



Best practices in current models mimicking drug permeability in the gastrointestinal tract - An UNGAP review

Joseph P. O'Shea^{a,*}, Patrick Augustijns^b, Martin Brandl^c, David J. Brayden^d, Joachim Brouwers^b, Brendan T. Griffin^a, René Holm^c, Ann-Christin Jacobsen^{c,e}, Hans Lennernäs^f, Zahari Vinarov^{b,g}, Caitriona M. O'Driscoll^a

^a School of Pharmacy, University College Cork, Cork, Ireland

^b Drug Delivery and Disposition, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Leuven, Belgium

^c Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Odense, Denmark

^d UCD School of Veterinary Medicine and UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

^e Department of Pharmacy, Uppsala University, Uppsala, Sweden

^f Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden

^g Department of Chemical and Pharmaceutical Engineering, Sofia University, Sofia, Bulgaria

ARTICLE INFO

Keywords:

Intestinal permeability
Oral absorption
In vitro models
In vivo models
In silico models, oral biopharmaceutics

ABSTRACT

The absorption of orally administered drug products is a complex, dynamic process, dependant on a range of biopharmaceutical properties; notably the aqueous solubility of a molecule, stability within the gastrointestinal tract (GIT) and permeability. From a regulatory perspective, the concept of high intestinal permeability is intrinsically linked to the fraction of the oral dose absorbed. The relationship between permeability and the extent of absorption means that experimental models of permeability have regularly been used as a surrogate measure to estimate the fraction absorbed. Accurate assessment of a molecule's intestinal permeability is of critical importance during the pharmaceutical development process of oral drug products, and the current review provides a critique of *in vivo*, *in vitro* and *ex vivo* approaches. The usefulness of *in silico* models to predict drug permeability is also discussed and an overview of solvent systems used in permeability assessments is provided. Studies of drug absorption in humans are an indirect indicator of intestinal permeability, but both *in vitro* and *ex vivo* tools provide initial screening approaches and are important tools for assessment of permeability in drug development. Continued refinement of the accuracy of *in silico* approaches and their validation with human *in vivo* data will facilitate more efficient characterisation of permeability earlier in the drug development process and will provide useful inputs for integrated, end-to-end absorption modelling.

1. Introduction

Oral drug administration continues to be the most common, convenient and economical route for drug therapy for patients (Mullard, 2020; Shahiwala, 2011). Oral bioavailability, therefore, remains a highly desirable property for molecules in development pipelines. Modern high throughput screening of compound libraries facilitates rapid lead identification of molecules with optimal pharmacodynamic and safety potentials. Once sufficient activity and safety have been demonstrated by a lead molecule, the focus of development will then shift to demonstrating high 'druggability' through assessing the key factors likely to impact drug absorption (Agoni et al., 2020; Benet et al., 2016; Egan and Lauri,

2002). Central to the concept of druggability is that new chemical entities must display sufficient absorption across the gastrointestinal tract (GIT) to achieve effective plasma concentration-time profiles. Modern small molecule drug candidates for oral absorption increasingly display high hydrophobicity and poor aqueous solubility. Consequently, some of the properties that provide optimal receptor binding may result in poor pharmacokinetic (PK) properties, including reduced and highly variable oral absorption and bioavailability (Bennett-Lenane et al., 2020; Bergström and Porter, 2016; Davies et al., 2020; Ditzinger et al., 2019). Therefore, the capacity to predict and measure a molecule's absorption, metabolism, excretion, and toxicity (ADMET) is of critical importance to drug product development and is central to the effective design of new

* Corresponding author.

E-mail address: joseph.oshea@ucc.ie (J.P. O'Shea).

<https://doi.org/10.1016/j.ejps.2021.106098>

Received 10 August 2021; Received in revised form 19 October 2021; Accepted 15 December 2021

Available online 22 December 2021

0928-0987/© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

chemical entities and their delivery systems.

Bioavailability (F) after oral administration is a function of the kinetic processes by which a molecule crosses the enterocyte epithelial cell layer into the portal vein, reaches the liver and ultimately the systemic circulation in its intact form (Fig. 1) (Wu et al., 1995). The bioavailability of a drug molecule is the product of the fraction of a dose absorbed (f_a), the fraction of intact drug escaping intestinal metabolism in the lumen and gut wall (f_g) and the fraction escaping hepatic first pass extraction (metabolism and/or biliary transport) (f_h), as described by (Eq. (1)) (Tozer, 2015; Wu et al., 1995):

$$F = f_a \times f_g \times f_h \quad (1)$$

Metabolism within both the gut wall and the liver is primarily mediated by the cytochrome P450 (CYP) superfamily (Fig. 1A & Fig. 1G), of which CYP3A alleles are the most abundant in the small intestine, with CYP2D, CYP1A, and CYP2C also playing a role (Benet et al., 1999; Paine et al., 2006; Thelen and Dressman, 2009; Thummel et al., 1997; Ungell, 2010). Eq. (1) can be further refined and described in relation to the extraction ratio of intestinal and hepatic metabolism (Eq. (2)) (Rautio et al., 2008; Wu et al., 1995).

$$F = f_a \times (1 - E_G) \times (1 - E_H) \quad (2)$$

where F is the bioavailability, and E_G and E_H are the fractions extracted by the gut wall and liver, respectively (Dahlgren and Lennernäs, 2019; Pond and Tozer, 1984; Rautio et al., 2008). The fraction of the administered dose that permeates the intestinal epithelium intact is, therefore, a crucial determinant of overall bioavailability. Prior to entering the systemic circulation and accessing its (non-GI) site of action, molecules delivered via the oral route must first demonstrate sufficient permeability and stability in the gut wall, followed by stability against liver enzymes.

The establishment of the Biopharmaceutic Classification System (BCS) and Developability Classification System (DCS), where drug absorption characteristics are categorised on the basis of solubility and

fraction absorbed (f_a), provided a framework to identify the key biopharmaceutical factors that influence *in vivo* performance; namely dose, solubility, permeability and dissolution rate (Amidon et al., 1995). Effective oral absorption of a drug molecule, with high bioavailability and low variability, requires that the molecule must display sufficient solubility and stability in the GI fluids as well as small intestine epithelial permeability (Section 2). The fundamental relationship between a permeability coefficient, a quantitative measure of the rate at which a molecule can cross a biological membrane (the intestinal epithelium in this case), and extent of absorption means that experimental models of permeability have regularly been used as a surrogate measure to predict f_a (Sinko et al., 1991; Volpe, 2010). The effective intestinal permeability (P_{eff}), is therefore, seen as one of the key biopharmaceutical parameters that determines the rate and extent of intestinal drug absorption (Di et al., 2020). Identifying the factors that contribute to determining a molecule's permeability is critical to understanding the drug absorption process and determination of these factors on the basis of accurate pre-clinical *in vitro*, *in silico* and/or *in situ* experimental techniques is crucial for an efficient drug development process (Amidon et al., 1995). The capability to screen drug candidates for effective permeability is, therefore, of paramount importance during drug product development.

The purpose of the current review, therefore, is firstly to provide an overview of drug absorption mechanisms in the GIT together with theoretical approaches underpinning drug absorption and permeability assessments. Secondly, we discuss approaches to determining permeability and/or permeability class as supported by the regulators (FDA, 2017; ICH, 2020). A discussion of solvent systems utilised in permeability screening is also included. Finally, a recent extension of the permeation assessment tool-box is discussed, which aims to reveal the interdependence between dissolution and permeation kinetics within a single experiment, developing insight into the mechanistic interplay behind, and the performance ranking of candidate-enabling formulations.

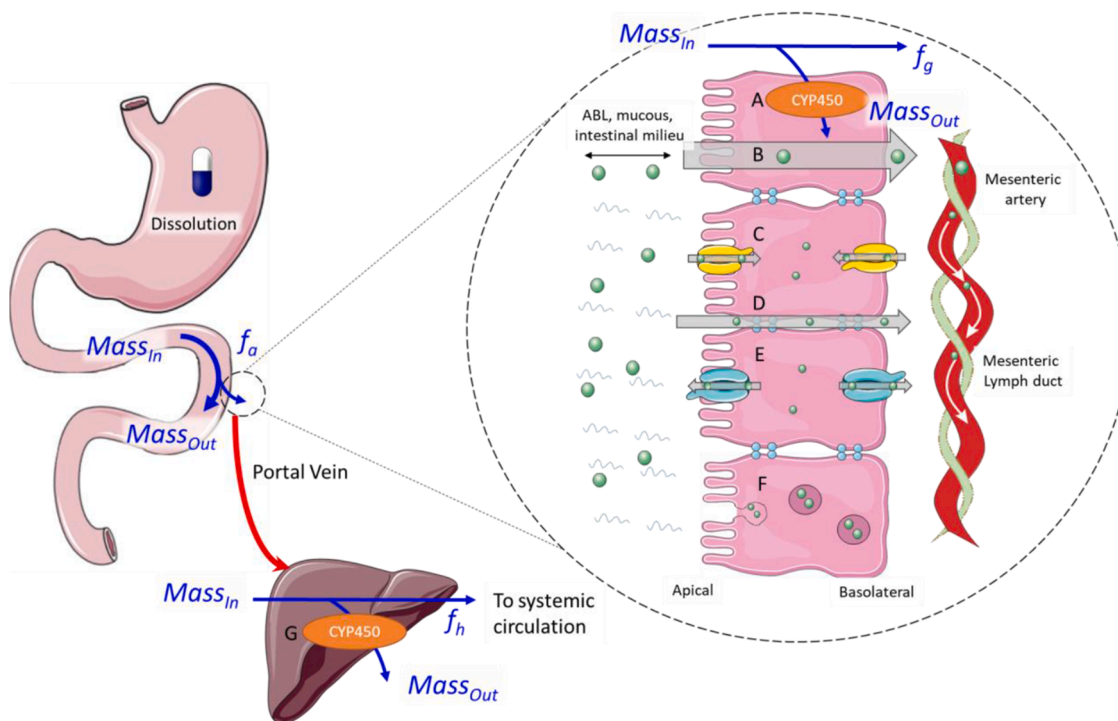


Fig. 1. Oral drug absorption process from the GIT. Schematic depiction of the three major processes (f_a , f_g and f_h) affecting absorption of drug from the site of administration to the systemic circulation i.e. oral bioavailability (F). Inset: schematic of mechanisms types of intestinal drug transport and metabolism; enteric metabolism (A); passive transcellular diffusion (B); Carrier-mediated uptake transport (C); Passive paracellular diffusion (D); Carrier-mediated efflux transport (E); Transcytosis (F); hepatic first pass metabolism (G) – adapted from (Griffin and O'Driscoll, 2007).

2. Fundamentals of gastrointestinal drug absorption

Absorption of orally administered drugs principally occurs in the small intestine, due to its unique anatomical properties, including the increased absorptive surface area attributed to the presence of microvilli, as well as the presence of specific drug transporters in the intestinal epithelium, which facilitate drug transport (Harwood et al., 2013; Helander and Fändriks, 2014; Sjögren et al., 2014; Varma et al., 2011; Wilson, 1967) and can occur by a variety of mechanisms, as shown in Fig. 1(B-F). These mechanisms of absorption can be broadly classified into (i) passive processes and (ii) carrier-mediated processes (Sugano et al., 2010).

Passive drug transport occurs via one of two primary mechanisms, passive transcellular diffusion (Fig. 1B) or passive paracellular diffusion (Fig. 1D). Passive transcellular diffusion occurs down a concentration gradient from the apical to the basolateral side of the intestinal epithelium according to Fick's first law. This energy independent process requires drug molecules to partition across the lipid bilayer in the apical plasma membrane of intestinal epithelial cells, and is strongly dependant on the physicochemical properties of the drug molecule (Section 3.2). Passive transcellular diffusion is the most common mechanism for small molecule drug absorption, but it exists in combination with other methods, most notably carrier-mediated absorption processes (Dahlgren and Lennernäs, 2019; Lennernäs, 2007; Matsson et al., 2005; Smith et al., 2014). Passive paracellular absorption involves movement of molecules through the narrow passages between intestinal epithelial cells. These narrow spaces, due to the presence of tight junctions, are designed to prevent the passage of hydrophilic molecules above a particular molecular weight, and therefore present a significant barrier to drug absorption (Artursson et al., 1993; P. Berben et al., 2018b; Salamat-Miller and Johnston, 2005). In addition, the total surface area of these paracellular spaces relative to the intestinal membrane, as a whole, is extremely low (~0.1%), so the overall contribution of paracellular diffusion in the drug absorption process is limited (Fagerholm et al., 1999; Xu et al., 2021).

Uptake of drug molecules by specific membrane transporters on the apical membranes of enterocytes facilitates drug uptake, typically against a concentration gradient (Fig. 1C). Carrier-mediated transport which occurs according to a concentration gradient is not energy-driven, thus is another type of passive process, termed facilitated uptake (Sugano et al., 2010). Energy dependant transport processes are also facilitated by membrane transporters of either the ATP-binding cassette (ABC) or solute carrier (SLC) super-families, and such processes are defined as active processes (Sugano et al., 2010; Tsuji and Tamai, 1996; Volpe, 2016). Carrier-mediated transport processes, either passive or active, may be subject to inhibition by both specific and non-specific interactions related to either drug-drug interactions, or the presence of food or food components (Di et al., 2012; Kell et al., 2013; O'Shea et al., 2019; Sugano et al., 2010). As these processes depend on the presence of specific membrane transporters and the capacity of drug molecules to act as substrates for these transporters, carrier-mediated processes are also more specific, both with regard to the molecules absorbed and cell types where absorption occurs (Sugano et al., 2010). In addition to these influx transporters, ATP-dependant efflux transporters, notably P glycoprotein (P-gp or also known as multidrug resistance protein 1 (MDR1, ABCB1) and the breast cancer resistance protein (BCRP, ABCG2) transport susceptible, mostly hydrophobic, drug molecules out of the cell (Fig. 1E). Efflux transporters can limit absorption of particular substrates by transporting them back to the lumen (Benet et al., 1999; Chan et al., 2004; Takano et al., 2006; Tannergren et al., 2003b; Zhang and Benet, 2001).

The complex interplay of these processes serves to determine the fraction of drug absorbed and, ultimately, the quantity of drug available within the body to exert its pharmacodynamic effect. A key element of determining intestinal absorption is assessment of a molecule's intestinal epithelial permeability.

3. Mathematical modelling and prediction of drug absorption and permeability

3.1. Theoretical models of drug permeability

In order to develop predictive models of permeability, and hence drug absorption, it is necessary to consider the mathematical descriptors of the absorption process (Griffin and O'Driscoll, 2007). P_{eff} quantifies the permeability across the intestinal membrane independent of the mechanism. The coefficient is measured based on the cylindrical nature of the intestinal segment, as represented in Fig. 2, where R is the radius of the small intestine, L is the length of the perfused segment and ΔC is the concentration gradient across the epithelial membrane (Johnson and Amidon, 1988).

Assuming sink conditions in the portal vein, the rate of drug absorption can be described according to Eq. (3) (Lennernäs et al., 1992):

$$\frac{dM}{dt} = A \cdot P_{eff} \cdot C_{lumen} \quad (3)$$

where A is the intestinal surface area and C_{Lumen} is the concentration of dissolved drug in the intestinal lumen. P_{eff} is calculated assuming the area and flow rate through the intestine is known. The methods of directly measuring P_{eff} in both humans and pre-clinical species are discussed in Section 4.2.1. Where it is difficult or impractical to measure P_{eff} directly *in vivo*, various techniques have been developed to determine a surrogate measure of P_{eff} through either *ex vivo* or *in vitro* approaches using non-cylindrical, polarised epithelia. Such approaches generate an apparent permeability co-efficient (P_{app}) by relating the rate of drug transfer (dM/dt) across the membrane barrier, which may be tissue, cell or non-cell based and can be related to human P_{eff} (Akamatsu et al., 2009; Artursson, 1990; Dahlgren and Lennernäs, 2019; Sjöberg et al., 2013), as outlined in Sections 4.2.2, 4.2.3 and 4.3 below.

The intestinal permeability is a critical biopharmaceutical property that affects the rate and extent of intestinal drug absorption, and the first order absorption rate constant (k_a) can be directly related to P_{eff} using Eq. (4) (Cao et al., 2008; Curatolo, 1987):

$$k_a = \frac{A}{V} * P_{eff} \quad (4)$$

where A is the surface area available for absorption and V is the volume. Implementing k_a allows estimation of the maximal absorbable dose (MAD), utilising Eq. (5):

$$MAD = S \cdot k_a \cdot V \cdot T \quad (5)$$

where S is drug solubility, k_a is absorption rate constant, V is intake of fluid (generally standardised as 250 mL), and T is transit time in the small intestine (~3–4 h). Such an approach has been suggested to identify rate limiting steps in absorption and guide molecule

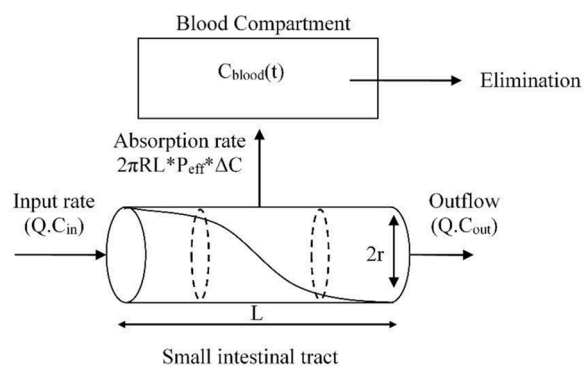


Fig. 2. Schematic diagram of the mass balance for intestinal perfusion adapted from (Johnson and Amidon, 1988) and (Ni et al., 1980).

developability and formulation choice (Butler and Dressman, 2010).

Combining Eqs. (4) and (5), this can be further simplified, according to the model proposed by Sun and co-workers (Sun et al., 2004), giving rise to Eq. (6).

$$MAD = P_{eff} \cdot S \cdot A \cdot T \quad (6)$$

In addition to the above, the concept of MAD, estimation of the P_{eff} may also allow for calculation of f_a , according to Eq. (7)

$$f_a = 1 - e^{-2P_{eff} \frac{T}{R}} \quad (7)$$

where T is the transit time in the human small intestine and R is the radius of small intestine (approx. 2 cm) (Amidon et al., 1995, 1988; Oh et al., 1993).

Using such approaches it is possible to quantify the likely rate and extent of drug absorption by accurate assessment of P_{eff} (Yu et al., 1996; Yu and Amidon, 1999). Reliable methods for its measurement are important during the drug development process. A more extensive and broader use of theoretical models in the future will require a productive collaboration of pharmacokinetic, physical chemistry, formulation, and physiology experts to improve oral absorption physiologically-based pharmacokinetic (PBPK) modelling (Sugano, 2021).

3.2. Estimating drug permeability from physicochemical characteristics

Absorption of drugs from the GIT is a complex, dynamic process dependant on a range of biopharmaceutical properties (Table 1).

The intestinal permeability of drugs is determined by a range of biopharmaceutical properties related to both the physiology and anatomy of the GIT itself as well as the formulation design, but it is profoundly impacted by the physicochemical properties of the drug molecules. The capacity of simplistic molecular and/or physicochemical descriptors to estimate drug permeability has been widely reported, with molecular weight, lipophilicity (LogP/ LogD), acid dissociation constant (pKa), polar surface area and hydrogen bonding potential commonly used as such simplistic predictors of either the magnitude or route of absorption (Bergström et al., 2003; Lipinski et al., 2001; Veber et al., 2002). The specific epithelial permeability mechanism used by molecules, as outlined in Section 2, is considered to be largely dependant on their physicochemical properties, and many attempts have been made to relate such properties to passive drug diffusion across biological membranes, and, therein, to predict the fraction absorbed from the GIT (Griffin and O'Driscoll, 2007). Traditionally, the rule of thumb is such that moderately lipophilic molecules tend to be transported by passive

Table 1
Biopharmaceutical factors affecting intestinal absorption.

Physicochemical Properties	Anatomical/ Physiological Factors	Formulation Factors
GI Solubility	Gastric emptying rate	Disintegration rate
Lipophilicity (Octanol/ water partition coefficient: LogP/ Octanol/ water distribution coefficient at specified pH: LogD)	Intestinal transit time/ motility	Dissolution rate
Acid dissociation constant (pKa)	Gut metabolism	Excipient effects e.g. solubilizers, permeation enhancers
Molecular weight	Membrane surface area	Release mechanism
H-bonding potential	Membrane receptors/ transporters and mechanisms	Supersaturation/ precipitation
Polar surface area	Intestinal metabolism Intestinal secretions – bile salts, mucous, enzymes Intestinal blood flow	

diffusion across enterocytes, while low molecular weight, hydrophilic molecules can be absorbed paracellularly (Artursson et al., 1993; Camenisch et al., 1996; Daugherty and Msrny, 1999).

Efforts to correlate diffusion across biological barriers, including across the intestinal epithelium, with physicochemical descriptors have long existed, ranging back to the pH-partition hypothesis proposed by Overton (Overton, 1899). This theory states that for an ionizable molecule, only the unionised form will diffuse across a lipophilic membrane, and though this is an overly simple description of the absorption process, it paved the way for further models of drug absorption (Griffin and O'Driscoll, 2007). The lipophilic nature of biological membranes has led to drug lipophilicity and hydrophilic-lipophilic balance (HLB) to be considered as a critical determinant of drug absorption (Ho et al., 1977; Ho and Higuchi, 1974; Milanetti et al., 2016; Seddon et al., 2009). The octanol/water partition coefficient (LogP) and/or the octanol/buffer partition coefficient at a selected pH (logD) are key indicators of lipophilicity, and are widely used as a crude estimate of a drug's capacity to diffuse across lipid bilayers, and hence as an estimation of intestinal permeation. Ho et al. demonstrated a sigmoid relationship between logP and fraction absorbed through an intestinal segment (Ho et al., 1977). However, the concept of a relationship between logP and membrane permeability has generally only been demonstrated for groups of structurally similar, passively absorbed molecules (Griffin and O'Driscoll, 2007). Hydrogen-bonding potential, which provides a measure of hydrophilicity, and surface charge characteristics of a molecule, including polar surface area (PSA), have also been used as predictors of passive membrane permeability and intestinal absorption, with theoretical models used to predict human intestinal absorption with reasonable success (Conradi et al., 1996; Diamond and Wright, 1969; Goetz et al., 2017; Palm et al., 1997, 1996; Winiwarter et al., 2003, 1998).

Perhaps the most widely implemented approach to the use of physicochemical descriptors in characterising intestinal drug absorption is Lipinski's rule of five (Ro5). It has been extensively used as a qualitative model of the capacity of molecules to be absorbed via the oral route (Lipinski et al., 2001). Recognising that for a drug molecule to cross a biological membrane it requires sufficiently small size to fit into phospholipid bilayers, sufficient lipophilicity to partition into the bilayer and exist in an unionised state. The Ro5 established limits on properties such as LogP, molecular weight (MW), and number of hydrogen donors (HBD) and acceptors (HBA), beyond which oral absorption is predicted to be limited. Compounds that violate the Ro5 are more likely to have poor oral absorption and these include compounds with greater than five HBD, greater than ten HBA, MW greater than 500 Daltons, a LogP greater than five. Lipinski's rule has prompted the discovery of several measures of 'drug likeness', such as that proposed by Veber et al., where the significance of molecular weight was questioned, while also proposing additional predictors of absorption of fewer than 10 rotatable bonds and a PSA less than 140 Å² (Veber et al., 2002). While such tools are simple, they continue to play a strong role in drug development, emphasizing the concept of 'druggability' or 'drug likeness.' Despite increased focus on successful oral delivery of drugs which possess properties which are outside the Ro5 (known as beyond Rule of 5 compounds; bRo5) and further scrutiny of the concept of "drug-like" properties, delivery of bRo5 compounds carries a higher development risk than those within the Ro5 (DeGoey et al., 2018; Di et al., 2020; Matsson et al., 2016; Shultz, 2019).

3.3. In silico methods of absorption and permeability prediction

The use of computational or *in silico* models to predict drug release, dissolution, permeability and absorption is now widely employed within the pharmaceutical industry and in regulatory agencies to support the drug development process. Computational models can be broadly classed into two differing approaches; simple models based on overall trends relating physicochemical properties to permeability and

involving broad classifications, and quantitative structure property relationships (QSPR), focusing on development of mathematical models to describe a correlation between molecular descriptors and the permeability (Cabrera-Pérez and Pham-The, 2018). The former are primarily based on simple physicochemical descriptors and include classification systems such as Lipinski's Ro5, described above.

The QSPR approaches are more complex mathematical, chemometric models used to develop a correlation between molecular descriptors and an ADMET property of interest (Bergström et al., 2016; Cabrera-Pérez and Pham-The, 2018). This approach has been widely used to create models that are predictive of intestinal absorption or permeability, through the use of either measured or derived molecular descriptors of drug molecules, with varying degrees of success (Bergström et al., 2016; Tropsha, 2010; Tropsha and Golbraikh, 2007; van de Waterbeemd and Gifford, 2003). The approach of such models is to:

- 1 Curate the experimental dataset including chemical structures and associated biopharmaceutical properties e.g. fraction absorbed, P_{app} , P_{eff}
- 2 Calculate molecular descriptors
- 3 Split into training, test and validation sets
- 4 Construct a statistical model using appropriate modelling technique – e.g. simple linear regression, multiple linear regression, multivariate analysis, partial least squares, neural networks, self-organising maps
- 5 Cross-validate the model with appropriate external validation sets and tools

When generating a predictive biopharmaceutical model, three factors are critical to the performance of the model; i) selection of the response or endpoint of interest and appropriate molecular descriptors, ii) a sufficiently large dataset and iii) appropriate selection of the correct mathematical/ statistical approach (Gozalbes et al., 2011; Tropsha, 2010; van de Waterbeemd and Gifford, 2003). An in-depth understanding of gastrointestinal physiology and physical chemistry is critical to reach *in vivo* relevant modelling and simulation results. Generating, obtaining and selecting the appropriate molecular descriptors is vital to ensure accuracy of the generated model. The capability of any model generated is dependant on the quality of the input data and its applicability domain, that is the chemical space occupied by the training set and which has been validated during model generation. In this sense this can be viewed as the calibration curve of the model, meaning that in order for reliable prediction to be made, molecules should be within this chemical space. While molecules sitting outside the chemical space can be investigated by generated models, such molecules are flagged to indicate that they are not well described by the training set (Bergström et al., 2016; Taskinen and Norinder, 2007). A large range of different molecular descriptors has been used to generate mathematically sound models of absorption and permeability, and a second element for consideration is the relationship between predictability, i.e. the mathematical robustness and accuracy of the model, and interpretability, i.e. where the molecular descriptors included in the model provide a mechanistic understanding of model performance, creating an intuitive model. These aims may, on occasion, coincide, but in most cases a balance is required to be struck between these two goals, and the development priorities of the model to be generated must be considered (Cabrera-Pérez and Pham-The, 2018; Norinder et al., 1997). Specific software packages are available to calculate thousands of molecular descriptors based on the 1D, 2D and 3D structure of molecules, and have recently been summarised (Cabrera-Pérez and Pham-The, 2018).

When considering permeability as the response variable, QSPR models have utilised various approaches to either model the fraction absorbed as a measure of intestinal absorption and permeability, to directly predict effective intestinal permeability or model a surrogate measure of permeability. A common approach is to indirectly model P_{eff} ,

through modelling of *in vitro* experimental assessments of permeability, most notably P_{app} across Caco-2 monolayers (Fujiwara et al., 2002; Gozalbes et al., 2011; Jung et al., 2006; Norinder et al., 1997; Palm et al., 1996; Pham-The et al., 2018, 2013; van De Waterbeemd et al., 1996; Wang et al., 2016), while P_{app} measurements across cell-free permeation assays have also been described (Akamatsu et al., 2009; Oja and Maran, 2015; Sun et al., 2017; Verma et al., 2007) (Section 4.3). The use of such approaches has prompted the question “Why model the model of human absorption?” (van de Waterbeemd and Gifford, 2003). It has repeatedly been demonstrated that it is possible to accurately model these processes using molecular descriptors, with reported accuracy of 0.39–1.43 \log_{10} P_{eff} units (Bergström et al., 2016; S. Palmer et al., 2015) (Bergström et al., 2016; Pham-The et al., 2018; Stenberg et al., 2001). The predictive capacity of these *in vitro* tools is discussed later.

In silico models which directly predict human P_{eff} have not been as widely investigated, due to the paucity of data relating to direct estimation of P_{eff} , particularly for low permeability compounds, and the datasets available for such direct models tend to be limited to a small number of drug molecules (Bergström et al., 2016). Examples of such approaches are described here and summarised in Table 2. Winiwarter et al. have studied the correlation of human intestinal permeability (P_{eff}) with hydrogen bonding parameters using Projection to Latent Structures Partial Least Squares analysis (PLS). Through their analysis, it was demonstrated that the combination of a hydrogen bond donor descriptor, a general hydrogen bonding descriptor and a lipophilicity descriptor enabled prediction of human intestinal permeability within 0.7 units, though the test set of four molecules was quite small (Winiwarter et al., 2003), while additionally hydrogen bond donors, polar surface area and log D (measured at either pH 5.5 or 6.5) provided a strong correlation with log P_{eff} (Winiwarter et al., 1998). Sun et al. have developed a QSPR model using multiple linear regression with a set of 30 compounds for which P_{eff} has been directly measured (Lennernäs, 2007; Sun et al., 2013). By using a training set ($n = 20$) and test set ($n = 10$) of molecules, a QSPR was developed based on seven parameters related to the size, topology, lipophilicity and charge of the molecule. The observed R^2 (0.78) and adjusted- R^2 (0.712) demonstrated high predictive performance, though the model was poorly capable of predicting the permeability of molecules with low permeability values (Sun et al., 2013). The model was further utilised to correlate human f_a with predicted P_{eff} ($R^2 = 0.717$), allowing identification of a cut-off P_{eff} value corresponding to high permeability (90% f_a) as per BCS classification (Amidon et al., 1995). The generated categorical model was capable of correctly categorising 70% of compounds according to their BCS permeability class (Sun et al., 2013). A notable feature of this model is that the parameters identified are largely reflective of passive permeability of compounds, as discussed in Section 3.2. This is a common feature of many QSPR models of permeability, which are somewhat more effective in predicting passive relative to carrier-mediated permeability. This may prove challenging for *in silico* prediction of P_{eff} in early drug development, where the mechanisms of drug transport may not yet be known. In a recent study, Lee et al. attempted to address the limitation of the relatively low number of molecules for which directly measured human P_{eff} values are available through prediction of rat intestinal P_{eff} , as measured by single pass intestinal perfusion (SPIP), by implementing a machine learning approach - Hierarchical Support Vector Regression (HSVR) (Lee et al., 2020). The resultant model demonstrated high correlation within the training set ($R^2 = 0.93$, $Q^2 = 0.84$) and reasonable predictability of the test set ($Q^2 = 0.75$ – 0.89 , RMSE = 0.26). A further validation was provided in the form of comparison of the ability of both the observed ($n = 7$; $r = 0.8$) and predicted ($n = 11$; $r = 0.79$) rat P_{eff} to accurately reflect that observed in humans, with both models performing similarly (Lee et al., 2020).

In addition to those in the published scientific literature, several commercial software packages are available to predict ADMET properties, including permeability (Cabrera-Pérez and Pham-The, 2018). A widely utilised commercial example of such is that of Simulations Plus®

Table 2
Summary of QSPR models predicting P_{eff} .

Model type	Method	Compound Datasets	Descriptors	Model performance		References
				Correlation	Prediction	
Regression	PLS	Training: 5 passively absorbed compounds with <i>in vivo</i> P_{eff} Test: 8 passively absorbed compounds with <i>in vivo</i> P_{eff}	1 HBD, PSA, LogD _{5.5} 2 HBD, PSA 3 HBD, PSA, CLogP	1 $Q^2 = 0.81$, $R^2 = 0.94$ 2 $Q^2 = 0.80$, $R^2 = 0.88$ 3 $Q^2 = 0.96$, $R^2 = 0.98$	1 $Q^2 = 0.90$, $R^2 = 0.93$ 2 $Q^2 = 0.82$, $R^2 = 0.85$ 3 $Q^2 = 0.85$, $R^2 = 0.88$	(Winiwarter et al., 1998)
Regression	PLS	13 passively absorbed compounds with <i>in vivo</i> P_{eff} measurements	1 29 descriptors 2 15 descriptors 3 HB, ΣQ_{H_i} , LogP 4 PSA, HBD, LogP 5 PSA, HBD 6 PSA, CWPSA _{HBD} 7 HBD, PSA/NPSA 8 HB, ΣQ_{H_i} , LogP _{Cr} 9 PSA, HBD, LogP _{Cr}	1 $R^2 = 0.960$, $Q^2 = 0.873$ 2 $R^2 = 0.953$, $Q^2 = 0.907$ 3 $R^2 = 0.952$, $Q^2 = 0.942$ 4 $R^2 = 0.945$, $Q^2 = 0.935$ 5 $R^2 = 0.864$, $Q^2 = 0.815$ 6 $R^2 = 0.863$, $Q^2 = 0.824$ 7 $R^2 = 0.855$, $Q^2 = 0.846$ 8 $R^2 = 0.947$, $Q^2 = 0.935$ 9 $R^2 = 0.945$, $Q^2 = 0.932$	Predict log P_{eff} to within 0.7 units	(Winiwarter et al., 2003)
Regression/ Classification	MLR	Training set: $n = 20$ Test set: $n = 10$	Log molecular mass, Shape index, Balaban topological index, HBD, Ionizational potential, Vamp octupole YXX, log P	$R^2 = 0.782$; adj- $R^2 = 0.712$; S.E. of the Estimate 1.816	1 $R^2 = 0.86$ 2 Correlation of Fa with predicted P_{eff} - $R^2 = 0.717$ 3 70% of drugs correctly classified as per BCS criterion	(Sun et al., 2013)
Regression*	HSVR	Training set: $n = 53$ Test set: $n = 13$	μ , LogD _{6.5} , LogP, HBD, n_{N+O} , Shadow- v , MR	$R^2 = 0.93$, $Q^2 = 0.84$, RMSE = 0.17	$Q^2 = 0.75-0.89$, RMSE = 0.26	(Lee et al., 2020)

Abbreviations: HB; Number of hydrogen bonding atoms (HBA + HBD), HBA; Number of hydrogen bond acceptor atoms, HBD; Number of hydrogen bond donor atoms, LogD; Partition coefficient between octanol and water at pH specified, LogP; Partition coefficient between octanol and water, LogP_{Cr}; Partition coefficient between octanol and water (calculated according to (Ghose and Crippen, 1987)), PSA; Polar surface area, PSA/NPSA – ratio of polar surface area to non-polar surface area, CWPSA_{HBD}; Sum of charge weighted surface area of all HBD-atoms, ΣQ_{H_i} ; Sum of the partial charges of all H-atoms attached to an O-, N-, or S-atom, μ ; Dipole moment of molecule, n_{N+O} ; Number of nitrogen and oxygen atoms, Shadow- v ; Ratio of largest to smallest molecular dimension, MR; Sum of molar refractivity of substituents. PLS; Partial Least Square regression, MLR; Multiple Linear Regression, HSVR; Hierarchical Support Vector Regression, RMSE; Root Mean Square Error.

* P_{eff} in rat measured by Single-pass intestinal perfusion.

(CA., USA) permeability model ($S+P_{eff}$), as outlined in ADMET™ Predictor, where a predictive model was developed through a combination of *in vivo* permeability measured in human subjects, *in situ* rat wall permeability, human jejunal P_{app} measured *ex vivo* and *in vitro*. Through a combination of regression analysis and neural networks, a predictive model was generated, which demonstrated a reasonable capacity to predict the passive permeability for a test set of 62 molecules. The model descriptors used in model generation were reflective of the relative lipophilicity, size and charge of the molecules, thus providing an intuitive model with readily interpretable variables (Simulations Plus, 2019).

As prediction of intestinal permeability is regularly carried out as a surrogate for prediction of the extent of absorption *in vivo*, a logical step is to attempt to directly model the fraction absorbed, often modelled as human intestinal absorption (Hou et al., 2006; Volpe, 2010). Models developed to predict fraction absorbed appear to be the most widely investigated indicator of intestinal permeability based on molecular descriptors (Basant et al., 2016; Cabrera-Perez et al., 2012; Klopman et al., 2002; Lee et al., 2020; Newby et al., 2013; Norinder and Bergström, 2006; Pérez et al., 2004; Suenderhauf et al., 2011; Wang et al., 2017; Wessel et al., 1998; Zhao et al., 2001). Several challenges exist when taking such an approach, which need to be considered during model generation. Firstly, the response dataset quality can be variable owing to the range of experimental methods used to calculate *fa* and the large variability associated with it. Secondly, *fa* will be affected by a range of complex biopharmaceutical factors *in vivo*, including

dissolution/solubility limitations, role of intestinal uptake and efflux transporters, GI motility and the complex and highly dynamic intestinal milieu, which can vary in response to the presence or absence of food. Such variability makes it difficult to derive a statistically and mechanistically sound model of permeability. Finally, owing to the fact that such models are by necessity derived from absorption data for approved pharmaceutical products, they may be biased towards active compounds that demonstrate high absorption, with those demonstrating limited oral bioavailability underrepresented due to the paucity of data detailing their fraction absorbed (Norinder and Bergström, 2006).

A further extension of rules of thumb and both categorical and quantitative QSPR models is in modelling overall bioavailability of a compound (Andrews et al., 2000; Fagerholm et al., 2021; Hou et al., 2007; Kim et al., 2014; Yoshida and Topliss, 2000). While the performance of such models is somewhat outside the scope of the current review, which focuses on intestinal permeability rather than bioavailability, and they have been reviewed by Cabrera-Pérez and Pham-The (2018) it is nevertheless important to consider them as extensions to the modelling and simulation toolkit relating to oral absorption and identify the role of *in silico*, *in vitro* and *ex vivo* models of permeability as inputs into such models. A significant challenge in QSPR models of intestinal absorption and bioavailability lies in the challenges of modelling the complex ADME processes from relatively simplistic molecular and physicochemical descriptors and the lack of a mechanistic approach. One suggestion to improve the predictive capabilities of such models is through incorporating *in vitro* or *ex vivo* measurements of

passive permeability, as determined by the methods outlined in Section 4, into the model and such models have routinely been proposed (Cabrera-Pérez and Pham-The, 2018; Esaki et al., 2019; Wang et al., 2008). However, while introduction of such a summary parameter may improve the overall predictive capacity of the model, this data may not always be available early during clinical development and the proposed models still lack a mechanistic predictive basis.

PBPK models are a potential alternative approach, designed to facilitate mechanistic, bottom-up predictions of oral bioavailability and simulation of plasma concentration-time profiles (Kostewicz et al., 2014). PBPK models are mathematical models which integrate knowledge of physiological processes, such as gastrointestinal transit times, intestinal luminal conditions and distribution related parameters, with compound physicochemical parameters, such as solubility, PSA, and lipophilicity, as well as formulation related characteristics such as dissolution rate, stability and release mechanism, in order to simulate complex ADME processes (Bergström et al., 2014; Jamei et al., 2009; Rowland et al., 2011). Such approaches have been widely used as computational tools in the prediction of oral absorption and for mechanistic investigation of drug-drug interactions, food effects on bioavailability, age and disease state related changes in absorption, setting of clinically relevant product specifications and formulation performance (Ahmad et al., 2020; Flanagan et al., 2016; Kaur et al., 2018; Kesiosoglou et al., 2016; Kostewicz et al., 2014; Lin and Wong, 2017; Pepin et al., 2016; Stillhart et al., 2019; Tistaert et al., 2019; Wang, 2019). A vital element of an accurate PBPK model is a reliable prediction or measurement of P_{eff} , which can be input either as an externally (*in vitro*, *ex vivo* or *in vivo* – Section 4) measured summary parameter or predicted from molecular descriptors as outlined above (Dahlgren et al., 2015). There are numerous commercial examples of PBPK models utilised in drug development, notably GastroPlus™, Simcyp™ and GI-SIM™, each with their own, proprietary, in-built model designed to predict absorption by correlation to human intestinal permeability, such as that outlined above for the $S+P_{eff}$ model used in ADMET™ Predictor, and incorporated within GastroPlus™ (Dahlgren et al., 2015; Sjögren et al., 2014). A typical approach is to initially use values predicted through QSPR methods, before replacing these values with experimentally derived measurements extrapolated to P_{eff} through *in vitro*–*in vivo* correlation (IVIVC), and thus refining the model as more data becomes available (Effinger et al., 2018; Suarez-Sharp et al., 2020). As these are mechanistic models, where *in vivo* pharmacokinetic data is available, a model may be refined to better reflect the observed data through a ‘top down’ approach, usually incorporating a sensitivity analysis to determine the effects of parameter adjustment on modelled outcomes (Stillhart et al., 2019). This iterative process of model refinement and integration of experimentally determined and *in vivo* measured biopharmaceutical properties and PK measures allows mechanistic understanding of the oral absorption process. It also facilitates identification of potentially limiting factors in oral absorption and can direct optimisation of drug candidate and formulation design (Cabrera-Pérez and Pham-The, 2018).

4. Experimental methods to determine drug permeability and permeability class

The capacity to predict permeability based on simple physicochemical or molecular descriptors is an attractive prospect in lead identification and optimisation during drug discovery. However, the predictive capacity of such approaches, particularly in a quantitative sense is limited. The complex biopharmaceutical processes that influence drug absorption within the GIT and transport into the systemic circulation require more than simplified, empirical relationships. Accurate techniques, capable of identifying the rate limiting steps to *in vivo* absorption are critical to understanding the gastrointestinal drug absorption. (Griffin and O'Driscoll, 2007). To this end, various approaches have been developed to characterise drug permeability within the human

GIT. The FDA, in their guidance on how to obtain biowaivers based on BCS classification have identified two major mechanisms to determine the permeability class; PK studies in humans and intestinal permeability. These classifications can be further subdivided, with PK studies comprising either mass-balance studies or absolute bioavailability studies (FDA, 2017). In their guidance, the FDA specifically identify *in vivo* or *in situ* intestinal perfusion studies in both humans and animal models, *in vitro* permeation studies using excised human and animal intestinal tissues and *in vitro* permeation studies across a monolayer of cultured epithelial cells as intestinal permeability methods utilised to determine permeability class, approaches described in the present review and considered in order of regulatory acceptability. It is important to note that where such approaches are used in regulatory filings, they are limited to molecules which are passively absorbed, due to the variability in transporter expression in isolated tissues and cell cultures (Giacomini et al., 2010). In addition, there is growing use of novel methods to further refine permeability determination, notably non-cellular based assays which are also discussed as part of this review (Berben et al., 2018a). In addition, the solvent systems used in these experimental approaches are critical to ensure accuracy and consistency. In particular, the requirement to maintain sink conditions while maintaining the viability and integrity of biomimetic barriers during *in vitro* and *ex vivo* studies is crucial and is discussed in Section 5. These models represent the most widely used current models of *in vivo* permeability in both drug development and in support of regulatory submissions and are the subject of the current best practice review. Novel techniques for permeability are continuously emerging, notably microfluidic (gut-on-a-chip), organoid and intestinal slice cultures, and have recently been well reviewed elsewhere, and are beyond the scope of the current review (Youhanna and Lauschke, 2021). These systems are, largely, developed as refinements of existing approaches, designed to improve user-friendliness, reproducibility or biorelevance, however the principles and theoretical basis on which they operate is largely similar to existing cellular and non-cellular based methods, in that they measure transport across a biomimetic membrane.

4.1. *In vivo* pharmacokinetic studies in humans

Investigation of the PK behaviour of any new compound is a standard part of all drug development programs, that will often be started with single ascending dose studies in human healthy volunteers followed by multiple ascending dose studies. Combined with studies in either healthy volunteers or patients, these constitute the clinical pharmacology package of a new drug application (NDA). From a regulatory point of view, a compound is considered to be highly permeable when the fraction absorbed from the GIT is greater than 85% of the administered dose (FDA, 2017; ICH, 2020), with the regulators preferred method for high permeability determination being *in vivo* PK studies. From a pharmaceutical perspective all these studies are highly relevant for the guidance of the formulation development, where in particular two studies stand out, the mass balance study and the absolute bioavailability study, including investigations of potential food effects. These will be described in further detail in the two following sections.

4.1.1. Mass balance studies

The human mass balance study, also often referred to as the absorption, distribution, metabolism, and excretion (ADME) study, is one of the clinical pharmacokinetic studies conducted as a part of a NDA (Coppola et al., 2019). The study is special in the context that a drug molecule containing a radioactive isotope, e.g. carbon 14 (Penner et al., 2009), is used for the dosage. In such cases attempts are made to collect as much of the radiolabel administered as possible. The radiolabel is collected in excreta, including urine, faeces, and other excreta as needed (e.g., expired air, sweat, etc.), while monitoring the exposure of radioactivity and drug in whole blood, plasma, cerebrospinal fluid (CSF) or other accessible tissues of interest, hence the term mass balance.

The primary objectives of a mass balance study are typically to;

- Identify and quantify circulating parent molecule and metabolite(s) including measure of their relative ratios
- Elucidate the primary elimination pathways of the medicinal product
- To determine the mass balance of drug related materials following administration

This information is important because it helps to define if other nonclinical or clinical investigations that might provide an accurate clinical pharmacological description of the new molecule are needed. The study should explore whether there are any metabolites contributing substantially to the safety profile of the drug substance that makes it necessary to conduct a specific non-clinical study, i.e. to evaluate the toxicity of the specific metabolite. The identification of the metabolites should also help to identify if there are any that may contribute to pharmacological activity and, hence, have a potential risk to cause drug interactions. Understanding the mass balance and the elimination pathways helps to understand the *fa* and clearance mechanism, i.e. determine if there is a specific need to investigate the molecule in subjects with organ impairment and/or define clinical drug–drug interaction (DDI) studies.

Not all administered radioactive materials are recovered in mass balance studies. The studies may include collection of urine and faeces over 14 days, but other elimination routes may be relevant and missed samples, analytical methods etc. can be an issue (Coppola et al., 2019; Penner et al., 2009). High recovery of radioactive material hence provides information about a well permeable/absorbed molecule, but low recovery could mean potential study errors and the difficulty in defining the reason.

4.1.2. Absolute bioavailability studies

A bioavailability (F) study provides an estimate of the fraction of the drug absorbed as well as information related to the PK of the drug, the effects of food on the absorption of the drug, dose proportionality or linearity in the PK of the active moieties and - when relevant - inactive metabolites (FDA, 2019).

The terms “absolute” and “relative” bioavailability refers to the design of the study. Absolute bioavailability is defined as the amount of drug from a formulation that reaches the systemic circulation relative to an intravenous administration, assuming that the intravenous (i.v.) dose is 100% bioavailable, according to Eq. (8).

Absolute bioavailability

$$F(\%) = \frac{AUC_{oral}}{AUC_{iv}} \times \frac{Dose_{iv}}{Dose_{oral}} \times 100 \quad (8)$$

Where AUC_{oral} and AUC_{iv} are the areas under the plasma concentration time profile following oral and intravenous administration, respectively, while $Dose_{oral}$ and $Dose_{iv}$ are the dose administered by each of these routes.

Relative bioavailability is the amount of drug absorbed from a formulation relative to that administered by any route other than i.v as per Eq. (9).

Relative bioavailability

$$F_{rel}(\%) = \frac{AUC_A}{AUC_B} \times \frac{Dose_B}{Dose_A} \times 100 \quad (9)$$

Where A and B, are both formulations administered by any extra-vascular route of administration.

Relative bioavailability can be measured if an i.v. formulation cannot be produced, e.g. due to very poor solubility. Another approach that may overcome the limitation of poor solubility, amongst other shortcomings of an absolute bioavailability study, is simultaneously dosing of subjects

with an orally therapeutic dose and a micro dose of i.v. ^{14}C marked molecule (Lappin, 2016; Lappin et al., 2006). This is often termed a microtracer Phase 0 study and defines an i.v. dose of an isotopically labelled drug and is typically $\leq 1\%$ of the therapeutic dose (Lappin, 2016). The approach has some advantages, for instance it is not necessary to develop a stable i.v. formulation of the drug and toxicology studies are not required (Beaumont et al., 2014; Lappin et al., 2013; Xu et al., 2014). Moreover, even poorly soluble drug substances can often be easily formulated as a solution for i.v. administration at very low doses (Lappin et al., 2006; Xu et al., 2014), and problems with non-equivalent clearance between the i.v. and the non-i.v. route are eliminated (Lappin et al., 2013).

For well absorbed compounds with similar clearance and tissue distribution between the i.v. and non- i.v. route a high absolute bioavailability is a clear indication for a high permeability across the relevant membrane. However, similar to the mass balance studies, if the bioavailability is low, it may not necessarily be due to poor permeability, as this could be driven by high first-pass metabolism. Information from the mass balance study or *in vitro* studies in hepatocytes can help elucidate if first pass metabolism is limiting. In summary, the absolute bioavailability study is an important study to define the formulation and to search for relevant administration routes.

4.2. Intestinal permeability methods

4.2.1. *In vivo* intestinal perfusion studies in humans

Intestinal P_{eff} , together with solubility, dissolution rate and gastrointestinal (GI) transit, is one of the key biopharmaceutical variables that determine the *in vivo* rate and extent of intestinal drug absorption and bioavailability following oral dosing (Amidon et al., 1995; Lennernäs et al., 1992). *In vivo* predictions of intestinal absorption require accurate determinations of P_{eff} . Different intestinal perfusion techniques have been developed over the last 70 years (Dahlgren et al., 2015; Sjögren et al., 2015).

4.2.1.1. Assay protocol. In short, four clinical intestinal perfusion systems have been developed and extensively used. They are the two open systems, double lumen (Double-L) and triple lumen (Triple-L), the semi-open proximal balloon (Prox-B) system, and the double-balloon system (Loc-I-Gut) (Fig. 3). The Double-L was the first system used to assess permeability by intestinal perfusion. It consists of two catheters separated by the test segment; one placed proximally to the test region within the intestine which is used to perfuse the test solution and the second, located distally, where sampling takes place. A key limitation to the Double-L system is its inability to control flow within the perfused segment. As a result, the infused solution can flow in either direction, resulting in reflux above the point of perfusion. Additionally, contamination of the test region with endogenous secretions proximal to the test segment may occur. To overcome some of these limitations, further systems were developed. The Triple-L setup was designed to limit the impact of the artifacts described above by creating a ‘mixing segment’ distal to the point of infusion and proximal to the test segment. Here the solution and GI fluids are mixed, and at the distal end of the mixing segment a baseline sample is taken. P_{eff} is calculated from a second outlet sample taken at the end of the test segment, which usually is 20–30 cm distal to the mixing segment (Dahlgren et al., 2019, 2015; Lennernäs, 1998; Sjögren et al., 2015). By measuring the concentration in and concentration out of the test segment, limiting the impact of this mixing effect, though control of flow was still not possible. The perfusate composition changes along both mixing regions, which makes it difficult to apply constant luminal and absorption conditions. The perfusate will also flow in both directions, which limits the estimation of the cylinder surface area in an open system. A further refinement was the development of the semi-open, Prox-B model, where an occluding balloon is inflated proximal to the test region, preventing reflux of the perfusate. A

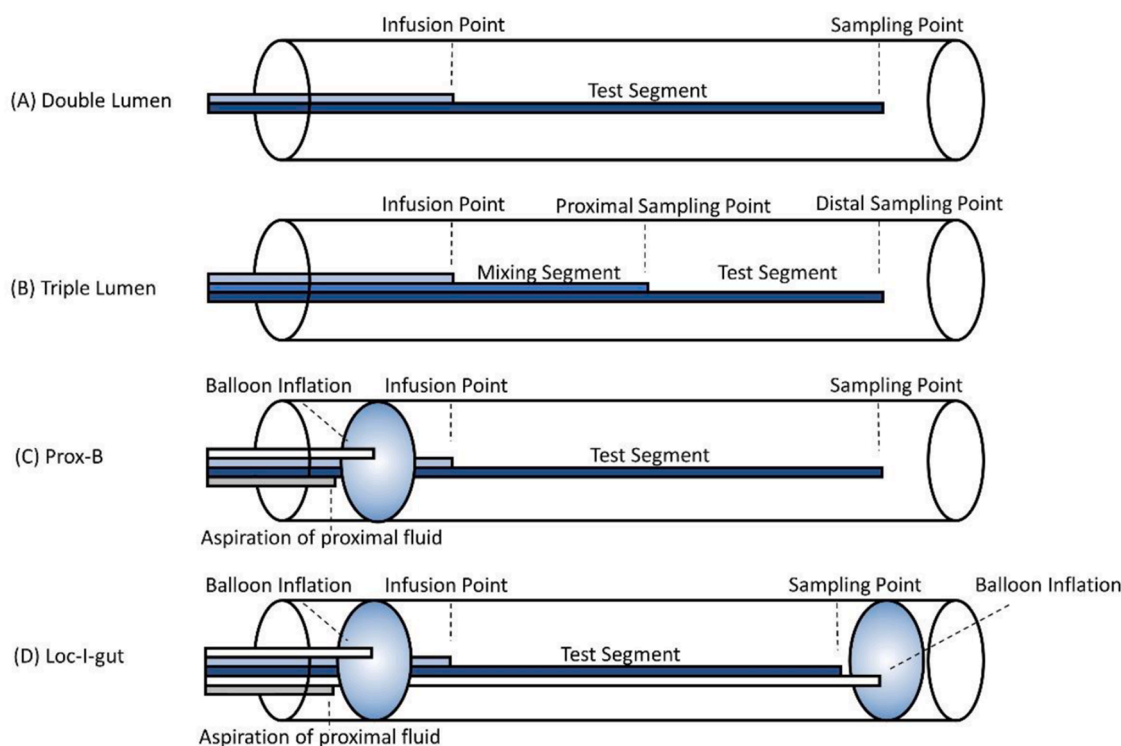


Fig. 3. Schematic representation of the clinical intestinal perfusion models. (a) The open double lumen (Double-L) perfusion system. (b) the open triple lumen (Triple-L) perfusion system. (c) The semi-open proximal balloon (Prox-B) perfusion system. (d) The double-balloon (Loc-I-Gut) perfusion system. Adapted from Dahlgren et al. (2015).

multi-lumen tube avoids this shortcoming with proximal contamination by using an occluding balloon proximal to the test segment. There is also a separate tube aboral to the occluding balloon to continuously drain and prevent proximal luminal contamination of the single-pass perfused test segment. This method decreases proximal leakage and therefore the luminal composition will be kept at equilibrium and drug permeability can be determined under well-defined conditions. However, these methods have the disadvantage that they have a low recovery of the non-absorbable volume marker, usually PEG 4000, and they also use a rather high single-pass perfusion rate (usually 5–20 mL/min) (Dahlgren et al., 2019, 2015; Lennernäs, 1998; Sjögren et al., 2015). To overcome this challenge, the double-balloon (Loc-I-Gut) perfusion system was developed, where occluding balloons compartmentalise a segment of intestine, preventing both proximal and distal contamination. This set-up facilitates high recovery (>95%) of nonabsorbable marker, while maintaining physiological flow rates of 2–3 mL/min - and is further discussed below (Dahlgren et al., 2015; Lennernäs, 1997; Lennernäs et al., 1992).

4.2.1.2. Considerations in study design. The most common approach to determine P_{eff} has been to calculate it based on the disappearance from a perfused intestinal segment. However, the Lennernäs group at Uppsala, Sweden have also validated a data analysis approach to calculate P_{eff} from appearance rate of the drug in plasma (Dahlgren et al., 2019, 2015; Lennernäs et al., 1992; Sjögren et al., 2015). It is necessary to monitor luminal and brush-border chemical stability/metabolism and to correct for binding to the tubing as these processes may strongly affect the accuracy for P_{eff} determinations for both disappearance and appearance methods (Lennernäs et al., 2002b, 1997a, 1994, 1992). With regard to disappearance methods, accurate calculation of P_{eff} requires consideration of the intraluminal hydrodynamics the perfusion experiment and varies with the design the chosen technique. For the open (double-L, triple-L) and semi-open (Prox-B), a parallel tube model is used, where the luminal drug concentration is assumed to decrease exponentially

along the perfused segment according to Eq. (10)

$$P_{eff} = Q_{in} \times \frac{(C_{in} - C_{out})}{A} \quad (10)$$

where Q_{in} is the flow rate, C_{in} and C_{out} are the concentrations of the substance entering and exiting the perfused segment, and A is the area available for permeation. Drug concentrations are sampled at the beginning and end of the perfused sample, as shown in Fig. 3, the flow rate is controlled through the experimental design and the area available for absorption is calculated by assuming the perfused region is a cylinder with radius, r , and length, L . The area can then be simply calculated using Eq. (11) (Komiya et al., 1980)

$$A = 2\pi.r.L \quad (11)$$

For the double-balloon (Loc-I-Gut) method, the most appropriate model is considered to be a well-stirred model, due to immediate mixing within the perfused area of the intestine, as described by Eq. (12) (Dahlgren et al., 2015; Lennernäs et al., 1997a)

$$P_{eff} = Q_{in} \times \frac{(C_{in} - C_{out})}{(C_{out} * A)} \quad (12)$$

Calculation of P_{eff} based on appearance in plasma requires deconvolution of plasma concentration-time profile followed by correction for first pass metabolism, in both enterocytes and hepatocytes, to allow calculation of the intestinal absorption rate (k_a), the fraction absorbed (f_a), thus allowing calculation of the remaining quantity of API and concentration with the lumen (Sjögren et al., 2015). Regional P_{eff} can subsequently be calculated using Eq. (13)

$$P_{eff} = \frac{k_a \times r}{(Amt_{Lumen} * 2)} \quad (13)$$

Where k_a is the calculated absorption rate, r is the radius of the small intestine and Amt_{lumen} is the remaining amount of API in the intestinal lumen. The derivation of this equation has been well-described by

Sjögren et al. (2015).

The intestinal P_{eff} is a direct measurement of the local absorption rate and reflects the transport velocity (cm/s) across the epithelial barrier (Amidon et al., 1995; Lennernäs et al., 1992). The enterocyte is the most common cell type in the epithelial barrier, which also contains a significant number of lymphocytes, mast cells, endocrine cells, goblet cells, macrophages and stem cells (localised to the crypts). P_{eff} for passively transcellularly transported drugs reflects the diffusion across the complex apical membrane into the cytosol and from there it diffuses across the basolateral membrane (Lande et al., 1995; Tannergren et al., 2004, 2003a, 2003b). Therefore, intestinal perfusion models, which measure the disappearance of the drug from the perfusion solution, directly describe uptake into the enterocyte. Intracellular metabolism, by CYP 3A4 and/or di- and tripeptidases (in the case of peptides), is/are not localized in the vicinity of the outer apical leaflet and are considered less likely to influence the disappearance rate (P_{eff}). Intracellular metabolism can be a further limitation to the bioavailability of the drug.

One of the main advantages of these techniques is the capacity to perfuse distal parts of the small intestine, as shown in several open-perfusion studies by Gramatté and co-workers (Gramatté, 1994; Gramatté et al., 1994; Gramatté and Oertel, 1999; Gramatté and Richter, 1994). In recent reports, the intestinal P_{eff} from these open-perfusion studies were calculated and in accordance with what was expected (Dahlgren et al., 2019, 2015; Sjögren et al., 2015). Single-pass perfusions with double-balloon system in both the proximal small intestine (Loc-I-Gut) and colo-rectal regions (Loc-I-Col®) have been reported earlier (Lennernäs et al., 2002a, 1995, 1992). When using Loc-I-Gut, a 10 cm segment is compartmentalised between two balloons, thereby enabling single-pass perfusion of a well-defined region of jejunum. One of the advantages with this design is that the occlusion of the test segment between two intraluminal balloons minimizes contamination with luminal fluids both proximally and distally into the perfused segment. In addition, the leakage out from the segment over the balloons is small, so the recovery of the non-absorbable marker is almost complete (Lennernäs et al., 2002a, 1995, 1992; Nyberg et al., 2007). These qualities enable control of the absorption conditions in the intestinal segment, and thus facilitate the study of mechanisms of transport and metabolism of xenobiotics and nutrients in the human intestine. Another important advantage is the ability to assess the degree of metabolism in the gut and liver by simultaneously determining the extent of absorption and bioavailability (Lennernäs et al., 1992; Nilsson et al., 1994; Tannergren et al., 2003a, 2003b). This cannot be done with the same accuracy using other perfusion techniques, since in those cases the degree of absorption cannot be estimated unless a radiolabelled molecule is used. The human jejunal permeability data obtained with the Loc-I-Gut system has been one of the cornerstones in the development and establishment of the BCS (Amidon et al., 1995; Lennernäs et al., 1992). In addition, human jejunal P_{eff} data also formed the basis of several *in silico* software, showing correlation with cell monolayer flux data and also with animal models for prediction of intestinal absorption (Fagerholm et al., 1999, 1996; Sjögren et al., 2013; Sun et al., 2002).

4.2.1.3. Ability to predict *in vivo* permeability. Human jejunal P_{eff} has been determined by applying the double-balloon system (Loc-I-Gut) and the data from these studies have been reported earlier (Dahlgren et al., 2019, 2015; Lennernäs et al., 1992; Sjögren et al., 2015). This provides a direct measurement of intestinal absorption, calculating P_{eff} regardless of transport mechanism in a fully integrated *in vivo* model incorporating physiological, biochemical and environmental factors (Lennernäs, 2007). Human P_{eff} values are determined in the most absorptive region, i.e. the proximal small intestine, but the P_{eff} has also been calculated from other more distal intestinal regions with perfusion data based on open (or semi-open) systems (Lennernäs, 1997; Sjögren et al., 2015; Winiwarter et al., 1998). It is clear that the data between the systems are comparable and that low permeability drugs have high intra- and

inter-individual variability in their P_{eff} determinations (Dahlgren et al., 2019, 2015). This is most likely explained by the fact that the disappearance calculations are sensitive to small differences in f_a during a single-pass perfusion. Additionally, the perfusion techniques themselves are by necessity invasive procedures and risk disturbing natural physiology, and intestinal motility in particular (Davis and Wilding, 2001).

Despite the limitations, human *in vivo* P_{eff} measured via perfusion techniques is the most relevant biopharmaceutical output capable of estimating fraction absorbed. As a result, it is used as a reference standard for the prediction of both rate and extent of human drug absorption (Lennernäs, 2007; Winiwarter et al., 1998). Over the last 30 years, the double balloon, Loc-I-Gut system has been the most widely used technique to assess permeability in proximal jejunum. These studies of jejunal P_{eff} were used to develop, validate, and implement the BCS system, now widely used as a drug development and regulatory tool. This was possible owing to the good correlation observed between P_{eff} , as measured via *in vivo* perfusion, and fraction absorbed *in vivo*, as shown in Fig. 4. On the basis of the regulatory definition of high permeability being defined as a fraction absorbed greater than 85%, compounds with a P_{eff} in proximal small intestine above approximately 1.5×10^{-4} cm/s are generally classified as high-permeability, though no definitive value is set (Dahlgren et al., 2015; Lennernäs, 2007).

4.2.2. *In vivo* / *in situ* intestinal perfusion in animal models

Since its introduction in 1958 (Schanker et al., 1958), the *in situ* intestinal perfusion technique in animal models has been a biorelevant and versatile tool to determine intestinal permeability and to explore mechanisms underlying drug absorption (Stappaerts et al., 2015a). The technique is usually performed in rats, but mice can be also used. In an anaesthetized animal, a laparotomy is performed to cannulate and clean a segment of the intestine before perfusing it with a solution (or suspension) containing the drug(s) of interest. Since any segment of the small or large intestine can be perfused, the technique is well suited to evaluate regional permeation. Usually, the perfusion solution passes once through the intestinal segment (i.e., single-pass perfusion), but when only a limited amount of molecule and/or perfusion medium is available, a closed-loop perfusion can be considered, in which case the perfusion solution is continuously recirculated (Caldeira et al., 2018), as represented in Fig. 5A and 5B. The absorption of the drug can be assessed based on either the disappearance of the drug from the perfusion solution, the appearance of the drug in the systemic circulation, or the appearance of the drug in the mesenteric vein that drains the blood from the perfused segment. As each of these approaches has its own strengths and limitations, a careful choice should be made depending on the molecule of interest and the research question (Fig. 6). *In situ* intra-intestinal instillations, a further simplification of the closed-loop

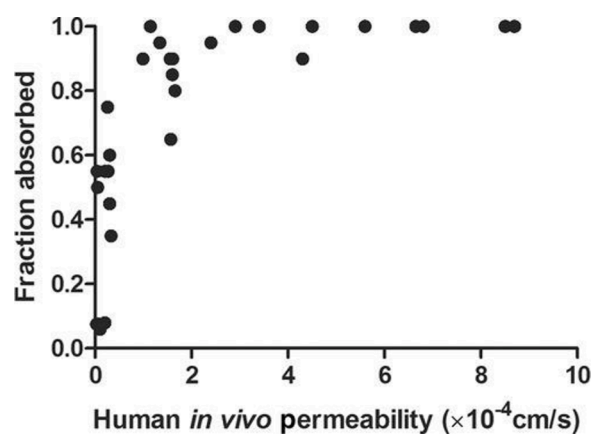


Fig. 4. Human *in vivo* permeability values (P_{eff}) determined by intestinal jejunum perfusion correlated to fraction absorbed (f_a) following oral administration. Reproduced from Dahlgren et al. (2015).

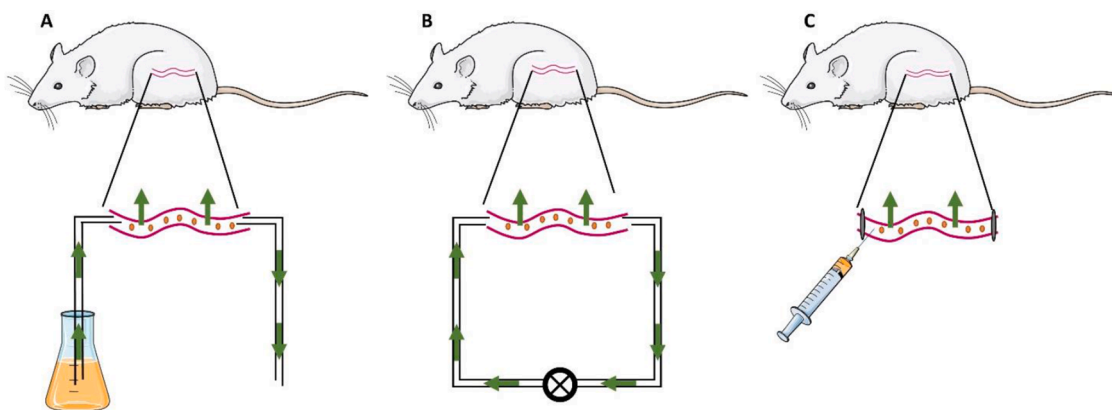


Fig. 5. Schematic representation of (A) open loop and (B) closed loop intestinal perfusion, and (C) intra-intestinal instillation in rats. Adapted from [Stappaerts et al. \(2015a\)](#).

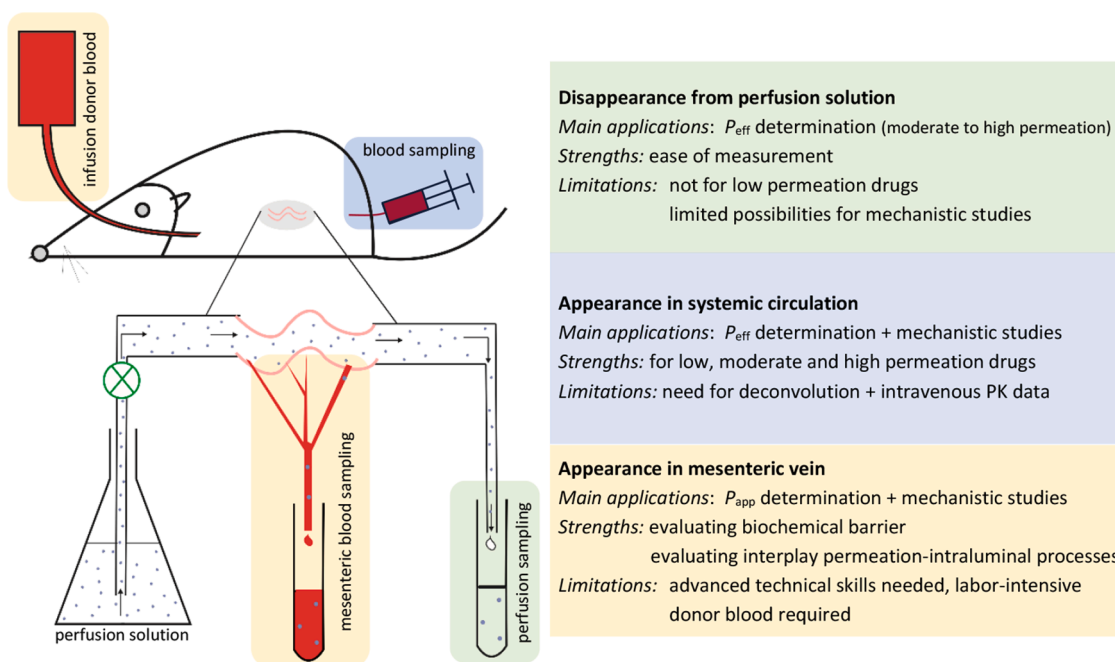


Fig. 6. Schematic representation of the *in situ* intestinal perfusion technique (single-pass) in rats, including a comparison between the different methods to assess permeation. The coloured areas are optional, depending on the selected method: drug disappearance from the perfusion solution (green), drug appearance in the systemic circulation (blue) or drug appearance in the mesenteric vein draining the perfused segment (yellow).

perfusion technique, where a fixed volume of solution is administered to an isolated intestinal segment may also be considered as an alternative approach to measure intestinal absorption (Fig. 5C) ([Cheng et al., 2010](#); [Maher et al., 2009](#); [Presas et al., 2018](#)). These models have proven useful in assessing intestinal epithelial transport and bioavailability (absolute or relative) following intestinal administration and are discussed in this context below, though such models lack the capacity to provide an estimation of P_{eff} .

4.2.2.1. Assay protocol. The disappearance of a drug from the perfusion solution is by far the easiest measure to determine the intestinal permeability for the drug in the perfused segment, as no blood samples need to be collected and analysed. P_{eff} is calculated from the decrease in drug concentration between the inlet (C_{in}) and the outlet (C_{out}) of the intestinal segment, taking into account the perfusion flow rate (F) and the radius (R) and length (L) of the perfused intestinal segment, according to Eq. (14):

$$P_{eff} = F \times \left(-\ln \left(\frac{C_{out}}{C_{in}} \right) \right) \times \frac{1}{2\pi RL} \quad (14)$$

Drug concentrations are most often quantified using HPLC along with an appropriate detection method (e.g. UV absorbance, mass spectroscopy) though direct UV absorbance or fluorescence has also been employed. The outlet concentration should be corrected for the water flux across the intestinal mucosa during perfusion, either gravimetrically or by using a non-absorbable marker in the perfusion solution ([Sutton et al., 2001](#)). The calculation further assumes that no other processes than permeation or water flux affect the drug concentration in the perfusion solution. Sinko et al. and Fagerholm and co-workers found correlations for passive drug permeability values between the rat intestinal perfusion and human single pass jejunal perfusion ([Amidon et al., 1988](#); [Fagerholm et al., 1996](#)). In rats, this experimental approach has been applied to determine the effective intestinal permeability for over 90 drugs ([Dubbelboer et al., 2019](#)). However, care should be taken to ensure that the difference between C_{in} and C_{out} can be accurately

quantified. For moderate to low permeability molecules, the difference can be small ($< 10\%$), resulting in an inaccurate assessment of P_{eff} (Dahlgren et al., 2019). In this case, one may attempt to increase the difference between C_{in} and C_{out} by extending the time available for absorption (e.g., increased segment length, reduced perfusion flow rate, or using a closed-loop instead of single-pass perfusion), but this is still usually insufficient for low permeability drugs.

Recently, Dahlgren et al. proposed the appearance of a drug in the systemic circulation following intestinal perfusion as a more accurate measure to determine the P_{eff} for low permeation drugs (Dahlgren et al., 2019). During the intestinal perfusion, blood samples are collected from the femoral artery to assess a systemic concentration-time profile. Deconvolution of this profile allows calculating the absorption rate, and thus the P_{eff} . A disadvantage is the need for i.v. PK data of the drug in the animal model to compensate for intestinal and hepatic first-pass extraction during deconvolution. The approach has been successfully applied to assess intestinal permeability in rats, dogs and humans (Ailiani et al., 2014; Dahlgren and Lennernäs, 2019; Sugano, 2021).

To avoid the need for intravenous PK data and deconvolution, intestinal permeability in perfusion models can also be determined based on a drug's appearance in the mesenteric vein that drains the blood from the perfused segment. The mesenteric vein is cannulated and blood samples are quantitatively collected at predefined time intervals. To maintain the hemodynamic balance in the animal, donor blood is supplied via the jugular vein. The P_{app} can then be determined based on the slope of the cumulative amount of drug appearing in the mesenteric blood samples over time (dQ/dt), taking into account the radius (R) and length (L) of the perfused intestinal segment, and the donor concentration in the perfusion solution (C_{donor}), according to Eq. (15):

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{C_{donor}} \times \frac{1}{2\pi RL} \quad (15)$$

This calculation assumes a stable donor concentration during perfusion, implying that the preferred perfusion flow rate (around 1 mL/min in rats) is typically higher in this method as compared to the flow rate during disappearance-based permeability determination (around 0.2 mL/min in rats). Obviously, the obtained apparent permeability values reflect drug permeation across the intestinal mucosa rather than uptake into the intestinal monolayer and cannot be directly compared to effective permeability values. Permeation evaluation based on mesenteric blood sampling is not straightforward to implement as a rapid screening tool in a drug discovery or development setting, as the cannulation of the fragile mesenteric vein requires advanced technical skills and sensitive and robust analytical assays for the drugs in blood or plasma are essential. From a research perspective, however, evaluating permeation based on drug appearance in the mesenteric vein rather than disappearance from the perfusion solution, opens additional possibilities for intestinal perfusion studies in animals (Stappaerts et al., 2015a). Firstly, the method can accurately evaluate not only high and moderate permeability drugs, but also low permeability drugs. Secondly, the method allows a deeper evaluation of the biochemical barrier function of the intestine on drug absorption, including the impact of transporters and metabolizing enzymes in enterocytes. Finally, the method can handle scenarios in which the luminal drug concentration is affected by processes other than permeation, including non-specific binding to perfusion tubing or the intestinal mucosa, as well as luminal drug degradation, dissolution or precipitation. In those cases, it is advised to determine the absorptive flux (J) (Eq. (16)), rather than the P_{app} , since the luminal drug concentration (C_{donor}) is not stable:

$$J = \frac{dQ}{dt} \times \frac{1}{2\pi RL} \quad (16)$$

4.2.2.2. Considerations in study design. Irrespective of the method of permeability assessment, the *in situ* intestinal perfusion approach in animals is highly technically demanding and many factors may affect

the outcome of the experiments. In a recent meta-analysis of 635 permeability determinations using intestinal perfusion in rats, Dubbelboer et al. observed high variability in permeability values obtained in different laboratories and even within the same laboratory (Dubbelboer et al., 2019). Although several factors (including rat strain, experimental method (single-pass versus closed-loop perfusion), pH of the perfusion solution, donor concentration, intestinal region) were explored, none could be identified to explain this variability. Therefore, permeation data from different intestinal perfusion studies should not be compared directly, unless reference molecules are included, such as atenolol (for paracellular transport) and metoprolol (for transcellular transport).

When planning *in situ* intestinal perfusion studies, several aspects need to be considered. Depending on the drug of interest and the purpose of the study, the method of permeability determination should be carefully selected and the experimental setup adjusted accordingly (see above and Fig. 6). Irrespective of the method, permeability calculations should be based on steady-state data (i.e., a constant absorption rate). During the perfusion experiments, care should be taken to maintain the normal physiology of the animal as much as possible. For this reason, ketamine/xylazine or inactin are preferred anaesthetic, as their impact on intestinal functions is limited (Ailiani et al., 2014; Saphier et al., 2020). The body temperature of the animal should be maintained by the use of a heating pad or an overhead lamp in combination with a rectal thermometer. To maintain the hemodynamic balance in the case of mesenteric blood sampling, blood pressure should be monitored throughout the experiment, and donor blood should be infused through the jugular vein at a similar rate as the mesenteric blood flow. At the end of the perfusion experiment, the surface area of the perfused intestinal segment (described as a smooth cylinder) needs to be assessed to allow calculation of the permeability coefficient (see Eqs. (14) and (15)). While the radius is usually estimated to be 0.2 cm for rat small intestine and 0.35 cm for rat colon, the segment length needs to be measured. Although this seems a trivial task, the elasticity of the intestine makes this measurement a source of variability and standardization between experiments is needed.

Apart from experimental factors, inter-subject differences may further increase the variability in permeation assessment using the intestinal perfusion technique in animal models. In mechanistic studies that compare the impact of multiple conditions on permeation, each tested in a different group of animals (e.g. drug-drug or drug-food interaction studies), these inter-subject differences may confound possible condition-dependant effects. To reduce this issue, a differential *in situ* perfusion experiment can be considered, in which the condition-dependant effect is evaluated within the same group of animals by exposing the perfused intestinal segment in each animal to a sequence of two or three conditions (Brouwers et al., 2010; Hanafy et al., 2001). The rat intestinal perfusion method is considered as very valuable as rat and human have similar drug intestinal absorption profiles and similar transport proteins expression patterns in the small intestine, while the two species have different expressions for intestinal tissue metabolizing enzymes (Cao et al., 2006).

4.2.2.3. Ability to predict *in vivo* permeability. Despite being technically complex and labour-intensive, the *in situ* intestinal perfusion technique in animals still has a prominent position in biopharmaceutical research and drug development, not only as a pure permeability assessment tool but recently also as a dynamic and integrated absorption tool. The close resemblance with the *in vivo* situation, including the intact membrane integrity, biochemical barrier functionality, physiological feed-back systems, blood flow and innervation, makes the *in situ* intestinal perfusion technique in animals the most physiologically relevant and versatile non-clinical tool to assess the intestinal permeation of drugs. Strong correlations between P_{eff} in rats and human fraction absorbed have been demonstrated (Lennernäs, 2014), supporting the predictive value of the technique and its role in the BCS classification and biopharmaceutical

selection of drug candidates (Caldeira et al., 2018; Dezani et al., 2017; Lozoya-Agullo et al., 2015b). The ability to perform regional permeation studies in the small and large intestine is important for the rational development of modified release formulations (Lozoya-Agullo et al., 2015a; Roos et al., 2017). In addition, numerous studies have reported on the use of the intestinal perfusion technique to better understand the interaction of drugs with the physical and biochemical barrier function of the intestinal mucosa, and to identify potential drug-drug, drug-food, and drug-excipient interactions (Stappaerts et al., 2015a). In this respect, the use of knockout mice, deficient for specific transporters or enzymes, extends the possibilities even further (Mols et al., 2009). Recently, intestinal perfusion in rats, combined with blood sampling, has become a versatile tool to investigate the dynamic interplay between intestinal drug permeation and intraluminal processes simulated in the perfusion media, such as drug dissolution, precipitation, solubilization and degradation. Examples include absorption studies with ester prodrugs, nanoparticles and lipid-based formulations (Presas et al., 2021; Roos et al., 2018; Stappaerts et al., 2015b; Yeap et al., 2013b, 2013a). The integration of intraluminal processes in the intestinal perfusion technique is possible thanks to the robustness of the perfused intestine to biorelevant media, as was illustrated by the use of human and simulated intestinal fluids as perfusion media to explore food-effects on drug permeability (Stappaerts et al., 2014). An extensive overview of applications of the *in situ* intestinal perfusion technique in animals can be found in recent reviews (Dahlgren and Lennernäs, 2019; Dezani et al., 2017; Stappaerts et al., 2015a).

4.2.2.4. *In situ* intra-intestinal instillations. The *in situ* intestinal perfusion methods, in either human or animal models have proven useful in measuring P_{eff} and to identify the mechanisms underlying drug absorption, and their robustness has led to their identification as suitable methods for determining permeability class by drug product regulators (Davitt et al., 2016; FDA, 2017). However, such studies can be difficult and cumbersome to perform, particularly during drug product development, where comparison of drug absorption from numerous formulation approaches may be required. *In situ*, intra-intestinal instillations have been used as a simpler alternative to intestinal perfusion studies in such cases, allowing determination of intestinal epithelial transport and absolute or relative bioavailability in development settings. In these instillation studies the intestinal segment of interest, often the jejunum and occasionally the colon, is isolated using occluding ligatures in anaesthetized rats, before a solution/suspension preparation of the formulation of interest is injected directly into the isolated intestinal segment – represented schematically in Fig. 5C (Aguirre et al., 2015; Maher et al., 2009). Such an administration approach maintains the benefit of *in situ* perfusion in providing a best-case scenario approach for delivery of formulations to the intestine, bypassing the stomach, which may be beneficial as an early screening tool for novel formulation platforms prior to oral gavage studies (Presas et al., 2018; Sladek et al., 2020). As with *in situ* intestinal perfusion, by-passing the stomach, and administration directly into the intestine allows direct measurement of intestinal epithelial transport as it reduces variability in exposure due to acid lability, gastric transit and digestion by pancreatic secretions (Taverner et al., 2015).

Following the intestinal instillation, a PK or pharmacodynamic (PD) response of interest is monitored for a fixed time-period in order to compare the extent of absorption of the administered preparations. Presas et al. used such an approach when assessing the potential for cyclodextrin based nanoparticles to deliver insulin glulisine (Presas et al., 2018) using an aqueous solution as a subcutaneous control in both cases. Determination of plasma concentrations of insulin glulisine following intestinal administration allowed calculation of the relative bioavailability (F_{rel}) according to Eq. (17)

$$F_{rel}(\%) = \frac{AUC_{installation} \times Dose_{control}}{AUC_{Control} \times Dose_{installation}} \times 100 \quad (17)$$

where $AUC_{installation}$ is the area under the concentration curve over the instillation period, four hours in this case, and $AUC_{Control}$ is the area under the plasma concentration versus time curve after injection of control formulation over the same time-period. The pharmacodynamic response was also monitored in this case, as measured by reduction in blood glucose following administration (Presas et al., 2018). Taverner et al. (2015) have further developed this approach to identify the mechanisms which limit the bioavailability of insulin as being either related to enteric or hepatic related extraction, by sampling both the systemic and portal circulation, allowing direct quantification of the extent of epithelial transport in the small intestine (Taverner et al., 2015). Overall, the intestinal instillation method facilitates characterisation of intestinal epithelial transport in drug development, prior to further formulation related challenges, such as the impact of gastric and pancreatic secretions.

4.2.3. *In vitro* permeation studies using excised human or animal tissues

Diffusion chambers to assay fluxes across isolated intestinal tissue mucosae from GI regions originate from the original Ussing chamber (Ussing and Zerahn, 1951), which was first used to measure electrogenic ion transport across epithelial tissue mucosae. Typically, chamber reservoirs have equal volumes of physiological Krebs's Henseleit (KH) buffer on the apical and basolateral sides of the intestinal epithelium, with a carbogen gas air-lift system to maintain a mixed circulation at a pH of 7.4, along with an outer water jacket to maintain 37 °C (Westerhout

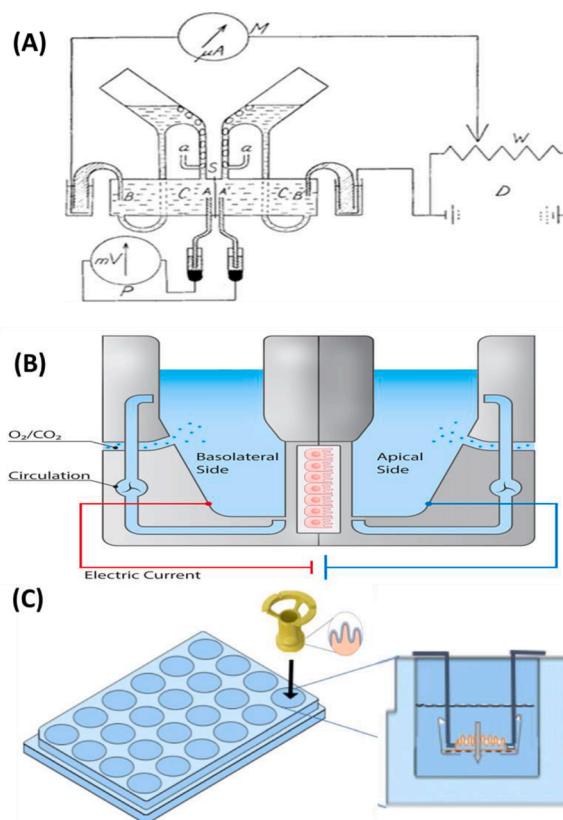


Fig. 7. Diagram of the key components of Ussing-type systems for epithelial ion transport and diffusion studies across isolated intestinal mucosae. (A) the original design used for frog skin by Ussing & Zerhan (1951), reproduced with permission. Abbreviations: A and A': voltage-sensing electrodes; a: air tubes; B and B': current electrodes; C: two half-chambers; S: isolated skin; D: battery; W: voltage clamp to zero (i.e., short-circuited); P: voltmeter; M: the microammeter. (B) a modern compact chamber design for drug diffusion with lower volumes of bathing fluids and single perspex materials. (C) TNO's InTESTine™ system designed for high throughput in a 24 well plate. Image reproduced from (Stevens et al., 2019) with permission.

et al., 2015). Fig. 7A shows Ussing's design for epithelial ion transport and how it has been modified for drug diffusion (7B). Measurement of electrophysiological parameters is recommended as part of flux protocols and these are monitored by an integrated Voltage Clamp system. Ag/AgCl voltage and current electrodes are set in agar or glass bridges and they provide real time measurement of open-circuit electrical potential (PD, mV) and short-circuit current (I_{sc} , $\mu\text{A}\cdot\text{cm}^{-2}$), from which transepithelial electrical resistance (TEER, $\Omega\cdot\text{cm}^2$) is calculated. Iterations for diffusion studies are designed with low donor and receiver side volumes and single surface perspex, important when using expensive molecules and in avoiding surface binding, respectively. Tissue mucosae are stripped of their longitudinal and circular muscle within 30 min of excision and are mounted between the two chamber halves, with window areas ranging from 0.2–1.2 cm^2 . This dissection allows the tissue to be stretched over the window pins and provides more reliable electrophysiological data than non-stripped tissue because the surface area calculation is more accurate. The system is designed to measure transepithelial flux from the apical to the basolateral side of the mounted tissue over a period of 120 min, while monitoring TEER, and then examining tissue histology at the end of the period. By using several donor-side concentrations, a molecule's P_{app} can be calculated from the slope of the linear part of the flux curve (following an initial lag phase) according to Eq. (18):

$$P_{app} = dQ/dt \times 1/A \cdot C_0 \quad (18)$$

where dQ/dt is the transport rate across the epithelium ($\text{mol}\cdot\text{s}^{-1}$), A is the surface area of the cell monolayer, and C_0 is the initial concentration apical donor compartment ($\text{mol}\cdot\text{ml}^{-1}$) (Kisser et al., 2017).

Mucosal sites to study electrophysiology and drug diffusion include duodenum, jejunum, ileum, and colon. These are sourced primarily from rats (Forner et al., 2017; McCartney et al., 2019), and mice (Feighery et al., 2008). Sources used less commonly are pigs (Aschenbach et al., 2002), dogs, rabbits and monkeys (Jezyk et al., 1992), as well as humans (Söderholm et al., 1998). In view of a study showing that the permeation enhancer, salcaprozate sodium (SNAC), acted in the stomach of ligated dogs to facilitate delivery of semaglutide (Buckley et al., 2018) (the basis of the mechanism of absorption of Rybelsus®, Novo-Nordisk, Copenhagen, Denmark), there is renewed interest in examining isolated stomach mucosae as a permeation enhancement site, where the dissection method for rat is available (Hopkins et al., 2002).

4.2.3.1. Assay protocol. Minor variations on the Ussing chamber protocol for intestinal tissue have been published by several labs. Differences in buffers, dissection methods, and chamber design are the main variants. A step-by-step protocol for murine tissue was published by Clarke (Clarke, 2009) in which chamber designs, trouble shooting, and electrophysiology interpretations were discussed. Kisser et al. (Kisser et al., 2017) also wrote a protocol for human intestinal tissue in which the emphasis was on drug transport and metabolism. For murine and human tissue, samples can be small and may require chamber adaptation by vendors or lab workshops. In respect of porcine and bovine sourcing from abattoirs, there is an emphasis on maintaining tissue in ice-cold buffer and reducing the time taken to mount tissue in chambers. Much of the literature on human tissue originates from the Artursson lab where they carried out nanoparticle uptake studies in human jejunal tissue in the Ussing system as part of the EU TRANS-INT FP7 project (Lundquist and Artursson, 2016). The technique is also useful to assess particle uptake by intestinal Peyer's patches from ovine and bovine sources where a mini-diffusion chamber was designed with a protocol using a horizontal format of the perfusion setup (Soni et al., 2006). Much focus has been on testing permeation enhancers on isolated human colon due to good access to tissue from colonoscopies in local hospitals (Fatah et al., 2020). Major contributions to assay development were made by the Ungell lab at Astra-Zeneca Pharma (Sweden), where 28 drugs were assessed for fluxes in Ussing chambers across four human intestinal

regions (Sjöberg et al., 2013). The use of the Ussing chamber by pharmaceutical companies for screening permeability of large numbers of molecules reflects their high analytical capacity, which typically exceeds that of academic labs.

Basal P_{app} value ranges for paracellular flux markers including [^{14}C]-mannitol, FITC-4000, and Lucifer Yellow are required when examining effects of permeation enhancers, as these values are established within and between labs. When KH or adapted buffers are used, high salt compositions need to be checked for compatibility with HPLC, uHPLC and LC-MS/MS, especially when used with peptides. Routine haematoxylin-eosin (H & E) histology to assess tissue damage can confirm if an enhanced flux is due to artefact or compromised tissue.

4.2.3.2. Considerations in study design

4.2.3.2.1. Advantages of the method. The versatile Ussing chamber system allows a series of molecules to be compared for P_{app} values across intestinal regions isolated from rats and humans. This gives comparative data, which can inform oral solid dosage formulation approaches. This aspect can be improved by using buffers more relevant to luminal compositions found in regions of the rat and human GI *in vivo* (Wuyts et al., 2015). Other uses are in the investigation of paracellular and transcellular mechanisms of permeability, screening of intestinal permeation enhancers (within concentration constraints), and effects on electrophysiology and histology. The technique is also useful to probe whether carrier-mediated transport across the epithelium is present (Gleeson et al., 2017), or whether P-glycoprotein and other efflux pumps play a role (Huang et al., 2018); which is important when assessing possible DDIs. Drug transport can be linked to expression and function of proteins in the different GI regions of different species. Additional information on functional changes in epithelial ion transport can be ascertained in the same assay. The technique can also be used to assess uptake of particulates by advanced imaging.

Generations of graduate students have been trained in the Ussing chamber technique. Skills to be practised are speed of consistent dissection of epithelial tissue from delicate small intestinal regions. Modern systems comprise banks of six chambers in a single unit and occupy much less lab space than the original Ussing system. Considerable assay expense is, however, associated with LC-MS/MS, use of ELISAs or radiolabelled payloads assayed through liquid scintillation counting to measure low concentrations of permeated molecules on the basolateral side of mucosae. An alternative lower cost assay option is to use fluorescently labelled payloads. It is important to rule out artefacts in fluxes from fractions of labelled materials obtained through metabolism or chemical degradation of the API in transit, so HPLC, uHPLC and LC-MS/MS are useful tools to confirm flux of unlabelled molecules. Troubleshooting requires establishing a mass balance so that all API is accounted for, showing that TEER is within an acceptable range for the tissue (thereby confirming accurate dissection), and that there are no physical leaks between the two chamber halves.

4.2.3.2.2. Limitations. Isolated intestinal mucosae are devoid of blood supply and have limited viability beyond 120 min in the chambers. Because the dissected tissues are delicate, the ability to withstand biorelevant buffers is low, although recent work suggests that modified fasted simulated small intestinal fluid (FaSSIF) with lower concentrations of bile salts and lecithin may be tolerated on the apical side of tissue mounted in chambers from specific small intestinal regions (Forner et al., 2017, 2016). Without such modifications, payload performance in the milieu of the GI tract will be difficult to model. The system has physiological deficiencies and cannot model dilution, spreading, and absorption present in the dynamic GI environment *in vivo*. The static feature means that intestinal permeation enhancers are presented in a best-case scenario in terms of maintaining apical-side concentration for an extended period, though this can provide useful initial screening information on permeation enhancers, such as SNAC, sodium caprate or glyceride/ macrogol based surfactants e.g. Labrasol®

ALF (caprylocaproyl macrogol-8 glycerides) (Maher et al., 2016; McCartney et al., 2019). Yet, because the tissue is delicate, the range of concentrations of most enhancers that can be tested have an upper limit in view of the membrane damage seen at high concentrations that induced permeability. Similarly, solvents cannot be used at concentrations higher than 1% v/v if artefacts in fluxes are to be avoided. Finally, the technique is based on solution-solution flux unlike the design of Franz Cells for transdermal delivery, so it is limited to admixtures in solution and not solid-dose formulations. Despite these limitations, fluxes across rat, canine, and human intestinal mucosae mounted in adapted diffusion chambers can relate well to absorption in humans, at least across small molecule datasets. The technique offers quite low throughput overall. A typical design would be to use four regions from a single rat in a four-hour protocol, repeated for a second rat the same day for an $N = 2$ for each region. Human and porcine tissues are obtained from hospital operations and abattoirs, respectively; such supplies can be irregular and are usually some distance from the lab, thereby reducing tissue quality and lowering throughput compared to lab animals. Von Erlach et al. (von Erlach et al., 2020) have established a robotic system based on 96-well plates to address low throughput for fresh porcine jejunal mucosae and for mucosae grown as explants for several days. They used this chamber design as a rapid screen of tens of excipients in pairs to boost flux of oxytocin without damaging tissue. Stevens and co-workers (Stevens et al., 2019) from TNO (The Netherlands) have also increased throughput for isolated human intestinal tissue mounted in a disposable 3D-printed two compartment systems designed for 6- and 24-well plates, the InTESTine™ system (Fig. 7C). The InTESTine™ system has also been utilised with mini-pig intestinal tissue in combination with biorelevant matrices, demonstrating that porcine jejunal tissue may be a suitable alternative to human intestinal tissue in such setups, though further characterisation of metabolism and transport in porcine intestinal tissue is necessary to improve predictability of such a set-up (Westerhout et al., 2014).

4.2.3.3. Ability to predict *in vivo* permeability. The ability to predict the *in vivo* permeability is central to the rationale for investment in the technology by the pharmaceutical sciences, both in academia and industry. Working with a limited range of small molecules, Lennernas and co-workers found correlations for passive drug fluxes between the Ussing method with rat intestinal tissue and human single pass jejunal perfusion (Lennernas et al., 1997b), but noted deviations in respect of carrier-mediated transport which is subject to species variation. Sjöberg et al. (Sjöberg et al., 2013) found a sigmoidal relationship between P_{app} values obtained in isolated jejunal and colonic mucosae and the fraction absorbed from those regions following oral administration of selected molecules to humans. Because data from humans was used in that study, carrier-mediated flux and efflux pumps could be compared and used for *in vivo* prediction. In respect of intestinal permeation enhancers, Dahlgren et al. (Dahlgren et al., 2018) reported that the Ussing method tended to over-predict efficacy of a selection of enhancers relative to rat single pass perfusion studies, reflecting limitations of the static system where concentrations of enhancers can be maintained for up to 120 min in the absence of the dilution and spreading seen *in vivo*. Table 3 summarises key attempts to relate flux data from isolated intestine mounted in Ussing chambers with human oral absorption.

4.2.4. *In vitro* permeation across a monolayer of cultured epithelial cells

The use of cultured Caco-2 monolayers of human intestinal epithelial cells grown on semi-permeable filters for the screening of drug fluxes was pioneered by the labs of Ron Borhardt (Hidalgo et al., 1989), Phil Burton (Hilgers et al., 1990) and Per Artursson (Artursson, 1990). Often forgotten is that the discovery and development of the Caco-2 cell line, from which such applications emerged, was originally made in Alan Zweibaum's lab in the 1980s (Pinto et al., 1983). The Pharmaceutical industry sought predictive preclinical screening tools to assess molecule

Table 3

Selection of studies relating flux data from Ussing chamber studies with isolated intestinal mucosae from several species to human oral absorption data.

Reference	Models and molecules	Relationship to <i>in vivo</i> bioassays
Lennernas et al. (1997)	Isolated rat proximal jejunum (P_{app}) versus human intestinal perfusion effective permeability (P_{eff}) <i>in vivo</i> ; 12 molecules, split between passive and carrier mechanisms of permeability	Matched rank order for passively-permeating molecules, but not for carrier-mediated. P_{eff} values were higher
Rozehnal et al. (2012)	P_{app} of 11 molecules across human small intestinal and colonic mucosae compared to F_a	Predicted absorption for poorly and moderately absorbed molecules
Sjöberg et al., (2013)	P_{app} of 25 molecules across human jejunal mucosae and 10 molecules across human colonic mucosae and compared them to fractionated absorbed from those regions in humans (F_a)	Sigmoidal relation between P_{app} and F_a in both regions. P_{app} values similar for highly permeable molecules in both regions, but lower in colon for highly polar ones
Wuyts et al. (2015)	P_{app} of rat intestinal mucosae mounted in human simulated fed- and fasted state buffers and compared to human F_a for 16 molecules	Strong correlation with human F_a using fasted state buffers, whereas fed-state buffer over-estimated F_a
Miyake et al. (2017)	Transport Index (TI, the sum of tissue and basolateral drug concentrations) measured in rat, dog, and monkey small- and large intestinal mucosae versus human F_a for FITC-dextran 4000 (FD-4), atenolol, and metoprolol	Good prediction between TI obtained in rat and dog mucosae for human F_a in both regions for the three drugs
Arnold et al. (2019)	P_{app} values cross porcine small intestinal mucosae in KH and biorelevant buffers compared to P_{eff} in humans for 11 molecules ranging across the Biopharmaceutical Classification System (BCS)	Good correlation for passively permeating molecules in all buffers; contribution of P-gp and Cytochrome P4503A4 demonstrated
Stevens et al. (2019)	P_{app} of 10 molecules across human intestinal tissue mounted in a miniaturised device (InTESTine™) for 24 well plates versus P_{app} in Ussing chamber and versus F_a in humans	Very close correlation between the two <i>in vitro</i> assays; good correlation with f_a in humans

permeability, assays that would yield more useful data than artificial membrane systems. The Caco-2 system is still a mainstay of early discovery programmes and is used to provide P_{app} values for series of molecules, mechanistic data on routes of permeation, cytotoxicity data, as well as information on interaction with P-gp, a potential source of DDIs. For flux studies across monolayers, a well-stirred apical-side reservoir reduces the unstirred water layer above the monolayer, which is otherwise an impediment to fluxes of permeable lipophilic molecules (Hidalgo et al., 1991; Karlsson and Artursson, 1992). Monolayers have also recently been used retrospectively to re-examine efficacy and mechanism of action of two intestinal epithelial permeation enhancers, SNAC and sodium caprate, following extensive clinical trial experience (e.g. (Twarog et al., 2020)).

Though originating from a colonic adenocarcinoma, Caco-2 cells express receptors and uptake carriers and transporters that better reflect small intestinal function. The gene expression profile and function of these proteins has been extensively analysed (Hayashi et al., 2008). Monolayer TEER values are electrically tighter than any region of the human GI tract and their tight junctions are especially sensitive to being opened by excipients and emulsifiers (Glynn et al., 2017), the biological relevance of which has yet to be ascertained. Other epithelial lines used to obtain similar information include the canine kidney MDCK epithelial line, as well as variations including multidrug resistance associated protein-transfected and carrier-transfected MDCK monolayers to probe drug-drug interactions and uptake pathways (e.g. (Sasaki et al., 2001)). Because Caco-2 monolayers do not comprise goblet cells, the unstirred

water layer above the monolayer does not contain protective mucus. A consequence is an increased sensitivity to cytotoxic effects of excipients compared to *in vivo*. This has led to co-culture variations in which Caco-2 cells were, for example, co-cultured with mucus-producing HT29-MTX cells in order to better mimic flux (Lozoya-Agullo et al., 2017), as well as with Raji B lymphocytes to convert Caco-2 cells to M-like cells of the Peyer's patch in order to study particle uptake (Beloqui et al., 2017).

4.2.4.1. Assay protocol. A definitive standard operating procedure for the Caco-2 bioassay for permeability studies was produced as a *Nature Protocol* by the Artusson lab (Hubatsch et al., 2007). Fig. 8A shows a cartoon of the typical monolayer on a filter. In the protocol, the authors emphasise bi-directional transport and efflux ratio calculations in examining the role of efflux pumps, basolateral side sampling details, calculation of P_{app} values, quality control, and troubleshooting in relation to assay of permeated molecules by HPLC and LC-MS. Useful notes were given on how to interpret flux data using the paracellular markers [14 C]-mannitol and Lucifer Yellow, the importance of maintaining sink conditions with a maximum of 10% flux on the basolateral side, use of < 1% w/v DMSO as a solvent for molecules added to the donor side to avoid flux artefacts. A more comprehensive discussion of solvent systems used in these models, including the use of biorelevant media is provided in Section 5 of this review. Inclusion of 4% bovine serum albumin in the receiving basolateral well can reduce non-specific binding of lipophilic drugs, but its inclusion can create an extra sink which has to be factored into P_{app} calculations.

4.2.4.1.1. Validation/acceptance criteria. Reference molecules are used as internal standards to establish a validated range of low, moderate, and high fluxes of passively transported molecules across Caco-2 monolayers. This is necessary because of large inter-lab variability in fluxes even for the same reference molecule. The 2017 FDA guidance for industry on "waivers of *in vivo* bioavailability and bioequivalence studies for immediate release solid oral dosage forms based on a BCS" gives an extensive set of molecules fulfilling criteria for high, moderate, and low permeability (mapped to f_a from human PK studies) (FDA, 2017). Reference molecules demonstrating negligible permeability are also needed to establish membrane integrity. The Guidance advises that these reference molecules should be used to establish and validate permeability assays in a lab, which applies to Caco-2 monolayers being used to provide P_{app} values for BCS Class I drug products in search of bioequivalents. A reference lab must therefore establish a set of P_{app} values based on a minimum of 20 molecules across a range of permeabilities to produce a correlation curve mapping P_{app} across monolayers with f_a in

humans.

More specifics in relation to use of Caco-2 monolayers were given in the subsequent ICH "harmonised guidelines on biopharmaceutics classification system-based bioequivalents", which came into effect in 2020 (ICH, 2020). Because Caco-2 have variable expression of uptake transporters (PepT1, OATP2B1, MCT1) and efflux pumps (e.g. P-gp, BCRP, MRP2), the ICH guideline also states that any flux data used to argue for bioequivalents is limited to passively permeable molecules. Nonetheless, a reference set of molecules is provided for which efflux ratios can be established, denoting functional expression of P-gp in monolayers: digoxin, vinblastine, paclitaxel, and rhodamine-123. The ICH guide requests that TEER values of monolayers be provided before and after molecule exposure, as part of QA to establish maintenance of monolayer integrity. TEER value ranges depend on the passage and clone used and range from 300 $\Omega \cdot \text{cm}^2$ up to 1800 $\Omega \cdot \text{cm}^2$. If studies are internally controlled, absolute TEER values are less important than relative changes comparing values to the starting TEER of the monolayer.

4.2.4.2. Considerations in study design

4.2.4.2.1. Advantages of the method. Caco-2 monolayers are useful in early discovery to assess basal permeability of a series of molecules. They are also of importance for industry in determining a molecule's P_{app} in respect of BCS Class I molecules as a route to a bioequivalent for expensive human studies. Flux assays are simple to carry out and analytical tools give reliable data in physiological buffers. Monolayers also give information on uptake pathways of molecules and particulates and these can be probed using pharmacological inhibitors and with advanced imaging. The Caco-2 cell line possesses many of the known intestinal uptake transporters, and assessment of permeability in both the presence and absence of transport inhibitors has been used to facilitate DDI assessment, while transfection with cDNA resulting in efflux transporter expression has also been carried out to identify substrates for and inhibitors of efflux *in vitro* (Sun et al., 2008). In addition, calculation of Caco-2 permeability has been utilised in prediction of *in vivo* pharmacokinetics as a vital input into PBPK models, as outlined in Section 3.3 (Kostewicz et al., 2014).

Caco-2 monolayers are a standard methodology that can be established in most labs with basic tissue culture expertise. Lamson et al. (Lamson et al., 2019) recently calculated that tissue culture maintenance of a 24-well plate of Caco-2 Transwells® for 21 days costs ~\$170 per plate, so consumable costs on a large project using many filters can be high. Analytical methods such as scintigraphy, fluorescence, confocal microscopy, HPLC, and LC-MS/MS need to be factored into costs.

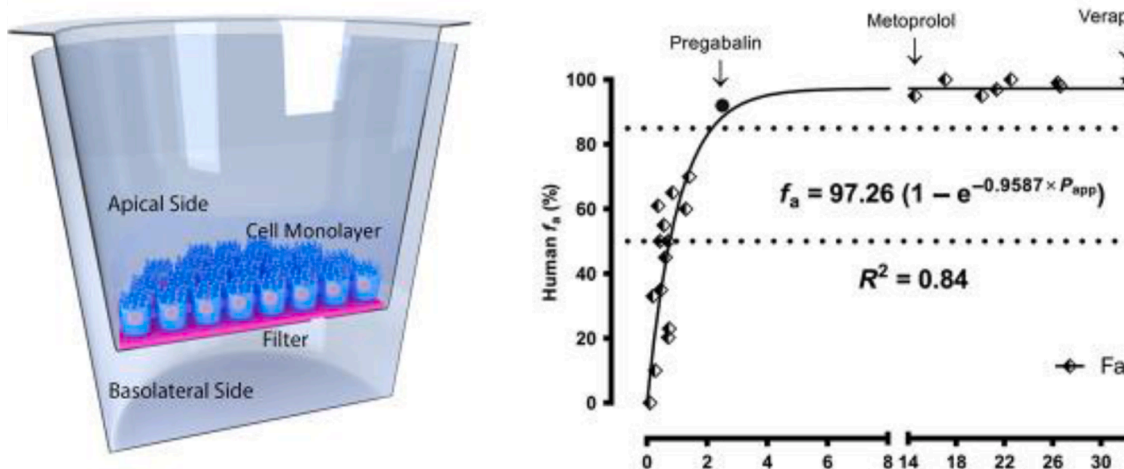


Fig. 8. (A) Cartoon of a Caco-2 monolayer grown on a semi-permeable filter. (B) Correlation of human fraction absorbed (f_a) from historical PK data and apparent permeability coefficients (P_{app}) determined in the Caco-2 model. Dotted line represents cut-off values for f_a of 50% and 85%. Graph reproduced from Jarc et al. (2019) with publisher permission.

Through-put can be adapted for robotics in 96 well and 356 well plates with automated TEER value capacity and sampling. Flux assays can be completed within 24 h using mature monolayers.

4.2.4.2.2. Limitations. One of the main limitations of the Caco-2 monolayer system is that it takes cells up to 21 days to differentiate, which requires logistical planning to keep throughput high. Efforts have been made to reduce this period by altering the culture medium composition to a 3-day system (Lamson et al., 2019). In addition to the exceptionally high TEER values of monolayers, Caco-2 tight junctions seem to be overly sensitive to potential tight junction openers, a result that can yield false positives in terms of screening for enhancers and cytotoxicity. Overall, monolayers have not been very predictive of enhancer efficacy in less reductive models, but a corollary is that if enhancers show no efficacy in the monolayer system, they are unlikely to do so in animal models. Finally, Caco-2 cells express carriers and efflux pumps to a variable extent compared to human intestinal tissue (Ölander et al., 2016), although some standardisation can be addressed by transfecting cells following knock-out of the defined genes of interest (Ye et al., 2020). There are also drawbacks for their use in metabolism studies, as Caco-2 do not express cytochrome P450 3A4, though this can be induced by culturing in the presence of Vitamin D3 (van Breemen and Li, 2005). The advantages and limitations of Caco-2 in permeability studies and intestinal transporter research have been summarised by Sun et al., (Sun et al., 2008). Overall, while industry uses *in silico* methods and PAMPA systems to estimate passive permeability, Caco-2 monolayers continue to be used in parallel for pre-clinical studies where they can provide additional biological mechanistic information on transport processes (Kerns et al., 2004).

4.2.4.3. Ability to predict the *in vivo* permeability/ correlations with *in vivo* data. The most important aspect is whether fluxes of molecules across Caco-2 relate to f_a from oral formulations in humans. Numerous academic and industrial labs have attempted to make such a correlation over several decades and the results show linkage, at least for highly passive-permeable molecules moving across the epithelium (Cheng et al., 2008; Sun et al., 2004). Yet, as more detail emerged on the variability of carrier and transporter expression in Caco-2, it became apparent that correlations in respect of molecules permeating by such pathways across the human intestine were unreliable.

Regulatory guidance documents have therefore encouraged oral drug developers to show that their in-house Caco-2 method is suitable as an *in vitro* permeability assay to allow permeability classification. One recent example (Jarc et al., 2019), demonstrated a correlation coefficient of 0.86 across 21 reference molecules versus human f_a (Fig. 8B), along with demonstration of P-gp function and expression of 84 transporters. Pertinent findings were that all molecules with f_a of >85% were accurately classified using the P_{app} value for metoprolol as the reference for highly permeable agents, whereas molecules with f_a < 84% tended to be underestimated according to the P_{app} values obtained. Reasons offered for the discrepancy for moderately permeable molecules were the restricted paracellular pathway in Caco-2 (underestimates the true flux of hydrophilic molecules), sequestration of lipophilic molecules by Caco-2 cells, non-specific binding, along with reduced expression of uptake carriers.

4.3. *In vitro* permeation using cell-free permeation tools

The permeability methods discussed in this section are all less labour intensive and time consuming than both *in vivo* and *in situ* methods as well as cellular methods. On the other hand, cell-free permeation tools cannot predict carrier-mediated transport. However, for the vast majority of drugs described in literature, passive (transcellular) transport is the primary pathway of intestinal absorption (Sugano et al., 2010). Even in cases with substantial contribution of carrier-mediated transport, cell-free permeation tools are regarded useful to clarify permeation

pathways when combined with other techniques (e.g. Caco-2) (Kerns et al., 2004). Cell-free permeation tools may be classified, according to their barrier type, into two classes: 1) biomimetic barriers that contain (phospho)lipids and 2) non-biomimetic barriers that are lipid-free. Biomimetic cell-free permeation tools include the parallel artificial membrane permeation assay (PAMPA) (Kansy et al., 1998), the phospholipid vesicle-based permeation assay (PVPA) (Flaten et al., 2006) and the Permeapad® model (di Cagno et al., 2015). In a broad sense, non-biomimetic cell-free permeation tools are based on dialysis membranes (molecular weight cut off (MWCO) <14 kDa). Examples are the artificial membrane insert (AMI) system (Berben et al., 2018a), poly (dimethyl siloxane) (PDMS) membranes (Lovering and Black, 1973; Sinko et al., 2017) or hollow fibre membrane modules (Blanquet et al., 2004) based on e.g. poly(ether sulfone) (Hate et al., 2017). Berben et al. recently provided an overview on cell-free permeation tools and their applications (Berben et al., 2018a).

With regard to their application in oral drug delivery research, cell-free permeation tools can be divided into two main categories, 1) permeability profiling of drug candidates (discussed in section 4.3.1) and 2) predictive biopharmaceutical characterisation of formulations (discussed in section 4.3.2.). The best practice conditions of these two applications are described separately because the aim and working hypothesis differ substantially. An overview of cell-free permeation barriers, their components, and construction/preparation is given in Table 4. Table 5 and Table 6 summarise the literature on predictive biopharmaceutical characterisation of formulations using cell-free permeation tools. Specifically, Table 5 summarises permeation devices/set-ups and Table 6 gives an overview of literature comparing the *in vitro* results to *in vivo* absorption data.

4.3.1. Permeability profiling of drug compounds using cell-free permeation tools

4.3.1.1. Assay protocol. The experimental set-up for permeability profiling using cell-free barriers is basically the same as for *in vitro* permeation studies using tissue or cell-based barriers. The set-up consists of a donor and a receiver/acceptor compartment separated by a barrier. Cell-free permeation set-ups come in a variety of geometries that range from small-volume devices (e.g. 96-well plates) to devices with larger volume (e.g. side-by-side cells). Independently of the barrier and the device, the P_{app} is typically derived from the amount of drug reaching the receiver within a given timeframe and is calculated using one of two main approaches. In the first approach, the permeability is calculated from a single measurement of the drug concentration in the receiver and the donor compartment after a given incubation time, may be expressed either as percent transport or as apparent permeability coefficient following correction for incubation time and membrane surface area (see e.g. (Wohnsland and Faller, 2001; Zhu et al., 2002)).

In the second approach, the drug amount in the receiver compartment is determined at several timepoints. The permeability is then derived from the slope of the linear part of the cumulative permeation curve (i.e. the cumulative amount of drug permeated per area vs time interval), which is referred to as (steady-state) flux. To calculate the P_{app} value, the steady-state flux is normalized by the (measured) drug concentration in the donor at the beginning of the experiment, as per Eq. (19) (see e.g. (P. Berben et al., 2018b; di Cagno et al., 2015; Flaten et al., 2006)).

$$P_{app} = \frac{J}{A(C_D - C_a)} \quad (19)$$

where J is the observed flux rate at steady-state, as assessed from the linear section of a permeated amount vs time graph, A is the surface area of the barrier and C_d and C_a are concentrations of solution in donor and acceptor chambers, respectively. Where sink conditions are maintained, the concentration of drug in the acceptor chamber can be negated, and

Table 4
Overview of cell-free permeation barriers, including their components and preparation method.*.

Category	Cell-free permeation tool	Components of the barrier	Preparation of the barrier	Reference(s)***
Biomimetic	PAMPA	Filter support (e.g. hydrophobic PVDF), (phospho) lipids dissolved in an organic solvent (e.g. 10% egg-lecithin in n-dodecane)**	The filter is impregnated with a solution of (phospho)lipids in an organic solvent (1–20% lipid content). Pre-dissolved phospholipid solution is available (Pion Inc., Billerica, MA, USA).	(Avdeef et al., 2001; Kansy et al., 1998; Sugano et al., 2001; Zhu et al., 2002)
	Hexadecane method (HDM)	Polycarbonate filter,	The filter is impregnated with 5% hexadecane in hexane, hexane is evaporated on standing (>10 min).	(Wohnslund and Faller, 2001)
	PAMPA	n-hexadecane		
	Precoated PAMPA	PVDF filter support, lipid/oil/lipid tri-layer	The tri-layer is formed by coating the filter stepwise with hexadecane (i.e. 'oil') and phospholipids dissolved in hexane, hexane is evaporated on standing. Available as ready-to-use format (Corning Inc., New York, USA).	(Chen et al., 2008)
	PVPA	Filter support (mixed cellulose ester), liposomes (from e.g. egg phosphatidylcholine)**	Small and large liposomes prepared by film hydration/extrusion are deposited stepwise on the filter support by centrifugation starting with the small liposomes. Alternatively, large liposomes are formed after depositing small liposomes by a freeze/thaw cycle.	(Flaten et al., 2006; Naderkhani et al., 2014)
	Mucus-PVPA	As PVPA, mucin	To prepare the mucus-PVPA, a suspension of mucin is pipetted onto the PVPA barriers directly before the experiment.	(Falavigna et al., 2018)
Non-biomimetic	Permeapad®	Two polymeric support sheets (e.g. cellulose hydrate), phospholipids (e.g. soy phosphatidylcholine)	Phospholipids are deposited in between two support sheets. The barrier has thus a 'sandwich' structure. Available as ready-to-use format (innoME GmbH, Espelkamp, Germany)	(di Cagno et al., 2015; Jacobsen et al., 2020)
	Cellulose membranes (e.g. AMI-system)	e.g. regenerated cellulose (MWCO <2 kDA) (used in AMI-system)	Ready-to-use, wetted before the experiment	(P. Berben et al., 2018b)
	PDMS membrane	poly(dimethyl siloxane)	Dependant on the pdms type. available as two-part elastomer kits consisting of a polymer base and a curing agent (e.g. sylgard 184, dow corning, midland, mi, usa). available as ready-to-use membranes (e.g. silastic®)	(Garrett and Chemburkar, 1968; Sinko et al., 2017)

* Abbreviations used in Table 4: AMI, artificial membrane insert, HDM, hexadecane method, MWCO, molecular weight cut-off, PAMPA, parallel artificial membrane permeability assay, PDMS, poly(dimethylsiloxane), PVDF, polyvinylidene fluoride, PVPA, phospholipid vesicle-based permeation assay.

** Variants of PAMPA and PVPA are reviewed in (Berben et al., 2018a).

*** References solely describe drug permeability profiling applications.

Table 5

Overview of (dissolution/permeation devices/set-ups used for biopharmaceutical characterisation of formulations including their geometries and employed barriers.

Setup	Brief description	Barrier	Area [cm ²]	Volume Donor [mL]	Volume Acceptor [mL]	A/V _{Donor} [cm ⁻¹]	Reference**
96-well plate	A 'sandwich' of two 96-well plates (i.e. a bottom plate and an insert plate). The insert plate comprises the membrane.	PAMPA	0.3	–	–	–	(Bendels et al., 2006)
		PAMPA-HDM	0.3	0.2	0.3	1.5	(Miller et al., 2012a)
		Precoated PAMPA	0.3	0.3	0.2	1.0	(Beig et al., 2015)
		Permeapad®	0.15	0.2	0.4	0.75	(Nazir et al., 2020)
		Dialysis membrane (i.e. Permeapad® Plain Plate)	0.15	0.3	0.2	0.5	(Jacobsen et al., 2019b)
Reusable 96-well high-throughput dialysis device (HTD 96b)	A 96-well plate assembled of 8 PTFE blocks. Each well is vertically separated into a donor and receiver by a dialysis membrane strip.	Dialysis membrane (MWCO 12–14 kDa)	–	0.15	0.15	–	(Holmstock et al., 2013)
AMI-system	The membrane is mounted between two plastic rings. The assembly is placed in a six-well plate.	Regenerated cellulose (MWCO 2 kDa or 25 kDa)	4.91	0.7	2	7.38	(P. Berben et al., 2018c)
Membrane filter inserts	Membrane filter inserts are placed in a carrier plate (6, 12 or 24-wells)	PVPA	–	–	–	–	(Kanzer et al., 2010)
		PVPA ±mucus	–	–	–	–	(Falavigna et al., 2018)
Vertical membrane flux cell	A vessel build on the μ Diss Profiler (Pion Inc.).	PAMPA	4.90	5	10	0.98	(Stewart et al., 2017b)
Franz-cell	A donor and a receiver cell separated by a barrier horizontally	PDMS (Silastic®)	1.6	1	5	1.6	(Pellett et al., 1994)
		Permeapad®	0.64	5	1	0.13	(Volkova et al., 2018)
		Cellulose membrane (MWCO 12–14 kDa)	1	8	2	0.13	(Fong et al., 2016)
Side-by-side diffusion cells	A donor and a receiver cell separated by a barrier vertically	PAMPA	0.64	3	3	0.21	(Ruponen et al., 2018)
		Permeapad®	1.77	7	6	0.25	(Sironi et al., 2017a)
		Hydrophilic cellulose hydrate membrane	1.77	5	5	0.35	(Sironi et al., 2017b)
		Regenerated cellulose (MWCO 6–8 kDa)	7.1	30	30	0.24	(Raina et al., 2015)
μ FLUX™	A side-by-side cell set-up with broad openings for UV-probes.	PAMPA	1.54	20	20	0.08	(Tsinman et al., 2018)
		Regenerated cellulose (MWCO 1 kDa)	1.54	18	18	0.09	(Borbás et al., 2018b)
PermeaLoop®	Receiver and donor vessels connected to a pump and permeation cells with a spiral-shaped interior via tubing.	Hydrophilic cellulose hydrate membrane	27.6	20	35	1.38	(Sironi et al., 2018)
		Permeapad®	27.6	20	35	1.38	(Ilie et al., 2020)
Tangential flow absorption model (TFAM)	Based on a tangential flow filtration (TFF) unit. A dissolution vessel, a dissolution buffer reservoir, a pump and a TFF unit connected with tubing.	Stabilized cellulose based membrane (Hydrosart®; MWCO 5 kDa)	50	100	–	0.5	(Haering et al., 2020)
Dissolution vessel combined with dialysis bag	A dialysis bag (receiver) is submerged into a dissolution vessel (donor).	Regenerated cellulose tubular membrane (MWCO 50 kDa)	–	200	5	–	(Hens et al., 2015)
Hollow fibre membrane module (absorptive dissolution testing apparatus)	A dissolution vessel, a receiver, a buffer reservoir and two pumps connected with tubing to a hollow fibre membrane module.	Dialysis membrane (polyethersulfone; MWCO 10 kDa)	115	100–400	flow-through	0.29–1.15	(Hate et al., 2017)
BioFLUX	USP 1 or 2 dissolution apparatus with incorporated absorption compartment	PAMPA	3.69	250	20	0.015	(Borbás et al., 2019)
MacroFLUX™	USP 1 or 2 dissolution apparatus with incorporated absorption compartment	PAMPA	3.80	1062	13	0.004	(Borbás et al., 2018a)
UTLAM absorption system	Based on rotating membrane diffusion cell	PDMS (Sylgard 184 kit)	21.8	250	70	0.09	(Sinko et al., 2020)
TNO's TIM-1 and tiny-TIM	A multicompartiment system simulating the gastro-intestinal tract.	Hollow fibre membrane dialysis type (MWCO 5.8 kDa)	–	–	–	–	(Blanquet et al., 2004)
		Hollow fibre membrane microfiltration type (pore size 50 nm)	–	–	–	–	(Dickinson et al., 2012)
		Polysulfone plasma filter (50 nm cut-off)	–	–	–	–	(Verwei et al., 2016)

* Abbreviations used in Table 5: AMI, artificial membrane insert, HDM, hexadecane method, MWCO, molecular weight cut-off, PAMPA, parallel artificial membrane permeability assay, PDMS, poly(dimethylsiloxane), PVPA, phospholipid vesicle-based permeation assay, UTLAM, ultrathin large-area membrane.

** As reference the first publication found in literature where a given device/set-up was used with a given barrier is provided.

thus equation can be simplified to Eq. (20)

$$P_{app} = \frac{J}{A(C_D)} \quad (20)$$

Since P_{app} is normalized by the donor starting concentration and surface area, P_{app} values obtained using different concentrations and different geometries should be directly comparable (provided that sink conditions are maintained and the thickness of unstirred water layer is comparable, see below). An advantage of the single time-point approach is the low number of samples making it suitable for high-throughput applications. Disadvantages are that a single measurement does not give information about a possible lag-time (i.e. time needed to reach steady-state flux) nor at which point sink conditions are exceeded.

When setting up a cell-free permeation assay for permeability profiling, the experimental parameters should be chosen such that sink conditions are ensured. This means that over the duration of the experiment, 1) the concentration of dissolved drug in the donor compartment should be maintained and 2) the concentration of dissolved drug in the receiver compartment is virtually 'zero'. To ensure that sink conditions are maintained, the following parameters should be considered carefully: media composition, starting concentration of the drug in the donor compartment, drug solubility in the donor and acceptor media, as well as sampling timepoint(s), sampling frequency and volume. Especially when the receiver volume to barrier surface area ratio is small or the drug is poorly soluble, sink conditions may collapse rapidly resulting in non-steady state permeation rates. Addition of surfactants or albumin to the receiver can help to reduce the concentration of (molecularly) dissolved drug and thus maintain sink conditions (Bermejo et al., 2004). Also, unspecific adsorption to plastic material or membrane retention, which often is observed with poorly soluble drugs, can be reduced by addition of surfactants/albumin, as further discussed in section 5.

Other experimental parameters that generally should be considered are: temperature (Sironi et al., 2017a; Vizserálek et al., 2014), pH (Flaten et al., 2008; Jacobsen et al., 2020; Kerns et al., 2004) and mixing conditions (Avdeef et al., 2004; Borregaard Eriksen et al., 2021; Jacobsen et al., 2020). Mechanical agitation is said to reduce the thickness of the so-called 'adherent/unstirred water layer' and is conducted using shaking, stirring, or pumping. Though an influence of stirring or shaking is not always observed on permeability (within limits of barrier integrity) (Flaten et al., 2007). Considering the many relevant experimental parameters, one should keep in mind that even when using the same cell-free permeation tool, permeability values may not be comparable in an absolute manner when the experimental conditions differ.

4.3.1.2. Considerations in study design. Poorly soluble drugs represent an extraordinary challenge when determining permeability. In these cases, the drug concentration in the donor side is low leading to even lower concentrations on the receiver side, thereby challenging quantification. Some researchers have suggested adding the drug in a water-miscible vehicle (e.g. DMSO-solutions) into the aqueous donor side and/or to use surfactants to overcome poor water-solubility. Several reports indicate, however, that P_{app} values from donor solutions containing significant amounts of solubilizing additives may be lower than those obtained with merely aqueous donors (Beig et al., 2012; Fischer et al., 2011a). With the aim of increasing *in vivo* relevance, the use of artificial intestinal fluids as the donor medium has been suggested. The authors of this review, however, do not recommend calculating P_{app} values in cases, where the donor contains both molecularly dissolved and solubilized drug (see Section 4.3.2).

When permeability profiling is conducted using additives e.g.

surfactants/co-solvents, the barrier stability in the presence of these additives should be validated. Barrier stability can be studied by e.g. measuring the electrical resistance (Fischer et al., 2011a) or by measuring the permeability of 'zero' permeability markers e.g. lucifer yellow (Liu et al., 2003) or calcein (Bibi et al., 2015). The stability of PAMPA, PVPA, Permeapad® and AMI-system in the presence of co-solvents, surfactants, other excipients, and biomimetic media has previously been reviewed (Berben et al., 2018a).

4.3.1.3. Ability to predict *in vivo* permeability. To attain a reliable cell-free assay for drug permeability profiling, the range of permeability values that typically are obtained using a given tool with given experimental conditions should be established. Such a 'calibration' is conducted using low and high permeability markers and is necessary to interpret permeability data. Typically, the permeability values are orders of magnitude lower than those from intestinal perfusion experiments (Dahlgren et al., 2015) but are in the same order of magnitude as Caco-2 permeability values (Zhu et al., 2002). The applicability of permeability profiling using cell-free tools is usually demonstrated by correlating the *in vitro* permeability to f_a . Commonly, this yields a hyperbolic correlation and has been reported for PAMPA (Kansy et al., 1998; Zhu et al., 2002), PVPA (Flaten et al., 2006) and Permeapad® (Jacobsen et al., 2020). This correlation consists of two regions, a steep slope region and a plateau region where low/moderate permeability compounds ($F_a=0-80\%$) and high permeability compounds are found ($F_a=80-100\%$), respectively. Thereby, drug compounds can only be categorized broadly with respect to their permeability. The absorption potential of compounds falling within the steep slope region is especially difficult to assess due to the very broad range of f_a values found in this region.

Even though the broad categorization into low and high permeability compounds is a limitation, the low cost and easy application justifies using cell-free permeation tools for permeability profiling in drug discovery. Ready-to-use tools or tools with a simple preparation technique and small-volume permeation devices are advantageous (see Table 4, 5 and 6,). Permeation devices in 96-well format are particularly suitable for (automated) high-throughput drug permeability assays with liquid handling systems (Flaten et al., 2009).

4.3.2. Cell-free permeation tools in formulation development

Within formulation development, cell-free permeation tools are used to investigate how excipients or formulations influence permeation behaviour. For this, permeation is often combined with dissolution to mimic the complete absorption process (Berben et al., 2018a; Boyd et al., 2019; Butler et al., 2019). Whereas drug permeability profiling is a well-established application (with PAMPA being conducted routinely by many pharmaceutical companies), formulation development using cell-free permeation tools is a less established application, even though it was first described in 1973 (Lovering and Black, 1973). Recently, interest in this application is increasing with a vast number of set-ups described in literature (see Table 5 and 6).

4.3.2.1. Assay protocol. As described in Section 4.3.1., the basic (dissolution/) permeation set-up for formulation development consists of a donor and a receiver/acceptor compartment separated by a cell-free barrier. Also, the formulation performance/excipient effect is evaluated from the amount of drug reaching the receiver over a given timeframe, which may be supported by dissolution data. However, a P_{app} value typically cannot be calculated because the drug concentration in the donor is not constant during the experiment and/or the drug is present in different 'dissolved' states (e.g. complexed drug or micelle bound

Table 6

Summary of literature comparing *in vitro* (dissolution/permeation) and *in vivo* absorption. The summary includes the (dissolution/permeation) set-up/device (see also Table 5) and barrier (see also Table 4) employed, the formulation(s) studied, and the *in vivo* model used for comparison.*.

Set-up/device	Barrier	Formulation	<i>In vivo</i> model	Reference
96-well plate	Precoated PAMPA	Nanosuspension of efavirenz	rabbit	(Patel et al., 2014)
	Precoated PAMPA	Lyophilized eprosartan mesylate nanosuspension	rat	(Shekhawat and Pokharkar, 2019)
	Permeapad®	Solid mono- and diacyl phospholipid dispersions of celecoxib with high phospholipid content	rat	(Jacobsen et al., 2021)
AMI-system	Dialysis membrane (i.e. Permeapad® Plain Plate)	Tadalafil ASD (Soluplus® matrix)	rat	(Jacobsen et al., 2019b)
	Regenerated cellulose (MWCO 2 kDa or 25 kDa)	Supersaturated loviride solutions, Sporanox® (itraconazole), Lipanthyl® (fenofibrate), Lipanthynano® (fenofibrate), Noxafil® (posaconazole)	human	(P. Berben et al., 2018c)
Reusable 96-well high-throughput dialysis device (HTD 96b)	Cellulose membrane (MWCO 12–14 kDa)	Nano- and micro-sized fenofibrate (Lipanthyl® and Lipanthynano®) in fasted and fed conditions.	human	(Hens et al., 2015)
Vertical membrane flux cell	PAMPA	Sporanox® and itraconazole ASDs at different levels of SIF	rat	(Stewart et al., 2017a)
Side-by-side diffusion cells	Permeapad®	Sub- and supersaturated LCM or MCM LBDDS containing celecoxib**	rat	(Ilie et al., 2020)
	Polyether sulfone (pore size 0.03 µm)	Crystalline and amorphous dispersions of carbamazepine (Eudragit® L100 matrix)	mice	(Warnken et al., 2018)
µFLUX™	Regenerated cellulose (MWCO 6–8 kDa)	Enzalutamide ASDs (HPMC-AS and PVPVA matrix)	rat	(Wilson et al., 2018)
	PAMPA	11 weakly basic drugs in normal and hypochlorhydric gastric conditions (pH-dependant drug-drug interactions; DDI)	human	(Zhu et al., 2016)
	PAMPA	Itraconazole ASDs, nanosuspensions and Sporanox®	rat	(Tsinman et al., 2018)
	PAMPA	Six cyclic peptides in the presence or absence of Labrasol®	rat	(Gadgil et al., 2019)
	PAMPA		rat	

Table 6 (continued)

Set-up/device	Barrier	Formulation	<i>In vivo</i> model	Reference
		Fenofibrate and megestrol acetate microcrystal- and nanocrystal suspensions	human	(Imono et al., 2020)
	PAMPA	Dipyridamole suspension, ketoconazole, Sporanox® oral solution and capsule (itraconazole)	human	(O'Dwyer et al., 2020)
PermeaLoop®	Permeapad®	Sub- and supersaturated LCM or MCM LBDDS containing celecoxib **	rat	(Ilie et al., 2020)
	Permeapad®	Dipyridamole granules containing an acidic modifier (fumaric acid)	rat	(Eriksen et al., 2020)
Dissolution vessel combined with dialysis bag	A dialysis bag (receiver) is submerged into a dissolution vessel (donor).	Nano- and micro-sized fenofibrate (Lipanthyl® and Lipanthynano®) in fasted and fed conditions.	human	(Hens et al., 2015)
BioFLUX	PAMPA	Sporanox® capsule and solution, SUBA-itraconazole capsule (Lozanoc/Itragerm)	human	(Borbás et al., 2019)
MacroFLUX™	PAMPA	Micardis®, generic telmisartan products	human	(Borbás et al., 2018a)
	PAMPA	IR tablets of GDC-0810 meglumine salt, GDC-0941 dimethylate salt and Compound A (a triprotic base) in normal and hypochlorhydric gastric conditions (pH-dependant DDI)	dog, human	(Li et al., 2018)
	PAMPA	Ritonavir ASD (HPMC-AS matrix) and Norvir®	dog	(Ellenberger et al., 2018)
ENA, a combined lipolysis/permeation set-up	PAMPA	Fenofibrate LBFs**	pig	(Hedge and Bergström, 2020)
TNO's TIM-1, tiny-TIM	Hollow fibre membrane dialysis type (MWCO 5.8 kDa)	Lyophilized <i>lactobacillus</i> (Bacilor®), paracetamol IR/SR tablets	human	(Blanquet et al., 2004)
	Hollow fibre membrane microfiltration type (pore size 50 nm)	AZD8055 solutions and suspensions, AZD8055 fumarate salt suspensions and tablet	human	(Dickinson et al., 2012)
	Hollow fibre membrane microfiltration type (pore size 50 nm)	Nine AstraZeneca compounds with >2 IR formulations.	dog, human	(Barker et al., 2014)

(continued on next page)

Table 6 (continued)

Set-up/device	Barrier	Formulation	<i>In vivo</i> model	Reference
	Hollow fibre membrane microfiltration type (pore size 50 nm)	Celecoxib IR tablets (fasted/fed)	human	(Lyng et al., 2016)
	Polysulfone plasma filter (50 nm cut-off)	Ciprofloxacin IR/SR tablets (Ciprobay®; Ciproxin®), posaconazole immediate suspension (Noxafil®), nifedipine IR capsule and SR tablets (Adalat®), fenofibrate (Lipanthyl® capsule, Lipanthylnano® tablet)	human	(Verwei et al., 2016)
	Hollow fibre membrane dialysis type (MWCO 5 kDa)	Zovirax® (acyclovir) tablets in the presence or absence of chitosan	human	(Kubbinga et al., 2019)
	Polysulfone plasma filter (50 nm cut-off)	A6197 sodium salt IR/SR tablets, mini-tablets, pellets	human	(Schilderink et al., 2020)
	Polysulfone plasma filter (50 nm cut-off)	Norvir® (ritonavir) tablets in normal and hypochlorhydric gastric conditions	human	(Van Den Abele et al., 2020)

* Abbreviations used in Table 6: ASD, amorphous solid dispersion, DDI, drug-drug interaction, HDM, hexadecane method, HPMC-AS, hydroxypropyl methylcellulose acetate succinate, IR, immediate release, LBDDS, lipid-based drug delivery system, LCM, long chain mono/di-glycerides, MCM, medium chain mixed glycerides, MWCO, molecular weight cut-off, PAMPA, parallel artificial membrane permeability assay, PDMS, poly(dimethylsiloxane), PVPVA, polyvinylpyrrolidone vinyl acetate, SIF, simulated intestinal fluid, SR, sustained release.

** The *in vitro* study included lipolysis.

drug). Instead, drug accumulated amounts/concentrations from a single or several measurement(s) are compared relatively to each other (see e. g. (P. Berben et al., 2018c; Jacobsen et al., 2019b; Kanzer et al., 2010; Tsinman et al., 2018)). Alternatively, a flux value (i.e. permeability not normalized according to the donor concentration) can be estimated for specific time intervals e.g. early or late measurements (Borbás et al., 2019). As can be seen from Table 5 and 6, various set-ups/devices have been used for (dissolution/permeation) studies with focus on formulation development. These range from simple 96-well plates to intricate hollow fibre membrane module set-ups (e.g. TNO gastric small intestinal model/TIM-1). In the context of device design, the geometric flexibility and stability of cell-free barriers is an advantage. The geometry of (dissolution/permeation) studies has gained increasing attention recently. Specifically, the surface area to donor volume ratio is regarded as an important factor to obtain a dynamic interplay between dissolution and permeation (Sironi et al., 2017a, 2017b) and thus *in vivo* relevant drug absorption rates (Boyd et al., 2019; Eliassen et al., 2020; Hate et al., 2017; Sinko et al., 2020; Sironi et al., 2018). As the experimental procedure highly depends on the device/set-up, the reader is referred to references given in Table 5 and 6 for more details.

When setting up a cell-free permeation assay for formulation development, the same experimental parameters as described in Section 4.3.1. should be considered (i.e. temperature, pH, mixing, sampling, receiver additives, donor media). As compared to drug permeability

Table 7

Composition of simple salt solutions and buffers used as transport media adapted from (Ingels et al., 2007).

Component	Hanks' balanced salt solution (HBSS)		HBSS-like-transport medium (TM)		Modified Krebs Bicarbonate Ringer's Solution (KBR)	
	g/L	mM	g/L	mM	g/L	mM
CaCl ₂ •2H ₂ O	0.19	1.29	0.19	1.29	0.18	1.22
KCl	0.40	2.72	0.40	2.72	0.37	2.52
KH ₂ PO ₄	0.06	0.41	0.06	0.41		
NaH ₂ PO ₄ •H ₂ O					0.05	0.34
MgCl ₂ •6H ₂ O	0.10	0.68	0.10	0.68	0.24	1.63
MgSO ₄ •7H ₂ O	0.10	0.68	0.10	0.68		
NaCl	8.00	54.42	8.00	54.42	6.54	44.49
NaHCO ₃	0.35	2.38	0.35	2.38	2.10	14.28
Na ₂ HPO ₄	0.05	0.34	0.05	0.34		
Na ₂ HPO ₄ •2H ₂ O					0.28	1.90
D-glucose	1.00	6.80	ad 25 mM		2.07	14.08
HEPES or MES		10*	10			
NaOH			ad pH 7.4 or 6.5		ad pH 7.4	
NaGlutamate					0.83	5.65
NaPyruvate					0.54	3.67
Na ₂ Fumarate					0.86	5.85

*Standard HBSS does not contain a buffer: HEPES or MES (10 mM) should be added.

Table 8

Recommendations for transport media composition (adapted from Ingels et al., 2007). Osmotic balance and pH in the systems is maintained by HBSS+HEPES (cell-free systems), TM (cell-based systems) or KBR (tissue-based systems). The composition of HBSS, TM and KBR is provided in Table 7.

Early screening for drug permeability ranking		
Cell-free system (AMI, PAMPA, PermeaPad)	Cell-based system	Tissue-based system
Donor: 2% DMSO*, pH = 6.5 Acceptor: Sink creating excipient**, pH = 7.4	Donor: 1% DMSO*, pH = 6.5 Acceptor: 1% DMSO*, pH = 7.4	Donor: 1% DMSO***, pH = 6.5 Acceptor: 1% DMSO***, pH = 7.4
Mechanistic studies Biorelevant study		Bidirectional transport mechanism studies
Donor: FaSSIF, pH = 6.5 Acceptor: 4% Albumin or 0.5% TPGS, pH = 7.4	Donor: 1% DMSO*, pH = 7.0 Acceptor: 1% DMSO*, pH = 7.0	Donor: 1% DMSO*, pH = 5-8 Acceptor: 1% DMSO*, pH = 7.4

*DMSO 1 or 2% can be replaced by dimethylacetamide 4-5% or 1-methylpyrrolidone 2.5%.

**0.5% TPGS can also be used.

***DMSO can be replaced by 0.3% Solutol.

profiling applications, the composition of the donor medium should be considered more carefully for formulation evaluation (see Section 5.2) as indicated by recent studies comparing different levels of simulated intestinal fluids (Stewart et al., 2017a). Additionally, it should be considered if the parameters affect the excipient(s) (e.g. pH-dependant solubility). To validate a permeation assay for formulation development, a 'calibration' with high and low permeability markers is often not necessary because only a single drug is studied, and formulations are compared relatively to each other. More importantly, the stability of the cell-free barrier in presence of the excipient(s)/formulation should be validated using a suitable method (see Section 4.3.1). Furthermore, the nature of the formulation (i.e., liquid/solid, complete dosage form/down-scaled dosage form, etc.) should be considered when choosing a cell-free permeation tool and designing the assay protocol.

4.3.2.2. *Ability to predict the in vivo permeability/ correlations with in vivo data.* Commonly, the usefulness of (dissolution/permeation assays for formulation development is demonstrated by correlating the permeation data (and/or dissolution data) to *in vivo* absorption (i.e. area under the plasma concentration curve and/or maximum plasma concentration). IVIVCs have been established using various set-ups/devices by correlating to *in vivo* absorption mainly from animal experiments (see Table 6). Compared to *in vitro* testing, animal experiments are expensive and laborious. Furthermore, a reduction of animal experiments is generally desired from an ethical point of view according to the 3R principles of reduce, replace and refine, as outlined in Article 4 of EU Directive 2010/63/EU (European Parliament, 2010). One of the most prominent ways in which the EU supports the protection of animals used for scientific research is the promotion of the 3Rs principle. The principle has been widely adopted by national and international policy makers, health and research agencies, research institutions and companies. While many multinational pharmaceutical companies actively promote the 3Rs principle in drug development research, by reporting numbers of animals in testing annually, encourage implementation of the 3R initiatives and seek to promote the use of non-animal alternatives whenever possible (Zane et al., 2019), over 10 million animals are used in scientific, medical and veterinary research annually in Europe alone (European Commission, 2021). Both economically and ethically, biopredictive *in vitro* testing of formulations is therefore an attractive alternative to traditional *in vivo* methods.

5. Donor- and acceptor-media for permeability assays

5.1. Solvent systems for permeability screening of drug compounds

A crucial aspect of all classical *in vitro* permeability models is the choice of the transport medium (Ingels et al., 2007; Ingels and Augustijns, 2003). Although frequently overlooked, the donor and acceptor media properties can have a dramatic impact on the outcome of a permeability study (Aungst et al., 2000; Bibi et al., 2015; Flaten et al., 2008; Saha and Kou, 2000; Yamashita et al., 2000). Sodium taurocholate (a common component of simulated small intestinal fluids) was shown to inhibit P-gp efflux in Caco2 monolayers, hence changing the measured permeation rate of cyclosporin A considerably (Ingels et al., 2002b). In another example, Saha and Kou showed that the application of solubilizing excipients to enhance drug solubility in the donor compartment can have a variable and unpredictable effect on drug permeation in Caco2 monolayers (Saha and Kou, 2000). If the goal is to perform a biorelevant permeability study, the conditions a drug encounters during absorption *in vivo* should be replicated as closely as possible: e.g. the media used in the donor compartment should correspond to the composition of human intestinal fluids, whereas the acceptor should represent the *in vivo* acceptor accurately, which acts as sink. In practice, a myriad of technical issues prevents the implementation of this concept (Ingels et al., 2007). Some of these include incompatibility between the barrier and the donor media (e.g. bile salts can compromise the integrity of Caco-2 monolayers) (Anderberg et al., 1992; Ingels et al., 2002b), whereas a biorelevant composition of the acceptor (e.g. albumin) results in laborious procedures for sample preparation prior to analysis, which reduces throughput and reproducibility. On the other hand, if the aim is to perform a bi-directional mechanistic study (e.g. transporter characterization), then the media at both sides of the barrier should be the same.

The issues outlined above were the reason for the historical use of simple buffers (see Table 7) as transport media (Mathieu et al., 1999; Walgren and Walle, 1999). Although the use of such a simple approximation of the physiological reality facilitated the throughput of the assay and resolved issues with analysis and barrier compatibility, it still had significant disadvantages. amongst those are the adsorption and non-specific binding of the drug to the barrier and/or the walls of the experimental setup (Augustijns et al., 1993; Helen Chan et al., 1996;

Krishna et al., 2001). These issues are even more pronounced for lipophilic drugs, for which the low solubility of the drug in the transport media results in a small concentration gradient across the barrier and difficult-to-impossible maintenance of sink conditions in the acceptor. Obviously, the contrast between the simple composition of the media used and the real *in vivo* conditions was also a significant downside of the approach.

To resolve the issues encountered when simple phosphate-based buffer solutions are used as transport media in permeability screening, and to bring the experimental conditions closer to the *in vivo* situation, a plethora of donor and acceptor media have been developed. For the purpose of the current review, the major components of the transport media are classified depending on their chemical properties as: (1) co-solvents, (2) surfactants and molecular containers and (3) biorelevant additives. These modified media enhance the properties of the simple buffered solutions by offering enhanced (apparent) solubility of the drug (critical for lipophilic drugs), reduced (non-specific) adsorption and/or increased biorelevance. In the following paragraphs, the main advantages, pitfalls, and representatives of each of the classes are briefly discussed. At the end of the section, recommendations for the composition of standard transport media are presented. A comprehensive review of the transport media components, including individual concentration ranges etc. is available in the literature (Avdeef, 2012; Ingels et al., 2007; Ingels and Augustijns, 2003). One should bear in mind that similar ingredients/additives may be used in combined dissolution-/permeation-assays, i.e. in settings where the drug is not or not completely dissolved at the start of the experiment; here, however the ingredients/additives play a different role. For details see paragraphs 4.3.2 and 5.2.

5.1.1. Co-solvents

A useful way to improve the solubility of poorly water-soluble drugs is to introduce co-solvents into the solution. Significant concentrations of co-solvent (up to 20%) are usually required to impact drug solubility (Ingels et al., 2007). In addition, considering that the magnitude of the solubility-enhancing effect depends on the physicochemical properties of both the drug and the solvent, it is difficult to define a "best" co-solvent. As a result, a diversity of co-solvents systems have been explored as transport medium and their scope of application, including working concentrations, have been described in detail elsewhere (Ingels et al., 2007; Ingels and Augustijns, 2003). One of the most widely accepted approaches is the use of DMSO as a co-solvent, as it is compatible with classical cell-based permeation models (Caco-2 cells) (Ingels et al., 2007). The concentrations of DMSO that are usually used are summarized in Table 8. It should be stressed that when using co-solvents one should always carefully consider their impact on the permeation model used: cell-based models and lipid-impregnated filters (PAMPA, Permeapad etc.) can be adversely affected by co-solvents, due to their effect on membrane proteins and lipid layer integrity. Another limitation of this method is that it is even further away from biorelevant conditions: (1) co-solvents are not present *in vivo* and (2) the mechanisms of increased solubility (solubilization in intestinal colloids) and sink conditions (binding to plasma proteins and blood flow) that operate *in vivo* are fundamentally different from the polarity-based concept applied here. Also, the concentration of the co-solvent and the used drug concentration should not be borderline in respect to drug solubility (in cases where the solvent is used only at the donor or acceptor side), in order to limit the possibility for precipitation due to equilibration of solvent concentration on both sides of the membrane.

5.1.2. Surfactants and molecular containers

Another approach to mitigate the poor drug solubility in simple buffer systems and resolve the issue with maintaining sink conditions in the acceptor chamber is to use surfactants or molecular containers in the media. Although both of these increase drug solubility, their mechanisms of action are different: surfactants self-assemble in colloidal

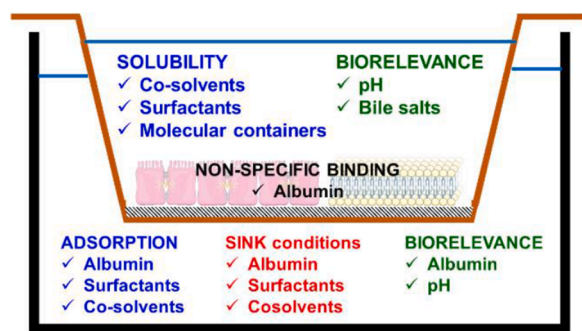


Fig. 9. Schematic overview of frequently encountered issues and proposed solutions for tissue-based, cell-based and cell-free *in vitro* permeability models (Ingels and Augustijns, 2003).

aggregates and solubilize drug molecules in the hydrophobic interior or in the palisade layer of the micelles (Vinarov et al., 2018), whereas the molecular containers – or complexing agents – are much larger molecules (oligomers) that incorporate the drug in their cavity (Fine-Shamir et al., 2017). The complexing excipients that have most frequently been used in transport media are cyclodextrins and their derivatives (Ingels et al., 2007), while more recently discovered compounds such as the acyclic cucurbit[n]urils (Ma et al., 2012) have not been evaluated yet. On the other hand, the structural diversity of the surfactants has prompted an equally diverse range of media being studied in the context of permeability assessment, including tocopherol PEG 1000 succinate (TPGS), polysorbates (Tween 20, 60, 80, 85), sodium lauryl sulfate (SLS), sodium oleate etc. In addition, the surface active polyethyleneglycol(PEG)-polypropyleneglycol(PPG) block copolymers of the Pluronic family (F68, L81 and P85) have also been evaluated.

The advantage of surfactants and complexing agents over the cosolvent approach lies in their different solubility-enhancing mechanism. The drug-surfactant or drug-complexing agent interactions required for solubilization, allow the use of typically lower concentrations of the additive. The mechanism of improving drug solubility is also closer to the drug solubilization observed *in vivo*. Drug adsorption to the walls of the experimental set-up can also be reduced in the presence of surfactants like Cremophor EL (Neuhoff, 2005; Udata et al., 2003). Nonetheless, the compatibility of these media with cell-based models should be carefully evaluated, due to the possible interactions with membrane fluidity and transporters (Ingels et al., 2007). The application of surfactants in the acceptor compartment (to achieve sink conditions), could cause analytical issues that should also be considered. Hence, surfactants which do not interfere significantly with analysis must be selected (TPGS has been shown to be useful for various drugs) (P. Berben et al., 2018b; Deferme et al., 2002; Ginski et al., 2000; Yu et al., 1999).

5.1.3. Biorelevant media: pH, intestinal fluids and proteins

5.1.3.1. pH considerations. The first item to consider when selecting a biorelevant medium for transport studies should be the pH in the donor and acceptor compartments. *In vivo*, a pH gradient exists on two sides of the intestinal barrier: the acidic microclimate on the apical side of the small intestinal epithelium is characterized by lower pH (5.8–6.3) (Lucas, 1983), compared to the pH at the basolateral side (pH = 7.4). Systematic studies have demonstrated an improved correlation to *in vivo* data when pH gradient conditions are used in the *in vitro* permeability setup, hence this is the recommended approach for ranking studies (Avdeef et al., 2007; Sugano et al., 2001; Yamashita et al., 1997).

However, the pH difference in the donor and acceptor compartments can impact the ionization and speciation of drugs with pKa in the same range (e.g. between 6 and 8), which will affect the concentration of the drug on one or both sides of the membrane, resulting in a change of the

permeation rate. This is especially problematic when performing bidirectional mechanistic studies on membrane transporters or efflux pumps and can lead to incorrect (“false efflux”) or difficult interpretation of the results (Neuhoff, 2005; Neuhoff et al., 2003; Ungell, 2002). Therefore, the use of identical pH at both sides of the barrier is advised when mechanistic studies are considered.

In terms of the pH compatibility of the barriers, the usual rule is also followed here: cell-free methods are the most robust and can sustain a wide variation of pHs (determined by the membrane material properties), followed by the lipid-impregnated membranes (acceptable pH from 4 to 10 for PAMPA) and cell-based methods (pH from 5 to 8 for Caco-2 monolayers), whereas the pH tolerance of excised tissue is not well defined (Ingels et al., 2007).

5.1.3.2. Intestinal fluid components. Bile salts are one of the main components of human intestinal fluids which are responsible for drug solubilization *in vivo*. Hence, the use of bile salts in the donor compartment was considered as a viable option to simultaneously resolve the issue with poorly water-soluble drugs and improve the biorelevance of the media. Several bile salt species and phospholipids have been shown to be compatible with lipid-impregnated membranes such as PAMPA and Permeapad® (Bibi et al., 2015; Markopoulos et al., 2013) and with cell-based models (Caco-2 cells), when passive diffusion and active absorptive transport were considered (Anderberg et al., 1992; Ingels et al., 2002b; Lo and Huang, 2000). However, care must be taken when assessing the impact of efflux pumps, as bile salts may have an inhibitory effect (Ingels et al., 2002b).

The effect of lipid digestion products on the membrane integrity of lipid-impregnated filters and cell-based models has not been systematically evaluated yet, hence one should be careful, especially considering the permeation-enhancing properties of medium-chain fatty acids on Caco-2 monolayers (Lindmark et al., 1998). The application of enzyme-containing digestion media adds another layer of complexity, in which unpredictable effects of the generated lipid digestion products can be added to those created by membrane or lipid layer degradation by lipases. Hence, due to the insufficient data available, such complex biorelevant media are currently not recommended as a best practice for standard cell-based or lipid-impregnated filter permeation studies. Similar issues could also be expected if HIF is considered as donor media, due to the presence of enzymes (and possibly lipid digestion products) therein. The viable options in cases where such highly biorelevant conditions are a priority for the permeation experiment, are either to use simpler, membrane-based permeation models (e.g. AMI (P. Berben et al., 2018b)), or more complex *ex vivo* (Ussing chamber; see Section 4.2.2) and *in situ* techniques (see Section 4.2.1).

5.1.3.3. Proteins. *In vivo*, sink conditions during drug absorption are achieved by two main factors: the constant blood flow and the binding of drug to plasma proteins. Hence, a biorelevant setup could include proteins in the acceptor compartment, which would both reduce the free drug concentration and facilitate the flux across the barrier. Serum albumin (whether present in the cell culture medium itself, or added on purpose) has been widely studied, and a concentration of 4% bovine serum albumin (BSA) has been proposed as biorelevant (Ingels et al., 2007). Although albumin decreases drug adsorption (Chan et al., 1996) and promotes drug partitioning from the cell layer to the acceptor (Krishna et al., 2001), it was also shown to affect the BCS ranking of highly lipophilic new drug candidates (Saha and Kou, 2000). In addition, the use of such high protein concentrations in the acceptor requires additional sample pre-treatment before analysis, which can impact the throughput and robustness of the method.

As an alternative to proteins, sink conditions could be maintained by using surfactants to solubilize the permeated drug molecules. A typically used surfactant in this case is 0.2–0.5% TPGS, which provides sufficient increase of drug solubility (ensuring sink conditions), often without

Table 9

Summary of advantages, disadvantages and method suitability requirements of experimental measures of permeability – adapted from (Volpe, 2010).

Class	Model	Advantages	Disadvantages	Parameters to standardize	Acceptance Criteria
Intestinal Tissue Based Methods	<i>In situ</i> perfusion	<ul style="list-style-type: none"> High biorelevance Maintains <i>in vivo</i> anatomy Can screen passive, active, efflux transport and intestinal metabolism 	<ul style="list-style-type: none"> Expensive, complex and cumbersome to perform Low throughput Limited suitability for LP molecules Requires surgery and anaesthesia Ethical considerations relating to human/animal studies 	<ul style="list-style-type: none"> Species utilised Intestinal region Perfusion buffer composition, osmolarity and pH Perfusion rate Drug analysis and Peff calculation 	<ul style="list-style-type: none"> Peff of non-absorbable marker Peff of highly permeable marker Peff of active transport marker
	<i>Ex vivo</i> tissue diffusion	<ul style="list-style-type: none"> Can utilise either human or animal tissue Retains intestinal anatomy/physiology Can screen for regional differences in permeability Can screen passive, active and efflux transport and intestinal metabolism 	<ul style="list-style-type: none"> Low throughput Maintenance of tissue viability Hydrodynamic/ flow considerations Buffer biocompatibility issues Tissue availability 	<ul style="list-style-type: none"> Animal species and age Fed/fast status of animal Anaesthesia regimen Stripped or unstripped tissue Intestinal region Time to equilibrium Diffusion buffer composition, osmolarity, and pH Monitoring of viability and integrity Oxygenation of buffer and mixing process Sink conditions and sampling method Drug analysis and Papp calculation 	<ul style="list-style-type: none"> Measure of tissue viability/integrity Transepithelial Papp of non-absorbable marker Papp of high and low permeability markers Papp of active transport marker
<i>In vitro</i>	<i>In vitro</i> cell monolayers	<ul style="list-style-type: none"> <i>In vitro</i> approach recognised by regulators (Caco-2 monolayers) Cell cultures/ co-cultures can be tailored to molecule of interest Increasing biorelevance in newer models (e.g. addition of mucus layer) Can screen passive, active and efflux transport 	<ul style="list-style-type: none"> Inter- and intra-laboratory variability Labour intensive Buffer and excipient biocompatibility Only one cell type Absence of mucous 	<ul style="list-style-type: none"> Cell clone and passage number Culture media composition Filter type, diameter, pore size Initial seeding density Feeding regimen Monolayer age Transport buffer composition and pH Transport temperature and time Co-solvent effects on cells Sink conditions and stirring process Sampling method Drug analysis and Papp calculation 	<ul style="list-style-type: none"> Measure of monolayer integrity Papp of non-absorbable marker Papp of high and low permeability markers Efflux of substrate molecule
	Cell free membranes	<ul style="list-style-type: none"> High throughput User friendly preparation Membrane composition can be tailored Increasing biorelevance of modern membrane Reduced concern regarding buffer biocompatibility 	<ul style="list-style-type: none"> Transport may vary based on membrane utilised Membrane dependant biorelevance Only measures passive diffusion 	<ul style="list-style-type: none"> Filter type, diameter, and pore size Transport buffer composition and pH Transport temperature and time Stirring process and sink conditions Sampling method Drug analysis and Papp calculation 	<ul style="list-style-type: none"> Papp of non-absorbable marker Papp of high and low permeability markers
<i>In silico</i>	QSPR Modelling approaches	<ul style="list-style-type: none"> Limited experimental data for model compound required High throughput Low cost Accessible/ open source 	<ul style="list-style-type: none"> Dataset to build model may be limited “Modelling the model of permeability” in the case of predicting P_{app} Limited ability to predict transport of carrier mediated compounds 	<ul style="list-style-type: none"> Molecular/ physicochemical descriptors utilised Modelling approach implemented e.g. MLR, PLS, HSVR Response/ output variables i.e. P_{eff}, P_{app}, F_{as}, F 	<ul style="list-style-type: none"> Correlation with training set Prediction of test/ validation set (e.g. RMSE, R^2)

significant analytical issues (Ingels et al., 2007). Another option is to use a non-specific “lipophilic sink”, as introduced in the double-sink PAMPA (DS-PAMPA) (Avdeef, 2012).

5.1.4. Recommendations

The overview provided above describes the diverse realm of possibilities, which is available when selecting transport media for a permeability study. The reason for the development of such a vast

number of options has been to tackle the various issues related to *in vitro* permeability testing (Fig. 9). While this allows for significant flexibility in the study design and facilitates the work of the experienced researcher, it is a major challenge to anyone new in the area of intestinal permeability studies. The matter is further complicated by the lack of standardised transport media, and one could easily get lost in the myriad of donor/acceptor combinations, which solve one or several issues commonly encountered during *in vitro* permeability testing. Additional

caution should be exercised when the experiment is performed at gradient conditions, where there is a difference in the chemical composition of the donor and the acceptor (e.g. solvent concentration, pH), as the equilibration kinetics and their impact on permeation have not yet been studied in detail.

To standardize the transport media composition across labs and facilitate comparative studies, a set of conditions to use in "standard" permeability studies should be available. Such a list was proposed by Ingels and co-workers, which still provides a useful guideline for setting up permeability studies in both academic and industrial conditions (Ingels et al., 2007). The purpose of the study (ranking, biorelevant or mechanistic) and the concentrations of the corresponding components in the donor and acceptor compartments are described in detail in Table 8. It should be stressed that to validate any permeation setup and to ensure comparison between different labs, reference compounds should always be characterized. For cell-free permeation methods, these should include at least one poorly and one highly permeable compound (passive diffusion). For cell- and tissue-based methods, the list could be expanded to accommodate active transport-dependant drug(s) and substrates of the efflux pumps. In a best-case scenario, a list of 10 to 20 reference compounds is suggested for model validation (FDA, 2017; Ingels et al., 2007).

Finally, the transport media suggested here may serve only as a guideline for researchers new to the field of intestinal absorption studies. To delve into the mechanisms of intestinal absorption and to provide results closer to the *in vivo* reality, it is necessary to advance the currently available experimental setups and transport media. However, one should be aware of the extensive validation that is required when any methodological changes are introduced, due to the multi-fold implications on drug permeation.

5.2. Donor-media for combined dissolution-/permeation-testing of formulations

When choosing a donor medium for combined dissolution-/permeation experiments, some groups argue that one should bear in mind, that different means to enhance the solubility of a poorly water-soluble drug impacts its permeability differently (summarised in (Buckley et al., 2013)): Additives suited to solubilize the drug compound (enhanced apparent solubility), typically do not affect or even reduce the compound's drive to overcome the membrane barrier. This holds true for non-ionic surfactants, bile salts, cyclodextrins or co-solvents (Beig et al., 2013; Fischer et al., 2011b; Frank et al., 2012b; Miller et al., 2012b, 2011). In contrast, formulations suited to generate a supersaturated state (enhanced concentration of molecularly dissolved drug) enhance the compound's drive to overcome the membrane barrier. Examples for the latter are amorphous solid dispersions, nanoparticles, weak bases within pH-modified formulations or under pH-jump conditions (Eriksen et al., 2020; Frank et al., 2012a; Sironi et al., 2017b). But, solubilizing and supersaturating conditions often co-exist and combined dissolution-/permeation can be useful to elucidate the mechanistic interplay (Frank et al., 2012a; Jacobsen et al., 2021, 2019b, 2019a) if properly designed. To this end, it is regarded counterproductive to add solubilizing agents to the donor beyond those that are part of the formulation. The only exception from this general rule are bile-salts if one wishes to mimic physiological luminal conditions. The acceptor medium should be selected such that maximum trans-barrier gradients are achieved and maintained throughout the whole experiment. In this respect the recommendations given in paragraph 5.1.4 in terms of solubilizing additives hold true for combined dissolution-/permeation setups as well.

6. Method comparability, validation and acceptance criteria

The range and variety of approaches outlined above, developed to assess drug permeability within the GIT is broad, with each approach

demonstrating its own unique characteristics and challenges. With such a range of approaches for permeability assessment, it is critical that a developed method is shown to be reliable, accurate and appropriate for the compound assessed and the likely route of absorption (Matsson et al., 2005; Volpe, 2010; Xu et al., 2021). Such characteristics are determined by the models' ability to reliably predict *in vivo* permeability, as described in the relevant section for each approach above. In addition to the ability to forecast *in vivo* permeability, sources of variation need to be considered both between and within the methods utilised. One of the most widely utilised applications for permeability assessment is in permeability class assignment (according to the BCS) in support of waivers for *in vivo* studies during drug development, as promoted by global regulators (Cardot et al., 2016; Davit et al., 2016; European Medicines Agency, 2010; FDA, 2017; ICH, 2020). According to the guidance, as directed by the seminal BCS paper by Amidon et al. (1995) high permeability is demonstrated when the fraction absorbed is >85%. Thus, while such compounds are designated as 'highly permeable', the criterion is in fact indirectly based on extent of absorption within the GIT (Benet and Larregieu, 2010), meaning that *in vivo* studies as outlined in Section 4.1 are the preferred method of determining permeability class (FDA, 2017; ICH, 2020). Such approaches allow the fullest consideration of the dynamic ADMET process *in vivo*, including regional differences in absorption, the complex luminal environment and the effects of first pass metabolism (Amidon et al., 2011; Bransford et al., 2020).

However, if permeability is to be determined via methods other than *in vivo* pharmacokinetic studies, the challenges of standardisation and method suitability are paramount. Permeability estimates for the same drugs will vary both between methods and between laboratories, regardless of the method implemented, thus it is critical that the suitability of the method is validated (Hidalgo, 2001; Ingels et al., 2002a; Volpe, 2010). This is particularly pertinent where permeability measurement via these mechanisms is used to support high permeability classification in a regulatory filing but applies also to the utility of permeability assessment during drug development. A general approach to method development, standardisation and validation was proposed by Volpe (Volpe, 2010, 2008). This approach is split in to three stages; method development, demonstration of IVIVC and permeability measurement of new compounds. The first step is to develop, optimise and parameterise the experimental method, taking into consideration the experimental variables which may affect assay performance, as outlined in the relevant section above and summarised in Table 9. In order for method suitability to be demonstrated, a range of model drugs need to be utilised to demonstrate a rank-order correlation between experimentally determined permeability values and fraction absorbed in humans. These model compounds should represent a range of permeability values, including non-absorbed markers (e.g. inulin, mannitol), low permeability (LP; e.g. famotidine, nadolol), moderate permeability (MP; e.g. furosemide, atenolol) and high permeability (HP; e.g. metoprolol, theophylline). Once the assay has been developed and demonstrated to be suitable, utilisation of the same experimental procedure allows for permeability measurement and classification of new compounds. Depending on the method utilised, additional validation may be required, such as assessment of cell monolayer or tissue integrity by means TEER measurement, or characterisation for the presence/absence or abundance of uptake or efflux transporters by means of application of known standards subject to transport via such mechanisms. Table 9 summarises the approach to methods parameterisation, the associated acceptance criteria as well as the advantages and disadvantages of *in situ*, *ex vivo*, *in vitro* and *in silico* methods of permeability screening.

7. Concluding remarks

Accurate assessment of a drugs intestinal permeability is of critical importance during the drug development process, serving to guide both

drug development and regulatory decisions. While such measurements may be inferred or estimated directly from physicochemical and molecular descriptors of molecule structure, *in vivo*, *in situ*, *ex vivo* and *in vitro* techniques continue to be the mainstay of intestinal permeability assessment. From a regulatory perspective, the concept of high permeability is intrinsically linked to the fraction of an administered dose that is absorbed and *in vivo* assessments of drug absorption or pharmacokinetic study in humans remain the gold standard in such an assessment. Over the past three decades, significant development has been made in optimising *in situ*, *ex vivo* and *in vitro* techniques as predictors of human intestinal permeability. Such models are essential tools for predictive assessment of intestinal permeability in drug development, and current trends are focussed on increasing the biomimetic nature of such models with improved clinical relevance and increased correlation with human *in vivo* data, along with the overall reduction in use of animal models in accordance with the 3Rs principle. In addition, continued refinement of *in silico* approaches and their validation with observed *in vivo* data to accurately predict drug permeability and transport mechanisms will allow for more efficient assessment in drug development. The integration of molecular and physicochemical based *in silico* modelling approaches with PBPK models will allow for more accurate prediction of drug absorption and disposition following oral administration. Overall, the use of intestinal permeability assessments has profoundly impacted the drug development and regulatory processes.

CRedit authorship contribution statement

Joseph P. O'Shea: Writing – original draft, Conceptualization, Writing – review & editing. **Patrick Augustijns:** Writing – original draft, Conceptualization, Writing – review & editing. **Martin Brandl:** Writing – original draft, Writing – review & editing. **David J. Brayden:** Writing – original draft, Writing – review & editing. **Joachim Brouwers:** Writing – original draft, Writing – review & editing. **Brendan T. Griffin:** Conceptualization, Writing – review & editing. **René Holm:** Writing – original draft, Writing – review & editing. **Ann-Christin Jacobsen:** Writing – original draft, Writing – review & editing. **Hans Lennernäs:** Writing – original draft, Writing – review & editing. **Zahari Vinarov:** Writing – original draft, Writing – review & editing. **Caitriona M. O'Driscoll:** Conceptualization, Writing – review & editing.

Acknowledgements

This article is based upon work from COST Action UNGAP (CA16205), supported by COST (European Cooperation in Science and Technology), funded by the Horizon 2020 Framework Programme of the European Union. DB is funded by the Science Foundation Ireland (SFI) CÚRAM Centre for Medical Devices, grant number 13/RC/2073_2 and also with support from the European Regional Development Fund. We thank Maciek Doczyk of the SFI CÚRAM Centre at NUI Galway for the artwork in Fig. 7 and Fig. 8. COD is funded by Science Foundation Ireland (SFI) CÚRAM Centre for Research in Medical Devices, grant number 13/RC/2073–2, by the SFI centre for Advanced Materials and BioEngineering Research, AMBER grant number 12/RC/2278 and SSPC grant number 12/RC/2275.

References

Agoni, C., Olotu, F.A., Ramharack, P., Soliman, M.E., 2020. Druggability and drug-likeness concepts in drug design: are biomodelling and predictive tools having their say? *J. Mol. Model.* <https://doi.org/10.1007/s00894-020-04385-6>.

Aguirre, T.A.S., Rosa, M., Coulter, I.S., Brayden, D.J., 2015. *In vitro* and *in vivo* preclinical evaluation of a minisphere emulsion-based formulation (SmPill®) of salmon calcitonin. *Eur. J. Pharm. Sci.* 79, 102–111. <https://doi.org/10.1016/j.ejps.2015.09.001>.

Ahmad, A., Pepin, X., Aarons, L., Wang, Y., Darwich, A.S., Wood, J.M., Tannergren, C., Karlsson, E., Patterson, C., Thörn, H., Ruston, L., Mattinson, A., Carlert, S., Berg, S., Murphy, D., Engman, H., Laru, J., Barker, R., Flanagan, T., Abrahamsson, B., Budhdeo, S., Franek, F., Moir, A., Hanisch, G., Pathak, S.M., Turner, D., Jamei, M.,

Brown, J., Good, D., Vaidhyathanan, S., Jackson, C., Nicolas, O., Beilles, S., Nguefack, J.-F., Louit, G., Henrion, L., Ollier, C., Boulu, L., Xu, C., Heimbach, T., Ren, X., Lin, W., Nguyen-Trung, A.-T., Zhang, J., He, H., Wu, F., Bolger, M.B., Mullin, J.M., van Osdol, B., Szeto, K., Korjamo, T., Pappinen, S., Tuunainen, J., Zhu, W., Xia, B., Daublain, P., Wong, S., Varma, M.V.S., Modi, S., Schäfer, K.J., Schmid, K., Lloyd, R., Patel, A., Tistaert, C., Bevernage, J., Nguyen, M.A., Lindley, D., Carr, R., Rostami-Hodjegan, A., 2020. IMI – oral biopharmaceutics tools project – evaluation of bottom-up PBPK prediction success part 4: prediction accuracy and software comparisons with improved data and modelling strategies. *Eur. J. Pharm. Biopharm.* 156, 50–63. <https://doi.org/10.1016/j.ejpb.2020.08.006>.

Ailiani, A.C., Neuberger, T., Brasseur, J.G., Banco, G., Wang, Y., Smith, N.B., Webb, A.G., 2014. Quantifying the effects of inactinvsulfurane anesthesia on gastrointestinal motility in rats using dynamic magnetic resonance imaging and spatio-temporal maps. *Neurogastroenterol. Motil.* 26, 1477–1486. <https://doi.org/10.1111/nmo.12410>.

Akamatsu, M., Fujikawa, M., Nakao, K., Shimizu, R., 2009. *In silico* prediction of human oral absorption based on QSAR analyses of PAMPA permeability. *Chem. Biodivers.* 6, 1845–1866.

Amidon, G.L., Lennernäs, H., Shah, V.P., Crison, J.R., 1995. A theoretical basis for a biopharmaceutical drug classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability. *Pharm. Res. An Off. J. Am. Assoc. Pharm. Sci.* 12, 413–420. <https://doi.org/10.1023/A:1016212804288>.

Amidon, G.L., Sinko, P.J., Fleisher, D., 1988. Estimating human oral fraction dose absorbed: a correlation using rat intestinal membrane permeability for passive and carrier-mediated compounds. *Pharm. Res.* 5, 651–654. <https://doi.org/10.1023/a:1015927004752>.

Amidon, K.S., Langguth, P., Lennernäs, H., Yu, L., Amidon, G.L., 2011. Bioequivalence of oral products and the biopharmaceutics classification system: science, regulation, and public policy. *Clin. Pharmacol. Ther.* 90, 467–470. <https://doi.org/10.1038/clpt.2011.109>.

Anderberg, E.K., Nyström, C., Artursson, P., 1992. Epithelial transport of drugs in cell culture. VII: effects of pharmaceutical surfactant excipients and bile acids on transepithelial permeability in monolayers of human intestinal epithelial (Caco-2) cells. *J. Pharm. Sci.* 81, 879–887. <https://doi.org/10.1002/jps.2600810908>.

Andrews, C.W., Bennett, L., Yu, L.X., 2000. Predicting human oral bioavailability of a compound: development of a novel quantitative structure-bioavailability relationship. *Pharm. Res.* 17, 639–644. <https://doi.org/10.1023/a:1007556711109>.

Arnold, Y.E., Thorens, J., Bernard, S., Kalia, Y.N., 2019. Drug transport across porcine intestine using an ussing chamber system: regional differences and the effect of P-glycoprotein and CYP3A4 activity on drug absorption. *Pharmaceutics* 11, 139. <https://doi.org/10.3390/pharmaceutics11030139>.

Artursson, P., 1990. Epithelial transport of drugs in cell culture. I: a model for studying the passive diffusion of drugs over intestinal absorptive (caco-2) cells. *J. Pharm. Sci.* 79, 476–482. <https://doi.org/10.1002/jps.2600790604>.

Artursson, P., Ungell, A.L., Löfroth, J.E., 1993. Selective paracellular permeability in two models of intestinal absorption: cultured monolayers of human intestinal epithelial cells and rat intestinal segments. *Pharm. Res.* 10, 1123–1129. <https://doi.org/10.1023/a:1018903931777>.

Aschenbach, J., Ahrens, F., Garz, B., Gäbel, G., 2002. Paracellular tightness and catabolism restrict histamine permeation in the proximal colon of pigs. *Pflügers Arch. Eur. J. Physiol.* 445, 115–122. <https://doi.org/10.1007/s00424-002-0897-7>.

Augustijns, P.F., Bradshaw, T.P., Gan, L.S.L., Hendren, R.W., Thakker, D.R., 1993. Evidence for a polarized efflux system in caco-2 cells capable of modulating cyclosporine a transport. *Biochem. Biophys. Res. Commun.* 197, 360–365. <https://doi.org/10.1006/bbrc.1993.2487>.

Aungst, B.J., Nguyen, N.H., Bulgarelli, J.P., Oates-Lenz, K., 2000. The influence of donor and reservoir additives on Caco-2 permeability and secretory transport of HIV protease inhibitors and other lipophilic compounds. *Pharm. Res.* 17, 1175–1180. <https://doi.org/10.1023/A:1026402410783>.

Avdeef, A., 2012. *ABSORPTION AND DRUG DEVELOPMENT: SOLUBILITY, PERMEABILITY, AND CHARGE STATE*. John Wiley & Sons.

Avdeef, A., Bendels, S., Di, L.I., Faller, B., Kansy, M., Sugano, K., Yamauchi, Y., 2007. PAMPA—critical factors for better predictions of absorption. *J. Pharm. Sci.* 96, 2893–2909. <https://doi.org/10.1002/jps.21068>.

Avdeef, A., Nielsen, P.E., Tsinman, O., 2004. PAMPA—A drug absorption *in vitro* model: 11. Matching the *in vivo* unstirred water layer thickness by individual-well stirring in microtitre plates. *Eur. J. Pharm. Sci.* 22, 365–374. <https://doi.org/10.1016/j.ejps.2004.04.009>.

Avdeef, A., Strafford, M., Block, E., Balogh, M.P., Chambliss, W., Khan, I., 2001. Drug absorption *in vitro* model: filter-immobilized artificial membranes: 2. Studies of the permeability properties of lactones in Piper methysticum Forst. *Eur. J. Pharm. Sci.* 14, 271–280. [https://doi.org/10.1016/S0928-0987\(01\)00191-9](https://doi.org/10.1016/S0928-0987(01)00191-9).

Barker, R., Abrahamsson, B., Kruusmägi, M., 2014. Application and validation of an advanced gastrointestinal *in vitro* model for the evaluation of drug product performance in pharmaceutical development. *J. Pharm. Sci.* 103, 3704–3712. <https://doi.org/10.1002/jps.24177>.

Basant, N., Gupta, S., Singh, K.P., 2016. Predicting human intestinal absorption of diverse chemicals using ensemble learning based QSAR modeling approaches. *Comput. Biol. Chem.* 61, 178–196. <https://doi.org/10.1016/j.compbiolchem.2016.01.005>.

Beaumont, C., Young, G.C., Cavalier, T., Young, M.A., 2014. Human absorption, distribution, metabolism and excretion properties of drug molecules: a plethora of approaches. *Br. J. Clin. Pharmacol.* 78, 1185–1200. <https://doi.org/10.1111/bcp.12468>.

Beig, A., Agbaria, R., Dahan, A., 2015. The use of captisol (SBE7-β-CD) in oral solubility-enabling formulations: comparison to HPβCD and the solubility-permeability

- interplay. *Eur. J. Pharm. Sci.* 77, 73–78. <https://doi.org/10.1016/j.ejps.2015.05.024>.
- Beig, A., Agbaria, R., Dahan, A., 2013. Oral delivery of lipophilic drugs: the tradeoff between solubility increase and permeability decrease when using cyclodextrin-based formulations. *PLoS One* 8, e68237. <https://doi.org/10.1371/journal.pone.0068237>.
- Beig, A., Miller, J.M., Dahan, A., 2012. Accounting for the solubility–permeability interplay in oral formulation development for poor water solubility drugs: the effect of PEG-400 on carbamazepine absorption. *Eur. J. Pharm. Biopharm.* 81, 386–391. <https://doi.org/10.1016/j.ejpb.2012.02.012>.
- Beloqui, A., Brayden, D.J., Artursson, P., Pr at, V., des Rieux, A., 2017. A human intestinal M-cell-like model for investigating particle, antigen and microorganism translocation. *Nat. Protoc.* 12, 1387–1399. <https://doi.org/10.1038/nprot.2017.041>.
- Bendels, S., Tsinman, O., Wagner, B., Lipp, D., Parrilla, I., Kansy, M., Avdeef, A., 2006. PAMPA–excipient classification gradient map. *Pharm. Res.* 23, 2525–2535. <https://doi.org/10.1007/s11095-006-9137-8>.
- Benet, L.Z., Hosey, C.M., Ursu, O., Oprea, T.I., 2016. BDDCS, the Rule of 5 and drugability. *Adv. Drug Deliv. Rev.* <https://doi.org/10.1016/j.addr.2016.05.007>.
- Benet, L.Z., Izumi, T., Zhang, Y., Silverman, J.A., Wachter, V.J., 1999. Intestinal MDR transport proteins and P-450 enzymes as barriers to oral drug delivery. *Journal of Controlled Release*.
- Benet, L.Z., Larregieu, C.A., 2010. The FDA should eliminate the ambiguities in the current BCS biowaiver guidance and make public the drugs for which BCS biowaivers have been granted. *Clin. Pharmacol. Ther.* 88, 405–407.
- Bennett-Lenane, H., O'Shea, J.P., O'Driscoll, C.M., Griffin, B.T., 2020. A retrospective biopharmaceutical analysis of >800 approved oral drug products: are drug properties of solid dispersions and lipid-based formulations distinctive? *J. Pharm. Sci.* <https://doi.org/10.1016/j.xphs.2020.08.008>.
- Berben, P., Bauer-Brandl, A., Brandl, M., Fallner, B., Flaten, G.E., Jacobsen, A.-C., Brouwers, J., Augustijns, P., 2018a. Drug permeability profiling using cell-free permeation tools: overview and applications. *Eur. J. Pharm. Sci.* 119 <https://doi.org/10.1016/j.ejps.2018.04.016>.
- Berben, P., Brouwers, J., Augustijns, P., 2018b. Assessment of passive intestinal permeability using an artificial membrane insert system. *J. Pharm. Sci.* 107, 250–256. <https://doi.org/10.1016/j.xphs.2017.08.002>.
- Berben, P., Brouwers, J., Augustijns, P., 2018c. The artificial membrane insert system as predictive tool for formulation performance evaluation. *Int. J. Pharm.* 537, 22–29. <https://doi.org/10.1016/j.ijpharm.2017.12.025>.
- Bergstr om, C.A.S., Charman, W.N., Porter, C.J.H., 2016. Computational prediction of formulation strategies for beyond-rule-of-5 compounds. *Adv. Drug Deliv. Rev.* <https://doi.org/10.1016/j.addr.2016.02.005>.
- Bergstr om, C.A.S., Holm, R., J rgensen, S.A., Andersson, S.B.E., Artursson, P., Beato, S., Borde, A., Box, K., Brewster, M., Dressman, J., Feng, K.-L., Halbert, G., Kostewicz, E., McAllister, M., Muenster, U., Thinnis, J., Taylor, R., Mullertz, A., 2014. Early pharmaceutical profiling to predict oral drug absorption: current status and unmet needs. *Eur. J. Pharm. Sci.* 57, 173–199. <https://doi.org/10.1016/j.ejps.2013.10.015>.
- Bergstr om, C.A.S., Porter, C.J.H., 2016. Understanding the challenge of beyond-rule-of-5 compounds. *Adv. Drug Deliv. Rev.* <https://doi.org/10.1016/j.addr.2016.05.016>.
- Bergstr om, C.A.S., Strafford, M., Lazorova, L., Avdeef, A., Luthman, K., Artursson, P., 2003. Absorption classification of oral drugs based on molecular surface properties. *J. Med. Chem.* 46, 558–570. <https://doi.org/10.1021/jm020986i>.
- Bermejo, M., Avdeef, A., Ruiz, A., Nalda, R., Ruell, J.A., Tsinman, O., Gonz alez, I., Fern andez, C., S anchez, G., Garrigues, T.M., Merino, V., 2004. PAMPA—a drug absorption in vitro model: 7. Comparing rat in situ, Caco-2, and PAMPA permeability of fluoroquinolones. *Eur. J. Pharm. Sci.* 21, 429–441. <https://doi.org/10.1016/j.ejps.2003.10.009>.
- Bibi, H.A., di Cagno, M., Holm, R., Bauer-Brandl, A., 2015. Permeapad™ for investigation of passive drug permeability: the effect of surfactants, co-solvents and simulated intestinal fluids (FaSSIF and FeSSIF). *Int. J. Pharm.* 493, 192–197. <https://doi.org/10.1016/j.ijpharm.2015.07.028>.
- Blanquet, S., Zeijdner, E., Beyssac, E., Meunier, J.-P., Denis, S., Havenara, R., Alric, M., 2004. A dynamic artificial gastrointestinal system for studying the behavior of orally administered drug dosage forms under various physiological conditions. *Pharm. Res.* 21, 585–591. <https://doi.org/10.1023/B:PHAM.0000022404.70478.4b>.
- Borb as, E., K ad ar, S., Tsinman, K., Tsinman, O., Csics ak, D., Tak acs-Nov ak, K., V olgyi, G., Sink o, B., Pataki, H., 2019. Prediction of bioequivalence and food effect using flux- and solubility-based methods. *Mol. Pharm.* 16, 4121–4130. <https://doi.org/10.1021/acs.molpharmaceut.9b00406>.
- Borb as, E., Nagy, Z.K., Nagy, B., Balogh, A., Farkas, B., Tsinman, O., Tsinman, K., Sink o, B., 2018a. The effect of formulation additives on in vitro dissolution-absorption profile and in vivo bioavailability of telmisartan from brand and generic formulations. *Eur. J. Pharm. Sci.* 114, 310–317. <https://doi.org/10.1016/j.ejps.2017.12.029>.
- Borb as, E., T ozs er, P., Tsinman, K., Tsinman, O., Tak acs-Nov ak, K., V olgyi, G., Sink o, B., Nagy, Z.K., 2018b. Effect of formulation additives on drug transport through size-exclusion membranes. *Mol. Pharm.* 15, 3308–3317. <https://doi.org/10.1021/acs.molpharmaceut.8b00343>.
- Borregaard Eriksen, Jonas, Jacobsen, Ann-Christin, Christensen, Katrine T ekker, Bauer-Brandl, Annette, Brandl, Martin, 2021. 'Stirred not Shaken!' Comparing Agitation Methods for Permeability Studies Using a Novel Type of 96-Well Sandwich-Plates. *Journal of Pharmaceutical Sciences* 111 (1), 32–40. <https://doi.org/10.1016/j.xphs.2021.06.006>.
- Boyd, B.J., Bergstr om, C.A.S., Vinarov, Z., Kuentz, M., Brouwers, J., Augustijns, P., Brandl, M., Bernkop-Schn ur, A., Shrestha, N., Pr at, V., M ullertz, A., Bauer-Brandl, A., Jannin, V., 2019. Successful oral delivery of poorly water-soluble drugs both depends on the intraluminal behavior of drugs and of appropriate advanced drug delivery systems. *Eur. J. Pharm. Sci.* 137, 104967 <https://doi.org/10.1016/j.ejps.2019.104967>.
- Bransford, P., Cook, J., Gupta, M., Haertter, S., He, H., Ju, R., Kanodia, J., Lennern as, H., Lindley, D., Polli, J.E., Wenning, L., Wu, Y., 2020. ICH M9 guideline in development on biopharmaceutics classification system-based biowaivers: an industrial perspective from the IQ Consortium. *Mol. Pharm.* 17, 361–372. <https://doi.org/10.1021/acs.molpharmaceut.9b01062>.
- Brouwers, J., Mols, R., Annaert, P., Augustijns, P., 2010. Validation of a differential in situ perfusion method with mesenteric blood sampling in rats for intestinal drug interaction profiling. *Biopharm. Drug Dispos.* <https://doi.org/10.1002/bdd.710> n/a/n/a.
- Buckley, S.T., B ekdal, T.A., Vegge, A., Maarbjer, S.J., Pyke, C., Ahnfelt-R onne, J., Madsen, K.G., Sch e le, S.G., Alantentalo, T., Kirk, R.K., Pedersen, B.L., Skyggebjerg, R.B., Benie, A.J., Strauss, H.M., Wahlund, P.-O., Bjerregaard, S., Farkas, E., Fekete, C., Sondergaard, F.L., Borregaard, J., Hartoft-Nielsen, M.-L., Knudsen, L.B., 2018. Transcellular stomach absorption of a derivatized glucagon-like peptide-1 receptor agonist. *Sci. Transl. Med.* 10, eaar7047. <https://doi.org/10.1126/scitranslmed.aar7047>.
- Buckley, S.T., Frank, K.J., Fricker, G., Brandl, M., 2013. Biopharmaceutical classification of poorly soluble drugs with respect to “enabling formulations”. *Eur. J. Pharm. Sci.* 50, 8–16. <https://doi.org/10.1016/j.ejps.2013.04.002>.
- Butler, J., Hens, B., Vertzoni, M., Brouwers, J., Berben, P., Dressman, J., Andreas, C.J., Schaefer, K.J., Mann, J., McAllister, M., Jamei, M., Kostewicz, E., Kesisoglou, F., Langguth, P., Minekus, M., M ullertz, A., Schilderink, R., Koziolek, M., Jedamzik, P., Weitschies, W., Reppas, C., Augustijns, P., 2019. In vitro models for the prediction of in vivo performance of oral dosage forms: recent progress from partnership through the IMI OrBiTo collaboration. *Eur. J. Pharm. Biopharm.* 136, 70–83. <https://doi.org/10.1016/j.ejpb.2018.12.010>.
- Butler, J.M., Dressman, J.B., 2010. The developability classification system: application of biopharmaceutics concepts to formulation development. *J. Pharm. Sci.* 99, 4940–4954. <https://doi.org/10.1002/jps.22217>.
- Cabrera-P erez, M.A., Pham-The, H., 2018. Computational modeling of human oral bioavailability: what will be next? *Expert Opin. Drug Discov.* <https://doi.org/10.1080/17460441.2018.1463988>.
- Cabrera-Perez, M.A., Pham-The, H., Bermejo, M., Alvarez, I.G., Alvarez, M.G., Garrigues, T.M., 2012. QSPR in oral bioavailability: specificity or integrality. *Mini Rev. Med. Chem.* 12, 534–550.
- Caldeira, T.G., Ruiz-Picazo, A., Lozoya-Agullo, I., Sa ude-Guimar es, D.A., Gonz alez- lvarez, M., de Souza, J., Gonz alez- lvarez, I., Bermejo, M., 2018. Determination of intestinal permeability using in situ perfusion model in rats: challenges and advantages to BCS classification applied to digoxin. *Int. J. Pharm.* 551, 148–157. <https://doi.org/10.1016/j.ijpharm.2018.09.022>.
- Camenisch, G., Folkers, G., van de Waterbeemd, H., 1996. Review of theoretical passive drug absorption models: historical background, recent developments and limitations. *Pharm. Acta Helv.* 71, 309–327. [https://doi.org/10.1016/S0031-6865\(96\)00031-3](https://doi.org/10.1016/S0031-6865(96)00031-3).
- Cao, X., Gibbs, S.T., Fang, L., Miller, H.A., Landowski, C.P., Shin, H.-C., Lennernas, H., Zhong, Y., Amidon, G.L., Yu, L.X., Sun, D., 2006. Why is it challenging to predict intestinal drug absorption and oral bioavailability in human using rat model. *Pharm. Res.* 23, 1675–1686. <https://doi.org/10.1007/s11095-006-9041-2>.
- Cao, X., Yu, L., Sun, D., 2008. Drug absorption principles. In: Krishna, R., Yu, L. (Eds.), *BIOPHARMACEUTICS APPLICATIONS IN DRUG DEVELOPMENT*. Springer US, Boston, MA, pp. 75–100. <https://doi.org/10.1007/978-0-387-72379-2.4>.
- Cardot, J.-M., Garcia Arieta, A., Paixao, P., Tasevska, I., Davit, B., 2016. Implementing the biopharmaceutics classification system in drug development: reconciling similarities, differences, and shared challenges in the EMA and US-FDA-recommended approaches. *AAPS J* 18, 1039–1046. <https://doi.org/10.1208/s12248-016-9915-0>.
- Chan, L.M.S., Lowes, S., Hirst, B.H., 2004. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. *Eur. J. Pharm. Sci.* 21, 25–51. <https://doi.org/10.1016/j.ejps.2003.07.003>.
- Chan, O.H., Schmid, H.L., Kuo, B.S., Wright, D.S., Howson, W., Stewart, B.H., 1996. Absorption of Cam-2445, and NK1 neurokinin receptor antagonist: in vivo, in situ, and in vitro evaluations. *J. Pharm. Sci.* 85, 253–257. <https://doi.org/10.1021/js9503338>.
- Chen, X., Murawski, A., Patel, K., Crespi, C.L., Balimane, P.V., 2008. A Novel Design of Artificial Membrane for Improving the PAMPA Model. *Pharm. Res.* 25, 1511–1520. <https://doi.org/10.1007/s11095-007-9517-8>.
- Cheng, K.-C., Li, C., Uss, A.S., 2008. Prediction of oral drug absorption in humans – from cultured cell lines and experimental animals. *Expert Opin. Drug Metab. Toxicol.* 4, 581–590. <https://doi.org/10.1517/17425255.4.5.581>.
- Cheng, W.-P., Thompson, C., Ryan, S.M., Aguirre, T., Tetley, L., Brayden, D.J., 2010. In vitro and in vivo characterisation of a novel peptide delivery system: amphiphilic polyelectrolyte–salmon calcitonin nanocomplexes. *J. Control. Release* 147, 289–297. <https://doi.org/10.1016/j.jconrel.2010.07.128>.
- Clarke, L.L., 2009. A guide to Ussing chamber studies of mouse intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.* 296, G1151–G1166. <https://doi.org/10.1152/ajpgi.90649.2008>.
- Conradi, R.A., Burton, P.S., Borchardt, R.T., 1996. Physico-chemical and biological factors that influence a drug's cellular permeability by passive diffusion. *БИОФИЗИКА В ДРУГ АКТІОН АНД ТОКСІСОЛОГІ*. Wiley Blackwell, pp. 233–252. <https://doi.org/10.1002/9783527614998.ch14>.
- Coppola, P., Anderson, A., Cole, S., 2019. The importance of the human mass balance study in regulatory submissions. *CPT Pharmacometrics Syst. Pharmacol.* 8, 792–804. <https://doi.org/10.1002/psp4.12466>.

- Curatolo, W., 1987. The lipoidal permeability barriers of the skin and alimentary tract. *Pharm. Res.* 4, 271–277. <https://doi.org/10.1023/a:1016432817415>.
- Dahlgren, D., Lennernäs, H., 2019. Intestinal permeability and drug absorption: predictive experimental, computational and in vivo approaches. *Pharmaceutics* 11, 411. <https://doi.org/10.3390/pharmaceutics11080411>.
- Dahlgren, D., Roos, C., Johansson, P., Tannergren, C., Lundqvist, A., Langguth, P., Sjöblom, M., Sjögren, E., Lennernäs, H., 2018. The effects of three absorption-modifying critical excipients on the in vivo intestinal absorption of six model compounds in rats and dogs. *Int. J. Pharm.* 547, 158–168. <https://doi.org/10.1016/j.ijpharm.2018.05.029>.
- Dahlgren, D., Roos, C., Peters, K., Lundqvist, A., Tannergren, C., Sjögren, E., Sjöblom, M., Lennernäs, H., 2019. Evaluation of drug permeability calculation based on luminal disappearance and plasma appearance in the rat single-pass intestinal perfusion model. *Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft für Pharm. Verfahrenstechnik e.V* 142, 31–37. <https://doi.org/10.1016/j.ejpb.2019.06.011>.
- Dahlgren, D., Roos, C., Sjögren, E., Lennernäs, H., 2015. Direct in vivo human intestinal permeability (peff) determined with different clinical perfusion and intubation methods. *J. Pharm. Sci.* 104, 2702–2726. <https://doi.org/10.1002/jps.24258>.
- Daugherty, A.L., Mrsny, R.J., 1999. Transcellular uptake mechanisms of the intestinal epithelial barrier Part one. *Pharm. Sci. Technol. Today* 2, 144–151. [https://doi.org/10.1016/S1461-5347\(99\)00142-X](https://doi.org/10.1016/S1461-5347(99)00142-X).
- Davies, M., Jones, R.D.O., Grime, K., Jansson-Löfmark, R., Fretland, A.J., Winiwarer, S., Morgan, P., McGinnity, D.F., 2020. Improving the accuracy of predicted human pharmacokinetics: lessons learned from the astrazeneca drug pipeline over two decades. *Trends Pharmacol. Sci.* 41, 390–408. <https://doi.org/10.1016/j.tips.2020.03.004>.
- Davis, S.S., Wilding, I.R., 2001. Oral drug absorption studies: the best model for man is man! *Drug Discov. Today* 6, 127–130. [https://doi.org/10.1016/S1359-6446\(00\)01653-6](https://doi.org/10.1016/S1359-6446(00)01653-6).
- Davit, B.M., Kanfer, I., Tsang, Y.C., Cardot, J.-M., 2016. BCS bio waivers: similarities and differences among EMA, FDA, and WHO requirements. *AAPS J* 18, 612–618. <https://doi.org/10.1208/s12248-016-9877-2>.
- Deferme, S., Van Gelder, J., Ingels, F., Van den Mooter, G., De Buck, S., Balzarini, J., Naesens, L., De Clercq, E., Kinget, R., Augustijns, P., 2002. Intestinal absorption characteristics of the low solubility thioacarboxanilide UC-781. *Int. J. Pharm.* 234, 113–119. [https://doi.org/10.1016/S0378-5173\(01\)00952-8](https://doi.org/10.1016/S0378-5173(01)00952-8).
- DeGoey, D.A., Chen, H.-J., Cox, P.B., Wendt, M.D., 2018. Beyond the rule of 5: lessons learned from AbbVie's drugs and compound collection. *J. Med. Chem.* 61, 2636–2651. <https://doi.org/10.1021/acs.jmedchem.7b00717>.
- Dezani, T.M., Dezani, A.B., de Silva, M.M.C., dos Reis Serra, C.H., 2017. In situ intestinal perfusion in rodents: future perspectives for application on absorption studies and classification of drugs. *Mini-Reviews Med. Chem.* 17, 746–757. <https://doi.org/10.2174/1389557516666160921145613>.
- di Cagno, M., Bibi, H.A., Bauer-Brandl, A., 2015. New biomimetic barrier Permeapad™ for efficient investigation of passive permeability of drugs. *Eur. J. Pharm. Sci.* 73, 29–34. <https://doi.org/10.1016/j.ejps.2015.03.019>.
- Di, L., Artursson, P., Avdeef, A., Benet, L.Z., Houston, J.B., Kansy, M., Kerns, E.H., Lennernäs, H., Smith, D.A., Sugano, K., 2020. The critical role of passive permeability in designing successful drugs. *ChemMedChem* 15, 1862–1874. <https://doi.org/10.1002/cmdc.202000419>.
- Di, L., Artursson, P., Avdeef, A., Ecker, G.F., Fallner, B., Fischer, H., Houston, J.B., Kansy, M., Kerns, E.H., Krämer, S.D., Lennernäs, H., Sugano, K., 2012. Evidence-based approach to assess passive diffusion and carrier-mediated drug transport. *Drug Discov. Today* 17, 905–912. <https://doi.org/10.1016/j.drudis.2012.03.015>.
- Diamond, J.M., Wright, E.M., 1969. Molecular forces governing non-electrolyte permeation through cell membranes. *Proc. R. Soc. London. Ser. B. Biol. Sci.* 172, 273–316. <https://doi.org/10.1098/rspb.1969.0022>.
- Dickinson, P.A., Abu Rmaileh, R., Ashworth, L., Barker, R.A., Burke, W.M., Patterson, C. M., Stainforth, N., Yasin, M., 2012. An investigation into the utility of a multi-compartmental, dynamic, system of the upper gastrointestinal tract to support formulation development and establish bioequivalence of poorly soluble drugs. *AAPS J* 14, 196–205. <https://doi.org/10.1208/s12248-012-9333-x>.
- Ditzinger, F., Price, D.J., Ilie, A.-R., Köhl, N.J., Jankovic, S., Tsakiridou, G., Aleandri, S., Kalantzi, L., Holm, R., Nair, A., Saal, C., Griffin, B., Kuentz, M., 2019. Lipophilicity and hydrophobicity considerations in bio-enabling oral formulations approaches - a PEARRL review. *J. Pharm. Pharmacol.* 71, 464–482. <https://doi.org/10.1111/jph.12984>.
- Dubbelboer, I.R., Dahlgren, D., Sjögren, E., Lennernäs, H., 2019. Rat intestinal drug permeability: a status report and summary of repeated determinations. *Eur. J. Pharm. Biopharm.* 142, 364–376. <https://doi.org/10.1016/j.ejpb.2019.07.005>.
- Effinger, A., O'Driscoll, C.M., McAllister, M., Fotaki, N., 2018. *Vitro and In Silico ADME Prediction. ADME Processes in Pharmaceutical Sciences.* Springer International Publishing, pp. 301–330. https://doi.org/10.1007/978-3-319-99593-9_13.
- Egan, W.J., Lauri, G., 2002. Prediction of intestinal permeability. *Adv. Drug Deliv. Rev.* 54, 273–289. [https://doi.org/10.1016/S0169-409X\(02\)00004-2](https://doi.org/10.1016/S0169-409X(02)00004-2).
- Eliassen, J.N., Berthelsen, R., Slot, A.L., Müllertz, A., 2020. Evaluating side-by-side diffusion models for studying drug supersaturation in an absorptive environment: a case example of fenofibrate and felodipine. *J. Pharm. Pharmacol.* 72, 371–384. <https://doi.org/10.1111/jph.13218>.
- Ellenberg, D.J., Miller, D.A., Kucera, S.U., Williams, R.O., 2018. Generation of a weakly acidic amorphous solid dispersion of the weak base ritonavir with equivalent in vitro and in vivo performance to norvir tablet. *AAPS PharmSciTech* 19, 1985–1997. <https://doi.org/10.1208/s12249-018-1060-x>.
- Eriksen, J.B., Messerschmid, R., Andersen, M.L., Wada, K., Bauer-Brandl, A., Brandl, M., 2020. Dissolution/permeation with PermeaLoop™: experience and IVIVC exemplified by dipyrindamole enabling formulations. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 154, 105532. <https://doi.org/10.1016/j.ejps.2020.105532>.
- Esaki, T., Ohashi, R., Watanabe, R., Natsume-Kitatani, Y., Kawashima, H., Nagao, C., Komura, H., Mizuguchi, K., 2019. Constructing an in silico three-class predictor of human intestinal absorption with Caco-2 permeability and dried-DMSO solubility. *J. Pharm. Sci.* 108, 3630–3639.
- European Commission, 2021. Summary Report on the statistics on the use of animals for scientific purposes in the Member States of the European Union and Norway in 2018.
- European Medicines Agency, 2010. CPMP/EWP/QWP/1401/98 Rev. 1 Committee for medicinal products for human use (CHMP) guideline on the investigation of bioequivalence.
- European Parliament, 2010. DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010 on the protection of animals used for scientific purposes.
- Fagerholm, U., Hellberg, S., Spjuth, O., 2021. Advances in predictions of oral bioavailability of candidate drugs in man with new machine learning methodology. *Molecules* 26. <https://doi.org/10.3390/molecules26092572>.
- Fagerholm, U., Johansson, M., Lennernäs, H., 1996. Comparison Between Permeability Coefficients in Rat and Human Jejunum. *Pharm. Res.* 13, 1336–1342. <https://doi.org/10.1023/A:1016065715308>.
- Fagerholm, U., Nilsson, D., Knutson, L., Lennernäs, H., 1999. Jejunal permeability in humans in vivo and rats in situ: investigation of molecular size selectivity and solvent drag. *Acta Physiol. Scand.* 165, 315–324. <https://doi.org/10.1046/j.1365-201x.1999.00510.x>.
- Falavigna, M., Klitgaard, M., Brase, C., Ternullo, S., Škalco-Basnet, N., Flaten, G.E., 2018. Mucus-PVPA (mucus phospholipid vesicle-based permeation assay): an artificial permeability tool for drug screening and formulation development. *Int. J. Pharm.* 537, 213–222. <https://doi.org/10.1016/j.ijpharm.2017.12.038>.
- Fattah, S., Ismaiel, M., Murphy, B., Rulikowska, A., Frias, J.M., Winter, D.C., Brayden, D. J., 2020. Salcaprozate sodium (SNAC) enhances permeability of octreotide across isolated rat and human intestinal epithelial mucosae in Ussing chambers. *Eur. J. Pharm. Sci.* 154, 105509. <https://doi.org/10.1016/j.ejps.2020.105509>.
- FDA, 2019. Bioavailability Studies Submitted in NDAs or INDs-General Considerations Guidance for Industry.
- FDA, 2017. Waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system guidance for industry.
- Feighery, L.M., Cochrane, S.W., Quinn, T., Baird, A.W., O'Toole, D., Owens, S.-E., O'Donoghue, D., Mrsny, R.J., Brayden, D.J., 2008. Myosin light chain kinase inhibition: correction of increased intestinal epithelial permeability in vitro. *Pharm. Res.* 25, 1377–1386. <https://doi.org/10.1007/s11095-007-9527-6>.
- Fine-Shamir, N., Beig, A., Zur, M., Lindley, D., Miller, J.M., Dahan, A., 2017. Toward successful cyclodextrin based solubility-enabling formulations for oral delivery of lipophilic drugs: solubility-permeability trade-off, biorelevant dissolution, and the unstirred water layer. *Mol. Pharm.* 14, 2138–2146. <https://doi.org/10.1021/acs.molpharmaceut.7b00275>.
- Fischer, S.M., Brandl, M., Fricker, G., 2011a. Effect of the non-ionic surfactant Poloxamer 188 on passive permeability of poorly soluble drugs across Caco-2 cell monolayers. *Eur. J. Pharm. Biopharm.* 79, 416–422. <https://doi.org/10.1016/j.ejpb.2011.04.010>.
- Fischer, S.M., Flaten, G.E., Hagesæther, E., Fricker, G., Brandl, M., 2011b. In-vitro permeability of poorly water soluble drugs in the phospholipid vesicle-based permeation assay: the influence of nonionic surfactants. *J. Pharm. Pharmacol.* 63, 1022–1030. <https://doi.org/10.1111/j.2042-7158.2011.01301.x>.
- Flanagan, T., Van Peer, A., Lindahl, A., 2016. Use of physiologically relevant biopharmaceutics tools within the pharmaceutical industry and in regulatory sciences: where are we now and what are the gaps? *Eur. J. Pharm. Sci.* 91, 84–90. <https://doi.org/10.1016/j.ejps.2016.06.006>.
- Flaten, G.E., Awoyemi, O., Luthman, K., Brandl, M., Massing, U., 2009. The phospholipid vesicle-based drug permeability assay: 5. development toward an automated procedure for high-throughput permeability screening. *JALA J. Assoc. Lab. Autom.* 14, 12–21. <https://doi.org/10.1016/j.jala.2008.04.002>.
- Flaten, G.E., Dhanikula, A.B., Luthman, K., Brandl, M., 2006. Drug permeability across a phospholipid vesicle based barrier: a novel approach for studying passive diffusion. *Eur. J. Pharm. Sci.* 27, 80–90. <https://doi.org/10.1016/j.ejps.2005.08.007>.
- Flaten, G.E., Luthman, K., Vasskog, T., Brandl, M., 2008. Drug permeability across a phospholipid vesicle-based barrier: 4. The effect of tensides, co-solvents and pH changes on barrier integrity and on drug permeability. *Eur. J. Pharm. Sci.* 34, 173–180. <https://doi.org/10.1016/j.ejps.2008.04.001>.
- Flaten, G.E., Skar, M., Luthman, K., Brandl, M., 2007. Drug permeability across a phospholipid vesicle based barrier: 3. Characterization of drug-membrane interactions and the effect of agitation on the barrier integrity and on the permeability. *Eur. J. Pharm. Sci.* 30, 324–332. <https://doi.org/10.1016/j.ejps.2006.11.017>.
- Fong, S.Y.K., Martins, S.M., Brandl, M., Bauer-Brandl, A., 2016. Solid phospholipid dispersions for oral delivery of poorly soluble drugs: investigation into celecoxib incorporation and solubility-in vitro permeability enhancement. *J. Pharm. Sci.* 105, 1113–1123. [https://doi.org/10.1016/S0022-3549\(15\)00186-0](https://doi.org/10.1016/S0022-3549(15)00186-0).
- Fomer, K., Holm, R., Morakul, B., Junyaprasert Varaporn, B., Ackermann, M., Mazur, J., Langguth, P., 2017. Dissolution and dissolution/permeation experiments for predicting systemic exposure following oral administration of the BCS class II drug clarithromycin. *Eur. J. Pharm. Sci.* 101, 211–219. <https://doi.org/10.1016/j.ejps.2017.02.003>.
- Fomer, K., Roos, C., Dahlgren, D., Kesiosoglou, F., Konerding, M.A., Mazur, J., Lennernäs, H., Langguth, P., 2016. Optimization of the Ussing chamber setup with excised rat intestinal segments for dissolution/permeation experiments of poorly

- soluble drugs. *Drug Dev. Ind. Pharm.* 43, 338–346. <https://doi.org/10.1080/03639045.2016.1251449>.
- Frank, K.J., Rosenblatt, K.M., Westedt, U., Hölig, P., Rosenberg, J., Mägerlein, M., Fricker, G., Brandl, M., 2012a. Amorphous solid dispersion enhances permeation of poorly soluble ABT-102: true supersaturation vs. apparent solubility enhancement. *Int. J. Pharm.* 437, 288–293. <https://doi.org/10.1016/j.ijpharm.2012.08.014>.
- Frank, K.J., Westedt, U., Rosenblatt, K.M., Hölig, P., Rosenberg, J., Mägerlein, M., Brandl, M., Fricker, G., 2012b. Impact of FaSSiF on the solubility and dissolution/permeation rate of a poorly water-soluble compound. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 47, 16–20. <https://doi.org/10.1016/j.ejps.2012.04.015>.
- Fujiwara, S.I., Yamashita, F., Hashida, M., 2002. Prediction of Caco-2 cell permeability using a combination of MO-calculation and neural network. *Int. J. Pharm.* 237, 95–105. [https://doi.org/10.1016/S0378-5173\(02\)00045-5](https://doi.org/10.1016/S0378-5173(02)00045-5).
- Gadgil, P., Alleyne, C., Feng, K.-I., Hu, M., Gindy, M., Buevich, A.V., Fauty, S., Salituro, G., Wen, J., Li, Y., Nofsinger, R., Sawyer, T.K., Buist, N., 2019. Assessing the utility of in vitro screening tools for predicting bio-performance of oral peptide delivery. *Pharm. Res.* 36, 151. <https://doi.org/10.1007/s11095-019-2682-8>.
- Garrett, E.R., Chemburkar, P.B., 1968. Evaluation, control, and prediction of drug diffusion through polymeric membranes III. Diffusion of barbiturates, phenylalkylamines, dextromethorphan, progesterone, and other drugs. *J. Pharm. Sci.* 57, 1401–1409. <https://doi.org/10.1002/jps.2600570828>.
- Ghose, A.K., Crippen, G.M., 1987. Atomic physicochemical parameters for three-dimensional-structure-directed quantitative structure-activity relationships. 2. Modeling dispersive and hydrophobic interactions. *J. Chem. Inf. Comput. Sci.* 27, 21–35. <https://doi.org/10.1021/ci00053a005>.
- Giacomini, K.M., Huang, S.-M., Tweedie, D.J., Benet, L.Z., Brouwer, K.L.R., Chu, X., Dahlin, A., Evers, R., Fischer, V., Hillgren, K.M., Hoffmaster, K.A., Ishikawa, T., Keppler, D., Kim, R.B., Lee, C.A., Niemi, M., Polli, J.W., Sugiyama, Y., Swaan, P.W., Ware, J.A., Wright, S.H., Yee, S.W., Zamek-Gliszczynski, M.J., Zhang, L., 2010. Membrane transporters in drug development. *Nat. Rev. Drug Discov.* 9, 215–236. <https://doi.org/10.1038/nrd3028>.
- Ginski, M.J., Gadre, A., Vatsaraj, N., Grover, U., Rhodes, C., 2000. Evaluation of cosolvent systems to facilitate Caco-2 permeability screening of poorly water soluble drug candidates. In: *AAPS Annual Meeting*.
- Gleeson, J.P., Brayden, D.J., Ryan, S.M., 2017. Evaluation of PepT1 transport of food-derived antihypertensive peptides, Ile-Pro-Pro and Leu-Lys-Pro using in vitro, ex vivo and in vivo transport models. *Eur. J. Pharm. Biopharm.* 115, 276–284. <https://doi.org/10.1016/j.ejpb.2017.03.007>.
- Glynn, A., Igra, A.M., Sand, S., Ilbäck, N.G., Hellenäs, K.E., Rosén, J., Aspenström-Fagerlund, B., 2017. Are additional effects of dietary surfactants on intestinal tight junction integrity an overlooked human health risk? – A mixture study on Caco-2 monolayers. *Food Chem. Toxicol.* 106, 314–323. <https://doi.org/10.1016/j.fct.2017.05.068>.
- Goetz, G.H., Shalaeva, M., Caron, G., Ermondi, G., Philippe, L., 2017. Relationship between passive permeability and molecular polarity using block relevance analysis. *Mol. Pharm.* 14, 386–393. <https://doi.org/10.1021/acs.molpharmaceut.6b00724>.
- Gozalbes, R., Jaciewicz, M., Annand, R., Tsaïoun, K., Pineda-Lucena, A., 2011. QSAR-based permeability model for drug-like compounds. *Bioorganic Med. Chem.* 19, 2615–2624. <https://doi.org/10.1016/j.bmc.2011.03.011>.
- Gramatté, T., 1994. Griseofulvin absorption from different sites in the human small intestine. *Biopharm. Drug Dispos.* 15, 747–759. <https://doi.org/10.1002/bdd.2510150903>.
- Gramatté, T., el Desoky, E., Klotz, U., 1994. Site-dependent small intestinal absorption of ranitidine. *Eur. J. Clin. Pharmacol.* 46, 253–259. <https://doi.org/10.1007/BF00192558>.
- Gramatté, T., Oertel, R., 1999. Intestinal secretion of intravenous talinolol is inhibited by luminal R-verapamil. *Clin. Pharmacol. Ther.* 66, 239–245. [https://doi.org/10.1016/S0009-9236\(99\)70031-7](https://doi.org/10.1016/S0009-9236(99)70031-7).
- Gramatté, T., Richter, K., 1994. Paracetamol absorption from different sites in the human small intestine. *Br. J. Clin. Pharmacol.* 37, 608–611. <https://doi.org/10.1111/j.1365-2125.1994.tb04312.x>.
- Griffin, B., O'Driscoll, C., 2007. Models of the Small Intestine, in: *Drug Absorption Studies*. Springer US 34–76. https://doi.org/10.1007/978-0-387-74901-3_2.
- Haering, B., Seyferth, S., Schiffter, H.A., 2020. The tangential flow absorption model (TFAM) – A novel dissolution method for evaluating the performance of amorphous solid dispersions of poorly water-soluble actives. *Eur. J. Pharm. Biopharm.* 154, 74–88. <https://doi.org/10.1016/j.ejpb.2020.06.013>.
- Hanafy, A., Langguth, P., Spahn-Langguth, H., 2001. Pretreatment with potent P-glycoprotein ligands may increase intestinal secretion in rats. *Eur. J. Pharm. Sci.* 12, 405–415. [https://doi.org/10.1016/S0928-0987\(00\)00195-0](https://doi.org/10.1016/S0928-0987(00)00195-0).
- Harwood, M.D., Neuhoof, S., Carlson, G.L., Warhurst, G., Rostami-Hodjegan, A., 2013. Absolute abundance and function of intestinal drug transporters: a prerequisite for fully mechanistic in vitro-in vivo extrapolation of oral drug absorption. *Biopharm. Drug Dispos.* <https://doi.org/10.1002/bdd.1810>.
- Hate, S.S., Reutzel-Edens, S.M., Taylor, L.S., 2017. Absorptive Dissolution Testing of Supersaturating Systems: impact of Absorptive Sink Conditions on Solution Phase Behavior and Mass Transport. *Mol. Pharm.* 14, 4052–4063. <https://doi.org/10.1021/acs.molpharmaceut.7b00740>.
- Hayeshi, R., Hilgendorf, C., Artursson, P., Augustijns, P., Brodin, B., Dehertogh, P., Fisher, K., Fossati, L., Hovenkamp, E., Korjamo, T., Masungi, C., Maubon, N., Mols, R., Müllert, A., Mönkkönen, J., O'Driscoll, C., Oppers-Tiemissen, H.M., Ragnarsson, E.G.E., Rooseboom, M., Ungell, A.-L., 2008. Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. *Eur. J. Pharm. Sci.* 35, 383–396. <https://doi.org/10.1016/j.ejps.2008.08.004>.
- Hedge, O.J., Bergström, C.A.S., 2020. Suitability of Artificial Membranes in Lipolysis-Permeation Assays of Oral Lipid-Based Formulations. *Pharm. Res.* 37, 99. <https://doi.org/10.1007/s11095-020-02833-9>.
- Helander, H.F., Fändriks, L., 2014. Surface area of the digestive tract-revisited. *Scand. J. Gastroenterol.* 49, 681–689. <https://doi.org/10.3109/00365521.2014.898326>.
- Helen Chan, O., Schmid, H.L., Kuo, B.-S., Scott Wright, D., Howson, W., Stewart, B.H., 1996. Absorption of Cam-2445, an NK1 neurokinin receptor antagonist: in vivo, in situ, and in vitro evaluations. *J. Pharm. Sci.* 85, 253–257. <https://doi.org/10.1021/js9503338>.
- Hens, B., Brouwers, J., Corsetti, M., Augustijns, P., 2015. Gastrointestinal behavior of nano- and micro-sized fenofibrate: in vivo evaluation in man and in vitro simulation by assessment of the permeation potential. *Eur. J. Pharm. Sci.* 77, 40–47. <https://doi.org/10.1016/j.ejps.2015.05.023>.
- Hidalgo, L.J., 2001. Assessing the absorption of new pharmaceuticals. *Curr. Top. Med. Chem.* 1, 385–401. <https://doi.org/10.2174/1568026013395010>.
- Hidalgo, L.J., Hillgren, K.M., Grass, G.M., Borchardt, R.T., 1991. Characterization of the unstirred water layer in Caco-2 cell monolayers using a novel diffusion apparatus. *Pharm. Res.* 8, 222–227. <https://doi.org/10.1023/A:1015848205447>.
- Hidalgo, L.J., Raub, T.J., Borchardt, R.T., 1989. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 96, 736–749. [https://doi.org/10.1016/0016-5085\(89\)90897-4](https://doi.org/10.1016/0016-5085(89)90897-4).
- Hilgers, A.R., Conradi, R.A., Burton, P.S., 1990. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. *Pharm. Res.* 7, 902–910. <https://doi.org/10.1023/a:1015937605100>.
- Ho, N.F.H., Higuchi, W.I., 1974. Theoretical model studies of intestinal drug absorption IV: bile acid transport at premicellar concentrations across diffusion layer-membrane barrier. *J. Pharm. Sci.* 63, 686–690. <https://doi.org/10.1002/jps.2600630508>.
- Ho, N.F.H., Park, J.Y., Morozowich, W., Higuchi, W.I., 1977. Physical model approach to the design of drugs with improved intestinal absorption. *Des. Biopharm. Prop. through prodrugs Analog.* 136–227.
- Holmstock, N., Bruyn, T.De, Bevernage, J., Annaert, P., Mols, R., Tack, J., Augustijns, P., 2013. Exploring food effects on indinavir absorption with human intestinal fluids in the mouse intestine. *Eur. J. Pharm. Sci.* 49, 27–32. <https://doi.org/10.1016/j.ejps.2013.01.012>.
- Hopkins, A.M., McDonnell, C., Breslin, N.P., O'Morain, C.A., Baird, A.W., 2002. Omeprazole increases permeability across isolated rat gastric mucosa pre-treated with an acid secretagogue. *J. Pharm. Pharmacol.* 54, 341–347. <https://doi.org/10.1211/0022357021778583>.
- Hou, T., Wang, J., Zhang, W., Wang, W., Xu, X., 2006. Recent advances in computational prediction of drug absorption and permeability in drug discovery. *Curr. Med. Chem.* 13, 2653–2667. <https://doi.org/10.2174/092986706778201558>.
- Hou, T., Wang, J., Zhang, W., Xu, X., 2007. ADME evaluation in drug discovery. 6. Can oral bioavailability in humans be effectively predicted by simple molecular property-based rules? *J. Chem. Inf. Model.* 47, 460–463. <https://doi.org/10.1021/ci6003515>.
- Huang, J., Guo, L., Tan, R., Wei, M., Zhang, J., Zhao, Y., Gong, L., Huang, Z., Qiu, X., 2018. Interactions between emodin and efflux transporters on rat enterocyte by a validated using chamber technique. *Front. Pharmacol.* 9, 646. <https://doi.org/10.3389/fphar.2018.00646>.
- Hubatsch, I., Ragnarsson, E.G.E., Artursson, P., 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat. Protoc.* 2, 2111–2119. <https://doi.org/10.1038/nprot.2007.303>.
- ICH, 2020. ICH M9 guideline on biopharmaceutics classification system-based biowaivers.
- Ilie, A.-R., Griffin, B.T., Brandl, M., Bauer-Brandl, A., Jacobsen, A.-C., Vertzoni, M., Kuentz, M., Kolakovic, R., Holm, R., 2020. Exploring impact of supersaturated lipid-based drug delivery systems of celecoxib on in vitro permeation across Permeapad® membrane and in vivo absorption. *Eur. J. Pharm. Sci.* 152, 105452. <https://doi.org/10.1016/j.ejps.2020.105452>.
- Imono, M., Uchiyama, H., Yoshida, S., Miyazaki, S., Tamura, N., Tsutsumimoto, H., Kadota, K., Tozuka, Y., 2020. The elucidation of key factors for oral absorption enhancement of nanocrystal formulations: in vitro-in vivo correlation of nanocrystals. *Eur. J. Pharm. Biopharm.* 146, 84–92. <https://doi.org/10.1016/j.ejpb.2019.12.002>.
- Ingels, F., Defemme, S., Delbar, N., Oth, M., Augustijns, P., 2002a. Implementation of the caco-2 cell culture model as a predictive tool for the oral absorption of drugs. In: *House evaluation procedures*. *J. Pharm. Belg.* 57, 153–158.
- Ingels, F., Defemme, S., Destexhe, E., Oth, M., Van den Mooter, G., Augustijns, P., 2002b. Simulated intestinal fluid as transport medium in the Caco-2 cell culture model. *Int. J. Pharm.* 232, 183–192. [https://doi.org/10.1016/S0378-5173\(01\)00897-3](https://doi.org/10.1016/S0378-5173(01)00897-3).
- Ingels, F.M., Augustijns, P.F., 2003. Biological, Pharmaceutical, and Analytical Considerations with Respect to the Transport Media Used in the Absorption Screening System, Caco-2. *J. Pharm. Sci.* 92, 1545–1558. <https://doi.org/10.1002/jps.10408>.
- Ingels, F.M., Ungell, A.-L., Augustijns, P.F., 2007. Selection of Solvent Systems for Membrane-, Cell- and Tissue-Based Permeability Assessment. *Biotechnol. Pharm. Asp.* https://doi.org/10.1007/978-0-387-69154-1_7.
- Jacobsen, A.-C., Ejskjær, L., Brandl, M., Holm, R., Bauer-Brandl, A., 2021. Do Phospholipids Boost or Attenuate Drug Absorption? In Vitro and In Vivo Evaluation of Mono- and Diacyl Phospholipid-Based Solid Dispersions of Celecoxib. *J. Pharm. Sci.* 110, 198–207. <https://doi.org/10.1016/j.xjps.2020.08.009>.
- Jacobsen, A.-C., Elvang, P.A., Bauer-Brandl, A., Brandl, M., 2019a. A dynamic in vitro permeation study on solid mono- and diacyl-phospholipid dispersions of celecoxib. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 127, 199–207. <https://doi.org/10.1016/j.ejps.2018.11.003>.

- Jacobsen, A.-C., Krupa, A., Brandl, M., Bauer-Brandl, A., 2019b. High-throughput dissolution/permeation screening -a 96-well two-compartment microplate approach. *Pharmaceutics*. <https://doi.org/10.3390/pharmaceutics11050227>.
- Jacobsen, A.-C., Nielsen, S., Brandl, M., Bauer-Brandl, A., 2020. Drug permeability profiling using the novel permeapad® 96-well plate. *Pharm. Res.* 37, 93. <https://doi.org/10.1007/s11095-020-02807-x>.
- Jamei, M., Turner, D., Yang, J., Neuhoﬀ, S., Polak, S., Rostami-Hodjegan, A., Tucker, G., 2009. Population-based mechanistic prediction of oral drug absorption. *AAPS J* 11, 225–237. <https://doi.org/10.1208/s12248-009-9099-y>.
- Jarc, T., Novak, M., Hevir, N., Rižner, T.L., Kreft, M.E., Kristan, K., 2019. Demonstrating suitability of the Caco-2 cell model for BCS-based biowaiver according to the recent FDA and ICH harmonised guidelines. *J. Pharm. Pharmacol.* 71, 1231–1242. <https://doi.org/10.1111/jphp.13111>.
- Jezyk, N., Rubas, W., Grass, G.M., 1992. Permeability characteristics of various intestinal regions of rabbit, dog, and monkey. *Pharm. Res.* 9, 1580–1586. <https://doi.org/10.1023/a:1015812408231>.
- Johnson, D.A., Amidon, G.L., 1988. Determination of intrinsic membrane transport parameters from perfused intestine experiments: a boundary layer approach to estimating the aqueous and unbiased membrane permeabilities. *J. Theor. Biol.* 131, 93–106. [https://doi.org/10.1016/s0022-5193\(88\)80123-1](https://doi.org/10.1016/s0022-5193(88)80123-1).
- Jung, S.J., Choi, S.O., Um, S.Y., Kim, J.I., Choo, H.Y.P., Choi, S.Y., Chung, S.Y., 2006. Prediction of the permeability of drugs through study on quantitative structure-permeability relationship. *J. Pharm. Biomed. Anal.* 41, 469–475. <https://doi.org/10.1016/j.jpba.2005.12.020>.
- Kansy, M., Senner, F., Gubernator, K., 1998. Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. *J. Med. Chem.* 41, 1007–1010. <https://doi.org/10.1021/jm970530e>.
- Kanzer, J., Tho, I., Flaten, G.E., Mägerlein, M., Höllg, P., Fricker, G., Brandl, M., 2010. In-vitro permeability screening of melt extrudate formulations containing poorly water-soluble drug compounds using the phospholipid vesicle-based barrier. *J. Pharm. Pharmacol.* 62, 1591–1598. <https://doi.org/10.1111/j.2042-7158.2010.01172.x>.
- Karlsson, J., Artursson, P., 1992. A new diffusion chamber system for the determination of drug permeability coefficients across the human intestinal epithelium that are independent of the unstirred water layer. *Biochim. Biophys. Acta - Biomembr.* 1111, 204–210. [https://doi.org/10.1016/0005-2736\(92\)90312-a](https://doi.org/10.1016/0005-2736(92)90312-a).
- Kaur, N., Narang, A., Bansal, A.K., 2018. Use of biorelevant dissolution and PBPK modeling to predict oral drug absorption. *Eur. J. Pharm. Biopharm.* 129, 222–246. <https://doi.org/10.1016/j.ejpb.2018.05.024>.
- Kell, D.B., Dobson, P.D., Bilsland, E., Oliver, S.G., 2013. The promiscuous binding of pharmaceutical drugs and their transporter-mediated uptake into cells: what we (need to) know and how we can do so. *Drug Discov. Today* 18, 218–239. <https://doi.org/10.1016/j.drudis.2012.11.008>.
- Kerns, E.H., Di, L., Petusky, S., Farris, M., Ley, R., Jupp, P., 2004. Combined application of parallel artificial membrane permeability assay and Caco-2 permeability assays in drug discovery. *J. Pharm. Sci.* 93, 1440–1453. <https://doi.org/10.1002/jps.20075>.
- Kesisoglou, F., Chung, J., van Asperen, J., Heimbach, T., 2016. Physiologically based absorption modeling to impact biopharmaceutics and formulation strategies in drug development—Industry case studies. *J. Pharm. Sci.* 105, 2723–2734.
- Kim, M.T., Sedykh, A., Chakravarti, S.K., Saiakhov, R.D., Zhu, H., 2014. Critical evaluation of human oral bioavailability for pharmaceutical drugs by using various cheminformatics approaches. *Pharm. Res.* 31, 1002–1014. <https://doi.org/10.1007/s11095-013-1222-1>.
- Kisser, B., Mangelsen, E., Wingolf, C., Partecke, L.I., Heidecke, C., Tannergren, C., Oswald, S., Keiser, M., 2017. The ussing chamber assay to study drug metabolism and transport in the human intestine. *Curr. Protoc. Pharmacol.* 77. <https://doi.org/10.1002/cpph.22>.
- Klopman, G., Stefan, L.R., Saiakhov, R.D., 2002. ADME evaluation. 2. A computer model for the prediction of intestinal absorption in humans. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 17, 253–263. [https://doi.org/10.1016/s0928-0987\(02\)00219-1](https://doi.org/10.1016/s0928-0987(02)00219-1).
- Komiya, I., Park, J.Y., Kamani, A., Ho, N.F.H., Higuchi, W.I., 1980. Quantitative mechanistic studies in simultaneous fluid flow and intestinal absorption using steroids as model solutes. *Int. J. Pharm.* 4, 249–262. [https://doi.org/10.1016/0378-5173\(80\)90140-4](https://doi.org/10.1016/0378-5173(80)90140-4).
- Kostewicz, E.S., Aarons, L., Bergstrand, M., Bolger, M.B., Galetin, A., Hatley, O., Jamei, M., Lloyd, R., Pepin, X., Rostami-Hodjegan, A., Sjögren, E., Tannergren, C., Turner, D.B., Wagner, C., Weitschies, W., Dressman, J., 2014. PBPK models for the prediction of in vivo performance of oral dosage forms. *Eur. J. Pharm. Sci.* <https://doi.org/10.1016/j.ejps.2013.09.008>.
- Krishna, G., Chen, K., Lin, C., Nomeir, A.A., 2001. Permeability of lipophilic compounds in drug discovery using in-vitro human absorption model, Caco-2. *Int. J. Pharm.* 222, 77–89. [https://doi.org/10.1016/s0378-5173\(01\)00698-6](https://doi.org/10.1016/s0378-5173(01)00698-6).
- Kubbinga, M., Augustijns, P., García, M.A., Heinen, C., Wortelboer, H.M., Verwei, M., Langguth, P., 2019. The effect of chitosan on the bioaccessibility and intestinal permeability of acyclovir. *Eur. J. Pharm. Biopharm.* 136, 147–155. <https://doi.org/10.1016/j.ejpb.2019.01.021>.
- Lamson, N.G., Ball, R.L., Fein, K.C., Whitehead, K.A., 2019. Thrifty, rapid intestinal monolayers (TRIM) using caco-2 epithelial cells for oral drug delivery experiments. *Pharm. Res.* 36. <https://doi.org/10.1007/s11095-019-2712-6>.
- Lande, M.B., Donovan, J.M., Zeidel, M.L., 1995. The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *J. Gen. Physiol.* 106, 67–84. <https://doi.org/10.1085/jgp.106.1.67>.
- Lappin, G., 2016. Approaches to intravenous clinical pharmacokinetics: recent developments with isotopic microtracers. *J. Clin. Pharmacol.* 56, 11–23. <https://doi.org/10.1002/jcph.569>.
- Lappin, G., Noveck, R., Burt, T., 2013. Microdosing and drug development: past, present and future. *Expert Opin. Drug Metab. Toxicol.* 9, 817–834. <https://doi.org/10.1517/1742525.2013.786042>.
- Lappin, G., Rowland, M., Garner, R.C., 2006. The use of isotopes in the determination of absolute bioavailability of drugs in humans. *Expert Opin. Drug Metab. Toxicol.* 2, 419–427. <https://doi.org/10.1517/1742525.2.3.419>.
- Lee, M.-H., Ta, G.H., Weng, C.-F., Leong, M.K., 2020. In silico prediction of intestinal permeability by hierarchical support vector regression. *Int. J. Mol. Sci.* 21, 3582. <https://doi.org/10.3390/ijms21103582>.
- Lennernäs, H., 2014. Regional intestinal drug permeation: biopharmaceutics and drug development. *Eur. J. Pharm. Sci.* 57, 333–341. <https://doi.org/10.1016/j.ejps.2013.08.025>.
- Lennernäs, H., 2007. Intestinal permeability and its relevance for absorption and elimination. *Xenobiotica*. <https://doi.org/10.1080/00498250701704819>.
- Lennernäs, H., 1998. Human Intestinal Permeability. *J. Pharm. Sci.* 87, 403–410. <https://doi.org/10.1021/js970332a>.
- Lennernäs, H., 1997. Human jejunal effective permeability and its correlation with preclinical drug absorption models. *J. Pharm. Pharmacol.* <https://doi.org/10.1111/j.2042-7158.1997.tb06084.x>.
- Lennernäs, H., Ahrenstedt, O., Hällgren, R., Knutson, L., Ryde, M., Paalzow, L.K., 1992. Regional jejunal perfusion, a new in vivo approach to study oral drug absorption in man. *Pharm. Res.* 9, 1243–1251. <https://doi.org/10.1023/a:1015888813741>.
- Lennernäs, H., Ahrenstedt, O., Ungell, A.L., 1994. Intestinal drug absorption during induced net water absorption in man; a mechanistic study using antipyrine, atenolol and enalaprilat. *Br. J. Clin. Pharmacol.* 37, 589–596. <https://doi.org/10.1111/j.1365-2125.1994.tb04309.x>.
- Lennernäs, H., Fagerholm, U., Raab, Y., Gerdin, B., Hällgren, R., 1995. Regional rectal perfusion: a new in vivo approach to study rectal drug absorption in man. *Pharm. Res.* 12, 426–432. <https://doi.org/10.1023/A:1016216905197>.
- Lennernäs, H., Gjellan, K., Hällgren, R., Graffner, C., 2002a. The influence of caprate on rectal absorption of phenoxymethylpenicillin: experience from an in-vivo perfusion in humans. *J. Pharm. Pharmacol.* 54, 499–508. <https://doi.org/10.1211/0022357021778772>.
- Lennernäs, H., Knutson, L., Knutson, T., Hussain, A., Lesko, L., Salmonson, T., Amidon, G. L., 2002b. The effect of amiloride on the in vivo effective permeability of amoxicillin in human jejunum: experience from a regional perfusion technique. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 15, 271–277. [https://doi.org/10.1016/s0928-0987\(02\)00005-2](https://doi.org/10.1016/s0928-0987(02)00005-2).
- Lennernäs, H., Lee, I.D., Fagerholm, U., Amidon, G.L., 1997a. A residence-time distribution analysis of the hydrodynamics within the intestine in man during a regional single-pass perfusion with Loc-I-Gut: in-vivo permeability estimation. *J. Pharm. Pharmacol.* 49, 682–686. <https://doi.org/10.1111/j.2042-7158.1997.tb06092.x>.
- Lennernäs, H., Nylander, S., Ungell, A.L., 1997b. Jejunal permeability: a comparison between the ussing chamber technique and the single-pass perfusion in humans. *Pharm. Res.* 14, 667–671. <https://doi.org/10.1023/a:1012121632357>.
- Li, J., Tsinman, K., Tsinman, O., Wigman, L., 2018. Using pH gradient dissolution with in-situ flux measurement to evaluate bioavailability and DDI for formulated poorly soluble drug products. *AAPS PharmSciTech* 19, 2898–2907. <https://doi.org/10.1208/s12249-018-1164-3>.
- Lin, L., Wong, H., 2017. Predicting oral drug absorption: mini review on physiologically-based pharmacokinetic models. *Pharm.* <https://doi.org/10.3390/pharmaceutics9040041>.
- Lindmark, T., Kimura, Y., Artursson, P., 1998. Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in Caco-2 cells. *J. Pharmacol. Exp. Ther.* 284, 362–369.
- Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 46, 3–26. [https://doi.org/10.1016/S0169-409X\(00\)00129-0](https://doi.org/10.1016/S0169-409X(00)00129-0).
- Liu, H., Sabus, C., Carter, G.T., Du, C., Avdeef, A., Tischler, M., 2003. In vitro permeability of poorly aqueous soluble compounds using different solubilizers in the pampa assay with liquid chromatography/mass spectrometry detection. *Pharm. Res.* 20, 1820–1826. <https://doi.org/10.1023/B:PHAM.000003380.44755.5a>.
- Lo, Y.-L., Huang, J.-D., 2000. Effects of sodium deoxycholate and sodium caprate on the transport of epirubicin in human intestinal epithelial Caco-2 cell layers and everted gut sacs of rats. *Biochem. Pharmacol.* 59, 665–672. [https://doi.org/10.1016/s0006-2952\(99\)00377-9](https://doi.org/10.1016/s0006-2952(99)00377-9).
- Lovering, E.G., Black, D.B., 1973. Drug permeation through membranes i: effect of various substances on amobarbital permeation through polydimethylsiloxane. *J. Pharm. Sci.* 62, 602–606. <https://doi.org/10.1002/jps.2600620412>.
- Lozoya-Agullo, I., Araújo, F., González-Álvarez, I., Merino-Sanjuán, M., González-Álvarez, M., Bermejo, M., Sarmento, B., 2017. Usefulness of Caco-2/HT29-MTX and Caco-2/HT29-MTX/Raji B coculture models to predict intestinal and colonic permeability compared to caco-2 monoculture. *Mol. Pharm.* 14, 1264–1270. <https://doi.org/10.1021/acs.molpharmaceut.6b01165>.
- Lozoya-Agullo, I., González-Álvarez, I., González-Álvarez, M., Merino-Sanjuán, M., Bermejo, M., 2015a. Situ perfusion model in rat colon for drug absorption studies: comparison with small intestine and caco-2 cell model. *J. Pharm. Sci.* 104, 3136–3145. <https://doi.org/10.1002/jps.24447>.
- Lozoya-Agullo, I., Zur, M., Wolk, O., Beig, A., González-Álvarez, I., González-Álvarez, M., Merino-Sanjuán, M., Bermejo, M., Dahan, A., 2015b. In-situ intestinal rat perfusions for human Fabs prediction and BCS permeability class determination: investigation of the single-pass vs. the Doluisio experimental approaches. *Int. J. Pharm.* 480, 1–7. <https://doi.org/10.1016/j.ijpharm.2015.01.014>.

- Lucas, M., 1983. Determination of acid surface pH in vivo in rat proximal jejunum. *Gut* 24, 734–739. <https://doi.org/10.1136/gut.24.8.734>.
- Lundquist, P., Artursson, P., 2016. Oral absorption of peptides and nanoparticles across the human intestine: opportunities, limitations and studies in human tissues. *Adv. Drug Deliv. Rev.* 106, 256–276. <https://doi.org/10.1016/j.addr.2016.07.007>.
- Lyng, E., Havenaar, R., Shastri, P., Hetsco, L., Vick, A., Sagartz, J., 2016. Increased bioavailability of celecoxib under fed versus fasted conditions is determined by postprandial bile secretion as demonstrated in a dynamic gastrointestinal model. *Drug Dev. Ind. Pharm.* 42, 1334–1339. <https://doi.org/10.3109/03639045.2015.1135935>.
- Ma, D., Hettiarachchi, G., Nguyen, D., Zhang, B., Wittenberg, J.B., Zavalij, P.Y., Briken, V., Isaacs, L., 2012. Acyclic cucurbit[n]uril molecular containers enhance the solubility and bioactivity of poorly soluble pharmaceuticals. *Nat. Chem.* 4, 503–510. <https://doi.org/10.1038/nchem.1326>.
- Maher, S., Msrny, R.J., Brayden, D.J., 2016. Intestinal permeation enhancers for oral peptide delivery. *Adv. Drug Deliv. Rev.* 106, 277–319. <https://doi.org/10.1016/j.addr.2016.06.005>.
- Maher, S., Wang, X., Bzik, V., McClean, S., Brayden, D.J., 2009. Evaluation of intestinal absorption and mucosal toxicity using two promoters. II. Rat instillation and perfusion studies. *Eur. J. Pharm. Sci.* 38, 301–311. <https://doi.org/10.1016/j.ejps.2009.07.011>.
- Markopoulos, C., Imanidis, G., Vertzoni, M., Symillides, M., Parrott, N., Reppas, C., 2013. In vitro and ex vivo investigation of the impact of luminal lipid phases on passive permeability of lipophilic small molecules using PAMPA. *Pharm. Res.* 30, 3145–3153. <https://doi.org/10.1007/s11095-013-1141-1>.
- Mathieu, F., Galmier, M.-J., Pognat, J.-F., Petit, J., Lartigue, C., 1999. Transepithelial transport of bepridil in the human intestinal cell line, Caco-2, using two media, DMEMc and HBSS. *Int. J. Pharm.* 181, 203–217. [https://doi.org/10.1016/s0378-5173\(99\)00027-7](https://doi.org/10.1016/s0378-5173(99)00027-7).
- Matsson, P., Bergström, C.A.S., Nagahara, N., Tavelin, S., Norinder, U., Artursson, P., 2005. Exploring the role of different drug transport routes in permeability screening. *J. Med. Chem.* 48, 604–613. <https://doi.org/10.1021/jm049711o>.
- Matsson, P., Doak, B.C., Over, B., Kihlberg, J., 2016. Cell permeability beyond the rule of 5. *Adv. Drug Deliv. Rev.* 101, 42–61. <https://doi.org/10.1016/j.addr.2016.03.013>.
- McCartney, F., Jannin, V., Chevrier, S., Boulghobra, H., Hristov, D.R., Ritter, N., Miolane, C., Chavant, Y., Demarre, F., Brayden, D.J., 2019. Labrasol® is an efficacious intestinal permeation enhancer across rat intestine: ex vivo and in vivo rat studies. *J. Control. Release* 310, 115–126. <https://doi.org/10.1016/j.jconrel.2019.08.008>.
- Milanetti, E., Raimondo, D., Tramontano, A., 2016. Prediction of the permeability of neutral drugs inferred from their solvation properties. *Bioinformatics* 32, 1163–1169. <https://doi.org/10.1093/bioinformatics/btv725>.
- Miller, J.M., Beig, A., Carr, R.A., Spence, J.K., Dahan, A., 2012a. A win-win solution in oral delivery of lipophilic drugs: supersaturation via amorphous solid dispersions increases apparent solubility without sacrifice of intestinal membrane permeability. *Mol. Pharm.* 9, 2009–2016. <https://doi.org/10.1021/mp300104s>.
- Miller, J.M., Beig, A., Carr, R.A., Webster, G.K., Dahan, A., 2012b. The solubility-permeability interplay when using cosolvents for solubilization: revising the way we use solubility-enabling formulations. *Mol. Pharm.* 9, 581–590. <https://doi.org/10.1021/mp200460u>.
- Miller, J.M., Beig, A., Krieg, B.J., Carr, R.A., Borchardt, T.B., Amidon, G.E., Amidon, G.L., Dahan, A., 2011. The solubility-permeability interplay: mechanistic modeling and predictive application of the impact of micellar solubilization on intestinal permeation. *Mol. Pharm.* 8, 1848–1856. <https://doi.org/10.1021/mp200181v>.
- Miyake, M., Koga, T., Kondo, S., Yoda, N., Emoto, C., Mukai, T., Toguchi, H., 2017. Prediction of drug intestinal absorption in human using the Ussing chamber system: a comparison of intestinal tissues from animals and humans. *Eur. J. Pharm. Sci.* 96, 373–380. <https://doi.org/10.1016/j.ejps.2016.10.006>.
- Mols, R., Brouwers, J., Schinkel, A.H., Annaert, P., Augustijns, P., 2009. Intestinal perfusion with mesenteric blood sampling in wild-type and knockout mice: evaluation of a novel tool in biopharmaceutical drug profiling. *Drug Metab. Dispos.* 37, 1334–1337. <https://doi.org/10.1124/dmd.109.026591>.
- Mullard, A., 2020. 2019 FDA drug approvals. *Nature Rev. Drug Discovery*. <https://doi.org/10.1038/d41573-020-00001-7>.
- Naderkhani, E., Isaksson, J., Ryzhakov, A., Flaten, G.E., 2014. Development of a Biomimetic Phospholipid Vesicle-based Permeation Assay for the Estimation of Intestinal Drug Permeability. *J. Pharm. Sci.* 103, 1882–1890. <https://doi.org/10.1002/jps.23954>.
- Nazir, I., Ghezzi, M., Asim, M.H., Phan, T.N.Q., Bernkop-Schnürch, A., 2020. Self-emulsifying drug delivery systems: about the fate of hydrophobic ion pairs on a phospholipid bilayer. *J. Mol. Liq.* 312, 113382. <https://doi.org/10.1016/j.molliq.2020.113382>.
- Neuhoff, S., 2005. REFINED IN VITRO MODELS FOR PREDICTION OF INTESTINAL DRUG TRANSPORT: ROLE OF pH AND EXTRACELLULAR ADDITIVES IN THE CACO-2 CELL MODEL. Uppsala University.
- Neuhoff, S., Ungell, A.-L., Zamora, I., Artursson, P., 2003. pH-dependent bidirectional transport of weakly basic drugs across caco-2 monolayers: implications for drug-drug interactions. *Pharm. Res.* 20, 1141–1148. <https://doi.org/10.1023/A:1025032511040>.
- Newby, D., Freitas, A.A., Ghafourian, T., 2013. Pre-processing feature selection for improved C&RT models for oral absorption. *J. Chem. Inf. Model.* 53, 2730–2742. <https://doi.org/10.1021/ci400378j>.
- Nilsson, D., Fagerholm, U., Lennernäs, H., 1994. The influence of net water absorption on the permeability of antipyrine and levodopa in the human jejunum. *Pharm. Res.* 11, 1540–1544. <https://doi.org/10.1023/A:1018941200575>.
- Norinder, U., Bergström, C.A.S., 2006. Prediction of ADMET properties. *ChemMedChem*. <https://doi.org/10.1002/cmdc.200600155>.
- Norinder, U., Österberg, T., Artursson, P., 1997. Theoretical calculation and prediction of Caco-2 cell permeability using MolSurf parametrization and PLS statistics. *Pharm. Res.* 14, 1786–1791. <https://doi.org/10.1023/A:1012196216736>.
- Nyberg, L., Månsson, W., Abrahamsson, B., Seidegård, J., Borgå, O., 2007. A convenient method for local drug administration at predefined sites in the entire gastrointestinal tract: experiences from 13 phase I studies. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 30, 432–440. <https://doi.org/10.1016/j.ejps.2007.01.005>.
- O'Dwyer, P.J., Imanidis, G., Box, K.J., Reppas, C., 2020. On the usefulness of two small-scale in vitro setups in the evaluation of luminal precipitation of lipophilic weak bases in early formulation development. *Pharmaceutics*. <https://doi.org/10.3390/pharmaceutics12030272>.
- O'Shea, J.P., Holm, R., O'Driscoll, C.M., Griffin, B.T., 2019. Food for thought: formulating away the food effect – a PEARRL review. *J. Pharm. Pharmacol.* 71. <https://doi.org/10.1111/jphp.12957>.
- Oh, D.M., Curl, R.L., Amidon, G.L., 1993. Estimating the fraction dose absorbed from suspensions of poorly soluble compounds in humans: a mathematical model. *Pharm. Res.* 10, 264–270. <https://doi.org/10.1023/a:1018947113238>.
- Oja, M., Maran, U., 2015. The permeability of an artificial membrane for wide range of pH in human gastrointestinal tract: experimental measurements and quantitative structure activity relationship. *Mol. Inform.* 34, 493–506. <https://doi.org/10.1002/minf.201400147>.
- Ölander, M., Wiśniewski, J.R., Matsson, P., Lundquist, P., Artursson, P., 2016. The proteome of filter-grown caco-2 cells with a focus on proteins involved in drug disposition. *J. Pharm. Sci.* 105, 817–827. <https://doi.org/10.1016/j.xphs.2015.10.030>.
- Overton, E., 1899. Ueber die allgemeinen osmotischen Eigenschaften der Zelle, ihre vermutlichen Ursachen und ihre Bedeutung für die Physiologie. S.L.
- Paine, M.F., Hart, H.L., Ludington, S.S., Haining, R.L., Rettie, A.E., Zeldin, D.C., 2006. The human intestinal cytochrome P450 "pie. *Drug Metab. Dispos.* 34, 880–886. <https://doi.org/10.1124/dmd.105.008672>.
- Palm, K., Luthman, K., Ungell, A.L., Strandlund, G., Artursson, P., 1996. Correlation of drug absorption with molecular surface properties. *J. Pharm. Sci.* 85, 32–39. <https://doi.org/10.1021/js950285r>.
- Palm, K., Stenberg, P., Luthman, K., Artursson, P., 1997. Polar Molecular Surface Properties Predict the Intestinal Absorption of Drugs in Humans. *Pharm. Res.* 14, 568–571. <https://doi.org/10.1023/A:1012188625088>.
- Patel, G.V., Patel, V.B., Pathak, A., Rajput, S.J., 2014. Nanosuspension of efavirenz for improved oral bioavailability: formulation optimization, in vitro, in situ and in vivo evaluation. *Drug Dev. Ind. Pharm.* 40, 80–91. <https://doi.org/10.3109/03639045.2012.746362>.
- Pellet, M.A., Davis, A.F., Hadgraft, J., 1994. Effect of supersaturation on membrane transport: 2. Piroxicam. *Int. J. Pharm.* 111, 1–6. [https://doi.org/10.1016/0378-5173\(94\)90395-6](https://doi.org/10.1016/0378-5173(94)90395-6).
- Penner, N., Klunk, L.J., Prakash, C., 2009. Human radiolabeled mass balance studies: objectives, utilities and limitations. *Biopharm. Drug Dispos.* 30, 185–203. <https://doi.org/10.1002/bdd.661>.
- Pepin, X.J.H., Flanagan, T.R., Holt, D.J., Eidelman, A., Treacy, D., Rowlings, C.E., 2016. Justification of drug product dissolution rate and drug substance particle size specifications based on absorption PBPK modeling for lesinurad immediate release tablets. *Mol. Pharm.* 13, 3256–3269.
- Pérez, M.A.C., Sanz, M.B., Torres, L.R., Avalos, R.G., González, M.P., Díaz, H.G., 2004. A topological sub-structural approach for predicting human intestinal absorption of drugs. *Eur. J. Med. Chem.* 39, 905–916. <https://doi.org/10.1016/j.ejmech.2004.06.012>.
- Pham-The, H., Cabrera-Pérez, M.Á., Nam, N.-H., Castillo-Garit, J.A., Rasulev, B., Le-Thi-Thu, H., Casanola-Martin, G.M., 2018. In silico assessment of ADME properties: advances in caco-2 cell monolayer permeability modeling. *Curr. Top. Med. Chem.* 18, 2209–2229. <https://doi.org/10.2174/1568026619666181130140350>.
- Pham-The, H., González-Álvarez, I., Bermejo, M., Garrigues, T., Le-Thi-Thu, H., Cabrera-Pérez, M.Á., 2013. The use of rule-based and QSPR approaches in ADME profiling: a case study on caco-2 permeability. *Mol. Inform.* 32, 459–479. <https://doi.org/10.1002/minf.201200166>.
- Pinto, M., Robine-Leon, S., Appay, M.-D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assman, P., Haffen, K., Fogh, J., Zweibaum, A., 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* 47, 323–330.
- Pond, S.M., Tozer, T.N., 1984. First-pass elimination basic concepts and clinical consequences. *Clin. Pharmacokinet.* <https://doi.org/10.2165/00003088-198409010-00001>.
- Presas, E., McCartney, F., Sultan, E., Hunger, C., Nellen, S., V Alvarez, C., Werner, U., Bazile, D., Brayden, D.J., O'Driscoll, C.M., 2018. Physicochemical, pharmacokinetic and pharmacodynamic analyses of amphiphilic cyclodextrin-based nanoparticles designed to enhance intestinal delivery of insulin. *J. Control. Release* 286, 402–414. <https://doi.org/10.1016/j.jconrel.2018.07.045>.
- Presas, E., Tovar, S., Cuñarro, J., O'Shea, J.P., O'Driscoll, C.M., 2021. Pre-clinical evaluation of a modified cyclodextrin-based nanoparticle for intestinal delivery of liraglutide. *J. Pharm. Sci.* 110, 292–300. <https://doi.org/10.1016/j.xphs.2020.10.058>.
- Raina, S.A., Zhang, G.G.Z., Alonzo, D.E., Wu, J., Zhu, D., Catron, N.D., Gao, Y., Taylor, L.S., 2015. Impact of solubilizing additives on supersaturation and membrane transport of drugs. *Pharm. Res.* 32, 3350–3364. <https://doi.org/10.1007/s11095-015-1712-4>.
- Rautio, J., Kumpulainen, H., Heimbach, T., Olyai, R., Oh, D., Järvinen, T., Savolainen, J., 2008. Prodrugs: design and clinical applications. *Nat. Rev. Drug Discov.* 7, 255–270. <https://doi.org/10.1038/nrd2468>.

- Roos, C., Dahlgren, D., Sjögren, E., Sjöblom, M., Hedeland, M., Lennernäs, H., 2018. Jejunal absorption of aprepitant from nanosuspensions: role of particle size, prandial state and mucus layer. *Eur. J. Pharm. Biopharm.* 132, 222–230. <https://doi.org/10.1016/j.ejpb.2018.09.022>.
- Roos, C., Dahlgren, D., Sjögren, E., Tannergren, C., Abrahamsson, B., Lennernäs, H., 2017. Regional intestinal permeability in rats: a comparison of methods. *Mol. Pharm.* 14, 4252–4261. <https://doi.org/10.1021/acs.molpharmaceut.7b00279>.
- Rowland, M., Peck, C., Tucker, G., 2011. Physiologically-based pharmacokinetics in drug development and regulatory science. *Annu. Rev. Pharmacol. Toxicol.* 51, 45–73. <https://doi.org/10.1146/annurev-pharmtox-010510-100540>.
- Rožehnal, V., Nakai, D., Hoepner, U., Fischer, T., Kamiyama, E., Takahashi, M., Yasuda, S., Mueller, J., 2012. Human small intestinal and colonic tissue mounted in the Ussing chamber as a tool for characterizing the intestinal absorption of drugs. *Eur. J. Pharm. Sci.* 46, 367–373. <https://doi.org/10.1016/j.ejps.2012.02.025>.
- Ruponen, M., Visti, M., Ojarinta, R., Laitinen, R., 2018. Permeability of glibenclamide through a PAMPA membrane: the effect of co-amorphization. *Eur. J. Pharm. Biopharm.* 129, 247–256. <https://doi.org/10.1016/j.ejpb.2018.06.007>.
- S. Palmer, D., Mišin, M., V. Fedorov, M., Llinas, A., 2015. Fast and General Method To Predict the Physicochemical Properties of Druglike Molecules Using the Integral Equation Theory of Molecular Liquids. *Mol. Pharm.* 12, 3420–3432. <https://doi.org/10.1021/acs.molpharmaceut.5b00441>.
- Saha, P., Kou, J.H., 2000. Effect of solubilizing excipients on permeation of poorly water-soluble compounds across Caco-2 cell monolayers. *Eur. J. Pharm. Biopharm.* 50, 403–411. [https://doi.org/10.1016/S0939-6411\(00\)00113-2](https://doi.org/10.1016/S0939-6411(00)00113-2).
- Salamat-Miller, N., Johnston, T.P., 2005. Current strategies used to enhance the paracellular transport of therapeutic polypeptides across the intestinal epithelium. *Int. J. Pharm.* 294, 201–216. <https://doi.org/10.1016/j.ijpharm.2005.01.022>.
- Saphier, S., Yacov, G., Wenger, A., Klausner, Z., Rosner, A., Goldvaser, M., Katalan, S., 2020. The Effect of Anesthetic Regimens on Intestinal Absorption of Passively Absorbed Drugs in Rats. *Pharm. Res.* 37 <https://doi.org/10.1007/s11095-020-02809-9>.
- Sasaki, M., Suzuki, H., Ito, K., Abe, T., Sugiyama, Y., 2001. Transcellular transport of organic anions across a double-transfected madin-darby canine kidney II cell monolayer expressing both human organic anion-transporting polypeptide (OATP2/SLC21A6) and multidrug resistance-associated protein 2 (MRP2/ABCC2). *J. Biol. Chem.* 277, 6497–6503. <https://doi.org/10.1074/jbc.m109081200>.
- Schanker, L.S., Tocco, D.J., Brodie, B.B., Hogben, C.A., 1958. Absorption of drugs from the rat small intestine. *J. Pharmacol. Exp. Ther.* 123, 81–88.
- Schilderink, R., Protopappa, M., Fleth-James, J., Vertzoni, M., Schaefer, K., Havenaar, R., Kulla, I., Metzger, M., Reppas, C., 2020. On the usefulness of compendial setups and tiny-TIM system in evaluating the in vivo performance of oral drug products with various release profiles in the fasted state: case example sodium salt of A6197. *Eur. J. Pharm. Biopharm.* 149, 154–162. <https://doi.org/10.1016/j.ejpb.2020.02.003>.
- Seddon, A.M., Casey, D., Law, R.V., Gee, A., Templer, R.H., Ces, O., 2009. Drug interactions with lipid membranes. *Chem. Soc. Rev.* 38, 2509–2519. <https://doi.org/10.1039/b813853m>.
- Shahiwal, A., 2011. Formulation approaches in enhancement of patient compliance to oral drug therapy. *Expert Opin. Drug Deliv.* <https://doi.org/10.1517/17425247.2011.628311>.
- Shekhawat, P., Pokharkar, V., 2019. Risk assessment and QbD based optimization of an Erosartan mesylate nanosuspension: in-vitro characterization, PAMPA and in-vivo assessment. *Int. J. Pharm.* 567, 118415 <https://doi.org/10.1016/j.ijpharm.2019.06.006>.
- Shultz, M.D., 2019. Two decades under the influence of the rule of five and the changing properties of approved oral drugs. *J. Med. Chem.* 62, 1701–1714. <https://doi.org/10.1021/acs.jmedchem.8b00686>.
- Simulations Plus, 2019. ADMET Predictor User Manual.
- Sinko, P.D., Gidley, D., Vallery, R., Lamoureux, A., Amidon, G.L., Amidon, G.E., 2017. In vitro characterization of the biomimetic properties of poly(dimethylsiloxane) to simulate oral drug absorption. *Mol. Pharm.* 14, 4661–4674. <https://doi.org/10.1021/acs.molpharmaceut.7b00798>.
- Sinko, P.D., Harris, S., Salehi, N., Meyer, P.J., Amidon, G.L., Amidon, G.E., 2020. Ultrathin, large-area membrane diffusion cell for pH-dependent simultaneous dissolution and absorption studies. *Mol. Pharm.* 17, 2319–2328. <https://doi.org/10.1021/acs.molpharmaceut.0c00040>.
- Sinko, P.J., Leesman, G.D., Amidon, G.L., 1991. Predicting fraction dose absorbed in humans using a macroscopic mass balance approach. *Pharm. Res.* 8, 979–988. <https://doi.org/10.1023/a:1015892621261>.
- Sironi, D., Christensen, M., Rosenberg, J., Bauer-Brandl, A., Brandl, M., 2017a. Evaluation of a dynamic dissolution/permeation model: mutual influence of dissolution and barrier-flux under non-steady state conditions. *Int. J. Pharm.* 522, 50–57. <https://doi.org/10.1016/j.ijpharm.2017.03.002>.
- Sironi, D., Rosenberg, J., Bauer-Brandl, A., Brandl, M., 2018. PermeaLoop™, a novel in vitro tool for small-scale drug-dissolution/permeation studies. *J. Pharm. Biomed. Anal.* 156, 247–251. <https://doi.org/10.1016/j.jpba.2018.04.042>.
- Sironi, D., Rosenberg, J., Bauer-Brandl, A., Brandl, M., 2017b. Dynamic dissolution-/permeation-testing of nano- and microparticle formulations of fenofibrate. *Eur. J. Pharm. Sci.* 96, 20–27. <https://doi.org/10.1016/j.ejps.2016.09.001>.
- Sjöberg, Å., Lutz, M., Tannergren, C., Wingolf, C., Borde, A., Ungell, A.L., 2013. Comprehensive study on regional human intestinal permeability and prediction of fraction absorbed of drugs using the Ussing chamber technique. *Eur. J. Pharm. Sci.* 48, 166–180. <https://doi.org/10.1016/j.ejps.2012.10.007>.
- Sjögren, E., Abrahamsson, B., Augustijns, P., Becker, D., Bolger, M.B., Brewster, M., Brouwers, J., Flanagan, T., Harwood, M., Heinen, C., Holm, R., Juretschke, H.P., Kubbanga, M., Lindahl, A., Lukacova, V., Münster, U., Neuhoff, S., Nguyen, M.A., Peer, A., Van, Reppas, C., Hodjegan, A.R., Tannergren, C., Weitschies, W., Wilson, C., Zane, P., Lennernäs, H., Langguth, P., 2014. In vivo methods for drug absorption - Comparative physiologies, model selection, correlations with in vitro methods (IVIVC), and applications for formulation/API/excipient characterization including food effects. *Eur. J. Pharm. Sci.* 57, 99–151. <https://doi.org/10.1016/j.ejps.2014.02.010>.
- Sjögren, E., Dahlgren, D., Roos, C., Lennernäs, H., 2015. Human in vivo regional intestinal permeability: quantitation using site-specific drug absorption data. *Mol. Pharm.* 12, 2026–2039. <https://doi.org/10.1021/mp500834v>.
- Sjögren, E., Westergren, J., Grant, I., Hanisch, G., Lindfors, L., Lennernäs, H., Abrahamsson, B., Tannergren, C., 2013. In silico predictions of gastrointestinal drug absorption in pharmaceutical product development: application of the mechanistic absorption model GI-Sim. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 49, 679–698. <https://doi.org/10.1016/j.ejps.2013.05.019>.
- Sladek, S., McCartney, F., Eskander, M., Dunne, D.J., Santos-Martinez, M.J., Benetti, F., Tajber, L., Brayden, D.J., 2020. An enteric-coated polyelectrolyte nanocomplex delivers insulin in rat intestinal instillations when combined with a permeation enhancer. *Pharm.* <https://doi.org/10.3390/pharmaceutics12030259>.
- Smith, D., Artursson, P., Avdeef, A., Di, L., Ecker, G.F., Faller, B., Houston, J.B., Kansy, M., Kerns, E.H., Krämer, S.D., Lennernäs, H., van de Waterbeemd, H., Sugano, K., Testa, B., 2014. Passive lipoidal diffusion and carrier-mediated cell uptake are both important mechanisms of membrane permeation in drug disposition. *Mol. Pharm.* 11, 1727–1738. <https://doi.org/10.1021/mp400713v>.
- Söderholm, J., Hedman, L., Artursson, P., Franzén, L., Larsson, J., Pantzar, N., Permert, J., Olaison, G., 1998. Integrity and metabolism of human ileal mucosa in vitro in the Ussing chamber. *Acta Physiol. Scand.* 162, 47–56. <https://doi.org/10.1046/j.1365-201x.1998.0248f.x>.
- Soni, J., Baird, A.W., O'Brien, L.M., McElroy, M., Callanan, J.J., Bassett, H.F., Campion, D., Brayden, D.J., 2006. Rat, ovine and bovine Peyer's patches mounted in horizontal diffusion chambers display sampling function. *J. Control. Release* 115, 68–77. <https://doi.org/10.1016/j.jconrel.2006.06.021>.
- Stappaerts, J., Brouwers, J., Annaert, P., Augustijns, P., 2015a. In situ perfusion in rodents to explore intestinal drug absorption: challenges and opportunities. *Int. J. Pharm.* 478, 665–681. <https://doi.org/10.1016/j.ijpharm.2014.11.035>.
- Stappaerts, J., Geboers, S., Snoeys, J., Brouwers, J., Tack, J., Annaert, P., Augustijns, P., 2015b. Rapid conversion of the ester prodrug abiraterone acetate results in intestinal supersaturation and enhanced absorption of abiraterone: in vitro, rat in situ and human in vivo studies. *Eur. J. Pharm. Biopharm.* 90, 1–7. <https://doi.org/10.1016/j.ejpb.2015.01.001>.
- Stappaerts, J., Wuyls, B., Tack, J., Annaert, P., Augustijns, P., 2014. Human and simulated intestinal fluids as solvent systems to explore food effects on intestinal solubility and permeability. *Eur. J. Pharm. Sci.* 63, 178–186. <https://doi.org/10.1016/j.ejps.2014.07.009>.
- Stenberg, P., Norinder, U., Luthman, K., Artursson, P., 2001. Experimental and computational screening models for the prediction of intestinal drug absorption. *J. Med. Chem.* 44, 1927–1937. <https://doi.org/10.1021/jm001101a>.
- Stevens, L.J., van Lipzig, M.M.H., Erpelinck, S.L.A., Pronk, A., van Gorp, J., Wortelboer, H.M., van de Steeg, E., 2019. A higher throughput and physiologically relevant two-compartmental human ex vivo intestinal tissue system for studying gastrointestinal processes. *Eur. J. Pharm. Sci.* 137, 104989 <https://doi.org/10.1016/j.ejps.2019.104989>.
- Stewart, A.M., Grass, M.E., Brodeur, T.J., Goodwin, A.K., Morgen, M.M., Friesen, D.T., Vodak, D.T., 2017a. Impact of drug-rich colloids of itraconazole and HPMCAS on membrane flux in vitro and oral bioavailability in rats. *Mol. Pharm.* 14, 2437–2449. <https://doi.org/10.1021/acs.molpharmaceut.7b00338>.
- Stewart, A.M., Grass, M.E., Mudie, D.M., Morgen, M.M., Friesen, D.T., Vodak, D.T., 2017b. Development of a biorelevant, material-sparing membrane flux test for rapid screening of bioavailability-enhancing drug product formulations. *Mol. Pharm.* 14, 2032–2046. <https://doi.org/10.1021/acs.molpharmaceut.7b00121>.
- Stillhart, C., Pepin, X., Tistaert, C., Good, D., Van Den Bergh, A., Parrott, N., Kesisoglou, F., 2019. PBPK absorption modeling: establishing the in vitro–in vivo link—industry perspective. *AAPS J* 21, 19. <https://doi.org/10.1208/s12248-019-0292-3>.
- Suarez-Sharp, S., Lindahl, A., Heimbach, T., Rostami-Hodjegan, A., Bolger, M.B., Ray Chaudhuri, S., Hens, B., 2020. Translational modeling strategies for orally administered drug products: academic, industrial and regulatory perspectives. *Pharm. Res.* 37 <https://doi.org/10.1007/s11095-020-02814-y>.
- Suenderhauf, C., Hammann, F., Maunz, A., Helma, C., Huwyler, J., 2011. Combinatorial QSAR Modeling of Human Intestinal Absorption. *Mol. Pharm.* 8, 213–224. <https://doi.org/10.1021/mp100279d>.
- Sugano, K., 2021. Lost in modelling and simulation? ADMET DMPK 9, 75–109. <https://doi.org/10.5599/admet.923>.
- Sugano, K., Hamada, H., Machida, M., Ushio, H., Saitoh, K., Terada, K., 2001. Optimized conditions of bio-mimetic artificial membrane permeation assay. *Int. J. Pharm.* 228, 181–188. [https://doi.org/10.1016/S0378-5173\(01\)00845-6](https://doi.org/10.1016/S0378-5173(01)00845-6).
- Sugano, K., Kansy, M., Artursson, P., Avdeef, A., Bendels, S., Di, L., Ecker, G.F., Faller, B., Fischer, H., Gerebtzoff, G., Lennernaes, H., Senner, F., 2010. Coexistence of passive and carrier-mediated processes in drug transport. *Nat. Rev. Drug Discov.* 9, 597–614. <https://doi.org/10.1038/nrd3187>.
- Sun, D., Lennernas, H., Welage, L.S., Barnett, J.L., Landowski, C.P., Foster, D., Fleisher, D., Lee, K.-D., Amidon, G.L., 2002. Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. *Pharm. Res.* 19, 1400–1416. <https://doi.org/10.1023/a:1020483911355>.
- Sun, D., Yu, L.X., Hussain, M.A., Wall, D.A., Smith, R.L., Amidon, G.L., 2004. In vitro testing of drug absorption for drug “developability” assessment: forming an interface

- between *in vitro* preclinical data and clinical outcome. *Curr. Opin. Drug Discov. Devel.* 7, 75–85.
- Sun, H., Chow, E.C.Y., Liu, S., Du, Y., Pang, K.S., 2008. The Caco-2 cell monolayer: usefulness and limitations. *Expert Opin. Drug Metab. Toxicol.* 4, 395–411. <https://doi.org/10.1517/17425255.4.4.395>.
- Sun, H., Nguyen, K., Kerns, E., Yan, Z., Yu, K.R., Shah, P., Jadhav, A., Xu, X., 2017. Highly predictive and interpretable models for PAMPA permeability. *Bioorganic Med. Chem.* 25, 1266–1276. <https://doi.org/10.1016/j.bmc.2016.12.049>.
- Sun, L., Liu, X., Xiang, R., Wu, C., Wang, Y., Sun, Y., Sun, J., He, Z., 2013. Structure-based prediction of human intestinal membrane permeability for rapid *in silico* BCS classification. *Biopharm. Drug Dispos.* 34, 321–335. <https://doi.org/10.1002/bdd.1848>.
- Sutton, S.C., Rinaldi, M.T., Vukovinsky, K.E., 2001. Comparison of the gravimetric, phenol red, and 14C-PEG-3350 methods to determine water absorption in the rat single-pass intestinal perfusion model. *AAPS PharmSci* 3, E25. <https://doi.org/10.1208/ps030325>. –E25.
- Takano, M., Yumoto, R., Murakami, T., 2006. Expression and function of efflux drug transporters in the intestine. *Pharmacol. Ther.* 109, 137–161. <https://doi.org/10.1016/j.pharmthera.2005.06.005>.
- Tannergren, C., Engman, H., Knutson, L., Hedeland, M., Bondesson, U., Lennernäs, H., 2004. St John's wort decreases the bioavailability of R- and S-verapamil through induction of the first-pass metabolism. *Clin. Pharmacol. Ther.* 75, 298–309. <https://doi.org/10.1016/j.clpt.2003.12.012>.
- Tannergren, C., Knutson, T., Knutson, L., Lennernäs, H., 2003a. The effect of ketoconazole on the *in vivo* intestinal permeability of fexofenadine using a regional perfusion technique. *Br. J. Clin. Pharmacol.* 55, 182–190. <https://doi.org/10.1046/j.1365-2125.2003.01722.x>.
- Tannergren, C., Petri, N., Knutson, L., Hedeland, M., Bondesson, U., Lennernäs, H., 2003b. Multiple transport mechanisms involved in the intestinal absorption and first-pass extraction of fexofenadine. *Clin. Pharmacol. Ther.* 74, 423–436. [https://doi.org/10.1016/S0009-9236\(03\)00238-8](https://doi.org/10.1016/S0009-9236(03)00238-8).
- Taskinen, J., Norinder, U., 2007. 5.26 - In Silico Predictions of Solubility. Elsevier, Oxford, pp. 627–648. <https://doi.org/10.1016/B0-08-045044-X/00279-0> in: Taylor, J.B., Triggler, D.J.B.T.-C.M.C.I.I. (Eds.).
- Taverner, A., Dondi, R., Almansour, K., Laurent, F., Owens, S.-E., Eggleston, I.M., Fotaki, N., Mrsny, R.J., 2015. Enhanced paracellular transport of insulin can be achieved via transient induction of myosin light chain phosphorylation. *J. Control. Release* 210, 189–197. <https://doi.org/10.1016/j.jconrel.2015.05.270>.
- Thelen, K., Dressman, J.B., 2009. Cytochrome P450-mediated metabolism in the human gut wall. *J. Pharm. Pharmacol.* 61, 541–558. <https://doi.org/10.1211/jpp.61.05.0002>.
- Thummel, K.E., Kunze, K.L., Shen, D.D., 1997. Enzyme-catalyzed processes of first-pass hepatic and intestinal drug extraction. *Adv. Drug Deliv. Rev.* [https://doi.org/10.1016/S0169-409X\(97\)00039-2](https://doi.org/10.1016/S0169-409X(97)00039-2).
- Tistaert, C., Heimbach, T., Xia, B., Parrott, N., Samant, T.S., Kesiosoglou, F., 2019. Food effect projections via physiologically based pharmacokinetic modeling: predictive case studies. *J. Pharm. Sci.* 108, 592–602. <https://doi.org/10.1016/j.xphs.2018.05.024>.
- Tozer, T.N., 2015. Chapter 5 pharmacokinetic principles relevant to bioavailability studies. *Principles and Perspectives in Drug Bioavailability*. S. Karger AG, pp. 120–155. <https://doi.org/10.1159/000400105>.
- Tropsha, A., 2010. Best practices for QSAR model development, validation, and exploitation. *Mol. Inform.* <https://doi.org/10.1002/minf.201000061>.
- Tropsha, A., Golbraikh, A., 2007. Predictive QSAR modeling workflow, model applicability domains, and virtual screening. *Curr. Pharm. Des.* 13, 3494–3504. <https://doi.org/10.2174/138161207782794257>.
- Tsinman, K., Tsinman, O., Lingmaneni, R., Zhu, S., Riebeschl, B., Grandeur, A., Juhnke, M., Van Eerdenbrugh, B., 2018. Ranking itraconazole formulations based on the flux through artificial lipophilic membrane. *Pharm. Res.* 35, 161. <https://doi.org/10.1007/s11095-018-2440-3>.
- Tsuji, A., Tamai, I., 1996. Carrier-mediated intestinal transport of drugs. *Pharm. Res.* 13, 963–977. <https://doi.org/10.1023/A:1016086003070>.
- Twarog, C., Liu, K., O'Brien, P.J., Dawson, K.A., Fattal, E., Illel, B., Brayden, D.J., 2020. A head-to-head Caco-2 assay comparison of the mechanisms of action of the intestinal permeation enhancers: SNAC and sodium caprate (C10). *Eur. J. Pharm. Biopharm.* 152, 95–107. <https://doi.org/10.1016/j.ejpb.2020.04.023>.
- Udata, C., Patel, J., Pal, D., Hejchman, E., Cushman, M., Mitra, A.K., 2003. Enhanced transport of a novel anti-HIV agent—Cosalane and its congeners across human intestinal epithelial (Caco-2) cell monolayers. *Int. J. Pharm.* 250, 157–168. [https://doi.org/10.1016/S0378-5173\(02\)00523-9](https://doi.org/10.1016/S0378-5173(02)00523-9).
- Ungell, A.-L., 2010. Preclinical evaluation of drug transport. *MOLECULAR BIOPHARMACEUTICS*. Pharmaceutical Press, pp. 225–254.
- Ungell, A.-L., 2002. Transport studies using intestinal tissue *ex vivo*. *CELL CULTURE MODELS OF BIOLOGICAL BARRIERS*. CRC Press, pp. 164–188. <https://doi.org/10.1201/9780203219935.ch11>.
- Ussing, H.H., Zerahn, K., 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* 23, 110–127. <https://doi.org/10.1111/j.1748-1716.1951.tb00800.x>.
- van Breemen, R.B., Li, Y., 2005. Caco-2 cell permeability assays to measure drug absorption. *Expert Opin. Drug Metab. Toxicol.* 1, 175–185. <https://doi.org/10.1517/17425255.1.2.175>.
- van De Waterbeemd, H., Camenisch, G., Folkers, G., Raevsky, O.A., 1996. Estimation of Caco-2 cell permeability using calculated molecular descriptors. *Quant. Struct. Relationships* 15, 480–490.
- van de Waterbeemd, H., Gifford, E., 2003. ADMET *in silico* modelling: towards prediction paradise? *Nat. Rev. Drug Discov.* <https://doi.org/10.1038/nrd1032>.
- Van Den Abeele, J., Kostantini, C., Barker, R., Kourentas, A., Mann, J.C., Vertzoni, M., Beato, S., Reppas, C., Tack, J., Augustijns, P., 2020. The effect of reduced gastric acid secretion on the gastrointestinal disposition of a ritonavir amorphous solid dispersion in fasted healthy volunteers: an *in vivo* - *in vitro* investigation. *Eur. J. Pharm. Sci.* 151, 105377. <https://doi.org/10.1016/j.ejps.2020.105377>.
- Varma, M.V., Ambler, C.M., Ullah, M., Rotter, C.J., Sun, H., Litchfield, J., Fenner, K.S., El-Kattan, A.F., 2011. Targeting Intestinal Transporters for Optimizing Oral Drug Absorption. *Curr. Drug Metab.* 11, 730–742. <https://doi.org/10.2174/138920010794328850>.
- Veber, D.F., Johnson, S.R., Cheng, H.-Y., Smith, B.R., Ward, K.W., Kopple, K.D., 2002. Molecular Properties That Influence the Oral Bioavailability of Drug Candidates. <https://doi.org/10.1021/jm020017n>.
- Verma, R.P., Hansch, C., Selassie, C.D., 2007. Comparative QSAR studies on PAMPA/modified PAMPA for high throughput profiling of drug absorption potential with respect to Caco-2 cells and human intestinal absorption. *J. Comput. Aided. Mol. Des.* 21, 3–22. <https://doi.org/10.1007/s10822-006-9101-z>.
- Verwei, M., Minekus, M., Zeijdner, E., Schilderink, R., Havenaar, R., 2016. Evaluation of two dynamic *in vitro* models simulating fasted and fed state conditions in the upper gastrointestinal tract (TIM-1 and tiny-TIM) for investigating the bioaccessibility of pharmaceutical compounds from oral dosage forms. *Int. J. Pharm.* 498, 178–186. <https://doi.org/10.1016/j.ijpharm.2015.11.048>.
- Vinarov, Z., Katev, V., Radeva, D., Tcholakova, S., Denkov, N.D., 2018. Micellar solubilization of poorly water-soluble drugs: effect of surfactant and solubilize molecular structure. *Drug Dev. Ind. Pharm.* 44, 677–686. <https://doi.org/10.1080/03639045.2017.1408642>.
- Vizserálek, G., Balogh, T., Takács-Novák, K., Sinkó, B., 2014. PAMPA study of the temperature effect on permeability. *Eur. J. Pharm. Sci.* 53, 45–49. <https://doi.org/10.1016/j.ejps.2013.12.008>.
- Volkova, T.V., Domanina, E.N., Kumeev, R.S., Proshin, A.N., Terekhova, I.V., 2018. The effect of different polymers on the solubility, permeability and distribution of poor soluble 1,2,4-thiadiazole derivative. *J. Mol. Liq.* 269, 492–500. <https://doi.org/10.1016/j.molliq.2018.08.062>.
- Volpe, D.A., 2016. Transporter assays as useful *in vitro* tools in drug discovery and development. *Expert Opin. Drug Discov.* 11, 91–103. <https://doi.org/10.1517/17460441.2016.1101064>.
- Volpe, D.A., 2010. Application of method suitability for drug permeability classification. *AAPS J* 12, 670–678. <https://doi.org/10.1208/s12248-010-9227-8>.
- Volpe, D.A., 2008. Drug permeability studies in regulatory bioequivalence applications. *DRUG ABSORPTION STUDIES*. Springer, pp. 665–680.
- von Erlach, T., Saxton, S., Shi, Y., Minahan, D., Reker, D., Javid, F., Lee, Y.-A.L., Schoellhammer, C., Esfandiary, T., Cleveland, C., Booth, L., Lin, J., Levy, H., Blackburn, S., Hayward, A., Langer, R., Traverso, G., 2020. Robotically handled whole-tissue culture system for the screening of oral drug formulations. *Nat. Biomed. Eng.* 4, 544–559. <https://doi.org/10.1038/s41551-020-0545-6>.
- Walgren, R.A., Walle, T., 1999. The influence of plasma binding on absorption/excretion in the caco-2 model of human intestinal absorption. *J. Pharm. Pharmacol.* 51, 1037–1040. <https://doi.org/10.1211/0022357991773366>.
- Wang, N.-N., Dong, J., Deng, Y.-H., Zhu, M.-F., Wen, M., Yao, Z.-J., Lu, A.-P., Wang, J.-B., Cao, D.-S., 2016. ADME properties evaluation in drug discovery: prediction of Caco-2 cell permeability using a combination of NSGA-II and boosting. *J. Chem. Inf. Model.* 56, 763–773. <https://doi.org/10.1021/acs.jcim.5b00642>.
- Wang, N.N., Huang, C., Dong, J., Yao, Z.J., Zhu, M.F., Deng, Z.K., Lv, B., Lu, A.P., Chen, A.F., Cao, D.S., 2017. Predicting human intestinal absorption with modified random forest approach: a comprehensive evaluation of molecular representation, unbalanced data, and applicability domain issues. *RSC Adv* 7, 19007–19018. <https://doi.org/10.1039/C6RA28442F>.
- Wang, Y., 2019. PBPK Current status and challenges: a regulatory perspective development of best practices in physiologically based pharmacokinetic modeling to support clinical pharmacology regulatory decision-making.
- Wang, Z., Yan, A., Yuan, Q., Gasteiger, J., 2008. Explorations into modeling human oral bioavailability. *Eur. J. Med. Chem.* 43, 2442–2452. <https://doi.org/10.1016/j.ejmech.2008.05.017>.
- Warnken, V., Puppulo, M., Hughey, J., Duarte, I., Jansen-Varmum, S., 2018. *In vitro*-*in vivo* correlations of carbamazepine nanodispersions for application in formulation development. *J. Pharm. Sci.* 107, 453–465. <https://doi.org/10.1016/j.xphs.2017.10.014>.
- Wessel, M.D., Jurs, P.C., Tolan, J.W., Muskal, S.M., 1998. Prediction of human intestinal absorption of drug compounds from molecular structure. *J. Chem. Inf. Comput. Sci.* 38, 726–735. <https://doi.org/10.1021/ci980029a>.
- Westerhout, J., van de Steeg, E., Grossouw, D., Zeijdner, E.E., Krul, C.A.M., Verwei, M., Wortelboer, H.M., 2014. A new approach to predict human intestinal absorption using porcine intestinal tissue and biorelevant matrices. *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 63, 167–177. <https://doi.org/10.1016/j.ejps.2014.07.003>.
- Westerhout, J., Wortelboer, H., Verhoeckx, K., 2015. Using Chamber, in: the Impact of Food Bioactives on Health. *Vitro and Ex Vivo Models*. Springer International Publishing, pp. 263–273. https://doi.org/10.1007/978-3-319-16104-4_24.
- Wilson, J.P., 1967. Surface area of the small intestine in man. *Gut* 8, 618.
- Wilson, V., Lou, X., Osterling, D.J., Stolarik, D.F., Jenkins, G., Gao, W., Zhang, G.G.Z., Taylor, L.S., 2018. Relationship between amorphous solid dispersion *in vivo* absorption and *in vitro* dissolution: phase behavior during dissolution, speciation, and membrane mass transport. *J. Control. Release* 292, 172–182. <https://doi.org/10.1016/j.jconrel.2018.11.003>.
- Winiwarter, S., Ax, F., Lennernäs, H., Hallberg, A., Pettersson, C., Karlén, A., 2003. Hydrogen bonding descriptors in the prediction of human *in vivo* intestinal permeability. *J. Mol. Graph. Model.* 21, 273–287. [https://doi.org/10.1016/s1093-3263\(02\)00163-8](https://doi.org/10.1016/s1093-3263(02)00163-8).

- Winiwarter, S., Bonham, N.M., Ax, F., Hallberg, A., Lennernäs, H., Karlén, A., 1998. Correlation of human jejunal permeability (in Vivo) of drugs with experimentally and theoretically derived parameters. A multivariate data analysis approach. *J. Med. Chem.* 41, 4939–4949. <https://doi.org/10.1021/jm9810102>.
- Wohnsland, F., Faller, B., 2001. High-throughput permeability pH profile and high-throughput alkane/water log p with artificial membranes. *J. Med. Chem.* 44, 923–930. <https://doi.org/10.1021/jm001020e>.
- Wu, C.-Y., Benet, L.Z., Hebert, M.F., Gupta, S.K., Rowland, M., Gomez, D.Y., Wachter, V. J., 1995. Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. *Clin. Pharmacol. Ther.* 58, 492–497. [https://doi.org/10.1016/0009-9236\(95\)90168-X](https://doi.org/10.1016/0009-9236(95)90168-X).
- Wuyts, B., Riethorst, D., Brouwers, J., Tack, J., Annaert, P., Augustijns, P., 2015. Evaluation of fasted and fed state simulated and human intestinal fluids as solvent system in the Ussing chambers model to explore food effects on intestinal permeability. *Int. J. Pharm.* 478, 736–744. <https://doi.org/10.1016/j.ijpharm.2014.12.021>.
- Xu, X.S., Jiang, H., Christopher, L.J., Shen, J.X., Zeng, J., Arnold, M.E., 2014. Sensitivity-based analytical approaches to support human absolute bioavailability studies. *Bioanalysis* 6, 497–504. <https://doi.org/10.4155/bio.13.318>.
- Xu, Y., Shrestha, N., Pr at, V., Beloqui, A., 2021. An overview of in vitro, ex vivo and in vivo models for studying the transport of drugs across intestinal barriers. *Adv. Drug Deliv. Rev.* 175, 113795. <https://doi.org/10.1016/j.addr.2021.05.005>.
- Yamashita, S., Furubayashi, T., Kataoka, M., Sakane, T., Sezaki, H., Tokuda, H., 2000. Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. *Eur. J. Pharm. Sci.* 10, 195–204. [https://doi.org/10.1016/s0928-0987\(00\)00076-2](https://doi.org/10.1016/s0928-0987(00)00076-2).
- Yamashita, S., Tanaka, Y., Endoh, Y., Taki, Y., Sakane, T., Nadai, T., Sezaki, H., 1997. Analysis of drug permeation across Caco-2 monolayer: implication for predicting in vivo drug absorption. *Pharm. Res.* 14, 486–491. <https://doi.org/10.1023/a:1012103700981>.
- Ye, D., Harder, A., Fang, Z., Weinheimer, M., Laplanche, L., Mezler, M., 2020. Characterization and validation of canine P-glycoprotein-deficient MDCK II cell lines for efflux substrate screening. *Pharm. Res.* 37, 194. <https://doi.org/10.1007/s11095-020-02895-9>.
- Yeap, Y.Y., Trevaskis, N.L., Porter, C.J.H., 2013a. The potential for drug supersaturation during intestinal processing of lipid-based formulations may be enhanced for basic drugs. *Mol. Pharm.* 10, 2601–2615. <https://doi.org/10.1021/mp400035z>.
- Yeap, Y.Y., Trevaskis, N.L., Quach, T., Tso, P., Charman, W.N., Porter, C.J.H., 2013b. Intestinal bile secretion promotes drug absorption from lipid colloidal phases via induction of supersaturation. *Mol. Pharm.* 10, 1874–1889. <https://doi.org/10.1021/mp3006566>.
- Yoshida, F., Topliss, J.G., 2000. QSAR model for drug human oral bioavailability. *J. Med. Chem.* 43, 2575–2585. <https://doi.org/10.1021/jm0000564>.
- Youhanna, S., Lauschke, V.M., 2021. The past, present and future of intestinal in vitro-cell systems for drug absorption studies. *J. Pharm. Sci.* 110, 50–65. <https://doi.org/10.1016/j.xphs.2020.07.001>.
- Yu, L., Bridgers, A., Polli, J., Vickers, A., Long, S., Roy, A., Winnike, R., Coffin, M., 1999. Vitamin E-TPGS increases absorption flux of an HIV protease inhibitor by enhancing its solubility and permeability. *Pharm. Res.* 16, 1812–1817. <https://doi.org/10.1023/A:1018939006780>.
- Yu, L.X., Amidon, G.L., 1999. A compartmental absorption and transit model for estimating oral drug absorption. *Int. J. Pharm.* 186, 119–125. [https://doi.org/10.1016/s0378-5173\(99\)00147-7](https://doi.org/10.1016/s0378-5173(99)00147-7).
- Yu, L.X., Lipka, E., Crison, J.R., Amidon, G.L., 1996. Transport approaches to the biopharmaceutical design of oral drug delivery systems: prediction of intestinal absorption. *Adv. Drug Deliv. Rev.* 19, 359–376. [https://doi.org/10.1016/0169-409x\(96\)00009-9](https://doi.org/10.1016/0169-409x(96)00009-9).
- Zane, P., Gieschen, H., Kersten, E., Mathias, N., Ollier, C., Johansson, P., Van den Bergh, A., Van Hemelryck, S., Reichel, A., Rotgeri, A., Sch fer, K., M llertz, A., Langguth, P., 2019. In vivo models and decision trees for formulation development in early drug development: a review of current practices and recommendations for biopharmaceutical development. *Eur. J. Pharm. Biopharm.* 142, 222–231. <https://doi.org/10.1016/j.ejpb.2019.06.010>.
- Zhang, Y., Benet, L.Z., 2001. The gut as a barrier to drug absorption: combined role of cytochrome P450 3A and P-glycoprotein. *Clin. Pharmacokinet.* <https://doi.org/10.2165/00003088-200140030-00002>.
- Zhao, Y.H., Le, J., Abraham, M.H., Hersey, A., Eddershaw, P.J., Luscombe, C.N., Boutina, D., Beck, G., Sherborne, B., Cooper, I., Platts, J.A., 2001. Evaluation of human intestinal absorption data and subsequent derivation of a quantitative structure–activity relationship (QSAR) with the Abraham descriptors. *J. Pharm. Sci.* 90, 749–784. <https://doi.org/10.1002/jps.1031>.
- Zhu, A.Z.X., Ho, M.-C.D., Gemski, C.K., Chuang, B.-C., Liao, M., Xia, C.Q., 2016. Utilizing in vitro dissolution-permeation chamber for the quantitative prediction of pH-dependent drug–drug interactions with acid-reducing agents: a comparison with physiologically based pharmacokinetic modeling. *AAPS J* 18, 1512–1523. <https://doi.org/10.1208/s12248-016-9972-4>.
- Zhu, C., Jiang, L., Chen, T.-M., Hwang, .K.-K., 2002. A comparative study of artificial membrane permeability assay for high throughput profiling of drug absorption potential. *Eur. J. Med. Chem.* 37, 399–407. [https://doi.org/10.1016/S0223-5234\(02\)01360-0](https://doi.org/10.1016/S0223-5234(02)01360-0).