been evaluated as potential models for penetration of compounds through the skin. The hydrophobic groups are intended to provide functional mimics for drug partitioning into lipids. Among these mixed-polarity membranes, foils of dimethylpolysiloxane (silicone rubber or Silastic) have been most extensively used (Garrett & Chembukar 1968; Cooper 1984; Jetzer et al 1986; Bosman et al 1996; Hou & Flynn 1997). These models, however, do not incorporate all the structural features needed to evaluate interactions occurring during transport across lipoidal intercellular channels.

Besides such solid membranes, organic liquid-supported membranes containing hydrocarbons, long-chain alcohols or isopropyl myristate have been investigated (Hadgraft & Ridout 1988; El Tayar et al 1991b; Santi et al 1991). Also, skin lipids (ceramides, palmitic acid, cholesterol, etc.) or liposomes comprising various skin lipids, have been tested (Kai et al 1993; Matsuzaki et al 1993). Several synthetic (mixed cellulose ester, poly(ethylene–vinylacetate), polypropylene, etc.) or natural materials (e.g. eggshell membrane) have been used to prepare artificial membranes. Such systems have been proposed as reasonable models for the epidermal barrier (Guy & Fleming 1979).

Other devices combine two or more different membranes into laminate or composite systems to mimic the hydrophilic and hydrophobic domains of the skin (Houk & Guy 1988). Such models, however, are not relevant because the desorption of solutes from the stratum corneum is linear with the square root of time, whereas linear relationships have been obtained with laminates. More recent experiments have used synthetic graft copolymers to model the heterogeneity of skin domains (Yamaguchi et al 1997; Feldstein et al 1998). These have proved useful as relevant models for quantitative evaluation of transdermal penetration.

Although skin-imitating membranes will continue to play a role in the future, artificial membranes should provide the most useful forecast of in-vivo transdermal delivery processes when:

- the passive diffusion barrier imposed by the stratum corneum is the major resistance to transport;
- the drug of interest is known to be metabolically inert and not specifically bound in viable skin;
- the formulation does not contain a permeability enhancer which can interact with the skin but not the membrane; and
- in-vivo experiments of similar design have been or can be performed and correlated with in-vitro results.

Cultured cells. Recent progress in the culture of cells from man has led to biological models of the skin growing on various dermal substrates (Michel et al 1995). One such model consists of de-epidermized derma devoid of living fibroblasts but with an intact basement membrane (Fartasch & Ponec 1994). This cutaneous model is discriminative in terms of absorption rates and yields the same ranking of permeants as normal skin from man. However, such cell cultures are more permeable than intact skin. Their lower content of ceramide, the most important class of lipid in barrier function, might be the cause for this quantitative difference (Régnier et al 1993).

Isolated tissues. Like biological membranes, isolated skin fragments from animals (e.g., rat, mouse, rabbit, squirrel, guinea-pig, snake and pig) (Jetzer et al 1986; Takahashi et al 1991, 1993; Borsiada et al 1992; Kai et al 1992) and man are frequently used (Morimoto et al 1992). Depending on the number of hair follicles seen in such skin preparations, three different phenotypic hair densities have been defined as hairy, fuzzy and hairless (Lauer et al 1995).

Among the many animal skins used, those from snakes and domestic pigs have been proposed as relevant models. Thus, the response of shed snake skin to lipophilicity and molecular weight mimics that of the stratum corneum from man more closely than that of hairless mouse skin (Itoh et al 1990). In addition, the rate of water evaporation of shed snake skin is comparable with that of skin from man. Nevertheless, the lack of an optimum correlation between skin from snake and man is probably because of differences between their lipids in structure and proportions. Otherwise, the domestic pig is perhaps the best skin model—it has been shown to have histological and physiological properties similar to those of skin from man. The epidermal lipid biochemistry of the pig has also been shown to be similar to that of man (Walker et al 1997). Although animal skin is more readily available than that from man, care must be taken when selecting a type of skin, because the method remains time-consuming and might be poorly reproducible (Houk & Guy 1988).

On the basis of the assumption that the stratum corneum represents the main barrier to skin penetration, solute permeability through the skin can also be determined by use of a diffusion cell in which the isolated stratum corneum is embedded as a membrane (Morimoto et al 1992). Although experiments to investigate the permeability of this horny layer have been conducted in-vitro, they do not differ markedly from an in-vivo situation, because this dead layer is also present in the living organism. However, such studies are conducted in water, resulting in artefactual hydration which promotes solute penetration (Flynn 1990). Therefore, any deduction made will not pertain directly to an in-vivo situation, but to an extensively hydrated skin. This drawback might be tolerable, given that under normal in-vivo conditions the skin is readily hydrated, particularly when a drug is applied as an aqueous gel or under occlusion (Barry 1983b).

Blood–brain barrier permeation

Most in-vitro models of the blood–brain barrier are based on cultured endothelial cells. In addition, preparations of brain microvessels are also used to study the properties of this barrier. Simpler models such as artificial membranes or subcellular fractions do not provide reliable predictions of blood-to-brain permeation. The relevant in-vitro models for assessment of blood–brain barrier passage are presented below in the chronological order of their development. The basic in-vivo methods used to assess blood–brain barrier permeability are also cited.

Isolated tissues (brain microvessels). The earliest work on in-vitro models of the brain endothelium used microvessel fragments isolated from the brain (van Bree et al 1992b). Some procedures used to separate microvessels employed mechanical dissociation followed by filtration. More successful cleaning of the vessels was achieved by means of enzymatic dissociation. Such preparations have proved useful in the identification of membrane receptors, and for study of some carrier-mediated transport phenomena (Abbott et al 1995; Joo 1995). However, morphological abnormalities and the impossibility of studying either polarized transport or transcellular movement renders this in-vitro system unsuitable for accurate transport studies.

Cultured cells. Isolated brain microvessels provide a good starting material for primary cultures (Joo 1993). Primary cultures of rat (Abbott et al 1995) or bovine (Audus et al 1990) brain microvessel endothelial cells, isolated either enzymatically or mechanically, retain many characteristics of the parent tissue, including morphology, specific blood–brain barrier enzyme markers, few pinocytic vesicles, no fenestrae and tight intercellular junctions. However, their main disadvantage, which limits studies of permeability and transport, is the difficulty of eliminating all non-endothelial contaminating cells, which can constitute up to 5–10% of the population. This might cause a defect or hole in the monolayer, because the endothelial cells are unable to grow over them. Although recent improvements in cultivation techniques seem to overcome these problems (Franke et al 1999), the preparation of primary cultures remains relatively expensive and time-demanding. For this reason, the use of immortalized cells has been investigated.

Endothelial cells can be immortalized by transfection with plasmids containing adenovirus genes (Roux et al 1994). Several such immortalized cell lines are known; they have been derived from brain capillary and microvascular endothelial cells from various sources, including mouse (Vinters et al 1985), rat (Abbott et al 1995), cattle (Isobe et al 1996) and man (Muruganandam et al 1997). However, if these immortalized cells are grown alone, they lose morphological and biological characteristics such as formation of tight junctions and expression of specific enzyme activity, and will need the presence of astroglial factors to maintain their blood–brain barrier properties (Arthur et al 1987; Roux et al 1994). Efficient in-vitro models of the blood–brain barrier thus require the co-culture of endothelial cells with astroglial cells, i.e. astrocytes (Dehouck et al 1990) or glioma cells (Hurst & Fritz 1996). It has been found that astrocytes can

even induce the differentiation of non-blood–brain barrier endothelial cells into endothelial cells with blood–brain barrier-like properties (Tio et al 1990; Hurst & Fritz 1996). Successful results have been obtained with immortalized umbilical vein endothelial cells from man (Hurst & Fritz 1996) and with bovine aortic endothelial cells (Isobe et al 1996).

In-vivo methods. Considerable progress has also been achieved with in-vivo methods of quantification of blood–brain barrier permeability and transport. Nowadays, five basic in-vivo methods are generally used to measure unidirectional transport into the brain. They are the intravenous administration technique, the indicator diffusion technique, the brain perfusion technique, the brain uptake index (BUI) technique and the single injection–external registration technique. Efflux from brain to blood can be measured with either the washout technique or the concentration profile technique (Smith 1989).

Physicochemical Determinants in Drug Permeation

Lipophilicity

Isotropic solvent systems. Parameters encoding relevant structural features and properties are needed for quantitative modelling of in-vivo or in-vitro biological data (QSAR) (Kubinyi 1995). Some physicochemical parameters have proved particularly useful for predicting drug permeation across biomembranes, but none has attracted as much interest in QSAR studies as lipophilicity (Kubinyi 1993).

The most common expression of lipophilicity is the logarithm of the *n*-octanol–water partition coefficient (log P_{oct}). The *n*-octanol–water biphasic system has been one of the most suitable models of the lipidic biological membranes, because of the analogies of *n*-octanol with lipids—its long alkyl chain and the polar hydroxyl group. The capacity of the hydroxyl group to be both hydrogen-bond donor and acceptor enables interaction with a large variety of polar groups in solutes. At the same time, this amphiprotic characteristic renders log P_{oct} unsuitable for assessing hydrogen-bond-donating capacity. Furthermore, the *n*-octanol–water system does not mimic the highly hydrophobic region of membranes.

Thus, different solvent systems yielding partitioning information complementary to *n*-octanol–water data have been suggested for the modelling of different membranes and tissues (van de Waterbeemd & Testa 1987; Leahy et al 1989, 1992a). Chloroform has been proposed as a hydrogen-bond-donor solvent, propylene glycol dipelargonate (PGDP) and di-*n*-butyl ether as hydrogen-bond acceptors, alkanes (e.g. *n*-heptane, cyclohexane, dodecane and, recently, 1,2-dichloroethane) as inert solvents. This has led to the concept of the so-called ''critical quartet'', namely a system of four solvents (amphiprotic, proton-donor, proton-acceptor and inert) for the general modelling of biological membranes (Leahy et al 1989, 1992a, b; Pagliara et al 1997a; Steyaert et al 1997).

Anisotropic solvent systems. The above mentioned isotropic biphasic solvent systems cannot adequately simulate the anisotropic characteristics of biological membranes. This has led to the development of anisotropic systems for lipophilicity determination which use bilayers formed from natural or artificial phospholipids (artificial membranes or liposomes). These systems, despite their own limitations, are of great value for the study of drug–membrane interactions and diffusion (Choi & Rogers 1990; New 1990; Herbette et al 1991; Mason et al 1991; Rogers & Choi 1993; Pauletti & Wunderli-Allenspach 1994; Austin et al 1995; Betageri et al 1996; Escher & Schwarzenbach 1996; Ottiger & Wunderli-Allenspach 1997; Avdeef et al 1998; Vaes et al 1998). It should be noted that the determination of partition coefficients in such anisotropic systems is now becoming easier, e.g. with immobilized artificial membrane (IAM) chromatography (Lambert 1993; Kaliszan et al 1994; Ong et al 1995, 1996; Pidgeon et al 1995; Barbato et al 1996, 1997; Beigi et al 1998; Caldwell et al 1998; Reichel & Begley 1998; Stewart et al 1998).

The lipophilicity of ionized species. Whatever the system (isotropic or anisotropic), the ionization state of a molecule must be taken into account if misleading interpretations or relationships are to be avoided. It has been demonstrated that log D (distribution coefficient) values measured at physiological pH are often a more pertinent parameter for QSAR studies than log P (partition coefficient) values (Hansch et al 1987; ter Laak et al 1994; Palm et al 1996).

The current dogma that ''only neutral molecules permeate membranes'' is slowly becoming suspect (Lee & Lippold 1995; Nakashima et al 1995) because of an increasing body of experimental evidence supporting ion partitioning (Chakrabarti

et al 1992, 1994; Pauletti & Wunderli-Allenspach 1994; Pirselova & Balaz 1994; Austin et al 1995; Depaula & Schreier 1995; Krämer et al 1998). Strategies in drug delivery also take advantage of ion partitioning (Hirvonen & Guy 1997; Kalia et al 1998b).

The importance of partitioning of ionic and zwitterionic species in isotropic and anisotropic biphasic media has recently been underlined in connection with their pharmacokinetic behaviour (Avdeef et al 1996; Abraham et al 1997; Pagliara et al 1997b, 1998; Reymond et al 1999a).

Some recent experimental techniques have been developed for determination of the lipophilicity of ionized species. The potentiometric method (Avdeef 1993) enables the determination of the distribution profile of an ionizable drug over the entire pH scale. Cyclic voltammetry (Reymond et al 1996b) generates a pH-potential representation (Reymond et al 1996a) which offers a global and direct visualization of all species present in a biphasic system and helps understanding of the mechanisms of transfer and partition of ionizable drugs. The two techniques have helped to clarify some important biological mechanisms, e.g. the export of protons from cells under acidosis as mediated by the anti-ischaemic drug trimetazidine (Reymond et al 1999b) or proton transfer assisted by the anti-inflammatory agent piroxicam (Reymond et al 1996c).

In addition, several models have also been developed for measurement of ion partitioning under biomimetic conditions, namely pH-dependent distribution profiles of solutes between phospholipid vesicles and aqueous buffers (Pauletti & Wunderli-Allenspach 1994; Austin et al 1995; Avdeef et al 1998), capillary electrophoresis (Esaka et al 1994; Kaliszan et al 1995), micellar electrokinetic chromatography (MEKC) capacity factors (Ishihama et al 1994, 1995; Smith & Vinjamoori 1995; Muijselaar 1997) and capacity factors measured on immobilized artificial membrane (IAM) columns (Barton et al 1997; Ducarme et al 1998).

The results so obtained clearly show that the behaviour of ionizable drugs in the body is controlled by the interactions of both neutral and ionic forms with biological membranes (Seydel et al 1994; Hellwich & Schubert 1995). The factors responsible for such interactions go well beyond lipophilicity. They include dynamic aspects of solute–membrane interactions (Perillo et al 1995), interactions with ion channels (Miyakoda et al 1995), and the influence of pH on membrane permeability (Ueda et al 1994) and on the ionization of bound solutes (pK_a shift) (Heberle et al 1994; Lau & McDonald 1995; Smith & Anderson 1995; Tamai et al 1995).

Hydrogen-bonding capacity

The correlation of hydrogen-bonding properties with permeation data is well known (Young et al 1988, 1993; LeCluyse et al 1991; Chikhale et al 1994; Paterson et al 1994; Abraham et al 1997; Jack 1997). Although lipophilicity measured in an isotropic system can be inadequate alone for prediction of permeability, the interest of lipophilicity-derived polar parameters must be appreciated. Numerous studies show that the difference between two different log P scales, generally *n*-octanol–water minus alkane–water ($\Delta\log P_{oct-alk}$), is a measure of the hydrogen-bonding capacity (Testa et al 1992). One of the most interesting and pioneering applications of QSAR for such parameters was the study by Young et al (1988, 1993) who showed that $\Delta\log P_{oct-cyc}$ (cyc = cyclohexane) is inversely related to brain penetration and is useful in the design of brain-penetrating drugs.

Indeed, $\Delta\log P_{oct-alk}$ is mainly an indicator of the hydrogen-bond donor capacity of a solute as demonstrated by its factorization into the so-called ''solvatochromic parameters'' (El Tayar et al 1991b). Thus, the limiting step in drug permeation across the blood–brain barrier might be the binding of the drugs to the lipid-rich layer of biomembranes by the donation of hydrogen-bonds. In fact, the hydrophilic part of lipids contains hydrogen-bonding acceptor groups, which might explain the very slow diffusion of strong hydrogen-bond donating solutes.

Another parameter accounting for the hydrogen-bonding capacity of a solute is the term Λ (El Tayar et al 1992; van de Waterbeemd & Kansy 1992). Factorizing lipophilicity into a cavity or volume term and a polarity term yields equation 1:

$$\log P = aV - \Lambda \qquad (1)$$

where V is the molar volume and Λ the global polarity of a given solute in a given solvent system (e.g. *n*-octanol–water or alkane–water). The coefficient a depends on the solvent system and on the mode of calculation of molar volumes.

Molecular size

Molecular size is believed to play a distinct role in permeation processes. The paracellular permeability of charged and neutral solutes has been successfully modelled in terms of size-restricted diffusion across negatively charged pores (Adson

et al 1994). It has been demonstrated that whereas some protonated amines permeate pores faster than neutral solutes of comparable size, the anions of weak acids permeate the pores at a slower rate. However, with increasing molecular size the ''sieving effect'' of the pores becomes increasingly discriminating and dominates over the influence of the electrical field.

A molecular sieve effect is also believed to promote transcellular diffusion. Diffusion coefficients across biological membranes have been shown to be highly dependent on molecular mass (Walter & Gutknecht 1986; Xiang & Anderson 1994; Bunge & Cleek 1995). Indeed, it has been found that the logarithm of blood–brain barrier permeation is linearly correlated with the logarithm of molecular mass (Levin 1980; Rim et al 1986). To account for the molecular size-dependence of brain penetration, the logarithm of permeability has also been correlated with the logarithm of molecular mass (Hansch et al 1987).

Aqueous solubility

Solubility and pH–solubility profiles are particularly useful means of identifying compounds likely to have absorption and distribution problems (Morelock et al 1994). Measuring thermodynamic solubility is laborious, time-consuming and requires large amounts of sample. Theoretical (Kamlet et al 1988; Nelson & Jurs 1994; Lee 1996; Breitkreutz 1998) and alternative experimental methods have been proposed, e.g. fast ''ranking'' techniques such as turbidimetry (Lipinski et al 1997) and potentiometry (Roseman & Yalkowsky 1973; Todd & Winnike 1994; Avdeef 1998). Because solubility is partly correlated with physicochemical parameters relevant to drug absorption, e.g. partition coefficients (Lewell 1992) and molecular surface properties (Murray et al 1995) (see below), a relationship between poor solubility and poor absorption can be expected and is often verified.

Conformational variability and molecular surface properties

The molecular flexibility of a solute and its potential conformational variability must be considered when evaluating drug permeation, given that some physicochemical properties vary among conformers. The conformational space should be systematically explored to evaluate the potential contribution of different conformers to permeation. Indeed, conformational facilitation of internal hydrogen bonds increases lipophilicity and reduces hydrogen-bond-donating capacity, and as a result facilitates membrane permeation, as seen for peptides, proteins (Kastin et al 1982; Roseman 1988; Raeissi & Audus 1989; Gray et al 1994) and various classes of drugs (Carrupt et al 1991a; Wright & Painter 1992; El Tayar et al 1993; Conradi et al 1994).

The solvent-accessible surface area (SASA) is also influenced by the three-dimensional molecular structure. The variation of this molecular surface enables the modelling of molecular properties which fluctuate with the geometry of a compound, as exemplified by the MLP (molecular lipophilicity potential) (Carrupt et al 1997), the MEP (molecular electrostatic potential) (Carrupt et al 1991b), the MHBP (molecular hydrogen bonding potential) and the polar surface area, all of which might be useful for rationalizing drug absorption and permeation.

A theoretical method based on the determination of dynamic surface properties has been proposed for prediction of intestinal absorption in man (Palm et al 1997a). Dynamic molecular surface properties take into account the three-dimensional shape of molecules and make allowance for their conformational flexibility. Starting from the molecular polar surface area (the area occupied by nitrogen, oxygen, sulphur, phosphorus and hydrogen atoms attached to heteroatoms) calculated for different energy-minimized conformers, a dynamic polar surface area (PSA_d) can be defined (Artursson et al 1996; Palm et al 1996, 1997a, b, 1998; Krarup et al 1998). The PSA_d is calculated as a statistical average in which the surface area of each low-energy conformer is weighted by its probability of existence (Palm et al 1997b).

One limitation is that the set of conformers calculated in the vacuum excludes a priori some conformers favoured in solution under biological conditions. This renders incomplete and misleading the description of the property space of a compound. Moreover, an average of gas-phase energies might not be relevant to the property space selected by a complex biological environment.

The variation of lipophilicity within the complete conformation space of a compound might be more informative in terms of drug absorption and permeation. Because the MLP enables computation of the (experimentally unmeasurable) lipophilicity of individual conformers (called their virtual lipophilicity), the difference between the most lipophilic and most polar conformers (the lipophilicity range) can itself be a suitable descriptor in drug design. For example, the variation in MEP and MLP of morphine-6-*O*-β-D-glucuronide, a major and highly active metabolite of morphine, within its property space of conformers and tautomers, showed that this metabolite can exist in molecular states (folded

conformers) with properties (lower polarity and higher lipophilicity) compatible with brain penetration (Carrupt et al 1991a; Gaillard et al 1994; Testa et al 1997).

Quantitative and Qualitative Approaches to the Prediction and Explanation of Passive Drug Absorption

Once in-vivo or in-vitro biological data, or both, have been obtained and physicochemical parameters determined, qualitative or quantitative relationships can be established. Many attempts have been made to explain and predict passive drug absorption directly from the physicochemical properties mentioned above (Artursson et al 1996).

Qualitative approaches

Qualitative models, a type of absorption–permeability alert procedure, can be a useful guide for medicinal chemists. A recent alert procedure called the ''rule of 5'' (Lipinski et al 1997) predicts that the absorption or permeation of a compound is more likely to be poor when it has more than five hydrogen-bond-donor groups, more than ten hydrogen-bond-acceptor groups, a molecular mass greater than 500 and a calculated log P (CLOGP) greater than 5. When two of these criteria are met, poor absorption or permeability is to be expected. This rule is based on a distribution of calculated properties among several thousand drugs belonging to different therapeutic classes, bearing in mind that quite a number of orally active drugs violate the rule. The rule of 5 can be considered as a ''wide spectrum'' qualitative model.

More modest qualitative approaches have also been proposed to model the permeation capacity of a single class of drug. For example, the brain-penetration capacity of histamine H_1-antagonists has been modelled with the two main physicochemical parameters influencing brain penetration, namely log $D^{7.4}{}_{oct}$ and $\Delta\log P_{oct-alk}$ (ter Laak et al 1994). In this model the potential for brain penetration of antihistamines was first estimated from their log $D^{7.4}{}_{oct}$ values. When log $D^{7.4}{}_{oct}$ was < 0 or > 3, penetration was negligible or hindered. For compounds with log $D^{7.4}{}_{oct}$ values between 0 and 3, penetration could occur if the compound had a weak hydrogen-bond-donor capacity ($\Delta\log P_{oct-alk} < 2$).

Quantitative approaches

Correlations of physicochemical properties with permeability, fraction of dose absorbed, or blood–brain uptake have led to a variety of mathematical relationships, e.g. linear (Young et al 1988; Burton et al 1996; Palm et al 1996), bilinear (Kubinyi 1993), parabolic (Penniston et al 1969; Timmermans et al 1977; Dearden & Patel 1978; Wils et al 1994b), or sigmoidal (Conradi et al 1996; Palm et al 1997a) depending on the experimental domain explored and the series investigated. Correlating a single predictor with the permeation of structurally unrelated compounds is seldom possible given the many mechanistic and physicochemical factors involved. Hence the need to combine several physicochemical properties into a single relationship, using multiple linear regression or even multivariate analyses (Levin 1980; Hansch et al 1987; LeCluyse et al 1991; Potts & Guy 1993).

Other theoretical descriptors related to lipophilicity, polarity, polarizability and hydrogen-bonding have also been used to model Caco-2 cell permeability or intestinal absorption (Norinder et al 1997, 1999). Even if good statistical models were obtained, the low number of compounds renders doubtful the general use of such approaches in the early phases of drug discovery.

When the relationship between biological data and physicochemical properties has been examined statistically and a good model obtained, it becomes possible to select drugs with optimum passive absorption characteristics. However, no reliable, universal predictive model exists. All too often, correlations break down when structural diversity is introduced. Also, many seemingly general models published in the literature are in fact statistically unsound and misleading because of overfitting or hidden intercorrelation between ''independent'' variables, or both. The absence of a broad predictive model for drug permeation is an actual problem, especially in the early preclinical phases of drug development, when candidates with potential drug absorption problems must be identified.

Conclusion

The aim of this review is to survey briefly how absorption and permeation problems can be approached. Thorough understanding of the biological characteristics of physiological barriers and of the mechanisms of transport is required if delivery problems are to be solved. The procedures for screening drug candidates have changed in recent years, and new in-vitro methods have shed some light on mechanisms of drug transport and led to some preliminary models of drug absorption and permeation.

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