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Review

Research and Prospects on the Evaluation of Drug Cocrystal Permeability

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Abstract: In developing new drugs, drug permeability assessment is crucial. Lead compounds exhibiting inadequate permeability often produce low bioavailability, rendering them inappropriate as drugs. The cocrystallization technique is a valuable tool for optimizing the physical and chemical properties of active pharmaceutical ingredients (APIs) and enhancing drug properties. This technique involves the introduction and weak interaction with cocrystal formers to produce supramolecular substances without altering the chemical structure of APIs, effectively improving their solubility and permeability and thereby significantly increasing their bioavailability. Consequently, drug cocrystal research has become a focal point for researchers in drug development. This study provides a comprehensive overview of four commonly employed methods for evaluating drug permeability and summarizes the applicability of each method to provide a reference for improving and refining the permeability evaluation method of drug cocrystals.

Keywords: permeability; bioavailability; cocrystallization; evaluating

1. Introduction

The Biopharmaceutical Classification System (BCS) is a scientific framework that classifies drugs based on their water solubility and intestinal permeability, primarily assessing their solubility, gastrointestinal permeability, and dissolution [1]. It categorizes drugs into four classes, as illustrated in Figure 1: I, II, III, and IV, based on their solubility and permeability differences.

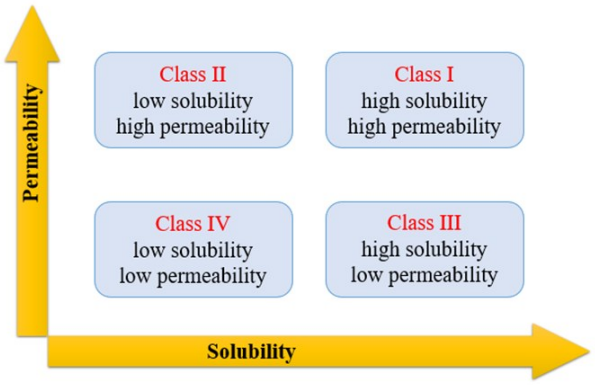

Figure 1. BCS classification.

Drug solubility is crucial in the design, preparation, and quality control of drug formulations, referring to the maximum amount of drug that can dissolve under specific conditions [2]. It is influenced by numerous factors such as molecular structure, drug polymorphs, particle size, and temperature. Drug permeability refers to the capability of drug molecules to traverse phospholipid bilayers, influenced by lipophilicity, hydrogen bonding, solute size, and ionization state [3].

The efficacy of oral medication depends on the permeability of the dissolved drug through the cell membrane [4]. In early drug screening and development stages, in vivo permeability models serve as decision-making tools for screening candidate drugs [5]. Currently, structural modification is frequently employed in the process of drug development to improve physicochemical properties. In clinical medication, strategies such as adding drug penetration enhancers, introducing ions, and employing transdermal patches are frequently utilized to enhance drug absorption. Compounds with inadequate permeability are unsuitable for further development. However, these compounds can be modified to improve their pharmacological properties, and cocrystallization technology is particularly well-suited for optimizing the physicochemical properties of such compounds.

The cocrystal classification based on their types can be traced back to 1922, and the discipline of crystal engineering was established in the 1970s. A review in 2004 explored the design and application of drug cocrystal [6,7]. Cocrystal preparation methods include solvent crystallization, spray, melting, pressure crystallization, and other methods. Drug cocrystal [8] is an important type of cocrystal containing at least one API. It is a multi-component crystal composed of cocrystal formers (CCFs) in a specific stoichiometric ratio, combining non-bonding interactions such as hydrogen bonding, van der Waals forces, and π-π stacking in a single lattice. It is characterized by the ability to enhance the physical and chemical properties of drugs without affecting their therapeutic efficacy [9,10]. As illustrated in Table 1, numerous drug cocrystals have been introduced to the market. Another survey exhibited that from approximately 4300 published articles on cocrystal, about 80% were about solubility, while only 2% were about permeability [7,11]. This disparity indicates a significant research gap in exploring cocrystals for improving permeability. However, these preliminary studies have provided valuable information and guidance, and future research will continue to develop upon this foundation.

Table 1. Currently marketed oral drug cocrystal preparations.

Trade Name	Components	Purpose
Beta-Chlor	Chloral hydrate:betaine (1:1)	Improve thermal stability
Depakote	Valproic acid:Sodium valproate (1:1)	Slow down the deliquescence rate
Cafcit	Caffeine:Citric acid (1:1)	Improve physical stability
Lexapro	Escitalopram:Oxalic acid (1:1)	Improve physical stability
Suglat	Ipragliflozin:L-proline (1:1)	Slow down the deliquescence rate
Entresto	Sacubitril:Valsartan (1:1)	Improve bioavailability
Odomzo	Sonidegib:Phosphoric acid (1:1)	Improve solubility
Steglatro	Ertugliflozin:L-pyroglutamic acid (1:1)	Slow down the deliquescence rate
Seglentis	Tramadol:Celecoxib (1:1)	Improve dissolution rate

For compounds with inadequate permeability, forming cocrystals and evaluating their permeability improvements is a practicable approach. The cocrystal formation may induce intermolecular interactions and/or polarity changes in chemical entities, potentially affecting drug permeability. BCS class I drugs, with high solubility and permeability, are seldom considered for cocrystal preparation due to their favorable properties. BCS class II drugs, such as apalutamide (APA), have low solubility and high permeability. Venkata et al. [12] developed a cocrystal of APA and methyl hydroxybenzoate (MP) in a 1:1 ratio through hydrogen bonding. Their experiments demonstrated significantly increased solubility and the intrinsic dissolution rate (IDR) of the cocrystal, making it a suitable choice for BCS class II drug development.

BCS class III drugs exhibit high solubility and low permeability. Ji et al. [13] synthesized one salt and three cocrystals with four dicarboxylic acids based on abacavir, achieving significant improvements in water

solubility, dissolution rate, and permeability compared to abacavir alone. The main factor influencing the permeability of abacavir and its salt and cocrystals is the concentration gradient, with secondary factors including the lipid-water partition coefficient, demonstrating the superiority of cocrystal formation in enhancing drug permeability.

In their transdermal drug delivery research, Dai et al. [14] constructed a cocrystal of 5-fluorouracil with hydroxybenzoic acid isomers and evaluated its permeability using Franz diffusion cells and a silica gel model. The results demonstrated enhanced membrane penetration and improved permeability, indicating that pharmaceutical cocrystal formation can effectively alter transdermal drug delivery permeability.

BCS class IV drugs, with limited solubility and permeability, include acetazolamide. Zhang et al. [15] formed a cocrystal with theophylline to enhance its properties, achieving significant permeability enhancements compared to acetazolamide alone, suggesting that cocrystal formation can offer innovative solutions for such drugs.

The drug cocrystallization approach can provide a promising foundation for developing compounds with permeability issues into clinically suitable drugs. This study summarized commonly employed methods for evaluating cocrystal permeability and elaborated their advantages and disadvantages.

2. Drug Permeability Evaluation Models

Researchers have developed various models for evaluating permeability, including in vivo or in-situ intestinal perfusion in animal models, in vitro human or animal intestinal tissue models, monolayer cultured epithelial cell models, and Franz diffusion cell methods [16–18]. The in-situ models closely mimic the human physiological conditions with fewer sampling points; however, it is costly, and the ex vivo cultivation time is limited, resulting in faster experiment completion. Therefore, this method is mainly used for preliminary research and falls outside the scope of this article [19]. The single-layer artificially cultured epithelial cell model primarily consists of Caco-2, derived from human colon cancer, and Madin-Darby Canine Kidney (MDCK) cells from Cocker Spaniel renal epithelial cells. Additionally, a parallel artificial infiltration model is available that simulates artificial lipid membranes as biofilms. This article mainly introduces the parallel artificial membrane permeability assay (PAMPA), Caco-2, MDCK, and Franz diffusing cell models. Figure 2 demonstrates the major membrane permeability mechanisms of drugs and the four models mentioned above.

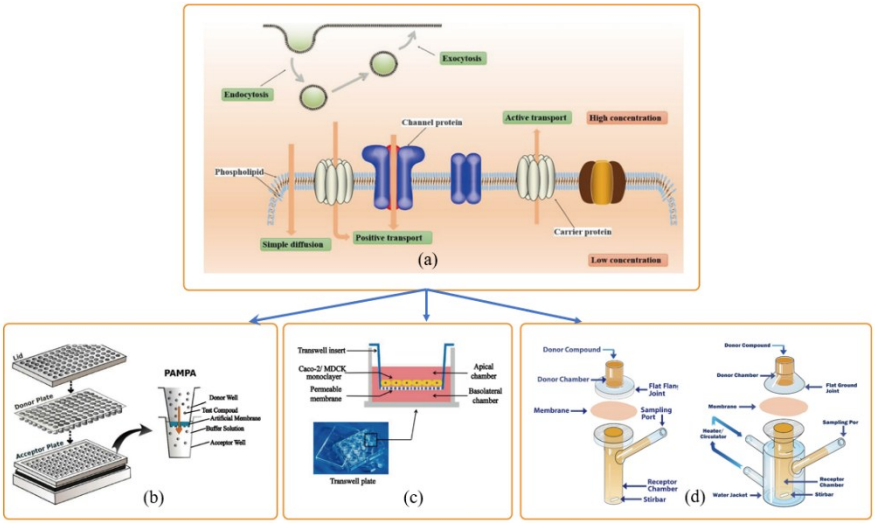

Figure 2. Drug permeability mechanism and four evaluation models: (**a**) major membrane permeability mechanisms; (**b**) PAMPA model [20]; (**c**) Caco-2 and MDCK model [21]; (**d**) Franz diffusing cells method [22].

2.1. PAMPA Model

The PAMPA model was initially developed by Kansy et al. [23] as a rapid method for determining passive transport permeability for early drug ADME (absorption, distribution, metabolism, excretion)

screening [24]. This model applies an organic solvent containing an artificial lipid membrane to a polyvinylidene fluoride or polycarbonate membrane in a 96-well plate. The lipid membrane forms a stable thin film at the center of the well, creating two compartments: the donor compartment, containing the test compound in a buffer solution, and the receptor compartment, containing an initial buffer solution. This configuration forms a sandwich-like structure containing three layers: donor compartment (bottom), artificial lipid membrane (middle), and receptor compartment (top). This technology can simultaneously form 96 infiltrating cells, improving evaluation throughput and reducing experimental and time costs. Consequently, the PAMPA model is widely employed in drug research [25]. However, due to its lack of transport proteins, it is unable to accurately predict drugs with active transport mechanisms. Recently, PAMPA technology has been actively developed and optimized, leading to various models for evaluating drug permeability.

2.1.1. PAMPA Models for Different Tissues

PAMPA-DS (Double Sink) Model for Intestinal Absorption

This model was initially developed by Winiwarter et al. [26] using human jejunal effective permeability (HJP) data from 8 training compounds out of 22 drugs. It can evaluate the drug permeability absorbed by the intestine. The lipid barrier of this model [27] is established by dissolving lipophilic molecules into the artificial lipid membrane and adding surfactants to the receptor compartment. The increased lipid concentration in this model is beneficial for evaluating drugs with poor water solubility.

PAMPA-BBB (Blood-Brain Barrier) Model for Brain Uptake

The BBB permeability can predict the speed at which compounds reach brain tissue. Screening BBB permeability is essential in early drug development stages. The artificial lipid membrane of this model is composed of brain fat. Li et al. [28] utilized 30 structurally diverse commercial drugs and 14 compounds from Wyeth Research Company to predict BBB permeability improvements using PAMPA experiments modified with pig brain fat. The experimental results indicated that the PAMPA-BBB model has the advantages of a high success rate, low cost, and significant reproducibility.

PAMPA-Skin Model for Skin Absorption

Dermal and transdermal drug therapies are gaining importance in drug development, necessitating the optimization of drug release and transdermal rates. The PAMPA-Skin model, developed by Sinkó et al. [29], is an in vitro experimental model designed to evaluate drug penetration through the skin. It simulates the characteristics of the stratum corneum to predict skin permeability. Recently, the PAMPA-Skin model has been widely applied, utilizing an artificial membrane composed of cholesterol, free fatty acids, and a ceramide-mimetic compound that simulates ceramide characteristics [30]. A patch experiment using the PAMPA-Skin model to investigate transdermal and local treatments demonstrated [31] that this method effectively reproduces the permeability differences among various drugs. It is particularly appropriate for patch testing and is recommended for permeability studies in developing transdermal treatment systems. Although the model offers advantages such as rapid and simultaneous detection of multiple formulations and quantification, it also has drawbacks, including manual sampling and non-automation.

2.1.2. Effect of Lipids on the PAMPA Model

Common lipid types reported in PAMPA models include egg lecithin, cholesterol, dialysis phosphatidylcholine, N-hexadecane, and various phospholipid mixtures. Seo et al. [32] investigated the permeability of five model compounds using six distinct lipids in PAMPA models. Their results revealed that compound permeability varied based on sensitivity to membrane lipid composition, with highly permeable compounds (permeability at least 0.2×10^{-6} cm/s) exhibiting sensitivity to membrane fluidity and ion pair effects. This observation aligns with the findings by Carrara et al. [33] observations that permeability mainly depends on the dodecane percentage. Palaiokostas et al. [34] systematically investigated the impact of layered and non-layered lipids on the passive permeation of 13 small molecules and drugs through molecular dynamics simulations. The results demonstrated that non-layered lipids can reduce the permeability of small

molecules (molecular weight <100 g/mol) and improve the permeability of larger molecules (>100 g/mol). Due to the lack of diffusion resistance in the blank membrane, the model initially employed organic solvents to coat the lipid membrane. However, recent findings suggest that organic solvents can interact with the supporting membrane, prompting efforts to modify the PAMPA model. Consequently, the lipid types and arrangements can impact the PAMPA model.

2.2. Caco-2 Cell Model

Oral administration is currently the preferred route of drug delivery, with bioavailability influenced by the physicochemical properties of the drug, metabolic transformation in the liver and intestine, and gastrointestinal tract conditions. Among these, drug transport through the intestinal epithelial cell barrier is a crucial factor in determining in vivo bioavailability [35]. Lipinski's Rule of Five (ROF) [36] provides a rough estimate of oral absorption of compounds based on their molecular weight, lipophilicity, and hydrogen bonding, although it is inapplicable for compounds undergoing active transport. In the intestine, a single layer of epithelial cells lines the intestinal wall, acting as a barrier that limits soluble drug absorption. The Caco-2 cell line originates from human colon adenocarcinoma cells. Caco-2 cells are seeded on a matrix with polycarbonate membranes and can spontaneously form a monolayer under appropriate culture conditions. This in vitro simulation of reconstructed epithelial cell monolayers can predict oral drug absorption. Initially employed in the late 1980s to simulate human intestinal absorption, the Caco-2 cell model has since become the most extensively used in vitro absorption model due to its simplicity and broad applicability. It can predict drug absorption and transport characteristics at the cellular level, expediting the screening and development of new drugs. However, significant inter-laboratories variations in cell culture generations, conditions, cell numbers, and monolayer integrity hinder direct comparison of permeability results [37]. Despite these limitations, the Caco-2 cell-based detection method provides effective and replicable data for drug development, surpassing the capabilities of non-biological and many other biological models.

2.2.1. Establishment of the Caco-2 Cell Model

The Caco-2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine serum albumin, small amounts of non-essential amino acids, L-glutamine, and 85 mg/L gentamicin at 37 °C. The environmental conditions were maintained at 5% CO_2 and 90% relative humidity, with cell passages occurring every five days. After 20–30 passages, the cells were implanted into Transwell inserts containing polycarbonate membranes, with the medium being replaced daily [38].

2.2.2. Indicators for Single-Cell Layer Integrity Testing

The integrity of a single-cell layer can be evaluated using the following five indicators [39]:

(1) Cell Morphology Examination: Optical or electron microscopy reveals the microvilli structure and dense single-cell layer tissue of the small intestine, providing insights into the integrity of the single-layer barrier. (2) Alkaline Phosphatase Activity: This enzyme, a marker for small intestinal brush border cells, can be detected at various cultivation stages, indicating an incomplete single-cell layer. (3) Transmembrane Electrical Resistance (TEER): The resistance of each monolayer can be measured using Ohm's law and impedance spectroscopy, reflecting the degree of monolayer fusion and tight junctions [40]. (4) Transmembrane Processes Observation: Fluorescent or radioactive markers such as inulin, mannitol, and fluorescein can be used to observe these processes. (5) Horseradish Peroxidase (HRP) Assay: The integrity of the Caco-2 cell model can be evaluated by determining the HRP amount passing through the cell monolayers, with higher amounts indicating poor integrity [41].

2.2.3. Advantages and Disadvantages of the Caco-2 Cell Model

The Caco-2 cell model, an in vitro analytical method for analyzing drug molecule absorption, provides valuable information on the ADME process of drugs crossing the small intestinal mucosa. Using human-derived Caco-2 cells, compared to the cells in the PAMPA model, does species differences, ensuring good reproducibility of results. The experimental conditions are easy to control, making the process more time-

efficient and cost-effective than animal studies. Moreover, the minimally required amount of drug facilitates high-throughput screening in drug development. Using human-derived Caco-2 cells, compared to the cells in PAMPA model, eliminates species differences, ensuring good reproducibility of results. This method can also enable the investigation of the exocytosis and active transport processes of drugs.

However, the Caco-2 cell model has several limitations: it requires a long culture cycle; the absence of mucin-producing cells leads to a deficiency in the mucus layer of the intestinal wall, a crucial barrier to drug absorption in humans; the lower expression level of absorption transporters compared to the small intestinal epithelium limits research on active drug transport; there is a lack of certain metabolic enzymes, such as cytochrome P450.

Liu Dong et al. [42] conducted comprehensive research to address these limitations, including introducing complementary DNA (cDNA) of metabolic enzymes into cells to compensate for low CYP450 levels, co-culturing with HT29-MTX goblet cells or covering them with mucin to address the lack of a mucus layer, developing novel Caco-2 cells with shorter cultivation cycles, modifying buffer composition to increase the solubility of lipophilic compounds, and reducing non-specific binding of lipophilic drugs.

2.3. MDCK Model

Although the Caco-2 cell model is known for its exceptional attributes, its relatively low throughput prompted Martin and Darby to establish the MDCK cell line in 1958 to reduce tissue culture time [43,44]. The MDCK cell line has since become a well-suited tool for permeability screening in drug development. Similar to Caco-2 cells, MDCK cells can differentiate into columnar epithelium and establish tight junctions when cultured on semi-permeable membranes [45].

MDCK cells are cultured in DMEM supplemented with 10% fetal bovine serum, non-essential amino acids, and antibiotics at 37 °C, 95% relative humidity, and 5% CO_2 [46]. Following media removal, the cells are washed twice with Ethylene Diamine Tetraacetic Acid (EDTA) physiological saline to remove all residual solutions. Trypsin is then added, and the cells are incubated at 37 °C for 10 min. Cells are counted using a hematology analyzer and diluted in DMEM to achieve the concentration of 2.5×10^5 cells/mL (Millicell-96 well plates, Millipore Sigma, New York, NY, USA) and 3×10^5 cells/mL (Millicell-24 well plates, Millipore Sigma). Permeability measurements are conducted on days three and four [47]. The integrity of MDCK cell monolayers can be evaluated by TEER values, and fluorescein sodium permeability can indicate cell differentiation. Only monolayers with TEER values >140 $\Omega \cdot cm^2$ and fluorescein sodium permeability $<0.22 \times 10^{-6}$ cm/s are considered intact [46]. However, since MDCK cells are derived from dogs rather than humans, their transport protein expression differs from human cells, potentially impacting data analysis.

2.4. Franz Diffusion Cells Method

To avoid first-pass effects, side effects, and poor patient compliance associated with oral administration, skin and transdermal drug delivery have emerged as potential alternatives to traditional drug delivery. Evaluating molecular transdermal absorption is critical in assessing transdermal drug delivery systems [48]. The Franz diffusion cell method is extensively used for in vitro transdermal absorption detection [49]. This method employs carriers such as mouse skin and artificial and ex vivo membranes. The Franz diffusion cell comprises two compartments, a donor and a receptor chamber, separated by the carrier, with the stratum corneum facing the donor chamber. A constant-temperature water bath and a magnetic stirrer in the receptor chamber maintain sink conditions. Akram et al. [50] evaluated the effects of five different enhancers on glibenclamide penetration through rabbit skin using a Franz diffusion cell. Similarly, Seo et al. [51] also compared the transdermal permeability of three types of nipagin esters at varying concentrations using hairless mouse whole skin and human cadaveric epidermis in a Franz cell model. The Franz diffusion cell can objectively reflect the drug release process of transdermal formulations. However, its low automation leads to a time-consuming process, and bubble formation after sampling in the receptor chamber may reduce the permeation area, limiting large-scale application.

The aforementioned methods each have unique advantages and can be employed to evaluate the drug permeability improvements due to cocrystal formation in drug cocrystal studies. They offer benefits in terms of objectivity, accuracy, and potential savings in resources and time. Existing research on PAMPA and Caco-2

methods to improve cocrystal permeability is limited. The Franz diffusion cell method, extensively studied [52,53], is the "gold standard" for evaluating drug permeability in transdermal drug delivery systems (TDDS) [54]. By employing diverse membrane materials, this method can also evaluate the permeability of some oral formulations frequently used in salt and cocrystal research.

3. Prediction Method for Drug Permeability

The prediction methods for drug permeability include the potential of mean force (PMF) [55], quantitative structure-permeability relationships (QSPRs) [56], and immobilized artificial membrane (IAM) chromatography [57], among others. The permeability of pharmaceutical cocrystals through cell membranes can be evaluated by the free energy required to cross the membrane. Meng et al. [58] employed molecular dynamics to simulate drug permeability and calculated the potential energy distribution of three drugs with varying permeability using the PMF method. The results revealed that low-permeability drugs have higher free energy, whereas high-permeability drugs exhibit the opposite. Drugs with hydrophobic groups can quickly pass through the cell membrane [59]. Consequently, the PMF method can effectively predict drug permeability. However, the lengthy calculation process limits its application.

Jung et al. [60] combined an in vitro Caco-2 model with QSPRs to evaluate the permeability of 20 structurally similar drugs. Their study demonstrated that this approach could successfully predict drug permeability based on the three-dimensional molecular structure of drugs containing carboxylic acid groups. Ong et al. [61] measured the solute capacity factors of 23 structurally unrelated compounds on an IAM column and compared their results with solute equilibrium distribution coefficients in a liquid liposome system, achieving a strong linear correlation ($r = 0.907$). This implies that solute distribution in liposome membranes can be modeled using IAM, and solute membrane distribution can be measured via chromatography on IAM surfaces.

Currently, the BBB model is the primary focus of research on predicting the pharmaceutical cocrystal permeability using artificial intelligence technology, with relatively limited exploration of other biological membrane barriers [62,63]. Developing predictive models for the BBB permeability of compounds can assist in developing drugs for the treatment of central nervous system diseases. Initially, machine learning could only predict compounds with high permeability. However, continuous improvements to the model have now enhanced the predicting accuracy of low-permeability compounds.

4. Prospects

The cell membrane permeability of drugs significantly impacts their efficacy, activity, and toxicity, making it a vital indicator in the early drug development stages. Given the limitations of the PAMPA, Caco-2, and MDCK models and the Franz diffusion cells method, it is essential to consider the characteristics of drugs and the required timeframe when selecting a model. Accordingly, establishing a new model for evaluating permeability could enhance applicability.

Numerous studies have demonstrated that drug cocrystals can significantly enhance the solubility of active pharmaceutical ingredients (APIs). However, the limiting step in drug efficacy is the absorption process of APIs. Currently, there is limited research on improving the permeability of cocrystals, which is the reason why regulatory authorities in various countries have differing views on cocrystals. The continued advancement and application of permeability evaluation methods for drug cocrystals and ongoing research into the underlying mechanisms will facilitate broader applications and developments of drug cocrystals.

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