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Biorelevant Setups:
An *In Vitro* Guide for Product Development

Dissertação no âmbito de Mestrado de Biotecnologia Farmacêutica orientada pelo Professor Doutor Sérgio Paulo de Magalhães Simões e pela Doutora Marta Cristina Filipe Simões, apresentada à Faculdade de Farmácia da Universidade de Coimbra

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The work presented in this thesis has been carried out under the supervision of Professor Sérgio Paulo de Magalhães Simões (PharmD, PhD, Associate Professor with habilitation in the Faculty of Pharmacy of the University of Coimbra), and Marta Cristina Filipe Simões (PharmD, PhD, Head of Formulation Development at Bluepharma – Indústria Farmacêutica, SA).

Agradecimentos

Ao longo desta caminhada, muitas foram as pessoas que tiveram um papel fundamental em todo este processo. Foram dois anos de aprendizagem, partilha de conhecimentos, momentos altos e baixos, nos quais tive a sorte de ter sempre a meu lado pessoas incríveis. Assim, não poderia deixar de agradecer a todos, pois sozinha não seria possível.

Em primeiro lugar, gostaria de agradecer aos meus orientadores. Ao Professor Sérgio, pela oportunidade que me concedeu ao permitir que desenvolvesse este projeto na Bluepharma. Tem sido um orgulho para mim, trabalhar nesta casa desde 2016, e poder agora colocar em prática, de maneira diferente o meu conhecimento. Foi incrível aprender ainda mais numa empresa que aposta continuamente na ciência e na inovação. À Marta Simões, o meu maior agradecimento: por toda a disponibilidade, pela confiança, pelos desafios, pelas ideias que partilhou comigo, pela motivação que me transmitia a cada *brainstorming,* por acreditar nos meus resultados e por me ensinar que, até os maus resultados são conhecimento adquirido. É sem dúvida, um exemplo de profissionalismo, exigência, entrega e rigor. Não podia ter tido mais sorte com a orientadora que escolhi. Obrigada por me fazeres crescer a todos os níveis e por me teres guiado, apoiado e orientado de maneira incansável!

Continuando na família Bluepharma, agradeço à Sandra Almeida e à Claúdia Arranja, por sempre incentivarem esta minha vontade de voltar a estudar. Obrigada por permitirem várias trocas de horários, pelos conselhos e pelo apoio. Agradeço ainda a todo o Grupo Analítico, em particular ao Apoio ao Desenvolvimento de Formulações, por toda a ajuda e por me continuarem a aturar nos dias de "férias". Às Dissoluções, um grupo muito especial que sempre me acompanhou. Obrigada pela companhia e por terem suportado o cheiro do Octanol dia após dia. Aos Formuladores do Desenvolvimento Galénico, pelas amostras fornecidas e pela disponibilidade em esclarecer sempre qualquer dúvida que me surgia.

Agradeço a todos os meus amigos, as amizades de sempre e a todas as que não são de sempre, mas serão para sempre. Quero agradecer de maneira particular ao Eduardo e à Margarida, por me ouvirem em qualquer altura, pelas explicações fora de horas, pelas horas de explicações e por me ajudarem a manter a calma em dias menos bons. Obrigada por serem uma espécie de *"Ying-Yang"* da minha vida, por todos os bons momentos que passamos e por me fizerem desligar um pouco da correria do dia-dia. Ao Daniel Branco e ao Daniel Martins, pela animação logo pela manhã, pelo bom humor nas mais pequenas coisas, pela ajuda e por

alinharem sempre nos meus desafios. Obrigada por demonstrarem sempre interesse e disponibilidade em ajudar e a aprender comigo. À Margô, à Padilha, à Loureiro e à Xana Roxo pelas excelentes conversas e pela motivação certa na hora certa. À Mariana, pela ponderação e pela calma. Obrigada pelos conselhos que me foste sempre transmitindo. À Maria, fico grata por seres aquela amiga completa. Agradeço-te pelo apoio incansável, por acreditares em mim (por vezes mais do que eu), e por me compreenderes de uma maneira tão especial. Obrigada pelo profissionalismo que transmites sempre e por todos os bons momentos que passamos dentro e fora do ambiente profissional. É tão bom trabalhar com pessoas assim! Agradeço também à Helena, à Vera e ao Tiago. Quando ensinamos, aprendemos ainda mais, obrigada por terem sido os melhores, por fazerem as melhores perguntas, por serem interessados e por me ouvirem sempre. Agradeço às minhas meninas, à Brigas, à Bea, à Catarina, à Marta e à Xana, pelas longas chamadas e por todos os momentos partilhados (muitos e sempre bons). Obrigada pelo apoio constante e por saber que cada uma à sua maneira estará sempre aqui. À Filipa, pelo exemplo que sempre me transmitiu. Ao Francisco e a Marta, pelas longas horas de viagens, aulas, estudo e escrita. Obrigada por me integrarem novamente num mundo que já não era o meu. Fazer esta caminhada com vocês foi muito bonito.

Agradeço a toda a minha família, pois, sem eles, sem o porto seguro e sem as minhas origens nada disto seria possível. Aos meus pais, Cecília e Gabriel, aqueles a quem, para além da vida, devo tudo o resto. Obrigada por me ensinarem que tudo vale a pena, que tudo o que fazemos nos acrescenta e pelo apoio e amor incondicional. Obrigada por serem sempre um exemplo que tenho orgulho em tentar seguir. Obrigada Mãe, por estar sempre aqui e por ser o meu exemplo de luta e dedicação. Obrigada Pai, por ser o meu "engenheiro" particular e por transformar sempre as minhas ideias em algo funcional. Agradeço ainda aos meus avós com os quais aprendi e aprendo dia após dia. Obrigada por continuarem a fazer parte do meu caminho e por me ensinarem que não há limites no amor. Obrigada primos e primas, os nossos primeiros amigos e, no meu caso, aqueles que sempre fizeram o papel de irmãos. Obrigada família, pela compreensão nas ausências, por aceitarem as minhas "visitas de médico" com o maior entusiasmo, por me ajudarem a crescer dia após dia, e por serem aqueles que acreditam em mim com todas as forças.

A todos aqueles que fizeram e fazem parte do meu caminho,

muito obrigada!

“Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.”

Marie Skłodowska Curie

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Abstract

The development of biorelevant dissolution tests has been a hot topic in research in the latest years, which reflects the challenge that most drug products currently under development represent, due to poor solubility or new technological approaches to dosage forms. Traditional dissolution testing is often used to predict the rate and extent to which a drug is released *in vivo*, and has an important role during drug development. However, this dissolution method is used in quality control testing, developed to achieve full drug release and to comply with compendial requirements. As a result, release dissolution methods are not necessarily biorelevant and their application in the early stages of product development can be very limited.

To improve the biopredictive power of dissolution testing, methods with higher biorelevant characteristics are needed. Applications include the study of food effects on the solubilization and absorption of orally administered drugs, identification of solubility limitations, and/or stability issues. The use of biorelevant setups can also have a great impact on product development, supporting the optimization of dosage form conditions and final product formulation, verified later by pharmacokinetic studies.

In this study, different biorelevant dissolution methods and permeability studies were tested, applied to challenging oral dosage forms, and mainly for Biopharmaceutical Classification System (BCS) class II and IV molecules. Models with aqueous biorelevant dissolution media with acidic and basic phases will be applied. To complete these models, dissolution phase changes were developed with permeability evaluation by different methods. Whenever possible, *in vitro* results were correlated with the results of clinical trials.

A theoretical dissolution/permeation method that simulates the entire gastro-intestinal tract were proposed, to successfully predict the *in vivo* performance of drug products and lower the risk of failure in bioequivalence studies.

Keywords: Dissolution, Biorelevant, *In vitro* studies, Poorly soluble drugs, Permeation models, IVIVC, Bioequivalence, Absorption, Oral administration, Oral solids, Product development, Analytical development.

Resumo

Um dos temas com maior foco na investigação científica dos últimos anos é o desenvolvimento de testes de dissolução com propriedades biorelevantes, refletindo o desafio que a maioria dos produtos farmacêuticos atualmente em desenvolvimento representam, devido à baixa solubilidade, ou a novas abordagens tecnológicas e formas farmacêuticas. Os testes tradicionais de dissolução, realizados em âmbito de controlo de qualidade ou de estudos de estabilidade, são desenvolvidos com o objetivo de se obter uma taxa de dissolução máxima ao longo do tempo, de modo a cumprir especificações e os requisitos compendiais. Como resultado, estes métodos não são necessariamente biorelevantes e a sua aplicação nas fases iniciais do desenvolvimento do medicamento pode ser muito limitada.

Para melhorar o poder biopreditivo dos testes de dissolução, são necessários métodos com melhores características biorelevantes. Essas características devem possibilitar o estudo do efeito dos alimentos na solubilização e absorção dos fármacos administrados oralmente, a identificação de problemas de solubilização, e/ou problemas de estabilidade. O uso de dissoluções biorelevantes pode ter um grande impacto no desenvolvimento do produto, apoiando a optimização da formulação do produto final, que é verificada posteriormente em estudos farmacocinéticos.

Neste estudo vão ser testados diferentes métodos de dissolução biorelevantes, incluindo modelos que simulam a passagem do medicamento do ambiente gástrico para o intestinal e ainda estudos de permeabilidade. Vão ser aplicados a formas farmacêuticas orais com propriedades desafiantes, como moléculas de classe II e IV do Sistema de Classificação Biofarmacêutica (BCS), entre outras. Os resultados obtidos *in vitro* serão, sempre que possível, correlacionados com resultados de ensaios clínicos, demonstrando a biorrelevância destes métodos.

Será ainda proposto um modelo teórico de dissolução/permeação que pretende simular todo o trato gastrointestinal, de modo a prever com sucesso o desempenho *in vivo* de medicamentos e reduzir o risco de insucesso nos ensaios de bioequivalência.

Palavras-Chave: Dissolução, Biorelevante, *In vitro*, fármacos pouco solúveis, Modelos de Permeação, IVIVC, Bioequivalência, Absorção, Administração oral, Sólidos orais, Desenvolvimento de produto, Desenvolvimento analítico.

Aim

Medicines have become essential. More and more people depend on drugs to survive or to be able to have a normal day and quality of life. Oral administration of drugs continues to be the preferable and most common form of drug therapy whether because it is the least invasive or the most widely available on the market.

Effective therapeutic concentrations are crucial and depend on the drug's biopharmaceutical properties, which will converge in good oral bioavailability. Oral bioavailability is defined as the fraction of an oral dose of the drug that reaches the systemic circulation and is the most common pharmacokinetic measure of drug candidate suitability for oral administration. Thus, the molecule solubility is of utmost importance, to reach the intestine and permeate. Drug absorption is therefore the result of a series of steps including the disintegration of the pharmaceutical form, followed by drug dissolution in the gastrointestinal tract and its absorption through the intestinal mucosa, and finally its introduction into the systemic circulation. This work aims to contribute to the field of bioprediction, by studying, developing, and implementing *in vitro* biorelevant setups for specific molecules, in order to understand their behavior in the gastrointestinal tract. Different setups were developed, designed to describe the *in vivo* performance of a drug after oral administration.

The development of biorelevant methodologies is an hot topic in today's pharmaceutical science, where solubility or permeability, and not rarely both, are more and more a limiting factor for absorption. These molecules are the scope of this work, where *in vitro* methods intend to obtain an accurate prediction of *in vivo* behavior. Although this correlation has been extensively studied for the last decade, there is still a lot to understand and it is still an enormous challenge for both academic and development scientists.

The ultimate goal of this work is to improve the success of clinical trials involving PK studies, namely bioequivalence studies. Simulations of how the drug will behave *in vivo* lead to additional knowledge and a deep understanding that can be applied sooner to further optimize drug products. Pharmaceutical development can benefit from scientific-driven methodologies, able to support critical formulation decisions, decrease the time-to-market, lower the investment, and avoid unnecessary human exposure to unoptimized drug products.

Outline of the thesis

This work is structured into five main chapters:

- CHAPTER 1 - Introduction

In this chapter, an overview of key concepts of the field of biorelevant methodologies is presented. A state-of-the-art is compiled to support the issues addressed during this work. The molecules under study in the experimental chapters are described, as well as the overall structure of the studies.

- CHAPTER 2 - Biorelevant dissolution studies

Biorelevant dissolution tests, such as phase changes or dissolutions in biomimetic media are presented and discussed.

- CHAPTER 3 - Dissolution/Permeation Studies

Dissolution/permeation setups and biphasic dissolutions are tested and the results are discussed in the scope of the properties of the assessed molecules.

- CHAPTER 4 - Conceptualization of a new biomimetic method

In this chapter, a new method is proposed for further application. Based on the knowledge and data obtained in the previous chapters, this chapter intends to discuss a novel methodology for testing dissolution and permeation along the gastrointestinal tract.

- CHAPTER 5 - Conclusions and Future Perspectives

Chapter 5 summarizes the main conclusions and future perspectives of this work.

Whenever possible, the results of the biopredictive setup are compared to *in vivo* results, to conclude its biopredictive accuracy.

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List of Abbreviations

API	Active Pharmaceutical Ingredient
AUC	Area Under the Curve
BCS	Biopharmaceutical Classification System
CI	Confidence Intervals
C_{max}	Maximum Concentration
CQ	Quality Control
EMA	European Medicines Agency
EU	Europe
FaSSGF	Fasted State Simulated Gastric Fluid
FaSSIF	Fasted State Simulated Intestinal Fluid
FeSSGF	Fed State Simulated Gastric Fluid
FeSSIF	Fed State Simulated Intestinal Fluid
GIT	Gastrointestinal tract
GMR	Geometric LSmeans Ratio
HPLC	High-Performance Liquid Chromatography

IVIVC	*In Vitro In Vivo* Correlation
JP	Japanese Pharmacopoeia
PBS	Phosphate Buffer Saline
Ph. Eur.	European Pharmacopoeia
PBPK	Physiologically Based Pharmacokinetic
RPM	Rotations Per Minute
SGF	Simulated Gastric Fluid
SSF	Simulated Salivary Fluid
USA	United States of America
USP	United States Pharmacopeia

1.1. Physiology of the gastrointestinal tract

The oral route remains the most common form of drug administration and is still the preferred *via* among patients [1, 2]. Oral administration must overcome physiological barriers (low solubility, permeation, and early degradation) to achieve an efficient and sustained absorption [3].

The gastrointestinal tract (GIT) is part of the digestive system along with several accessory organs (teeth, tongue, and glandular organs such as the salivary glands, liver, gallbladder, and pancreas) [4]. It comprises the oral cavity, pharynx, esophagus, stomach, small, and large intestine. All these organs work together to mechanical and chemically process, digest, and absorb food and drugs. It also works to promote the secretion of water, acids, enzymes, and the excretion of waste products [4].

The purpose of the oral cavity is to perceive food material before it is ingested and process it by the action of the tongue, teeth, and the palatal surface. There is also the secretion of saliva from the glands that also plays a key role in the bioavailability of certain drugs. The tongue, in addition to its mechanical function, secretes mucins and lingual lipase. The lingual lipase has a broad pH and breaks down lipids (mainly triglycerides). A pH of 3.5 to 6 allows lingual lipase to function even in the acidic environment of the stomach [5].

The primary function of the esophagus is to empty food material or drugs into the stomach through waves of contraction of its longitudinal, circular muscles, known as peristalsis. The smooth muscles of the esophagus are arranged circularly and longitudinally and aid in peristaltic movement during swallowing [6]. When the food or the drug reaches the stomach, it can be temporarily stored and broken down mechanical and chemically by the action of the stomach acids and enzymes.

The stomach's ability to store food results from its ability to change size. The smallest curvature of the stomach is approximately 10 cm long, and the largest is approximately 40 cm [3]. The characteristics of the stomach are important as drugs suffer changes in this area of the GIT. The hydrochloric acid produced by the parietal cells maintains the pH of the stomach between 1.5 and 2.0 during fasting [5]. This acidity has several functions such as denaturing

proteins, destroying microorganisms, and activating pepsin (protein-digesting enzyme secreted by the principal cells).

After the stomach, the small intestine is reached. This is where 90% of the food absorption occurs, and the same applies to drugs. The small intestine is subdivided into three parts called the duodenum, the jejunum, and the ileum [7]. The duodenum receives the whole mass that comes from the stomach, as well as digestive material from the pancreas and liver. The jejunum is where most of the digestion and chemical absorption takes place.

Finally, the ileum has also digestion and absorption functions [7]. The mucosa of the small intestine has multiple microvilli that increase the surface area exponentially for optimal absorption.

There are extensive networks of capillaries within the villi that transport the absorbed nutrients and drugs into the hepatic portal circulation [7]. The small intestine also releases hormones and enzymes that are important for the absorption process. The following table lists the most well-described hormones and the cells responsible for their production.

Table 1: *Small intestine hormones and production cells. Adapted from [8, 9].*

Hormones	Production Cell	Local of production
Gastrin	G Cells	Upper small intestine
Cholecystokinin	I Cells	
Secretin	S Cells	
Motilin	M Cells	
Gastric inhibitory peptide	K Cells	Small intestine
Somatostatin	D Cells	
Pro-glucagon	L Cells	Distal ileum and colon

In what concerns enzymes, there is the excretion of lipase for the digestion of fats, peptidase for peptide breakdown, sucrase, maltase, and lactase for the breakdown of sucrose, maltose, and lactose, respectively [10].

In addition to enzymes and hormones, there are a couple of glands, the Brunner's glands, found mainly in the duodenum, which produce bicarbonate buffer for acid neutralization [4]. This buffer plays a key role in maintaining the intestine characteristics.

Beyond the intestine, the entire gastrointestinal lumen has long been shown to be buffered by bicarbonate, which maintains a pH gradient. The secretion of bicarbonate in the mucus creates a pH gradient with a near-neutral pH at the epithelial surfaces of the stomach and duodenum, providing the first line of mucosal protection against the luminal acid [11]. Because of that, under normal physiological conditions, the mucosal bicarbonate barrier is sufficient for the protection of the gastric mucosa against acid and pepsin and is even more effective in the duodenum.

Among all the accessory organs, the liver is the largest organ of the GIT and the largest gland in the human body. It has numerous functions, but as an accessory organ of the digestive system, it produces bile that emulsifies fats and various lipids for complete and functional digestion [4].

Finally, the unabsorbed and undigested food material proceeds to the large intestine. The peristaltic motion of the large intestine promotes the movement of stool into the rectum [4]. Like food, drugs follow the same pathway and are also influenced by the physiology of the GIT. Therefore, it is crucial to understand how they interact and change according to the characteristics of each specific digestion phase of the GIT.

1.2. Interactions drug – gastrointestinal tract

Each region of the GIT has very specific characteristics, depending on a series of factors such as enzyme activities, fluid composition, different morphologies, and different transporters. These variables influence the drug product behavior in the GIT, command disintegration, drug dissolution, and permeability [7], and consequently affect the bioavailability of the drug.

The main physiological parameters that influence drug behavior in the GIT are gastric emptying rate, pH, transit times in the different intestinal segments, intestinal surface area, epithelial permeability, as well as the amount of intestinal enzymes and transporter expression [12]. The effect of each main parameter on the drug behavior and the local of absorption is briefly described below.

pH

The pH of luminal fluids is acidic in the stomach (pH 1.5-3.5), and increases to approximate pH 5-6 in the duodenum and pH 7-8 in the distal jejunum and ileum, before dropping to pH 6 in the colon, with some inter-subject variability [13]. When the degree of ionization of certain molecules is altered by variations in pH, their dissolution and therefore permeation behavior may change completely [14]. For example, for an extended-release drug product, if the drug's degree of ionization changes, it can accelerate dissolution, compromising its main objective, the gradual action over time.

Transit times

Gastric emptying time is approximately 45 minutes after ingestion of a glass of water (250 mL) but can take more than 6 hours after ingestion of a very caloric meal [14, 15]. The transit time in the small intestine is usually constant between 4.3 and 4.6 hours, while the colon transit time may vary between ~18 and 34.2 hours [16]. In some cases, the drug may remain longer or shorter in each of the mentioned zones of the GIT, which may affect its bioavailability.

Intestinal surface area

The intestinal area influences absorption. Absorption occurs through passive permeation (paracellular or transcellular) or active absorption through intestinal transporters. The type of transport depends on the characteristics of the intestinal membrane and the physicochemical properties of the molecule. As a rule of thumb, polar molecules are absorbed via passive transport in the upper small intestine, while apolar drugs are actively transported through the intestine [15].

Transporter expression

The transporters differ throughout the GIT. While some are expressed in the entire GIT (e.g., monocarboxylate transporter 1 and organic cation transporter 1), others are found only in specific locations. For instance, peptide transporter protein 1 is located mainly in the jejunum, and organic cation transporter 2B1 (OATP2B1) is located mostly in the colon [17].

Cytochrome P450 enzymes

The cytochrome P450 enzymes play an important role in drug interaction with the GIT. Their activity is essential for the metabolism of many drugs. The ones that participate most actively in this process are CYP3A4 and CYP2D6. These enzymes can be induced or inhibited by drugs.

Some of these changes can culminate in adverse reactions or therapeutic failures [18]. These enzymes are expressed mainly in the liver but can also be found in the small intestine [19].

Volumes

The volume of gastrointestinal fluids determines the local drug concentrations. Therefore, these volumes will alter the dissolution performance as well as the driving force for permeation [20].

In the fasted stomach, resting gastric fluid volumes range between 25 and 45 mL. The volume in the fed state depends on the volume of food ingested and the time after ingestion. In the small intestine, the volume in the fasted state is usually around 43 ± 14 mL. In the ascending colon, the volume for the fasted state was 22.3 ± 7.7 mL and 29.9 ± 10.8 mL for the fed state [20, 21].

Fluids composition

In addition to these characteristics, the composition of bile salts and pancreatic enzymes can affect the solubility and dissolution rate, especially for hydrophobic drugs.

The composition of intestinal fluids changes during intestinal transit due to digestion and absorption processes, as well as due to the secretion of bile and pancreatic fluids into the intestinal lumen. According to the literature, these are the typical GIT fluids' concentrations [22]:

- Fasted state:

In the duodenum → 2.5 mM to 5.9 mM

In the jejunum → 1.4 mM to 5.5 mM

- Fed state:

In the duodenum → 3.6 mM to 24.0 mM

In the jejunum → 4.5 mM to 8.0 mM

Many of these characteristics vary with age, gender, food type, and several diseases like infections, among others. Consequently, during product development, changes that can occur

in the GIT should be taken into account to understand what influence they may have on the mode of action for which the drug is intended.

Usually, in bioequivalence studies, drugs are studied and tested on healthy adult subjects who have these parameters quite similar and stable [12]. However, this is not true for all populations.

1.3. BCS Classification

Two key characteristics of molecules are solubility and permeability. The Biopharmaceutical Classification System (BCS) categorizes the molecules regarding these characteristics into four classes. The BCS is widely used to evaluate the potential effects of the formulation on the oral bioavailability of the drug in humans [24]. According to solubility and permeability, Amidon *et al.* devised this classification system, which categorizes drug substances into one of four BCS classes [2]:

Class I: high solubility, high permeability

Class II: Low solubility, high permeability

Class III: high solubility, low permeability

Class IV: low solubility, low permeability

This classification was later adopted by all regulatory authorities and is now a guide in drug development teams.

Solubility is classified based on how the molecule behaves in certain aqueous media. A drug is considered highly soluble when the highest dose is soluble in 250 mL of aqueous media with a pH between 1.2 and 8.0 [25]. Permeability classes are indirectly related to drug absorption in humans. A substance is considered highly permeable when the extent of absorption in humans is determined to be 85% or more of the administered dose based on a mass balance determination or in comparison to an intravenous dose [24, 26].

The main goals of BCS are to improve the efficiency of drug development and to meet the challenges of formulation design. It is also possible to support the prediction of *in vivo*

pharmacokinetic performance as well as permeability and solubility or to request a biowaiver of *in vivo* bioequivalence studies (for BCS I and III) [27].

For orally administered drugs, a low solubility represents an obstacle to efficient absorption, and this becomes a bigger challenge when low permeability is added. BCS has significantly affected the way the pharmaceutical industry and new drug development proceeds. It promoted the development of tools to understand the *in vivo* performance focused on BCS II or BCS IV compounds, and supported the research on advanced drug delivery strategies [24].

1.4. Importance of the dissolution method

Dissolution is a physicochemical test in which the drug substance is released into an aqueous solution. It can also be called *in vitro* release and is mandatory to control the quality of most oral solids [28]. The dissolution method is also used to predict the release of the drug in the body and consequently to assess how much will be available for absorption *in vivo* [29].

In the industry, dissolution tests are used to control quality and monitor the formulation of the final product [28, 29]. The dissolution test is also key to detecting changes in formulation or manufacturing process, and discriminating promising formulations from unpromising ones [7, 28, 29]. For this reason, it is crucial at all stages of the product development process and needs to be robust and reproducible [26, 28].

The factors affecting drug dissolution can be explained by the Noyes-Whitney equation (Equation 1), proposed in 1897 [30]. This equation is based on the Nernst-Brunner modifications [10].

$$\frac{dc}{dt} = \frac{DA}{V\delta}\,(C_S - C)$$

Equation 1

In this equation, C is drug concentration, D is drug diffusion coefficient, A is drug surface area available for dissolution, δ is the thickness of the hydrodynamic boundary layer, C_s is the equilibrium solubility of the Active Pharmaceutical Ingredient (API) at the solid-liquid interface and V is the volume of the dissolution medium. Each parameter of this equation is influenced by physiological and physicochemical characteristics are summarized in the next table.

Table 2: *Physicochemical and physiological parameters influencing drug dissolution in the GIT. Adapted from [27, 34].*

Characteristics	Physicochemical parameter	Physiological parameter
Drug diffusivity	Molecular size	The viscosity of luminal contents, the diffusivity of mixed micelles
Drug Surface Area	Particle size, wettability	Surfactants in gastric fluids and bile
Solubility	Particle size, crystal structure, pKa	pH, buffer capacity, bile, food
Boundary layer thickness	-	Mobility pattern, Flow rate
Drug Concentration	Particle size, wettability, solubility	Permeability
Volume of solvent	-	Secretions, co-administered fluid, all GI fluids

The dissolution process takes place in two steps (Figure 1). First, the drug product disintegrates and disaggregates, and only then does the process of drug dissolution in the liquid medium occurs. This ends up in a dissolution profile that shows how much drug is dissolved per unit of time. For biomimetic dissolutions, it is important to ensure that the *in vitro* experiment resembles *in vivo* conditions so that the dissolution test can be an effective surrogate [31].

On the left side of Figure 1, the conventional *in vitro* dissolution test is schematized, while on the right side of Figure 1, what happens *in vivo* is described. Once the API is in solution, the absorption process can take place; the drug substance passes from the gastrointestinal lumen into the circulatory system, where it can achieve the receptor sites to exert a biological response [28].

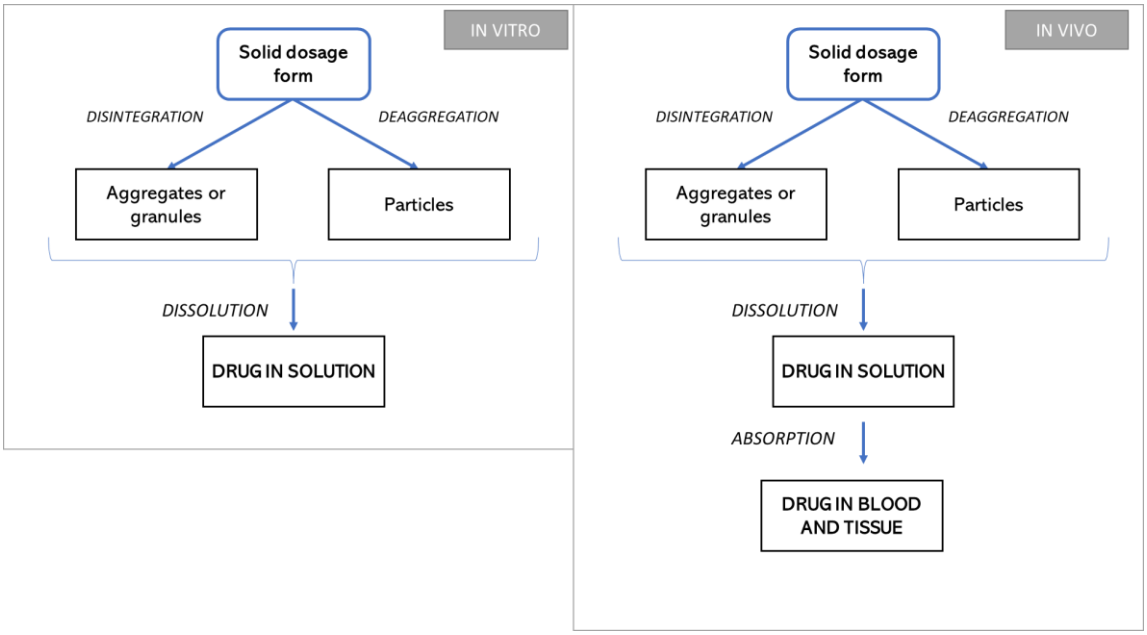

Figure 1: *In vivo versus in vitro steps of the dissolution process.*

1.5. Quality Control (QC)/Release Dissolution vs Biorelevant Dissolution

QC/release dissolution methods are conventional methods that provide information on the drug dissolution in an aqueous solution. Compendial dissolution media, used in QC dissolutions, are simple buffers or solutions to maintain the pH and ionic strength [11]. According to the pharmacopeias, the pH of a dissolution medium should be between 1.2 and 6.8 [32].

Combinatorial chemistry, high-throughput screening, and genomics generate a large number of new chemical entities with therapeutic potential [45]. However, these new molecules have high molecular weights and lipophilicity. These characteristics result in poor water solubility which affects the bioavailability of orally administered drugs. Thus, poor water solubility not only limits the biological application of the drug but also challenges its pharmaceutical development. In addition, many of these molecules are also poorly permeable [37].

Therefore, it becomes imperative to develop biorelevant methods and strategies to predict the behavior of these molecules *in vivo*. These methods are called biorelevant dissolution methods and have advantages over conventional dissolution tests. Accordingly, biorelevant dissolutions can be used to guide formulation development, identify food effects on drug release and bioavailability of orally administered drugs, and also to identify solubility limitations and stability problems [33].

Biorelevant dissolutions have become critical for successful product development and the constant concern of most industries and scientists. This can be proven by the number of papers published in recent years related to "biorelevant dissolution media", as in the last decade approximately 260 articles related to this topic were published, while in the previous decade (2001-2011) only 68 had been published.

The characteristics of these methods should mimic the physiological conditions of the GIT that affect drug dissolution. Characteristics such as pH conditions, buffer capacity, osmolarity, surface tension, viscosity, the chemical composition of the GIT, temperature, hydrodynamics, and transit times should be evaluated and applied [34].

One of the main goals of these methods is to generate a successful *In Vitro In Vivo* correlation (IVIVC) [11, 34], as the number of clinical studies might eventually be reduced. In addition to financial advantages, it decreases the exposure of humans to formulations that are not fully fit for their intended purpose [26]. BCS Class II and IV molecules are the special focus of biorelevant methods, where relevant information collected with these specialized setups may drastically affect the course of development projects.

1.6. Dissolution Apparatus

One of the key steps in dissolution method development is the choice of dissolution apparatus. Whether it is a release or a biorelevant dissolution, this selection needs a thorough evaluation of the molecule. The United States Pharmacopeia (USP), the European Pharmacopoeia (Ph. Eur.), and the Japanese Pharmacopoeia (JP) describe different types of apparatus [35].

The apparatus, associated with the imposed rotation, create specific thermodynamics in the dissolution vessel that is essential for a good simulation of biorelevant conditions. Moreover, other parameters such as the medium composition, pH, osmolality, viscosity, and surface tension are important in the development of release methods but are crucial in biorelevant methods [28]. USP approved seven apparatuses that could be used in the QC of drug products [36]. They are summarized in Table 3 with the common rotation speed and the usual dosage forms tested.

Table 3: *Comparison of compendial dissolution apparatuses. Adapted from [9, 37].*

USP Apparatus	Description of the apparatus	Rotation Speed	Dosage Forms
I	Basket	100-150 RPM	Immediate, delayed and extended released tablets or capsules
II	Paddle	25-150 RPM	
III	Reciprocating cylinder	5-35 DPM	Immediate and extended released tablets or capsules
IV	Flow-though cells	NA	Extended released tablets or capsules and poorly soluble drugs
V	Paddle over disk	25-50 RPM	Transdermal systems
VI	Cylinder	NA	
VII	Reciprocating Holder Apparatus	30 RPM	Extended released tablets or capsules Transdermal systems

RPM, rotations per minute.

Additional information on these apparatuses is provided in the following paragraphs.

Apparatus I – Basket

A rotating basket method that provides a steady stirring motion in a large vessel with 500 to 1000 mL of dissolution medium, immersed in a temperature-controlled water bath (Figure 2). The basket method is simple, robust, and easily standardized [36].

Apparatus II – Paddle

In the paddle apparatus method (Figure 2), the paddle replaces the basket as the source of agitation. As with the basket apparatus, the shaft is located no more than 2 mm at any point from the vertical axis of the vessel and rotates without significant vibration [36].

Most tablets and capsules use Apparatus I or II. These two apparatuses were developed through the 1960s and adopted by the USP in the 1970s [36].

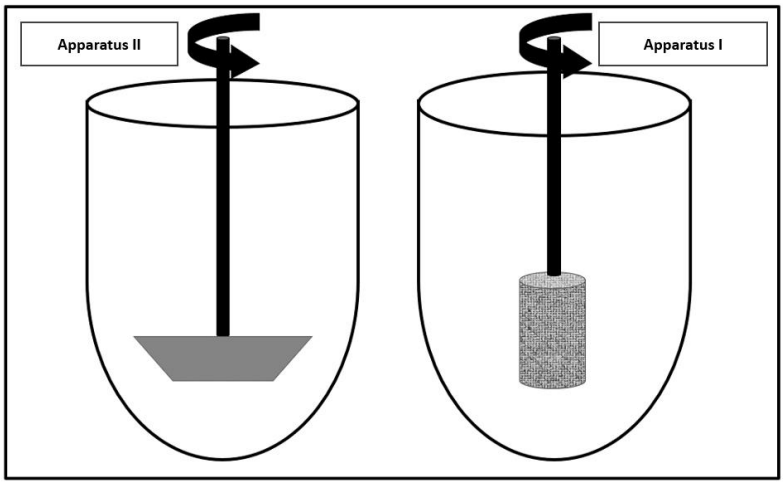

Figure 2: *Simplifying scheme of apparatus I and II.*

Apparatus III – Reciprocating cylinder

This method is based on the disintegration method. It has cylindrical outer glass containers and another set of inner glass cylinders. It requires stainless steel fittings to fit the tops and bottoms of the cylinders [38]. The action produced by the movement of these cylinders carries the tested product through a constantly moving medium [36]. Figure 3 shows how this method works.

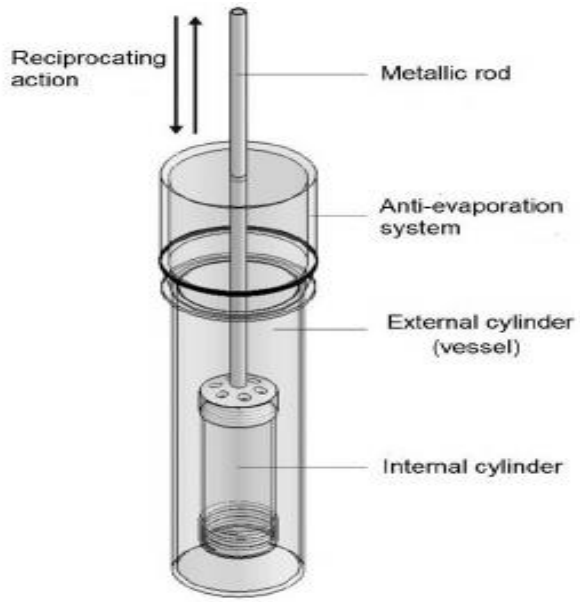

Figure 3: *Simplifying scheme of the reciprocating cylinder. Adapted from [2].*

Apparatus IV – Flow-through cells

This apparatus consists of a reservoir containing a dissolution medium, a pump that forces the movement of the medium, and a water bath that keeps the dissolution medium at 37°C. The cell, made of a transparent and inert material, is placed vertically with a filtration system that prevents the escape of undissolved particles from the top of the cell. The bottom of the cell is filled with small glass beads [33].

USP Apparatus IV can work in an open or closed system (Figure 4). Different flow rates and temperatures may also be applied. Since there is a wide range of cell types, this method can be used in a wide range of pharmaceutical forms, from tablets, powders, suppositories, or even soft capsules [36].

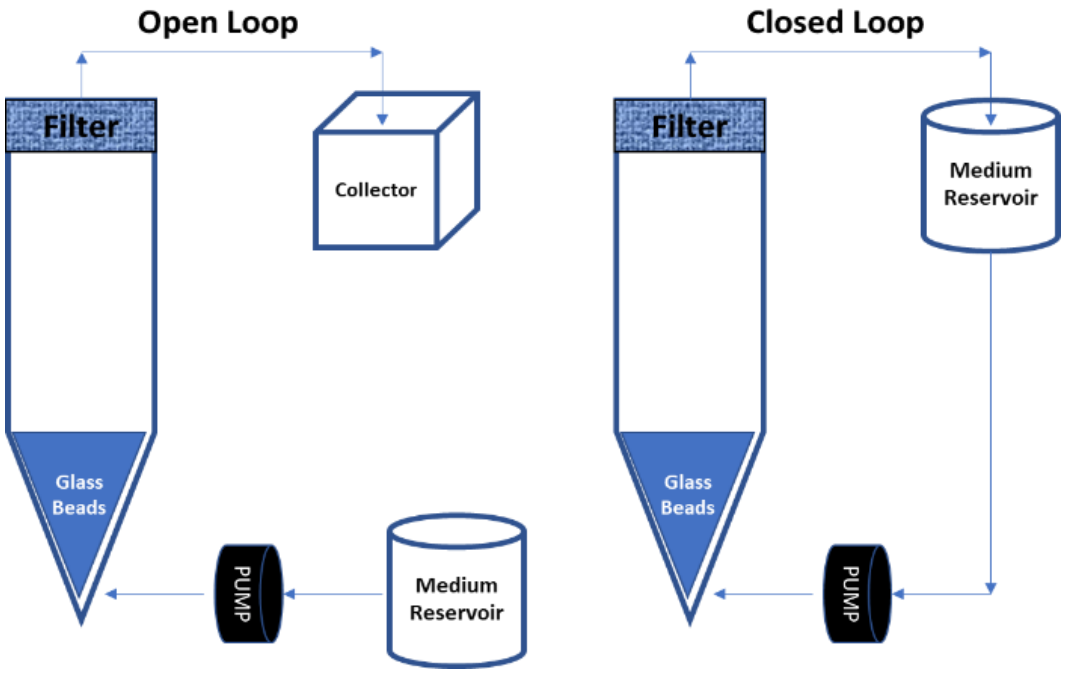

Figure 4: *Simplifying scheme of Flow-through Cells.*

Apparatus V – Paddle over Disk

The Paddle over Disk method is derived from Apparatus II with the addition of a stainless steel disk assembly (Figure 5). This disk is aimed at holding the transdermal system at the bottom of the vessel, which is the dosage form for which this method is used [36].

Apparatus VI – Rotating Cylinder

This system is a modification of the basket apparatus (USP Apparatus 1), as it uses the same vessel assembly from Apparatus 1 and replaces the basket with a stainless steel cylinder stirring element (Figure 5). Like Apparatus V, the VI is used mainly to test transdermal patches [36].

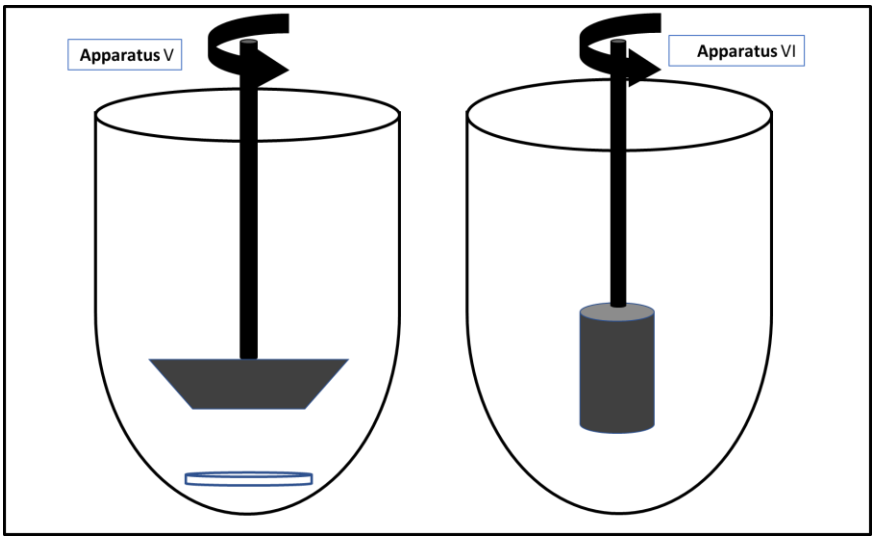

Figure 5: *Simplifying scheme of Paddle over Disk (Apparatus V) and Rotating Cylinder (Apparatus VI).*

Apparatus VII – Reciprocating Holder Apparatus

Apparatus VII consists of a set of volumetrically calibrated solution containers made of glass or other inert material, a motor, and a drive. The solution containers are partially immersed in a water bath of a convenient size that allows a constant temperature. This apparatus simulates the biorelevant conditions for the skin, GIT, and tissue required for transdermal, oral drugs, and implants with biorelevant temperature agitation rates and retention times. The holder has a stroke length of 20 mm and agitates at 5 to 40 dips per minute. Following a designated time, the dosage form is transported from one row to the next one. It accommodates a variety of dosage form holders including reciprocating disks, angled disks, spring holders, stent holders, and pointed rods [39].

1.7. Biorelevant Media

Routine QC media lack essential components of the human GIT such as bile acids, lecithin, and other components. Due to this limitation, they are not able to fully mimic the behavior of the drug after administration. Biorelevant dissolution media are therefore needed to increase similarity to the fluids of the GIT.

Biorelevant dissolution tests designed with appropriate simulated media and hydrodynamic conditions are useful from the early stages of product development to final drug product characterization. They allow the characterization of the biopharmaceutical performance of the drug product, including solubility issues, whether the food effect may influence its absorption and even the potential for precipitation on gastric emptying.

In highly soluble compounds (BCS class I and III), dissolution is usually not a bottleneck and the compendial media are commonly enough to obtain the necessary information. However, for poorly soluble compounds, the medium selection is expected to play an important role in their dissolution behavior. For weak acids in the fasting state, dissolution is usually slow in the stomach and in many cases not complete before the compound reaches the small intestine. For poorly soluble weak bases administered in the fasting state, dissolution is expected to be mainly in the stomach since they are more soluble at lower pH values [34].

After food intake, the small intestine provides an environment with a higher solubilization capacity for poorly soluble compounds due to the natural surfactants and may lead to an improved oral bioavailability compared to the fasting state [33]. These natural surfactants, such as bile salts, lecithin, free fatty acids, and monoglycerides are present in the gastrointestinal fluids [40]. The bile salts promote the formation of micellar solutions increasing the solubilization of the drug [41]. Accessory products of digestion, for example, phospholipids and fatty acids, have a major impact on intestinal solubility and consequently on the bioavailability of poorly soluble drugs. A normal adult diet contains about 150 g of lipids, 95% of which are long-chain triglycerides, and 4-8 g of phospholipids mainly composed of lecithin [42], which act as solubility enhancers. An ultrastructural characterization of the fluid indicates that it is composed of a range of micellar, vesicular, colloidal, and lipid droplet systems [40]. The proportion of each of these components depends on the fed or fasting state.

As the physiology of the GIT is quite varied, it is necessary to apply different biorelevant media for each phase. In addition, it is extremely important to consider if the fed or fasted state is the goal of the assessment. In summary, for a correct selection of the medium, it is necessary

to consider the composition and hydrodynamics in the GIT but also the API characteristics and other formulation properties.

Understanding the extensive composition of the GIT fluids is crucial to developing the correct composition and concentration of biorelevant media. There are already commercial ready-to-use preparations for these biorelevant media, and several others described in the literature simulating each of these states. In the following paragraphs, an overview of these media and their composition is provided.

FaSSGF (Fasted State Simulated Gastric Fluid), FeSSGF (Fed State Simulated Gastric Fluid) FaSSIF (Fasted State Simulated Intestinal Fluid), and FeSSIF (Fed State Simulated Intestinal Fluid)

FaSSGF, FeSSGF, FaSSIF, and FaSSGF are dissolution media that simulate human gastrointestinal fluids. These dissolution media are part of a set of products developed and marketed by the company *Biorelevant* (London, UK). Initially known as SIF powder, it contains natural surfactants (bile salts and phospholipids) present in the gut to simulate gastrointestinal fluids more accurately than conventional dissolution media [43]. This powder also considers the fed or fasting phase.

Based on the SIF powder, this company has been dedicated to producing many ready-to-use compositions as similar as possible to GIT fluids. *Biorelevant* has developed new versions of these powders improving the biorelevance and accuracy of its results [44]. The preparation of the biorelevant media from these powders is intended to be very easy and error-free since dilutions are enough to get the final media. In addition to all the products, the company publishes numerous papers in the field of biorelevant methods.

FaSSGF and FeSSGF, are mainly considered to simulate gastric fluids. The principal differences in their composition are summarized in Table 4.

Until the final compositions were reached, many improvements were necessary to be able to simulate the gastric fluids. FaSSGF, a dissolution medium that mimics the actual gastric composition in the fasted state according to published physiological data, was developed in 2005 [33]. This medium has a pH of 1.6 and contains physiologically relevant amounts of pepsin, bile salts, and lecithin to obtain a surface tension close to what is found *in vivo*. It appears to be more biorelevant than the previously proposed media (e.g. Simulated Gastric

Fluid (SGF), which contains Triton) as it only comprises natural components, recovered from the stomach in fasting conditions [42].

After food ingestion, the conditions in the stomach can vary greatly depending on the composition of the meal. Initially, the composition of the gastric fluid will be close to the composition of the meal concerning pH, osmolality, and surface tension. The pH is increased to values of 3 to 6 [28]. With the secretion of gastric acids and gastric emptying, the values return then to those of the fasting state [42].

Table 4: *Composition of biorelevant medium that simulates stomach conditions.*

Composition	FaSSGF	FeSSGF (Early)	FeSSGF (Middle)	FeSSGF (Late)
Sodium Taurocholate (mM)	0.08	-	-	-
Lecithin (mM)	0.02	-	-	-
Pepsin (mg/mL)	0.1	-	-	-
Sodium Chloride (mM)	34.2	148	273.02	122.6
Hydrochloric Acid (mM)	25.1	-	-	-
Acetic Acid (mM)	-	-	17.12	-
Sodium Acetate (mM)	-	-	29.75	-
Milk:Buffer	-	1:0	1:1	1:3
pH	**1.6**	**6.4**	**5**	**3**
Osmolality (mOsm/kg)	120	559	400	300
Buffer Capacity (mmol/L.ΔpH^{-1})	-	21.33	25	25

The intestinal lumen is where most phenomena of dissolution and absorption occur. Biorelevant intestinal media were also developed to simulate the intestine in a fasted state, and also after the ingestion of food [36]. FaSSIF takes into account the solubilizing capacity of the intestinal fluids in a pre-prandial state. It reflects the *in vivo* pH, osmolality, surface tension, and buffer capacity. More recently, due to some improvements and new physiological information, FaSSIF-V2 has been developed [42]. The characteristics of these media are summarized in Table 5

As in the stomach, the composition of the fluids in the upper small intestine in the fed state depends on the type of food ingested, although to a lesser extent than in the stomach. There are changes in both hydrodynamics and intraluminal volume with a meal [33]. The pH changes, as well as osmolality and buffer capacity. FeSSIF was developed to reflect these changes. This medium can simulate the fluids of the upper small intestine ensuring that characteristics such as the amount of bile salts and phospholipids, pH, osmolarity, and buffer capacity are as accurately simulated as possible [11].

In summary, FeSSGF and FeSSIF-V2 are suitable for predicting drug dissolution in the fed stomach and upper small intestine, respectively, while FaSSGF and FaSSIF-V2 are recommended for predictive dissolution studies in the fasting state.

Table 5: *Composition of biorelevant medium that simulates intestinal lumen.*

Composition	FaSSIF	FaSSiF V2	FeSSIF (Early)	FeSSIF (Middle)	FeSSIF (Late)	FeSSIF-V2
Sodium Taurocholate (mM)	3	3	10	7.5	4.5	10
Lecithin (mM)	0.75	0.2	3	2	0.5	2
Dibasic Sodium Phosphate (mM)	28.65	-	-	-	-	-
Sodium Hydroxide (mM)	8.7	34.8	52.5	65.3	72	81.65
Sodium Chloride (mM)	105.85	68.62	145.2	122.8	51	125.5
Glyceryl Monooleate (mM)	-	-	6.5	5	1	5
Sodium Oleate (mM)	-	-	40	30	0.8	0.8
Maleic Acid (mM)	-	-	28.6	44	58.09	55.02
pH	**6.5**	**6.5**	**6.5**	**5.8**	**5.4**	**5.8**
Osmolality (mOsm/kg)	270	180	400	390	240	390
Buffer Capacity ($mmol/L.\Delta pH^{-1}$)	12	10	25	25	15	25

Bicarbonate buffer

The gastrointestinal lumen has long been shown to be buffered by bicarbonate, which maintains the pH gradient [11]. This is because pancreatic secretions are composed of several digestive enzymes and a large volume of sodium bicarbonate solution. The bicarbonate ions are thus important in neutralizing the acidity of the contents coming from the stomach [45].

In the duodenum, carbonic acid dissociates rapidly into carbon dioxide (CO_2) and water. The CO_2 can be absorbed into the systemic circulation and released through the respiratory system. A neutral sodium chloride solution is left in the duodenum and the acidic contents of the stomach are then neutralized by the bicarbonate [11, 42].

Thus, the use of bicarbonate buffer has been of increasing interest for the development of suitable biorelevant *in vitro* media. However, reproducing this buffer *in vitro* has many challenges and requires the use of sophisticated equipment [11] to keep the pressure of CO_2 in the solution constant. One example of equipment that could stabilize the medium is the pHysio-grad® device. It can monitor and adjust the pH value of commonly used hydrogen carbonate buffers, enabling a precise pH adjustment in the range of pH 5.5 to 8.3. It can precisely simulate desired pH gradients within the intestinal pH range and can be used in various pharmacopoeial and non-compendial dissolution test apparatuses [31]. The next scheme summarizes how this device works.

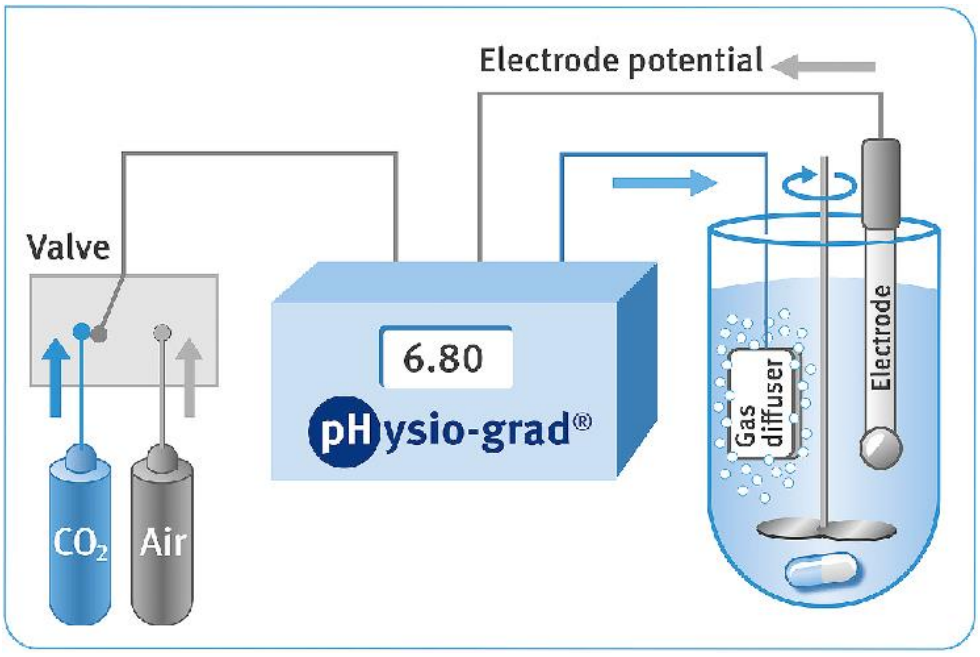

Figure 6: *Simplifying scheme of pHysio-grad®. Adapted from [48].*

MCIllvain buffer

Some authors consider citrate and phosphate buffers to be biosimilar buffer components [46,47,48]. McIlvaine buffer solutions are mixtures of phosphate and citrate buffer systems, which facilitate comparable *in vivo* buffering capabilities. This happens due to the pKa values of the citrate/phosphate combination, which are similar to the *in vivo* pKa values in physiological pH values, as summarized in Table 6.

Table 6: *Overview of pKa values comparing McIlvaine buffer and physiological conditions. Adapted from [49, 52].*

Component	McIlvain Buffer		Human Buffer		
	Citrate	**Phosphate**	**Carbonate**	**Phosphate**	**Organic Acid**
pKa	-	2.2	-	2.2	-
	3.1	-	3.5	-	-
	4.8	-	-	-	5
	6.4	-	6.4	-	-
	-	7.2	-	7.2	-
	-	12.2	10.3	12.2	-

Another advantage of this buffer is that it allows working with a wide pH range avoiding the addition of other components. Bile salts can also be added to make this buffer even more biorelevant.

1.8. Combined models of dissolution-permeation

In addition to simulating dissolution, the permeation process may also be assessed *in vitro*. Several models were developed to allow a simulation of the permeation and, consequently, an evaluation of drug absorption [49]. These models are summarized according to the literature [50] in Figure 7.

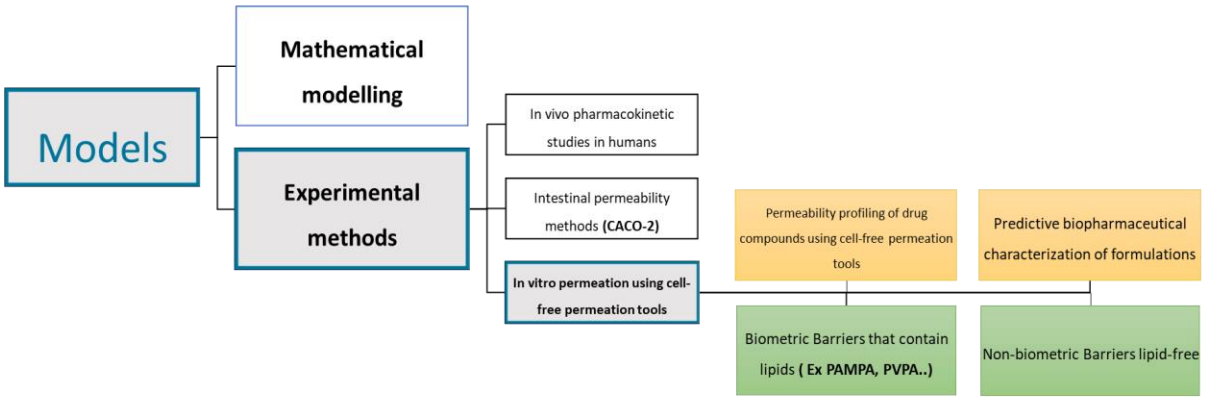

Figure 7: *Types of models mimicking drug permeability in the gastrointestinal tract.*

Permeation models may be classified into two large groups: mathematical and experimental models [50]. The experimental models can be divided into *in vivo* models that study pharmacokinetics in humans, models based on the study of the gut (*in vivo* and *in situ*), and *in vitro* permeation models using cell-free permeation tools.

Focusing on the latter, it can be subdivided according to their applicability, i.e., they may be used to determine the permeation profile of the drug or to build a biopharmaceutical prediction. Alternatively, these methods can also be further subdivided according to the type of barrier (lipidic/non-lipidic). The models applied in this thesis fall into the subtypes that predict biopharmaceutical characterization of formulations using biometric barriers.

Accurate assessment of the intestinal permeability of a drug is of paramount importance. Although such measurements can be inferred or estimated directly from physicochemical and molecular characteristics, *in vivo*, *in situ*, *ex vivo*, and *in vitro* techniques remain the basis of intestinal permeability assessment [33]. From a regulatory point of view, the concept of high permeability is 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 for such assessment [51]. The combination of *in vitro* and *in vivo* data may lead to the construction of IVIVCs [37]. Moreover, the continued refinement of *in silico* approaches to accurately predict drug permeability and transport mechanisms, and their validation with observed *in vivo* data will allow for a more efficient evaluation of drugs. One of the most well-known *in silico* modeling for pharmacokinetic prediction is the Physiologically-based Pharmacokinetic models (PBPK), a recent hot topic in the literature [31].

1.9. Justification of selected case studies

The BCS captures two of the most significant factors that influence oral absorption of drugs, solubility, and intestinal permeability. Solubility is the bottleneck for the absorption of BCS II compounds, and permeability is added for BCS IV molecules. In these cases, advanced *in vitro* approaches are needed to better understand and simulate what happens *in vivo*. This includes the development of biorelevant methods as described in this chapter.

In this work, biorelevant setups will be developed for real industry case studies, which are projects integrated into the product development team of *Bluepharma – Indústria Farmacêutica, SA*. Challenging molecules are the scope of this work, mainly BCS II and IV, where *in vitro* results are intended to be correlated with *in vivo* data. Some characteristics of the molecules and final products are summarized in Table 7. The aim is to gather a better understanding of their behavior *in vivo* and investigate improved setups for performance characterization for an enhanced prediction of *in vivo* results.

The experimental work was divided into two chapters. In Chapter 2, five molecules are studied, evaluating their dissolution, supersaturation and solubility through phase change tests (pH variation) using dissolution media and other setups under biorelevant conditions. The pH variation and the composition of biorelevant dissolution media are intended to simulate the GIT.

The biorelevant medium was mainly chosen based on the OrBiTo (Oral Biopharmaceutics Tools) decision tree. This decision tree was developed to help in the selection of the most appropriate *in vitro* methodology for release testing of solid oral dosage forms [52]. Other decision trees are available, developed by European Federation of Pharmaceutical Industries and Associations (EFPIA) partners, Bayer (based on the volume needed to dissolve the human dose and the drug relative oral bioavailability), or Boehringer Ingelheim (based on the estimated human dose and dose number) [53]. Each tree has its specific approach due to the context and experience of each company. The combined evaluation of dissolution in biorelevant conditions and permeation is explored in Chapter 3 with four model drugs. Biphasic dissolution setups equipped with permeation membranes or dialysis bags were used and the results are interpreted in light of known pharmacokinetic profiles.

Table 7: *Characteristics of the molecules and final products under study. All the values presented are approximate for confidentiality reasons, as well as the source of the reported values.*

Drug/ Product code	Characteristic						
	Molecule				Final Product		
	BCS class	*pKa*	*Acid/Base*	*LogP*	*Oral Solid Drug*	*Bioavailability (%)*	*Tmax (h)*
A	II	12	Weak acid	6	Capsules with microtabs	40-50	1-4
B	II / IV	4	Weak acid	6	Film-coated tablets	50	2-6
C	II	5; 10	Weak base	4	Film-coated tablets	20	1-8
D	I / III	0,1;15	Weak base	4	Film-coated tablets	90	1-4
E	IV	12	Weak base	5	Capsules	30	0.5-4
F	II / III	9	Weak base	3	Film-coated tablets	30-40	1-4
G	III	5	Weak base	1	Orodispersible tablets	15	3

As can be seen from the table, BCS Class I and III molecules were also used. The choice of these molecules is related to the challenges inherent in the formulation development. For D, the release method did not prove to be discriminatory and was far from biorelevant, while for G, the pharmaceutical form differed from the form of the reference product (orodispersible tablets *versus* film-coated tablets).

2.1. Introduction

Dissolution methods have been traditionally used for QC purposes, and therefore the application of dissolution to predict the biopharmaceutical performance of oral dosage forms needs to address several extra features [11]. Over the last 20 years, different *in vitro* setups have been developed to evaluate supersaturation, drug dissolution, and precipitation. Some models apply phase changes and pH variations to predict the behavior of the drug during the transition from the stomach to the intestine [54]. One such model uses two dissolution vessels with USP Apparatus II connected by a peristaltic pump to predict intestinal supersaturation and precipitation [55].

However, due to the complexity of supersaturation and precipitation, multiple factors can affect the behavior of drugs *in vivo*, as detailed in Chapter 1. More complex setups such as TIM-1 and tiny-TIM have been developed to cope with these challenges [56]. These systems simulate the conditions in the stomach and the three compartments of the small intestine (duodenum, jejunum, and ileum). The compartments are connected by peristaltic valves that control gastric emptying, intestinal transit, and ileal emptying. It was also possible to control simulated gastric secretion, duodenal, jejunal, and ileal secretion. In these models, biorelevant media, already described above, are used.

This chapter was based on single-phase (i.e., aqueous) biorelevant dissolutions, focusing mainly on pH variations, to understand the behavior of the molecule in the different microenvironments of the GIT. The molecules used in this part of the study were coded A, B, C, D, and E. Their main characteristics have been summarized in Table 7. The *in vitro* results are intended to be compared whenever possible with the results obtained *in vivo*.

2.2. Materials and methods

All developed analyses were divided into two parts. The dissolution setup includes the sample preparation on dissolution followed by subsequent quantification. In all dissolution tests, the medium temperature was adjusted to 37 (±0.5)°C. Data acquisition and HPLC control were performed using Empower™ 3 Chromatography Data Software (Waters, Milford, MA USA), in all the methods detailed below. All solutions used in this part of the experiment were prepared according to the USP, except for FaSSIF which was prepared according to the manufacturer's directions (Biorelevant, London, UK).

Each setup was based on a sequence of development *in vitro* tests that will not be described for sake of simplification and discussion of final dissolution results. These *in vitro* tests included QC dissolutions, solubility tests to ensure sink conditions, pH tests to maintain the target pH at each phase, simulation tests at different concentrations, filtration tests, and linearity calculations. All these tests allowed, among other conditions, the selection of dissolution media and specific volumes, pH to be evaluated, and the need for centrifugation or filtration steps before HPLC quantification. These evaluations were carried out individually, for each model drug under study, as well as for each setup, when required.

Model drug A

- Dissolution setup

The dissolution experiments were performed using USP Apparatus II equipped with 1 L vessels, stirred at 50 rotations per minute (RPM). This test was divided into two phases with a pH change at 30 minutes. Initially, samples were placed in the vessels with 500 mL of 0.025M HCl pH 1.6. After 30 minutes, 500 mL of double concentrated FaSSIF was added and the pH was adjusted to 6.5 with 1 M NaOH. Manual samples of 10 mL were withdrawn at 30, 45, 60, 75, and 90 minutes, with replacement with fresh equivalent volume. The samples were then filtered with on-line 10 µm and then syringe 0.45 µm filters.

- Quantification

The quantification of the samples was done by High-Performance Liquid Chromatography (HPLC). The analytical column was a reversed-phase Apex ODS (100 x 4.6mm, 5µm; Avantor - VWR, Radnor, Pensilvânia, EUA). The method was isocratic and the mobile phase consisted of methanol (MeOH) and ultrapure water at 53:47, v/v. The flow rate was set to 1.5 mL/minute and the chromatographic run lasted 7 minutes. An injection volume of 15 µL was chosen. The

column was maintained at 30 °C. The molecule was detected spectrophotometrically at its maximum wavelength of 245 nm. Quantification was performed against a calibration prepared with a standard solution at 100% of the dissolved drug.

Model Drug B

- Dissolution setup

The dissolution experiments were done using Apparatus II equipped with USP 1 L vessels, stirred at 50 RPM. This test was divided into two phases with a pH change at 30 minutes. Initially, samples were placed in the vessels with 500 mL of 0.025M HCl pH 1.6. After 30 minutes, 500 mL of double concentrated FaSSIF was added and the pH was adjusted to 6.5 with 1M NaOH. Manual samples of 8 mL were withdrawn at 30, 35, 40, 45, 50, 60, 75, and 90 minutes, with replacement with fresh volume. The first time point samples (30 minutes) were centrifuged at 4000 RPM for 15 minutes and the other samples were filtered with on-line 35 µm and syringe 0.45 µm filters.

- Quantification

The quantification of the samples was done by HPLC. The analytical column was a reversed-phase X-Bridge C18 (50 x 4.6mm; 3.5µmw; Waters Milford, MA). The method was isocratic and the mobile phase consisted of 10 mM sodium acetate pH 4.5 (Ph. Eur) and acetonitrile (ACN) at 50:50, v/v. The flow rate was set to 1.5 mL/minute and the chromatographic run lasted 7 minutes. An injection volume of 5 µL was chosen for the acid stage (time point of 30 minutes) and 10 µL for the buffer stage. The column was maintained at 35°C. The molecule was detected spectrophotometrically at its maximum wavelength of 415 nm. Quantification was performed against two calibrations prepared with a standard solution at 100% of the dissolved drug, both at buffer and acid stages.

Model Drug C

- Dissolution setup

The dissolution experiments were done using USP 1 L vessels and Apparatus II, stirred at 50 RPM. Phase change occurred at 30 minutes. Samples were placed in the vessels with 500 mL of 0.025M HCl pH 1.6, and at 30 minutes, 500 mL of double concentrated FaSSIF was added and the pH adjusted to 6.5 with 1M NaOH. Manual samples of 4 mL were withdrawn at 5, 10,

15, 20, 30, 35, 40, 45, 50, 60, 75, and 90 minutes, with replacement of the removed volume. All samples were filtered with on-line 10 µm and syringe 0.45 µm filters.

- Quantification

The quantification was done by HPLC. The analytical column was a reversed-phase Purospher Star RP18E (150 x 4.6mm, 3µm; Sigma Aldrich, St. Louis, Missouri, EUA). The method was isocratic and the mobile phase consisted of 10 mM ammonium acetate pH 4.0 (Ph. Eur) and MeOH at 60:40, v/v. The flow rate was set to 1.0 mL/minute and the chromatographic run lasted 4 minutes. An injection volume of 10 µL was chosen. The column was maintained at 40°C. The molecule was detected spectrophotometrically at its maximum wavelength of 268 nm. Quantification was performed against one calibration prepared with a standard solution at 100% of the dissolved drug.

Model Drug D

- Dissolution setup

The dissolution experiments were performed using USP 1 L vessels and Apparatus II, stirred at 50 RPM. pH change occurred at 30 minutes. Initially, samples were placed in the vessels with 500 mL of 0.025M HCl pH 1.6, and at 30 minutes, 500 mL of double concentrated FaSSIF was added and the pH was adjusted to 6.5 with 1M NaOH. Manual samples of 5 mL were withdrawn at 5, 10, 15, 30, 45, 60, 75, 90, and 120 minutes, with medium replacement. All samples were filtered with on-line 45 µm and syringe 0.45 µm filters.

- Quantification

The quantification of the samples was done by HPLC. The analytical column was a reversed-phase X-Terra RP18 (250 mm x 4.6 mm; 5 µm; Sigma Aldrich, St. Louis, Missouri, EUA). It used a gradient method detailed in Table 8. Mobile phase A was a 10 mM potassium dihydrogen phosphate buffer pH 3.0 (USP) and mobile phase B consisted of ACN and MeOH at 70:30, v/v. The flow rate was set to 1 mL/minute and the chromatographic run lasted 20 minutes. An injection volume of 5 µL was chosen for the acid stage (time points of 5, 10, 15, and 30 minutes) and 10 µL for the buffer stage. The column was kept at 35°C. The molecule was detected by UV at 349 nm. Quantification was performed against two calibrations prepared with a standard solution at 100% dissolved drug, at both the buffer and acid stages.

Table 8: *Description of HPLC gradient method applied to product D.*

Time (minutes)	Mobile Phase A (%)	Mobile Phase A (%)
0	90	10
15	20	80
16	90	10
20	90	10

Model Drug E

For this molecule, two strategies were designed with pH changes similar to what happens in GIT. Both were quantified by the same HPLC method.

- Dissolution Setup

Test 1: The dissolution experiments were performed using USP 200 mL dissolution vessels with mini-paddles, stirred at 50 RPM. This test was divided into two phases with a pH change at 15 minutes. Samples were placed in the vessels with 200 mL of 0.025M HCl pH 1.6, and after 15 minutes, 100 mL was transferred to another vessel (1 L) with the assistance of a peristaltic pump, with an emptying speed of 6.75 mL/minute. After 50 minutes, the flux of the peristaltic pump was decreased to 3.25 mL/minute. The 1 L vessel contained initially 200 mL of FaSSIF pH 6.5. Manual samples of 3 mL were withdrawn at 5, 10, 15, 20, 30, 35, 40, 45, 50, 60, 75, 90, 105, and 240 minutes, with medium replacement with fresh volume. All samples were filtered with on-line 10 µm and syringe 0.45 µm filters.

Test 2: The dissolution experiments were done using USP 200 mL vessels with mini-paddles, at a stirring rate of 50 RPM. This test was divided into two phases with a pH change. Samples were placed in the vessels with 30 mL of 0.025M HCl pH 1.6. After 20 minutes, 15 mL of quadruple concentrated FaSSIF was added, and the pH was adjusted to 5.5 with 1M NaOH. At 90 minutes, 1M NaOH was added to increase the pH to 6.5. Manual samples of 2 mL were withdrawn at 20, 30, 60, 90, 105, and 240 minutes, with replacement by fresh medium. All samples were filtered by on-line 10 µm and syringe 0.45 µm filters. All samples were diluted 25 times with 0.025M HCl.

- Quantification

The quantification of the samples was done by HPLC. The analytical column was a reversed-phase X-Terra MS C8 (50 x 4.6mm; 2.5um; Waters Milford, MA). The method was isocratic and the mobile phase consisted of 0.001M HCl pH 3.0 (Ph. Eur) and ACN at 67:33, v/v. The flow rate was set to 0.5 mL/minute and run for 4 minutes. An injection volume of 5 µL was chosen. The column was maintained at 40°C. The molecule was detected by UV at 310 nm. Quantification was performed against one calibration prepared with a standard solution at 100% of dissolved drug.

2.3. Results and discussion

Model Drug A

This model intended to simulate fasting conditions contemplating the disintegration of the tablet in the gastric environment and its subsequent dissolution in the intestinal environment. The phase transition chosen (pH 1.6 to pH 6.5) simulates the passage from the stomach to the intestine during gastric emptying. Due to the estimated average time for this emptying to occur, the experiment was performed for 30 minutes in HCl pH 1.6. There was only one sampling point in the acid phase as the drug was not expected to solubilize (very poorly soluble, pKa of 12 and weak acid). This control sample was collected at 30 minutes, showing a zero dissolved percentage.

The intestinal environment was simulated for 60 minutes. Although the T_{max} value occurs at 1-4 hours, the test was carried out for 90 minutes because it intended to simulate intestinal dissolution and not permeation. The high LogP of 6 provides high lipophilicity to the molecule so the limiting step is not permeation, but solubility (translated into BCS class II).

Different formulations were produced throughout the development process, with different characteristics, in order to mimic as much as possible the reference product. For the biorelevant study of product A, two test products were selected (Figure 8). This figure shows the dissolution profile for the three characterized products.

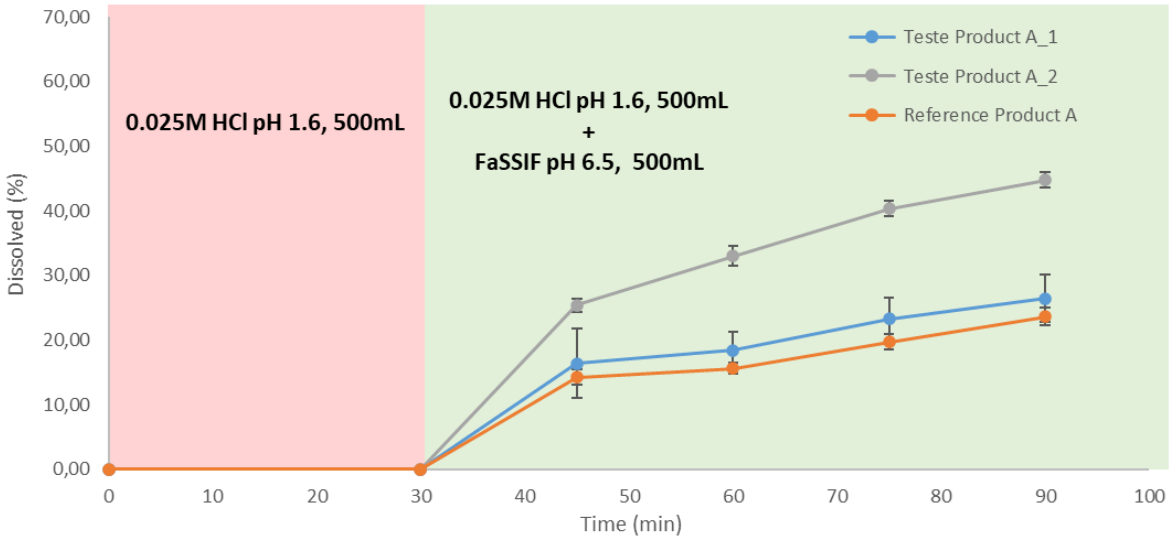

Figure 8: *Biorelevant Dissolution in HCl pH 1.6 500 mL + FaSSIF pH 6.5 500 mL for products Test A_1 (n=12) and A_2 (n=12) versus Reference Product (n=6).*

According to this method, a huge discrepancy between the two tests indicates that the Product A_1 test was the promising one. However, before the clinical trial, there was insufficient evidence to ensure that this method was biorelevant. Other tests, such as dissolutions at different pH or even dissolutions in routine medium indicated similarity in the profiles of both tests against the reference product. The final test product selected for *in vivo* study was the A_2 test.

The clinical study was a single-center, single-dose, open-label, laboratory-blinded, randomized, two-sequence, two-treatment, two-period crossover study in 52 healthy male and female subjects, under fasting conditions. The tendency for the test product to be above the reference product was also observed in the clinical trial either in the area under the curve (AUC) or in the maximum concentration (C_{max}). The study's results showed that the Test-to-Reference Geometric LSmeans Ratio (GMR) obtained for both C_{max} and AUC laid within the 80.00% - 125.00% acceptance interval but at around 120%. Moreover, the upper bound of the 90% Confidence Intervals (CI) for both C_{max} and AUC fell outside the 125.00% acceptance limit. Following the European Medicines Agency's (EMA) applicable guideline, bioequivalence between test and reference products could not be inferred under fasting conditions.

These results are in line with the ones obtained *in vitro*. The tendency to be over-bioavailable was seen in this method, later proven to be biorelevant, or considered at least highly promising. Accordingly, after verification and comparison of these data with clinical data, this method was closer to biorelevance validation, which would require additional clinical data of

alternative test products. The developed method would allow the project to move forward with a reformulation toward a bioequivalent test product.

Model Drug B

As a poorly soluble drug and a weak acid, the model drug B is dissolved *in vivo* only after stomach emptying. QC method results at pH 6.8 with the addition of artificial surfactants do not reflect the pharmacokinetics profile, as the transition from the stomach to the intestine seems to be critical. The rationale for dissolution setup development was similar to model drug A since the goal was to study a fasting environment as well. In the acidic medium, the tablet disintegrates, but model drug B is not dissolved. Therefore, almost any dissolved drug is observed until 30 minutes. From then on, the test follows pH 6.5, simulating the intestinal phase after gastric emptying. For weak acids, dissolution is minimal in the stomach and the dissolution rate increases as the drug reach the less acidic intestinal region. Consequently, at this point, it was possible to distinguish the behavior of the test from the reference products (Figure 9).

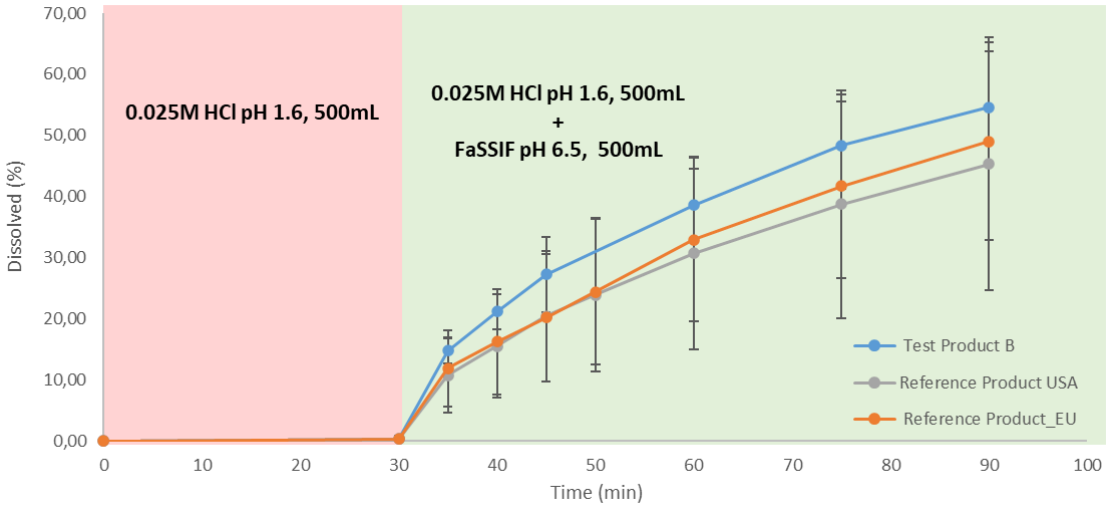

Figure 9: *Biorelevant Dissolution in HCl pH 1.6 500 mL + FaSSIF pH 6.5 500 mL for Product Test B (n=10) and two Reference Products (USA, n=8 and EU, n=9).*

All products show large variability, probably explained by the fact that tablets do not disintegrate similarly during the acidic phase. This phenomenon leads to a lower contact area between the product and the medium. This variability makes it difficult to conclude the similarity between the reference and the test products, as the error bars overlap. However, there is a tendency for the test product to be faster dissolved than the reference products.

This product was subjected to a three-arm pilot clinical study, where the same test product was compared with two reference products, one from the EU and the other acquired in the USA market. The clinical study was a single-centered, single-dose, open-label, laboratory-blinded, randomized, six-sequence, three-treatment (Test, Reference-1 (USA), and Reference-2 (EU)), three-period crossover pilot study in 14 (Reference_1) and 16 (Reference_2) healthy male and nonpregnant, non-lactating female volunteers under fasting conditions. The Test-to-Reference-1 (USA) GMR calculated for the drug ln-transformed C_{max} and AUC were within the 80.00% to 125.00% bioequivalence acceptance interval but both at around 118%. The results of the Test-to-Reference-2 (EU) showed a GMR calculated for the C_{max} and AUC at approximately 105%, but after the removal of some outliers, the same trend as before was observed, with both ratios around 114%.

The tendency for the tests to be above the reference product was in line with the *in vitro* experiments. These results support the validation of the previous method as biorelevant. Still, it will be necessary to improve this method in order to decrease variability and also to include permeation simulation. Due to the BCS of this molecule, it is crucial to evaluate permeation as it may be a limiting step in absorption. An alternative model was discussed in Chapter 3.

Model Drug C

The final product C has two different strengths. Figure 10 summarizes the results for the lower strength, while Figure 11 contains the results for the higher one.

The release profiles in the acid phase (the first 30 minutes) of the test and the reference products were practically overlapping in both strengths. In the next phase, which simulates the intestinal environment, some differences and a few collection times with large variability are observed. Over time, the two profiles become closer and more similar. The variability verified at 30 to 40 minutes in the reference product can be explained by the tablets sticking to the bottom of the vessel during the acid phase. As a week base with pKa of approximately 5, precipitation after gastric emptying could be expected. This precipitation limits bioavailability by preventing absorption. This precipitation occurs to almost the same extent in the test and in the reference products. The low bioavailability reported for this molecule is in line with the results obtained in this dissolution, for both strengths.

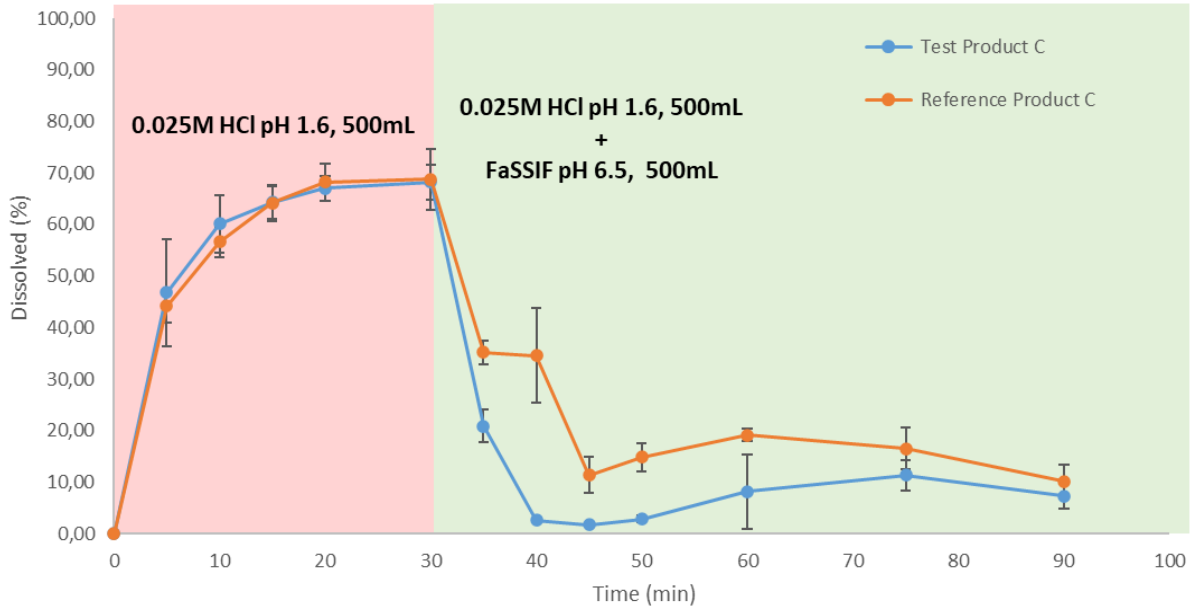

Figure 10: *Dissolution in HCl pH 1.6 500mL + FaSSIF pH 6.5 500 mL for lower strength of Product Test C (n=12) versus Reference Product (n=12).*

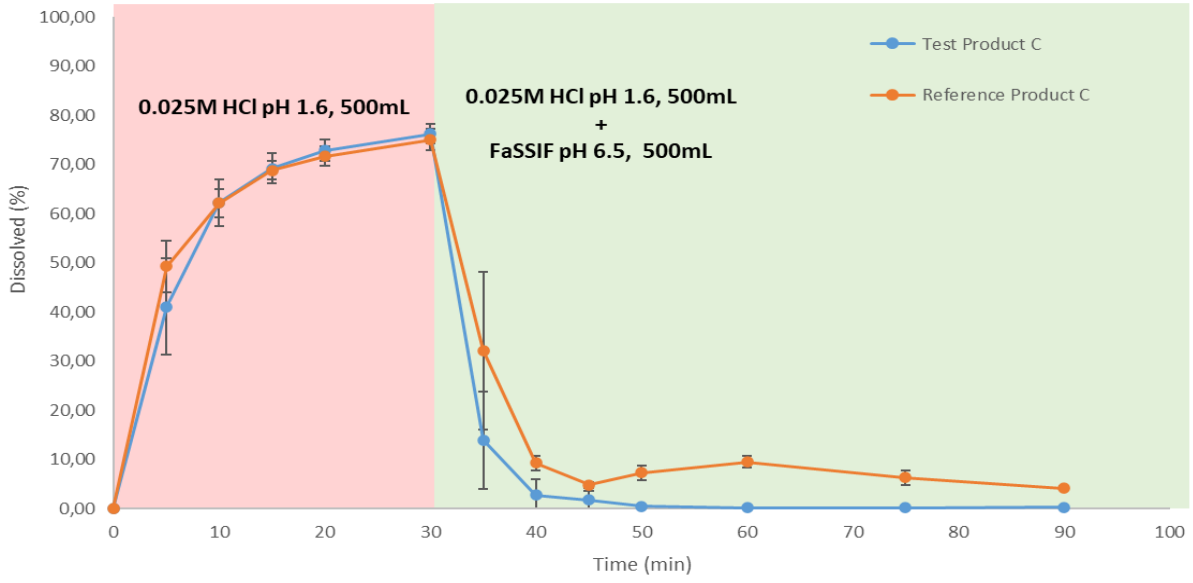

Figure 11: *Dissolution in HCl pH 1.6 500 mL + FaSSIF pH 6.5 500 mL for higher strength of Product Test C (n=12) versus Reference Product (n=12).*

The clinical study for the lower strength was a single-center, single-dose, open-label, laboratory-blinded, randomized, two-sequence, two-treatment, two-period crossover study in 116 healthy male and nonpregnant female subjects, under fasting conditions. The study results rounded 100% for both C_{max} and AUC (Test-to-Reference GMRs). Following EMA

applicable guidelines, bioequivalence between test and reference products could be inferred under fasting conditions.

The clinical study with the highest strength was a single-center, single-dose, open-label, laboratory-blinded, randomized, two-sequence, two-treatment, two-period crossover study in 122 healthy male and nonpregnant female subjects, under fasting conditions. The results obtained were similar to those of the lowest strength, and bioequivalence between test and reference products could be inferred as well.

The overlap of the profiles in the acid phase may justify the similarity obtained in the clinical trials as well as the 100% value for C_{max}. With the evaluation of the results of this clinical study along with the *in vitro* data, it is possible to infer on the biorelevance of this method.

Model Drug D

Drug D is a highly soluble drug (BCS class I or III) and a fast dissolution is typically observed at different pH. However, different release profiles from the reference product were observed in pH 4.5 and 6.8, and an additional investigation was required in biomimetic conditions.

As observed in Figure 12, dissolved percentages of 100% right after 20 minutes were obtained, with no precipitation in FaSSIF pH 6.5. The profiles are practically overlapping with some initial variability, related to the initial tablet disintegration.

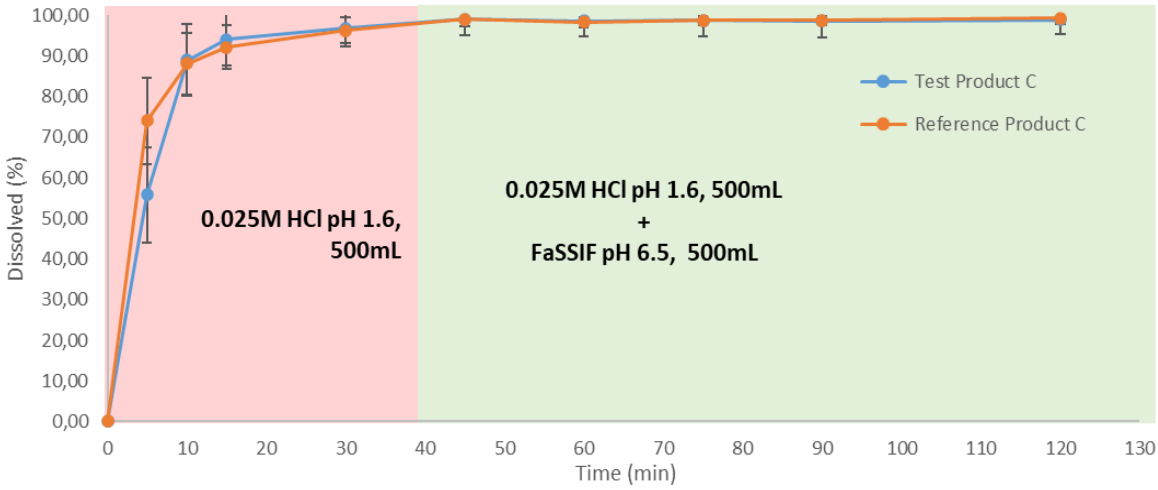

Figure 12: *Biorelevant Dissolution in HCl pH 1.6 500 mL + FaSSIF pH 6.5 500 mL for Product Test D (n=12) versus Reference Product (n=3).*

The results of the clinical trial (at around 100% for both C_{max} and AUC) were in line with these results. The pilot clinical study was a single-center, single-dose, open-label, laboratory-blinded, randomized, two-sequence, two-treatment, two-period crossover study in 18 healthy male and nonpregnant female subjects, under fasting conditions.

Therefore, the initial differences observed in Ph. Eur. standard buffers of pH 4.5 and 6.8 were shown not relevant for the model drug product D, as long as an initial acid phase occurs, where the drug product is fully disintegrated and dissolved. Precipitation due to supersaturation *in vivo* in the passage from the stomach to the intestine seems not to be an issue. All these characteristics potentiate a high bioavailability as expected (Table 7).

Model Drug E

This phase change experiment on model drug E intended to mimic the fasted state, as a new bioequivalence study was planned. Test 1 was conducted on two small-scale batches compared to the reference product (Figure 13).

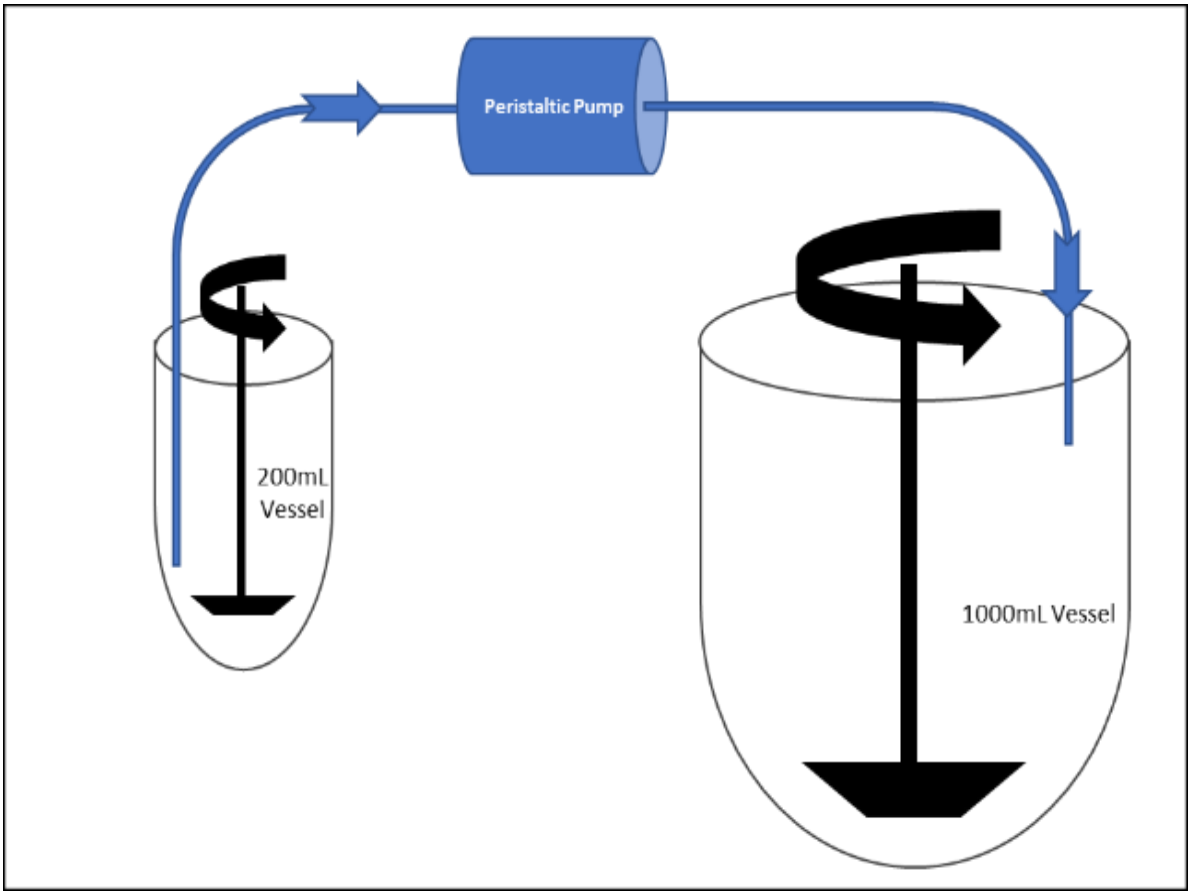

Figure 13: *Scheme of the dissolution setup used in test 1 of product E.*

The results depicted in Figure 14 reflect relevant differences between the two prototypes (1 and 2) but only in the acid phase. As a weak base with very low drug solubility, the drug immediately precipitates as soon as it contacts a higher pH, which occurs similarly for the three characterized products. Regardless of the flow of the peristaltic pump, the results remained constant. An alternative strategy in the pH transition was needed to avoid the full precipitation of the sample. There was no available *in vivo* data at the time of this experiment for comparison.

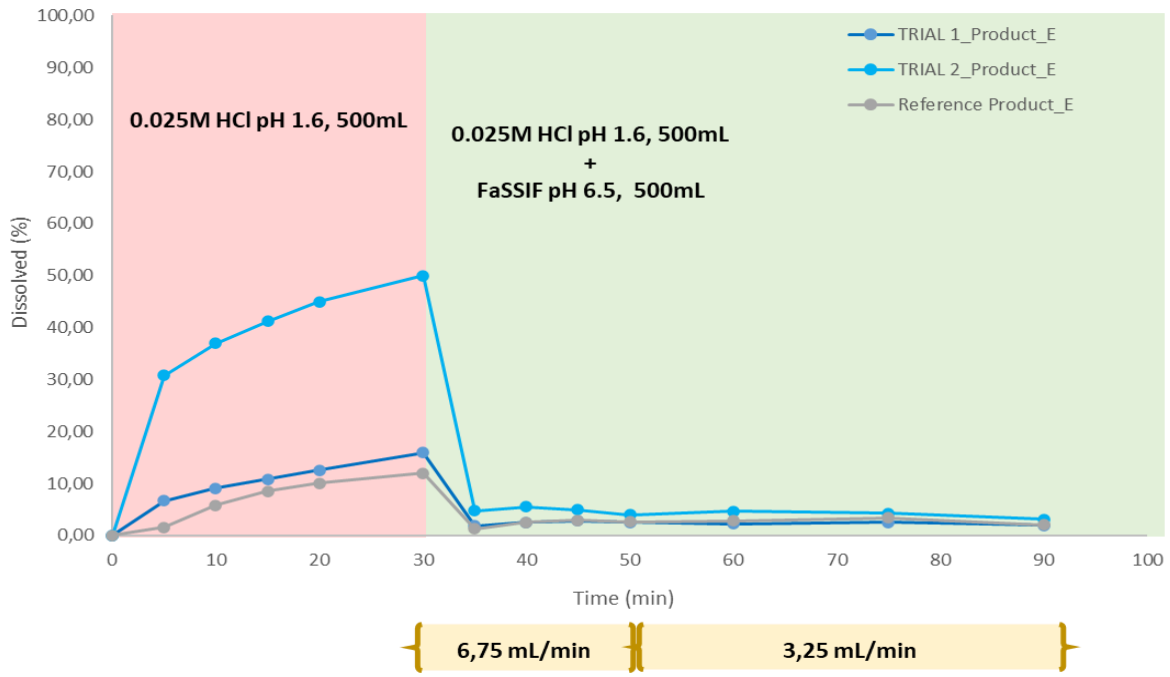

Figure 14: *Dissolution in HCl pH 1.6 500 mL + FaSSIF pH 6.5 500 mL for Product Test E (Trial 1, n=1 and Trial 2, n=1) versus Reference Product. The pH change was performed with a peristaltic pump with two different fluxes.*

A new test was designed to understand the impact of applying lower volumes, mimicking as close as possible the volumes of the gastric fasting state (test 2). In addition, a gradual transition of pH was performed to avoid the full precipitation in the intestinal-like phase. This transition is more close to what happens *in vivo* since the pH does not change immediately from 1.6 to 6.5. As observed in Figure 14, it was indeed an adequate technique, as the sample did not precipitate completely at pH 5.5. However, due to the reduced volume used, drug dissolution was not complete. The capsule disintegrated but a strong conning effect occurred, resulting in very low dissolved percentages.

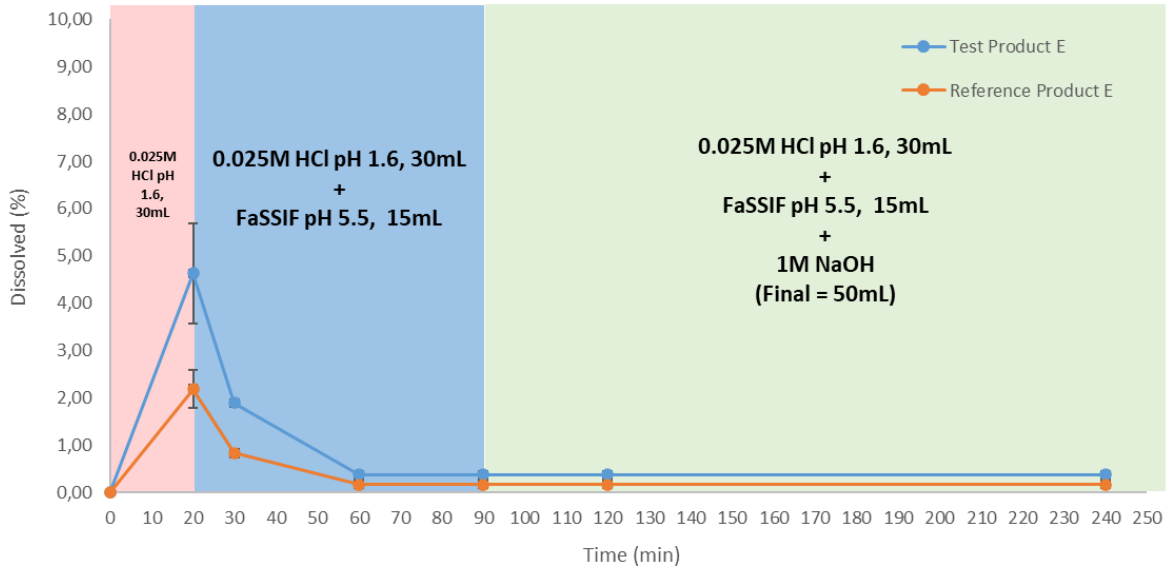

Figure 15: *Dissolution in HCl pH 1.6 30 mL + FaSSIF pH 6.5 15 mL for Product Test E (n=3) and Reference Product (n=3). The pH change was performed gradually.*

For molecules with similar properties to model drug E, the gradual pH change strategy may be considered to improve precipitation issues. However, the experiment as designed was considered not adequate to predict *in vivo* results due to the very low drug release. An alternative strategy is explored in Chapter 3.

2.4. Conclusion

These biorelevant methods based on phase changes allowed the simulation of the behavior of the drug in the stomach and its passage to the intestine. They allowed the early detection of differences between the test and the reference products. It was possible to collect knowledge of product trends and assess whether the test product dissolution is slower or faster than the innovator in specific biomimetic conditions.

The release methods (QC) reflect completely different conclusions. As already mentioned in the introduction, the purpose of a QC method is that it is discriminatory and allows detecting differences in some critical parameters of the product quality or detecting changes on some parameters of the manufacturing process. As observed in Figure 16, where the same products are analyzed by QC, almost all pairs of products look similar, whether it is known from the clinical results, in some cases, that this is not true.

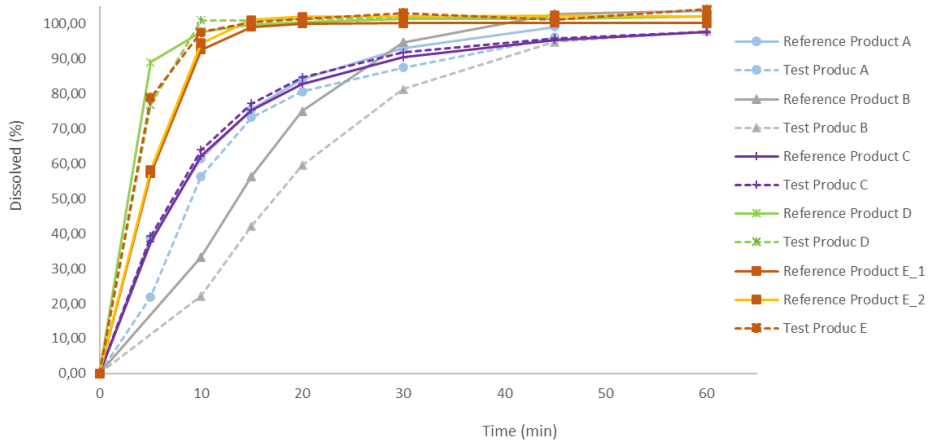

Figure 16: *QC methods applied to molecules A, B, C (higher strength), D, and E, and their respective reference products (all n=12). Although not detailed in the graph, the dissolution conditions are product-specific.*

These findings supported formulation scientists in their decisions, changed scientific perspectives, and redefined strategies when the results were significantly different from expected. When the results show similarity between the products, the decision to move into the clinical trials is performed with greater confidence. However, although the tablet is dissolving similarly or not to the innovator, these phase change methods are not able to evaluate how permeation will occur. Dissolution/permeation setups were developed to integrate permeation data into the assessment of the results. These setups are described in Chapter 3.

3.1. Introduction

Dissolution setups that use techniques to simultaneously simulate permeation and dissolution are useful to mimic the behavior of the drug *in vivo*. These setups complement the information obtained through dissolution with pH change [57], as described in the previous chapter.

The dissolution of an oral drug and its subsequent permeation through small intestinal cells are the two critical processes in drug absorption, each of which could be a rate-limiting step. These parameters are essential to the determination of the bioavailability of the drug [58]. Most of the models initially developed did not consider permeation. However, excipients can modulate both the drug dissolution and its permeation. Their combined evaluation in the final drug product is essential [58].

Currently, several models are described as able to evaluate these two processes [60]. As referred to in the introductory chapter (Figure 7), these models may be divided into those that study pharmacokinetics *in vivo*, *in vitro* permeation models without the use of cells, and models that assess intestinal permeability [50].

Within this last subtype, the continuous dissolution system/Caco-2 and the flow through-cell system/Caco-2 should be highlighted. This setup is composed of two chambers (one acceptor and one donor, simulating respectively the intestine and the bloodstream) separated by a permeation membrane composed of a monolayer of Caco-2 cells [61]. Some authors claim that these approaches have disadvantages, as Caco-2 cells take 21 days to differentiate and Caco-2 tight junctions appear to be overly sensitive. There are also drawbacks to its use in metabolism studies since Caco-2 does not express cytochrome P450 3A4, although this can be induced by culturing in the presence of Vitamin D3 [62].

Concerning cell-free models, these can be categorized according to their applicability or barrier type. In this chapter, setups with various types of barriers were tested with the aim of bio-predictively characterizing formulations, while building knowledge. These barriers can be biometric (e.g. PermeaPad® membranes) or non-biometric (e.g. dialysis membranes) [63]. PermeaPad® barriers are robust, easy to store, and allow measurements over a wide pH range and high additive concentrations. These barriers can be used in conventional Franz cells, side-by-side diffusion cells, or other configurations [64].

Setups consisting of dialysis membranes were based on the concept of diffusion, i.e., the net movement of molecules from areas of high to lower concentration until equilibrium is reached. In dialysis, a low molecular weight saline solution is placed side by side with a buffer solution separated by a semi-permeable barrier, the dialysis membrane [65]. It is composed of regenerated cellulose and may have different pore sizes. The concentration differential between the sample and the dialysate facilitates the diffusion of small molecules through the membrane, while molecules that are larger than the membrane pores are retained on the sample side. These models have been further applied to nanoformulations or liposomal formulations in *In Vitro* Release (IVR) assays [66].

Other known setups with numerous applications used octanol or decanol to predict permeation and consequent absorption of the drug. These setups are called two-phase models [67] and simulate drug dissolution and absorption in the GIT by implementing an immiscible organic phase acting as an absorbent sink over the aqueous solution. Octanol mimics fatty tissues within the body and simulates the *in vivo* absorption process when used together with a physiologically relevant aqueous buffer [68, 69]. These studies further state that in a two-phase dissolution setup a customized double paddle should be used to ensure agitation in both phases [69]. The advantages of these setups include the easy handling and the use of a single container for the analysis. Previous studies have reported that these methods were able to differentiate formulation parameters such as dosage strength, the effect of excipients, drug precipitation, drug loading, and particle size [41, 70-73].

The setups described in this chapter were based on these previously presented techniques and aim to complement the data obtained by the phase change methods (Chapter 2). The results obtained with the different methods are discussed and compared with clinical results when available.

3.2. Materials and Methods

Each model drug was divided between dissolution setup and quantification. The dissolution setup includes the sample preparation on dissolution followed by subsequent quantification. For drugs already tested in Chapter 2, the quantification was performed with the same HPLC method. In such cases, reference were be made to the relevant description. Data acquisition and HPLC control were performed using Empower™ 3 Chromatography Data Software (Waters, Milford, MA USA), in all the methods detailed below. In all dissolution tests, the medium temperature was adjusted to 37 (±0.5)°C. All solutions used in this part of the experiment were prepared according to the USP, except for FaSSIF, which was prepared according to the manufacturer's directions (Biorelevant, London, UK).

Each configuration was based on a sequence of *in vitro* development tests that will not be described in the scope of this work. In addition to the tests performed in the previous chapter, which helped to outline the conditions for the models, solvent solubility tests, pH tests to maintain the target pH at each stage, filtration tests, and linearity calculations were performed. All these tests allowed, among other conditions, the selection of dissolution media and specific volumes, the evaluation of pH, and the need for centrifugation or filtration steps before HPLC quantification. These evaluations were performed individually, for each model drug under study, as well as for each configuration, when necessary.

<u>Model Drug B</u>

Method and setup optimization were been carried out to obtain less variable and more reproducible data. Three different setups were tested.

- Dissolution setup

Test I: Biphasic dissolution experiments were done using 200 mL vessels with modified mini-paddles. A second propeller was adapted to the blade to ensure the homogenization of the two phases. A stirring rate of 50 RPM was used.

This test was divided into three phases according to pH. Initially, the tablets were placed in 40 mL of 0.025M HCl pH 1.6. After 30 minutes, 10 mL of four-times concentrated McIlvaine buffer containing a shot of surfactants was added, and the pH was adjusted to 5.5 (to ensure a gradual pH transition). At 120 minutes, the pH was once again adjusted to 6.5 with NaOH 162 g/150 mL. In the first 30 minutes, the dissolution occurs with aqueous phase only. 50 mL

of octanol was added at this time point to create the organic phase. Manual samples of 2 mL were withdrawn at 15, 30, 45, 90, 120, 130, 150, 180, 240, 300, and 360 minutes from the aqueous phase, with replacement with fresh volume. The same volume and time points were applied to the organic phase, starting at 45 minutes. All samples were filtered with on-line 45 µm and syringe 0.45 µm filters. Organic samples were diluted 50 times with the mobile phase for HPLC analysis.

Test 2: The differences between this test and the previous one were the volume of each phase, as it started with 400 mL instead of 40 mL, and the used vessel (1 L USP instead of 200 mL). After 30 minutes, 100 mL of concentrated McIlvaine buffer and 500 mL of octanol were added. Apparatus II was also modified by adding a second paddle for organic phase movement. The time points and the pH changes were the same as in Test 1. The solutions were prepared following the same procedures, and the samples were manipulated and quantified with the same parameters.

Test 3: In this test, only 200 mL of octanol were added instead of the 500 mL as in Test 2. All remaining parameters were kept constant

- Quantification

Same method as described in Chapter 2.

<u>Model Drug E</u>

- Dissolution setup

Three different setups were developed based on biphasic dissolution and dialysis membrane.

Test 1: These biorelevant dissolution analyses were done using USP 1 L vessels and Appatarus II, stirred at 50 RPM. Initially, the tablets were placed in 250 mL of 0.025M HCl pH 1.6. After 20 minutes, 250 mL of double concentrated FaSSIF was added and the pH was adjusted to 6.5 with 1N NaOH. Five dialyzes devices with 100KDa porosity (Float-A-Lyzer® devices, Spectrum&Repligen), with 2 mL of phosphate buffer saline (PBS) pH 7.4 each, were added to each vessel at this time point. The selected porosity was based on the size of the molecule under study as recommended in the manufacturer's guide for use. The devices were prepared one day before their use. One device was used by time-point. Manual samples of 5 mL were withdrawn from the vessel at 5, 10, 15, 20, 30, 60, 90, 120, and 240 minutes with the replacement of the removed volume. From the 30 minutes time point, 1 mL was withdrawn

from each dialysis device. The devices were kept in the vessel after the respective harvest to avoid the hydrodynamic disturbance of the setup. All samples were filtered with on-line 10 µm and syringe 0.45 µm filters.

Test 2: The difference from the previous one is that a gradual change of pH is performed. Instead of a transition from pH 1.6 to pH 6.8, the pH value is adjusted to 5.5 at 20 minutes, and later at 90 minutes adjusted to 6.5. All other parameters remained constant.

Test 3: Octanol was used as the organic phase. This analysis was done using 1 L USP vessels. A second propeller was adapted to Apparatus II to ensure the homogenization of the two phases, at a stirring rate of 50 RPM. Initially, the tablets were placed in 250 mL of 0.025M HCl pH 1.6. After 20 minutes, 250 mL of double concentrated FaSSIF was added and the pH was adjusted to 5.5 with 1N NaOH; 500 mL of octanol was also added at this point. At 90 minutes, the pH was adjusted to 6.5 with 1N NaOH. Manual samples of 5 mL were withdrawn from the aqueous phase at 20, 30, 60, 90, and 240 minutes with the replacement with fresh volume. From the organic phase, 5 mL was withdrawn at 30, 60, 90, 120, and 240 minutes. All samples were filtered with on-line 10 µm and syringe 0.45 µm filters. Organic samples were diluted 50 times with the mobile phase.

Test 4: The differences between this experiment and the previous one were the volume of each phase, as it started with 40 mL instead of 400 mL, and the dissolution vessel (200 mL instead of USP 1L). After 30 minutes, 10 mL of four times concentrated FaSSIF and 50 mL of octanol were added. Double mini-paddles were applied to the apparatus. The time points and the pH changes occurred as described in test 3. The solutions were prepared following the same procedures and the samples were manipulated and quantified with the same parameters.

- Quantification

Same method as described in Chapter 2.

<u>Model Drug F</u>

- Dissolution setup

This analysis was done using USP 1L vessels. A second propeller was adapted to Apparatus II to ensure the proper homogenization of each phase, stirred at 50 RPM. Initially, the tablets were placed in 250 mL of FeSSGF Middle (prepared according to Table 4) with 250 mL of purified water. After 120 minutes, 100 mL of six-times concentrated FeSSIF was added and

the pH was adjusted to 5.5 with 1N NaOH, as well as 200 mL of octanol. Manual samples of 5 mL were withdrawn from the aqueous phase at 20, 40, 60, 120, 135, 150, 180, 240, and 300 minutes with the replacement of the removed volume. From the organic phase, 5 mL was also withdrawn starting at 135 minutes timepoint. Samples of the aqueous phase were centrifuged for 10 minutes at 30 000 RPM. Organic samples were filtered with on-line 10 µm and syringe 0.45 µm filters and then diluted 50 times with the mobile phase.

- Quantification

The quantification of the samples was done by HPLC. The analytical column was a reversed-phase X-Terra RP18 (250 mm x 4.6 mm; 5 µm; Sigma Aldrich, St. Louis, Missouri, EUA). The method was isocratic and the mobile phase consisted of phosphate buffer pH 9.0 and ACN at 25:75, v/v. The flow rate was set to 1.4 mL/minute and the chromatographic run lasted 5 minutes. An injection volume of 20 µL was chosen. The column was maintained at 40 °C. The molecule was detected spectrophotometrically at its maximum wavelength of 210 nm. Quantification was performed against a calibration prepared with a standard solution of the molecule at 100% dissolved drug.

Model Drug G

- Dissolution setup

Test 1: Test samples were placed in 10 mL of simulated saliva fluid (SSF) and agitated for one minute in the rotating tube revolver. Then, dissolution proceeded in USP 1L vessels, stirred at 50 RPM with paddles. The samples were transferred to the vessel containing 490 mL of 0.025 M HCl pH 1.6 and, after 30 minutes, 500 mL of double concentrated FaSSIF was added (pH adjusted to 6.5 with 1N NaOH). At this time, a permeation device that includes a PermeaPad® membrane was also added per vessel. The device contains 25 mL of PBS and was submerged in the final volume of 1000 mL.

Manual samples of 5 mL were withdrawn from the vessel at 15, 30, 45, 60, 90, 120, and 150 minutes with the replacement of the removed volume. From the permeation device, 1mL was withdrawn starting at 45 minutes, also with the replacement of the removed volume. All samples were filtered with on-line 10 µm and syringe 0.45 µm filter.

Test 2: Test samples were placed in 10 mL of simulated saliva fluid (SSF) and agitated for one minute in the rotating tube revolver. Then, dissolution proceeded in USP 1L vessels, stirred at 50 RPM with paddles. The samples were transferred to the vessel containing 490 mL of

0.025 M HCl pH 1.6 and, after 30 minutes, 500 mL of double concentrated FaSSIF was added (pH adjusted to 6.5 with 1N NaOH). Reference product samples were placed directly into the vessel with 500 mL of 0.025M HCl pH 1.6. Manual samples of 5 mL were withdrawn from the vessel at 15, 30, and 45 with the replacement of the removed volume. After this time, 25 mL of each vessel were transferred to the permeation device that includes a PermeaPad® membrane adapted to the 1L vessel. A volume of 1000 mL of PBS was added and manual samples of 5 mL were withdrawn from the vessel at 15, 30, 45, 60, 75, 90, and 120 minutes. All samples were filtered with on-line 10 µm and syringe 0.45 µm filters.

- Quantification

The quantification of the samples was done by HPLC. The analytical column was a reversed-phase X-Terra RP18 (250 mm x 4.6 mm; 5 µm; Sigma Aldrich, St. Louis, Missouri, EUA). The gradient method detailed in Table 9 was applied. Mobile phase A was a 10 mM ammonium acetate buffer pH 10.0 and mobile phase B was ACN. The flow rate was set to 1 mL/minute and the chromatographic run lasted 20 minutes. An injection volume of 10 µL was chosen. The column was maintained at 30°C, and quantification was performed by UV at 282 nm, against a calibration prepared with a standard solution at 100% dissolved drug.

Table 9: *Description of HPLC gradient method from Drug G.*

Time (minutes)	Mobile Phase A (%)	Mobile Phase A (%)
0	70	30
8	70	30
9	50	50
12	50	50
13	70	30
20	70	30

3.3. Results and Discussion

Model Drug B

Test 1 intended to simulate the dissolution and permeation of drug B, adding information to the results obtained in the previous chapter. The test started with small volumes to make the experiment as biorelevant as possible. The use of the McIlvaine buffer instead of FaSSIF was suggested by some authors because it is considered an inexpensive and biorelevant buffer. Surfactants were needed due to the low solubility of this molecule. It contains lecithin and taurocholate at the same concentration as in FaSSIF (Table 5).

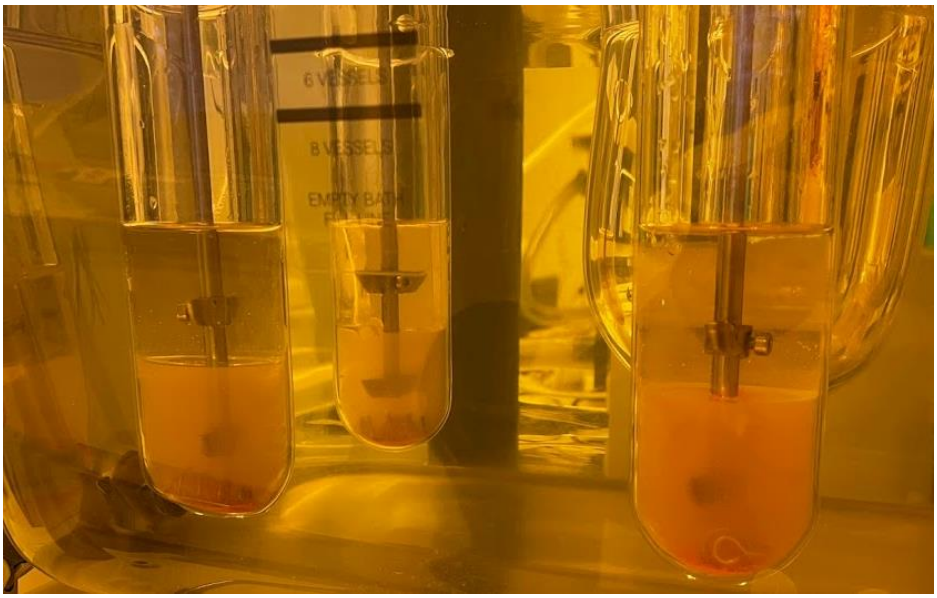

Figure 17: *Biphasic dissolution Test 1 of drug B. Image taken after 30 minutes of analysis. It depicts the 50 mL of aqueous and 50 mL of organic phases.*

Two different batches were subjected to the same setup, as shown in Figure 18. In this initial phase, no reference products were tested since the method was not optimized. As observed on the dotted lines, the aqueous phase has practically no drug dissolved. In HCl pH 1.6, the tablet disintegrates but never dissolves. After this time point, the dissolved percentage slightly increases due to the surfactants. Despite the very low solubility (BCS class II/IV), permeation occurs. In both tests 1 and 2, these values are low but a trend is seen. It was known that test 2 had better performance and a higher dissolved percentage was expected. Considering that the volume of the organic phase is sufficient to guarantee sink conditions, the limiting factor for permeation may have been the surface contact area between phases. Thus, in the next tests, vessels with larger capacities were used. The strategy of gradual pH change was kept.

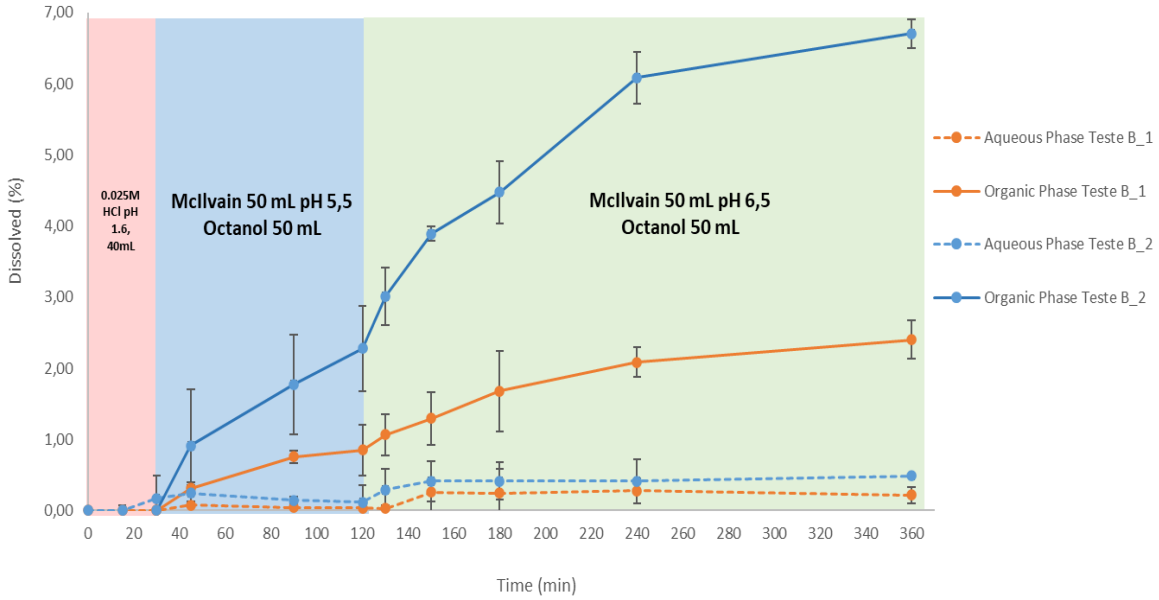

Figure 18: *Biphasic dissolution for two tests of drug B (n=6). Aqueous phase (40 mL in pH 1.6, 50 mL in pH 5.5 and 6.5) and organic phase (50 mL) in a pH profile from 1.6 to 6.5.*

The experiments in the 1 L (Figure 19) vessels allowed the volume to increase by 10 times and a higher contact area between phases (test 2).

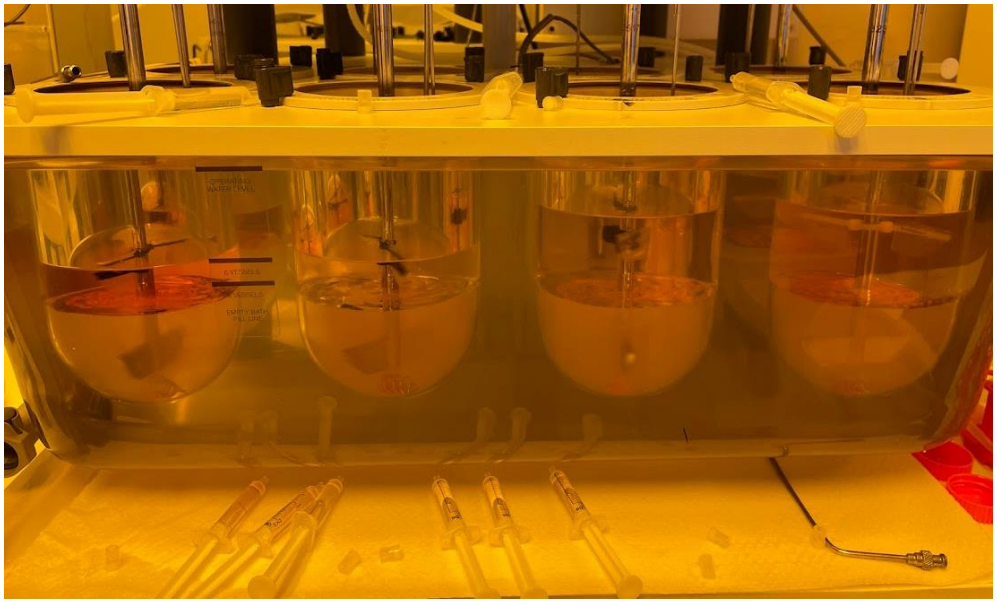

Figure 19: *Biphasic dissolution for tests 2 and 3. The first two vessels represent test 3 and the last two represent Test 2. Image taken after 45 minutes of analysis. They include 500 mL of aqueous phase and 200 mL (test 3)/500 mL (test 2) of organic phase.*

The reference product was also tested and the results are shown in Figure 20. The higher volumes allowed the increase in the dissolved percentages, thus obtaining a better analysis and higher confidence in the results. The variability of both the test and the reference product may be due to tablet disintegration in the first 30 minutes. To decrease the variability, sinkers were

used to avoid the tablets to stick to the bottom of the vessel. A tendency of the test batch to be above the reference product is seen.

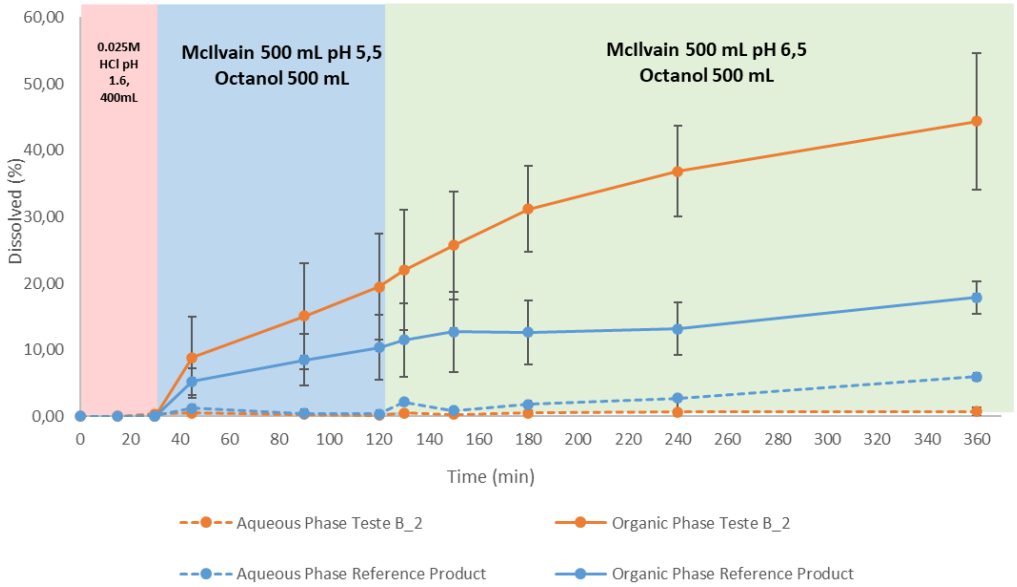

Figure 20: *Biphasic dissolution for Test B_2 (n=12) and Reference Product (n=6). Aqueous Phase (400 mL in pH 1.6 and 500 mL in pH 5.5 and 6.5) and organic phase (500 mL) in a pH profile from 1.6 to 6.5.*

To check on the impact of the organic phase in the permeation of model drug B, the amount of octanol was decreased (test 3). This analysis was only performed with the test product B_2 (Figure 21). Although there seems to be a slight tendency for the volume decrease to lead to a higher amount of drug dissolved, the results were not conclusive due to very high variability, as observed in the error bars.

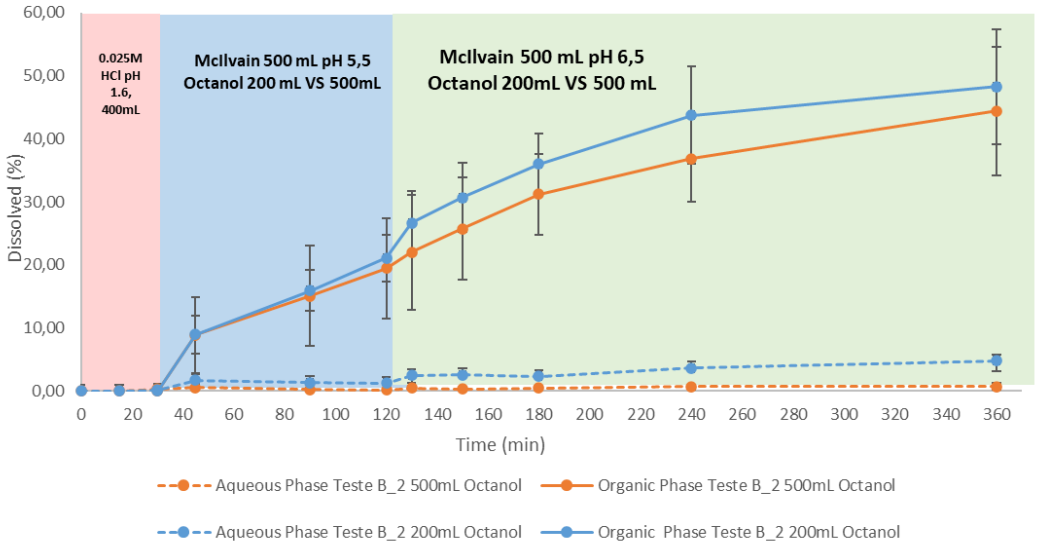

Figure 21: *Biphasic dissolution for TestB_2 (n=3) with different volumes at the organic phase (500 mL or 200 mL) in a pH profile from 1.6 to 6.5.*

Considering the results of test 2 in Figure 20, they were in line with the bioequivalence results already discussed in Chapter 2, both in terms of trend and variability. The Test-to-Reference GMRs for C_{max} and AUC were around 115%. Variability was observed also *in vivo* and was higher in the test than in the reference product. Despite the tendency and variability, this method does not reflect what was seen *in vivo,* since the differences were too large (contrary to what was observed in the clinic). This may be related to two causes. Model drug B is a substrate for BCRP (an efflux pump), which is not possible to mimic through the octanol phase. The efflux pump decreases the amount of drug that permeates *in vivo,* explaining the discrepancy between *in vitro* and *in vivo* results. Another reason for this discrepancy may be that the method still requires optimization.

Model drug E

The goal of test 1 with the dialysis membranes (Figure 22) was to add relevant information to the phase change dissolutions already described in Chapter 2, including permeation, relevant for a BCS class IV drug.

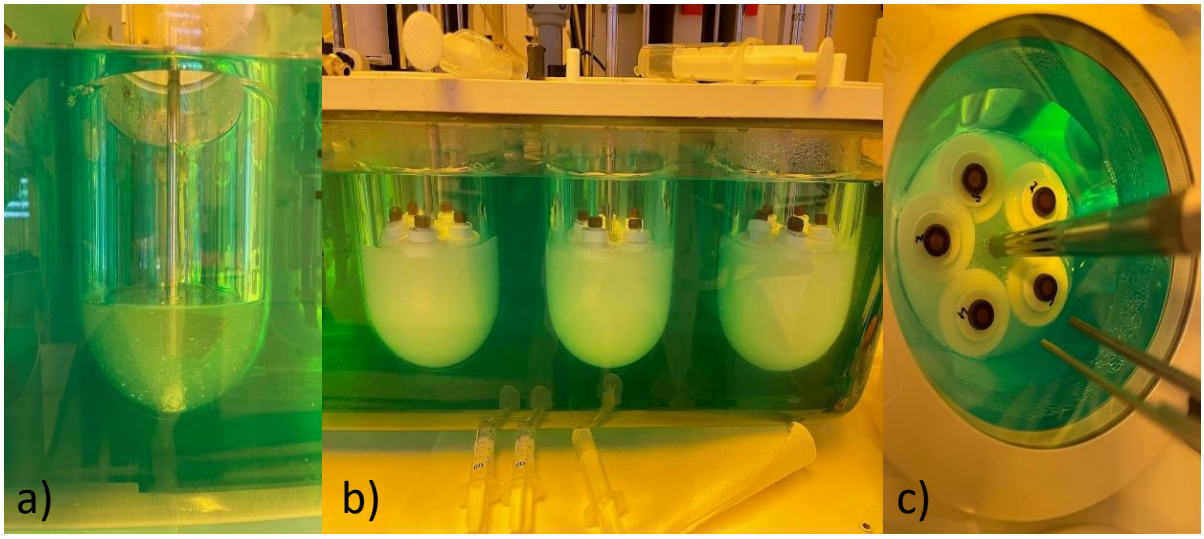

Figure 22: *Dissolution setup of Test 1; a) after 15 minutes of analysis (250 mL HCl pH 1.6); b) after 30 minutes of dissolution (500 mL of aqueous phase and five dialysis devices); c) the top at 30 minutes.*

However, no permeation of the drug into the dialysis membranes occurred, as observed in the following figure. This can be explained by the low solubility of this drug at pH 6.5, leading to precipitation. If the drug is not solubilized, it cannot permeate through the membrane pores. After the pH change, the dissolution medium in the vessel was extremely turbid, and a lot of precipitated powder at the bottom of the vessel was seen at the end of the experiment, justifying the obtained results.

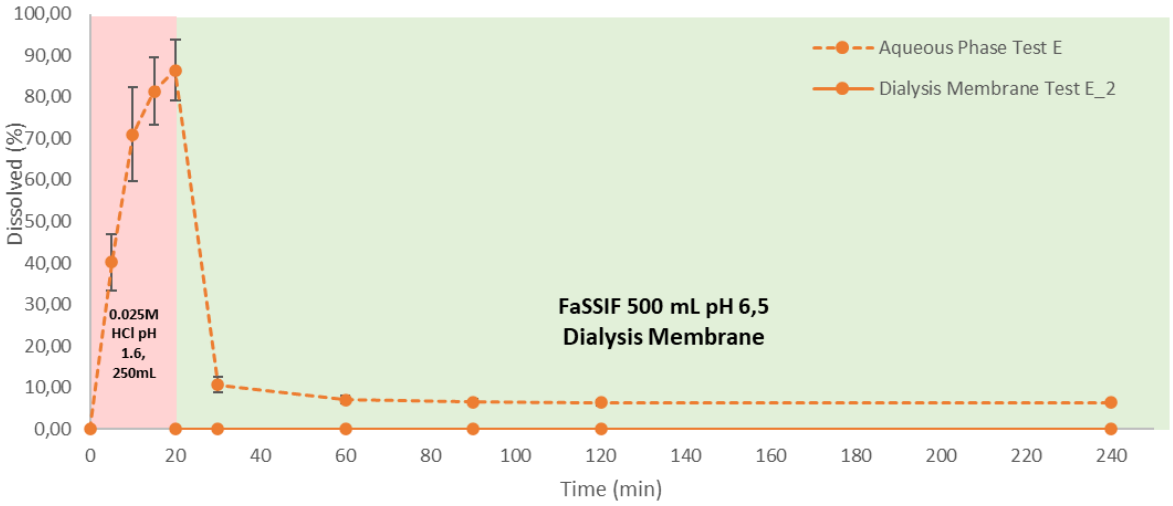

Figure 23: *Dissolution for Test E (n=6) with dialysis membranes added at 20 minutes.*

For this test, membranes with a pore size a hundred times the size of the molecule under study were chosen, to ensure that the pore size would not be a limiting factor. Even so, permeation was not observed and a different strategy was needed. A gradual pH change was applied to verify if drug precipitation in the intestinal environment could be at least partially avoided (test 2). In the aqueous acidic phase, only a confirmatory sample was collected at 20 minutes to ensure that the drug was dissolving as expected (Figure 24)

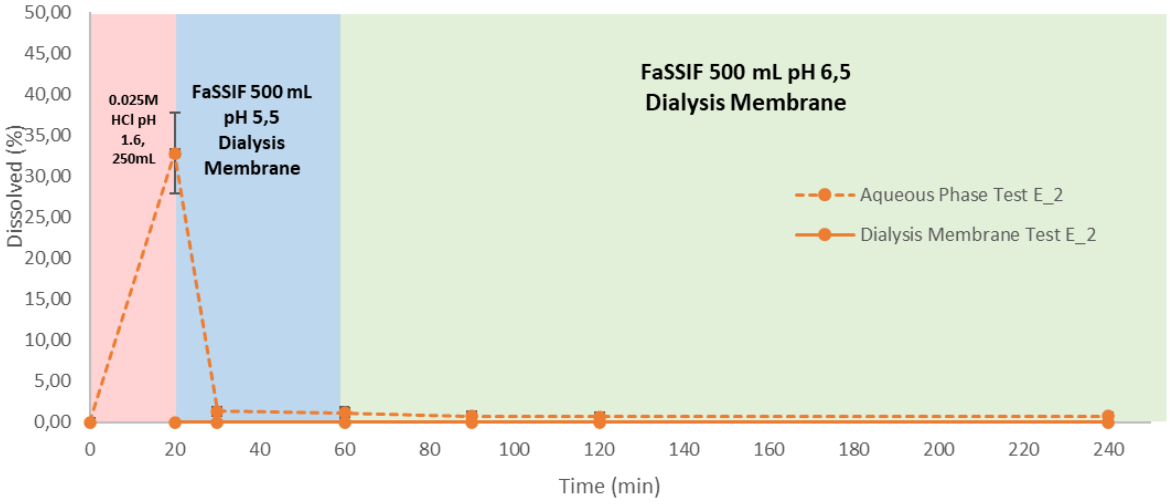

Figure 24: *Dissolution for Test E(n=6) with dialysis membrane added at 20 minutes and a gradual pH shift.*

Despite the gradual pH change, drug precipitation occurred again and no permeation through the dialysis membrane was obtained. The drug precipitation after the addition of the FaSSIF medium may influence its physical state, leading to aggregates that make permeation through the dialysis membrane impossible.

Other hypotheses for the absence of permeation were considered. The drug can interact with the membrane resulting in its retention on the outer side. Moreover, drug permeation may be dependent on the type of dialysis applied, normal or reverse, since the characteristics of the membrane material and its porosity may influence the access of the drug. In this case, reverse dialysis was applied.

Finally, these results may also be explained by the permeation rate, which may be much slower when compared to the dissolution rate. If this is the case, permeation was not evaluated at the most adequate time points. In future experiments, a model with normal dialysis or an increase in the experiment time may be tested.

Due to the reported difficulties, alternative approaches were then explored. An organic phase with octanol as a permeation indicator was tested (test 3, Figure 25). A gradual pH change was applied and several products were analyzed for comparison, including two batches of the reference product (Figure 26).

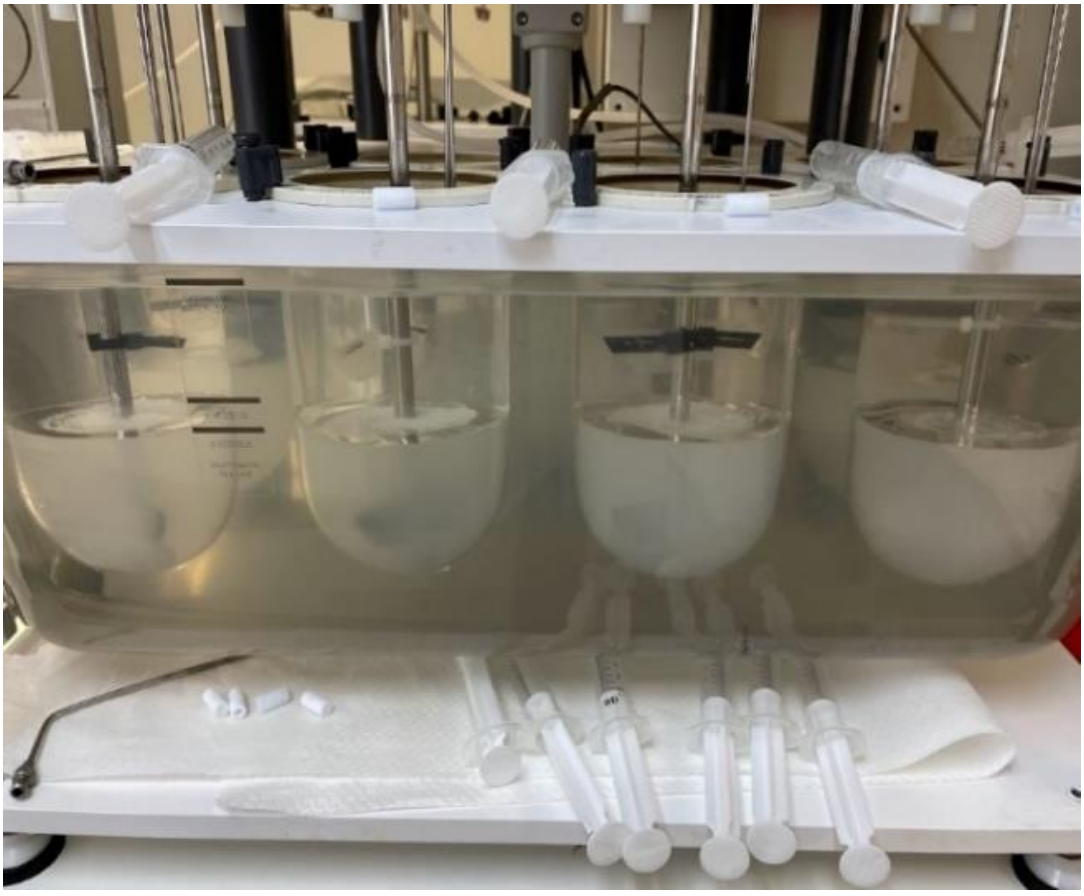

Figure 25: *Biphasic dissolution for Test 3 of drug E. Image taken after 30 minutes of analysis. It includes 500 mL of aqueous phase and 500 mL of octanol.*

The initial phase, in which the capsules are in the acidic medium of pH 1.6, was intended to simulate their behavior in the stomach under fasted conditions (the same state of the clinical trial). After 20 minutes, the expected time for gastric emptying to occur, a FaSSIF solution was added and the pH was adjusted to 5.5 to maintain the intestinal conditions. At this stage, octanol is added to promote permeation, and that is where differences start to become evident. Later at 90 minutes, the pH is again adjusted, to 6.5. The two reference products demonstrated similar behavior. On the contrary, the two test products showed to be quite distinct.

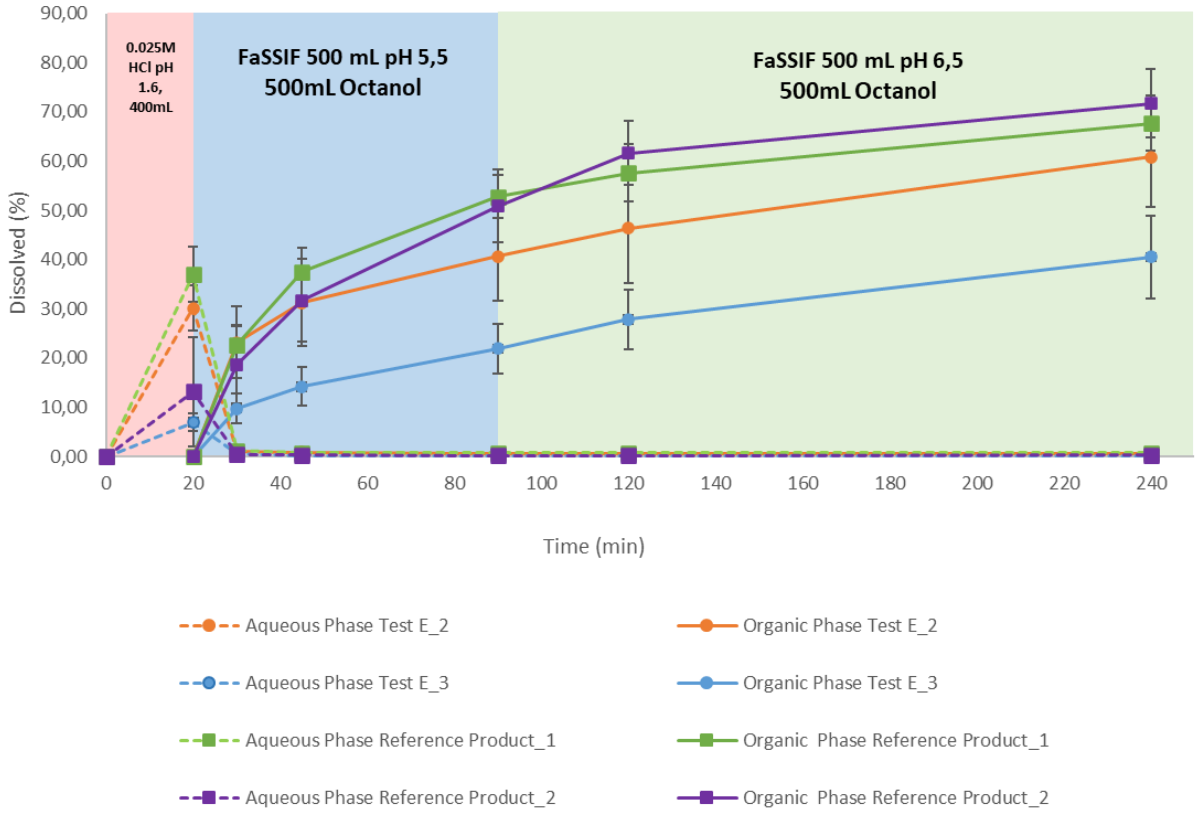

Figure 26: *Bifasic dissolution for Test E_2, Test E_3 (n=12) and reference products (n=6 each) with gradual pH shift. Aqueous phase (400 mL in pH 1.6 and 500 mL in FaSSIF) and organic phase (500 mL).*

Evaluating the acid phase, test product 2 and reference product 1 were very similar. As for test product 3 and reference product 2, they were also very close, although test product 3 had a lower dissolution at 20 minutes. The test_E_3 is an aged test product and was characterized to verify if the method could detect different performances. The lower dissolved percentage of test 3 was indicative of product degradation over time or capsule cross-linking. During dissolution, it was possible to observe the cross-linking phenomenon resulting from the interaction of the capsule gelatine with the formulation. This interaction hinders the solubilization of the capsule and the consequent dissolution of the formulation.

Test E_2 was tested *in vivo* with Reference Product 1 and similarity was not proved. The study was a single-center, single-dose, open-label, laboratory-blinded, randomized, two-sequence, two-treatment, two-period crossover pilot study in healthy 18 male and nonpregnant female volunteers under fasting conditions. The Test-to-Reference GMR obtained for C_{max} rounded 100%, but for the AUC was approximately 85%, failing to prove bioequivalence. Comparing the area under the dissolution curves of these products, a similar value to *in vivo* is obtained (about 85%). Therefore, this method seems to be biorelevant for the prediction of AUC.

Moreover, it is possible to observe in the graph an overlapping of the profiles of the reference product 1 and Test_2 at 30 minutes (about 25%), which may correspond to the C_{max} observed *in vivo* of about 100%. This method also showed the potential to be biopredictive for C_{max}.

An additional conclusion may be taken from these results. The variability of the reference is considerably smaller than the variability of the test product, reflected in the error bars. This is in line with what was observed *in vivo*, as the coefficient of variation of both AUC and C_{max} were higher in the test when compared to the reference product. The difference was around 10 - 20%. Despite the difficulties in permeation, this method seems to be able to mimic what happens *in vivo*.

To investigate the impact of volumes on the predictive power of the method, the previous setup was adapted for 10 times smaller volumes (test 4). The results are summarized in the following figure.

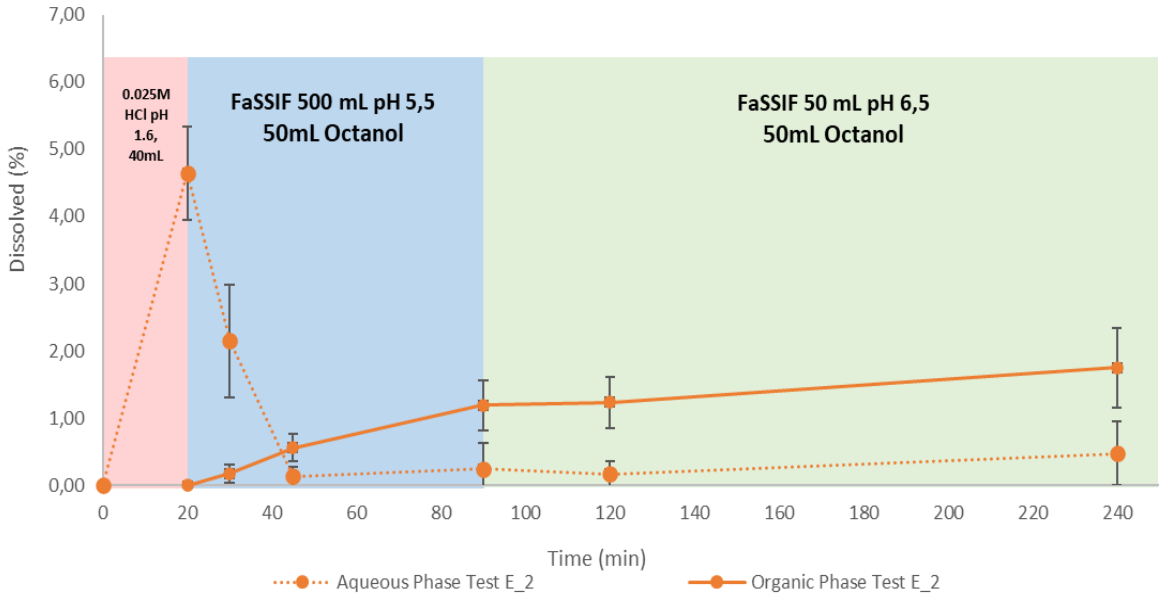

Figure 27: *Dissolution for Test E (n=3) with gradual pH shift. Aqueous phase (40 mL in pH 1.6 and 50 mL in FaSSIF) and organic phase (50 mL).*

The permeation rate was much lower than previously obtained as well as the dissolution in the aqueous phase. Due to the results obtained, the reference product was not tested in these conditions.

It is concluded that the surface area between the two phases is a factor to be taken into account when developing biorelevant methods for molecules with similar characteristics. Test 3 was then selected as the biomimetic dissolution setup for this drug product.

Model Drug F

After collecting evidence from the previous molecules, there was another challenging product that could benefit from biphasic dissolution. This drug product has been reformulated and tested in humans, but it continued with two major problems to solve.

No formulation seemed to be bioequivalent to the reference product, but most of all, there was no *in vitro* method that showed biopredictive power to support the product development team. Over the years of product development, more than 15 different setups were tested. In this case, the fed condition in the clinical study was considered the most challenging, and a new *in vitro* setup was pursued. The results are summarised in the Figure 28.

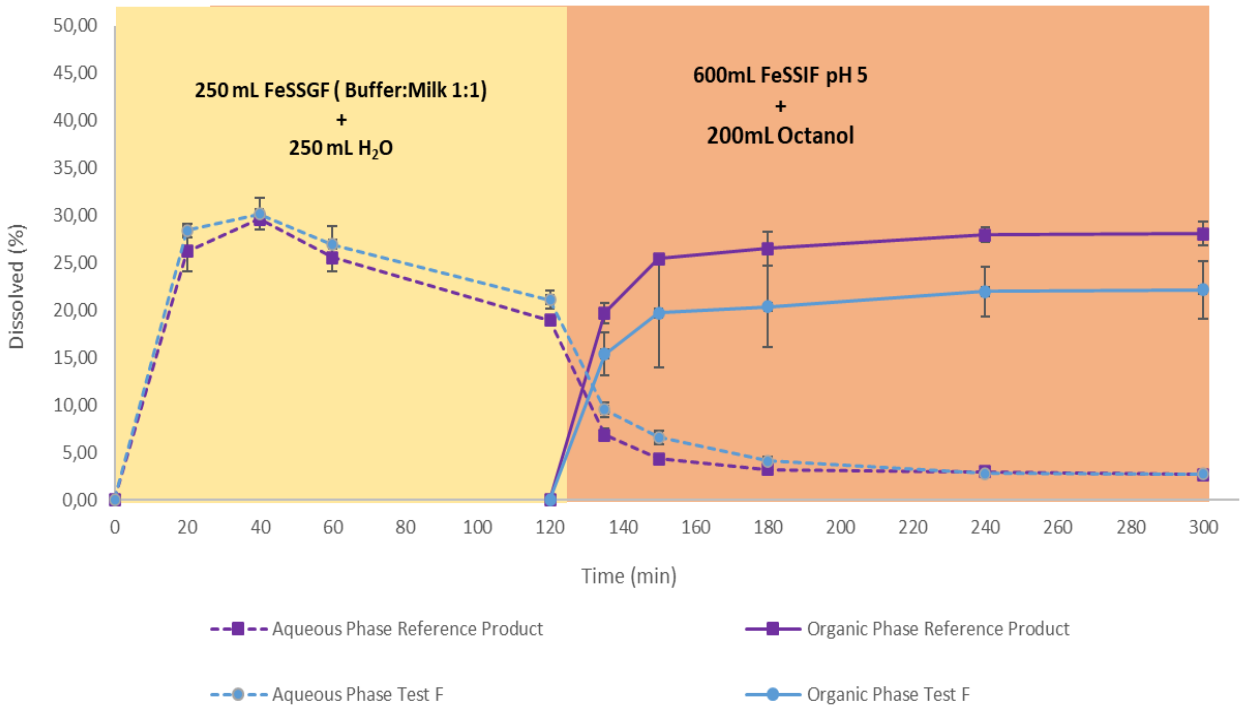

Figure 28: *Bifasic dissolution for Test F (n=3) and Reference Product (n=3) in fed state. Aqueous phase (500 mL in acid and 600 mL in FeSSIF) and organic phase (200 mL).*

Dissolution was initiated with FeSSGF at a 1:1 buffer:milk ratio (fresh milk), to simulate an intermediate fed state. Right at the beginning of the dissolution, 250 mL of purified water was added to simulate the volume ingested by the subject in the oral administration, as performed in clinical studies. The acid phase took 120 minutes to simulate the gastric environment after a meal. A FeSSIF solution was added to simulate the passage to the intestine, and octanol was to evaluate the permeation.

The QC method (pH 6.8) showed similarity between the test and the reference product. This similarity was also observed in the aqueous phase of the biphasic dissolution, but in the organic phase, relevant differences were observed. When gastric emptying occurs, the differences start to be noticed. The maximum dissolved percentages are also in line with the bioavailability described for this product.

The clinical study was a single-center, single-dose, open-label, laboratory-blinded, randomized, two-sequence, two-treatment, two-period crossover pilot study in 24 healthy male and nonpregnant female volunteers under fed conditions. The Test-to-Reference GMR for both AUC and C_{max} rounded 75%. Consequently, the 90% CI for the Test-to-Reference GMR of C_{max} and AUC_{0-t} were not within the 80.00% to 125.00% acceptance interval. The clinical study showed that after a single dose under fed conditions, the test product presented a lower absorption in comparison to the reference product.

The result of the clinical study is in line with the result of the octanol phase of the *in vitro* experiment. Permeation occurred to a greater extent in the reference than in the test product. Another interesting correlation was the variability. This parameter was more noticeable in the test product than in the reference product, which was also in line with the results obtained in the clinical study.

After the conclusions taken from the *in vitro* setup mimicking the fed state, this method could be considered promising for a strong biopredictive power. Further testing of product F with other prototypes should be performed to prove the biorelevance of the developed setup.

Model Drug G

In this case, the test product is orodispersible while the reference product is not. Therefore, any biorelevant method has to take this particularity into account. Accordingly, the samples of the test product were exposed to an initial treatment that simulates the disintegration of the tablet in the mouth. The samples were placed in a shaker with 10 mL of SSF for one minute simulating the interaction of saliva and the oral cavity (Figure 29).

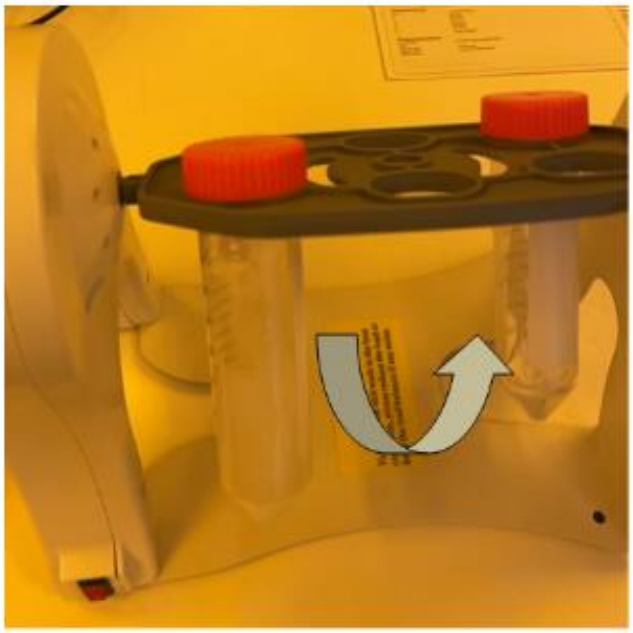

Figure 29: *Agitation of the product in 10 mL of SSF for 1 minute.*

The 10 mL of SSF led to the full disintegration of the orodispersible tablet, which was then placed in the dissolution vessel containing 500 mL of acidic medium mimicking the stomach. To avoid turbulence in the vessels, the permeation devices were already in place (but not submerged in the HCl, Figure 30 and Figure 31). Then, at 30 minutes, 500 mL of FaSSIF were added, simulating the passage into the intestinal environment and covering the permeation devices with PermeaPad® membranes (test 1).

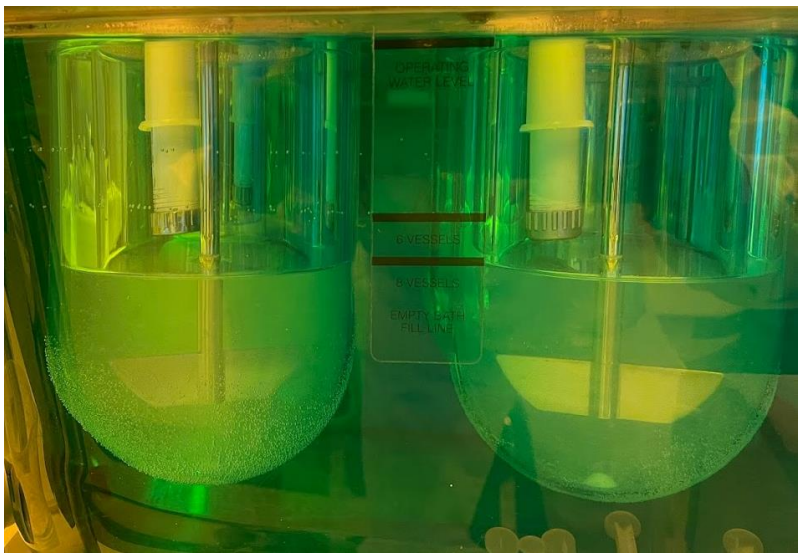

Figure 30: *Dissolution setup 1 at 20 minutes for drug G (500 mL HCl pH 1.6 and permeation devices with PBS).*

Figure 31: *Dissolution setup 1 for product G; a) the top of permeation devices; b) the devices c) image taken after 45 minutes from the beginning (1000 mL of FaSSIF).*

During the development of this product, dissolutions had already been made in the QC medium (pH 1.2), showing a slow release rate. This behavior was not in line with the reference product which had a full release in 5 minutes. The developed biorelevant setup ensured a very fast release as seen for the reference product (Figure 32). In the first 5 minutes, a 100% dissolved drug was obtained due to the total disintegration of the drug product in SSF. This is a good indicator of the performance of the method and also of the future performance of the product *in vivo*.

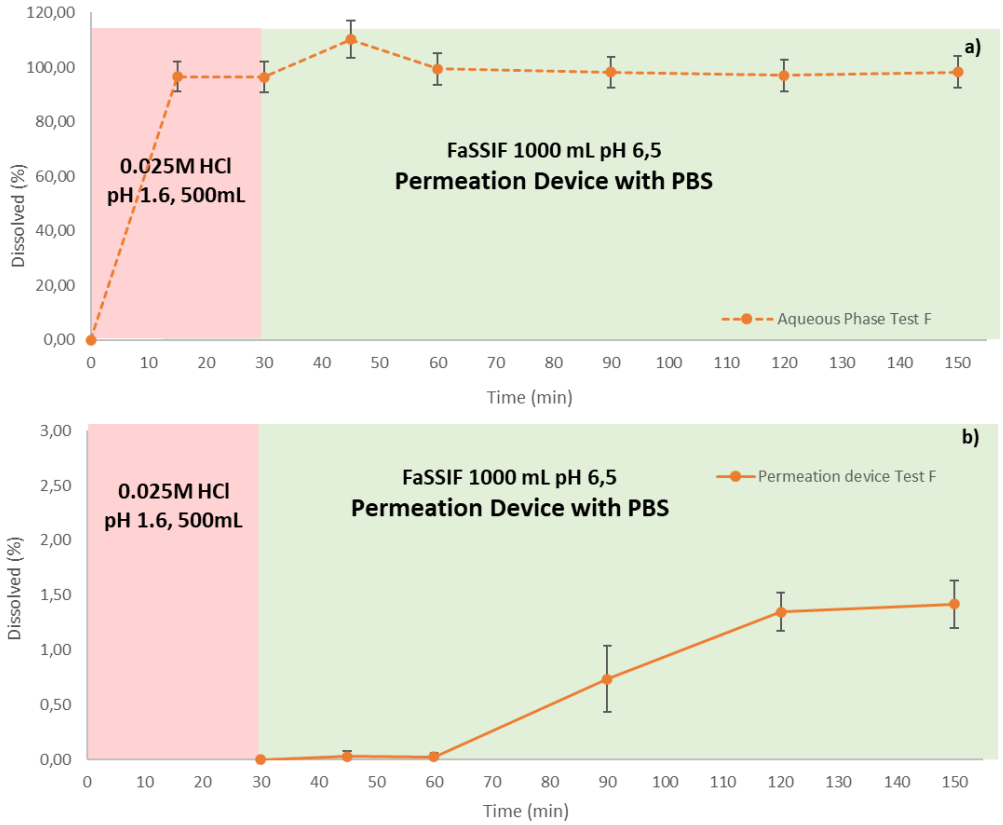

Figure 32: *Biorelevant dissolution for product G (n=6); a) dissolution profile in the aqueous medium; b) permeation rate in the permeation device with PBS.*

As seen in the aqueous phase profile, there was an abnormal increase in the dissolved percentage at 45 minutes. This time point occurs after the addition of FaSSIF at 30 minutes (first collection). Several possibilities for this occurrence were considered, among them the possible interference of the components of the medium in the chromatographic method. However, the peak obtained in the chromatogram was pure. It was then realized that this interference was due to the use of the same filter in both the 30 and the 45 minutes time points.

As the concentration in the solution decreases drastically to half, it would be necessary to change filters at this point to avoid contamination by previous residues in the syringe filter. Therefore, in future experiments of this type, both the cannula and syringe filters need to be changed.

The permeation that occurred was quite low. Although the bioavailability of the reference product is low, such low values were not expected. These values suggest that the method should be optimized. The diameter of the membranes may be a limiting factor in permeation rate as was the contact surface in permeation in octanol dissolutions. To test this hypothesis, different membranes should be tested in the same setup. Moreover, there was no constant

agitation within the device, which may also affect permeation. This method is still to be optimized, and alternative approaches are needed. However, the aqueous phase already provided a lot of useful information about the product behavior.

A new experiment to improve permeation was performed, where the permeation system was inverted (test 2). This drug has a high volume of distribution, and the volume of 25 mL of PBS in test 1 might not have been enough. The system was then inverted, using 1000 mL of PBS in the dissolution vessel, while the permeation setup had the 25 mL of the dissolution solution with a dissolved amount of approximately 100%.

Initially, the test product suffered the initial treatment in SSF simulating its disintegration in the mouth, and the resulting solution was inserted in a vessel simulating the gastric environment in the fasted state (pH 1.6). The reference product was placed directly into the vessel and both were subjected to a phase transition simulating gastric emptying to the intestine. The results obtained are shown in Figure 33.

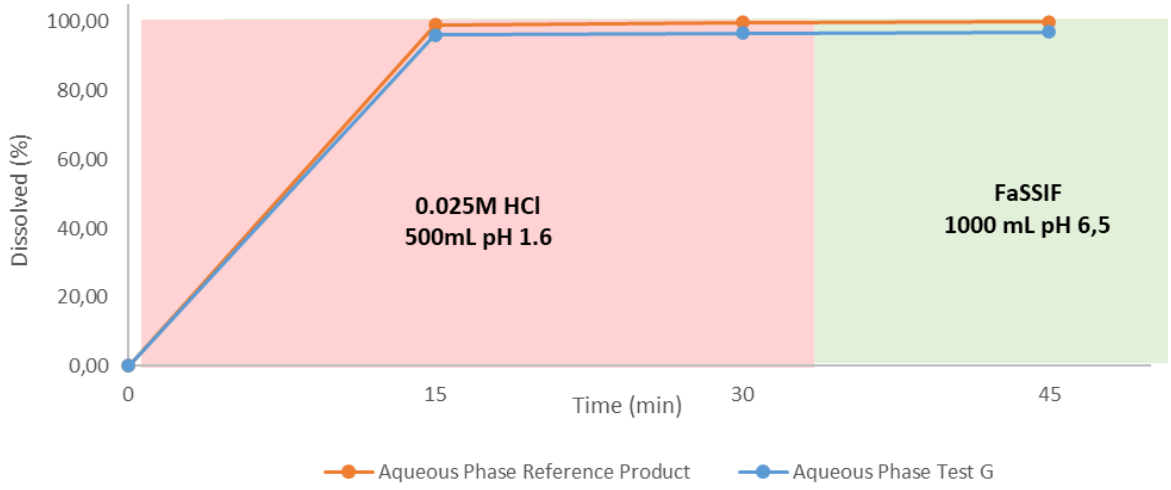

Figure 33: *Dissolution for product G (n=6) and reference product (n=6) with a pH transition.*

After 45 minutes, 25 mL of each vessel were collected and placed inside the permeation device with the incorporated membrane already adapted to the vessel. The vessel contained 1000 mL of PBS and the dissolution was initiated with the collection of samples at 15 minutes. The results for this part of the assay are shown in Figure 34.

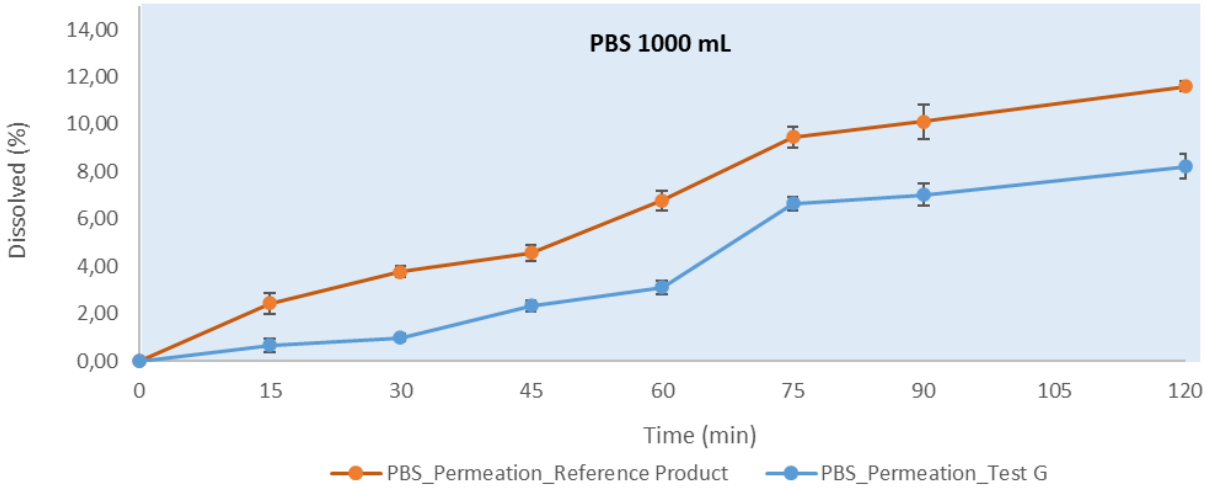

Figure 34: *Biorelevant dissolution for product G (n=6) and reference product (n=6) with a permeation device into a vessel with 1000 mL of PBS.*

Permeation is indeed the limiting factor for BCS III molecules, in line with the reported 15% of the bioavailability of this drug. The maximum results obtained (8% for the test product and 12% for the reference product) are in line with this value. The samples taken from the permeation device at the end of the analysis as a control had an average of 90% for the test product and 86% for the innovator, which supports the permeation values obtained. Moreover, there was a tendency for the reference product to permeate more than the test product, something to take into consideration during product development.

No clinical results are known at the date of this work. However, this setup is supporting the prediction of the behavior *in vivo* of the reference product as well as the different prototypes of the test product under development. Clinical data, as soon as available, will allow the optimization of the setup parameters to improve the biopredictive power. For now, this method is serving as a guide in the development of the final formulation.

3.4. Conclusion

All the results presented show that the selection of the dissolution medium and specific setup is very important in the development of this type of method. It is essential to take into account the pH ranges studied, the concentration of the media, and also the composition of each phase. It is also required to understand the clinical study that is foreseen for the product, for instance, fasted or fed conditions so that the entire strategy is designed with the correct goal. Then volumes, pH, dissolution medium composition, analysis times, and critical time points are selected to obtain the most biorelevant method possible.

Despite the different characteristics of all the molecules studied, the model that showed more reproducible and more similar results to those obtained *in vivo* was the octanol design. For molecules B, E, and F, correlations between test and reference products similar to the results obtained *in vivo* were obtained.

For poorly soluble drugs, the developed setups are very relevant for the project, since the solubility in biorelevant conditions can be predicted, and the organic phase allows the drug to permeate over time, allowing the study of permeation as well.

The tests with the dialysis membranes need further optimization and probably a different strategy, which may involve changing to normal dialysis, testing another type of membrane, or even prolonging the experiment to understand better the permeation rate. The same happened with the setup with the Permeapad® membranes. The low permeation obtained does not allow taking conclusive results, but it is a promising technique that may support several formulations as long as it is adjusted to the characteristics of the products. It was possible to observe a tendency with these methods that, after optimization, may lead to a stronger correlation between the products that may be tested.

For BCS Class II and IV, whose permeation is critical, models like the ones discussed may be vital for successful bioequivalences. Even ensuring a similar behaviour during the gastric and intestinal phases, differences may arise during drug permeation. Despite all the opportunities for improvement discussed, these models are an asset in the development of oral solids. They show unequivocally that it is not enough to assess precipitation and solubility in the stomach and intestine. They enable a global evaluation of the drug's behaviour and allow conclusions to be drawn which would not be possible otherwise.

Thorough knowledge of the chemical properties of the API and excipients is essential for the correct application of these models. They allow also the identification of key parameters that are likely to influence the performance of the final drug product and improved know-how on the formulation and process, as well as greater confidence in the prototypes selected for clinical studies.

The knowledge collected during the execution of this work allowed the design of a general biomimetic model to be applied to different products with different properties. The main difference from previous models is that steps were added to ultimately study the behavior of the drug along the largest possible portion of the gastrointestinal tract.

In this method, most parts of the GIT that influence the release and absorption of the drug are intended to be included. The interference of several variables in the same test can be evaluated, reducing variability and increasing the confidence in the results. The whole model should be connected to a temperature control system, and the pH should be tightly controlled as well as the desired concentration by adjusting them at each step of the process. Samples from each of the simulators can be collected, as represented in Figure 35.

The following phases were considered in the model, due to the influence on the bioavailability of drugs: mouth/oesophagus, stomach, and Intestine (duodenum and jejunum).

Mouth and oesophagus

The first part of the GIT that contacts the oral solid matter is the mouth. To mimic the disintegration and initial digestion in the mouth, different approaches can be considered according to the pharmaceutical form. If it is a buccal dosage form, initial treatment with SSF is relevant. They are solid pharmaceutical forms that disintegrate rapidly in the oral cavity and that may be swallowed without the need for concomitant administration of water. In this case, they can be evaluated in small volumes of SSF for a short period, aided with agitation to simulate tongue movement. This procedure may be applied to oral dispersible films, or powders for oral administration.

In the case of standard oral administration (like in capsules or tablets), they can be introduced into the dissolution system along with 250 mL of water by a 20 to 25 cm tube simulating the oesophagus, connected to a peristaltic pump for flux control. At this stage, the SSF may be complemented by adding buccal enzymes through secretory chambers associated with the device that simulates the mouth (e.g, drop counting device).

Several parameters may be modulated, such as the pH, the buffer capacity, the viscosity, and the flow rate of this setup. The tube content is then transferred to a container (dissolution

vessel) that simulates the desired gastric conditions. For standard oral dosage forms (tablets or capsules), the passage through the mouth is very short and the test may be simplified by starting with 250 mL of water added to the dissolution medium to simulate the gastric environment.

Stomach

This stage is critical to assessing solubility and subsequent precipitation after gastric emptying. The first main decision is the conditions to mimic, which may be fed or fasted. The volume and composition of the dissolution medium should be adjusted accordingly. For instance, in the fed state, the dissolution time is adjusted to the longer gastric emptying time (100 - 120 minutes), and volumes are adjusted to what is described in the literature for the post-meal phases (500-900 mL). For the fasting state, the dissolution time is shortened (about 20 minutes) to be in accordance with gastric emptying, as well as the composition and pH that also requires adjustments.

Visual observation of this stage is extremely important as it helps to understand the results that will be obtained. Understanding how the capsule or tablets coating behave helps in formulation decisions such as the selection core composition, the percentage of coating, or the type of capsule to be applied in the final formulation.

Once again, a reservoir that simulates gastric secretions may be attached to this phase. The solutions resulting from this phase are then transported to another dissolution vessel using a peristaltic pump with a predetermined flow simulating gastric emptying.

Intestine (duodenum and jejunum)

The dissolution vessels into which the previously prepared solutions are transferred contain a medium that simulates intestinal fluids. A gradual pH change may be performed to ensure the evaluation of both the duodenum and jejunum. The conditions of the medium should also be adjusted according to the feeding state, which includes the time of dissolution. A third secretory chamber may be attached to release intestinal enzymes over time. The media described in Table 5 may be applied, or the McIlvaine buffer, adjusting the concentration and composition of the surfactants. Other dissolution media may be considered, developed with the specific characteristics intended to be studied.

If permeation is not needed, a simple phase change may be performed. This approach is useful for BCS I molecules, whose permeation is not a limiting factor. Apart from these molecules,

this shorter method can be considered for molecules whose uptake implies active transport, as a correct simulation *in vitro* may be too complex or even not possible. When permeation is to be assessed *in vitro*, different options may be considered, such as the addition of octanol or another organic layer, the insertion of dialysis bags, or incorporating permeation devices with PermeaPad®, Caco-2 membranes, or others.

The methodology should have the physicochemical characteristics of the molecule into consideration. In case of a very high molecular weight, dialysis bags and membrane setups are not recommended due to limiting pore size. For BCS II or IV drugs, octanol seems to be one of the most promising methods and should be considered first-line, due to solubility constraints. However, any BCS molecule can be studied by this setup. For BCS I or III drugs, the dialysis membranes or the devices with Permeapad® or Caco-2 may be considered.

The application of this model offers several advantages compared to other systems already on the market, which intend to simulate dissolution/permeation, but are complex and very expensive (some of them briefly explained in the last chapters). The goal of this biomimetic setup is to have a reliable method, easy to reproduce in the laboratory with relatively low costs. The use of compendial dissolution vessels and common peristaltic pumps allows this setup to be associated with any dissolution equipment with only a few changes, like a couple of peristaltic pumps, and vessels of different volumes and characteristics. It is an open system that allows aliquots to be collected at different stages of the process, enabling quantification throughout the entire simulated GIT. The addition of secretory chambers also allows the simulation of a number of GIT diseases, such as the lack of production of a certain enzyme.

Besides the evaluation of solubility, precipitation, and permeation along the GIT, this model may have several applications once validated. By changing parameters, reflux situations may be simulated (by changing the orientation of the peristaltic pumps), but also the effect of certain foods or diets (by changing the composition of the chosen dissolution media), the effect of the deficiency or excess production of various enzymes, or the increase or decrease in the transit times. All the changes introduced in the parameters must be supported in the most up-to-date literature, and require an in-depth study of the more adequate composition of the fluids to be applied in each situation.

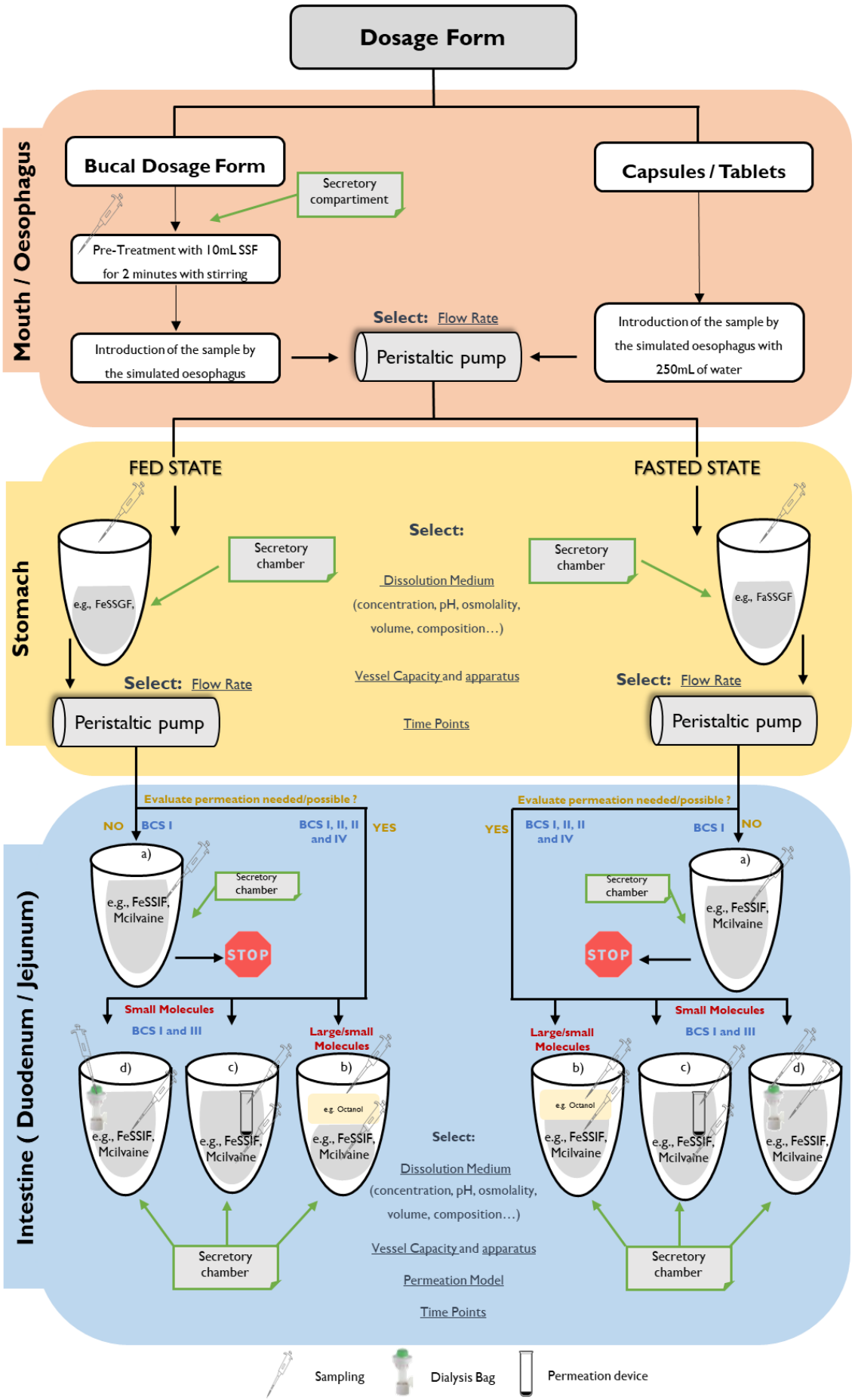

Figure 35: *Theoretical concept of a new biomimetic method; in the intestinal phase, a) pH change setup, b) biphasic dissolution with organic and aqueous phase, c) permeation device adapted to different membranes, d) dialysis bag.*

The development of oral solid forms tends to be more challenging as the new molecules are more lipophilic. BCS Class II and IV molecules were the focus of this work. In this type of drugs, the bioprediction is challenging and release methods are not enough to understand the behaviour of these formulations *in vivo*.

This work showed the importance of biorelevant methods to integrate the development of oral solid formulations. Molecules whose permeability and solubility become limiting factors in their performance require analytical characterization beyond release methods. These biorelevant methods intend to simulate the entire GIT by subjecting the drug to environments similar to *in vivo*. Aqueous buffers are usually not enough to predict the performance of the drug *in vivo*, and the study of biorelevant dissolution media with compositions similar to oral and gastrointestinal fluids is required.

Besides a correct selection of biorelevant media, in more challenging cases a whole setup should be designed, to translate as much as possible to *in vivo* events: gastric emptying times, simulation of fed state or fasted state, volumes of each phase, and permeation conditions. The evaluation of solubilization by itself helps to understand the performance of the drug, but the combination with techniques that estimate, among others, supersaturation and permeability, boosts the predictive power of these analytical methods. It is therefore essential to apply biorelevant methods during product development.

The results of this study demonstrate that it is possible to correlate *in vivo* data with the results of biorelevant methods, which will always have to be confirmed with further product testing or be supported by IVIVC. These methods were able to differentiate different formulations of the same product, showing trends and even some very close correlations with the main parameters evaluated in the clinical studies, such as C_{max} and AUC.

Although these setups were mostly tested on late-stage formulations, the highest value may be provided if applied in the early screening phase, where they can support the selection of excipients, compression forces, or even eliminate doomed formulations at the early stages of development. Biorelevant setups are therefore an essential tool to identify problems in premature development and to provide assurance in the final prototype. These methods can discriminate and eliminate, along with other analyses, formulations that would possibly fail *in*

vivo. This avoids subjecting individuals to clinical trials and provides enormous financial savings for companies. By combining these methods with other *in silico* techniques and the knowledge of formulation and analytical teams, it is possible to guide the development of oral solids to increase success in clinical studies. With these approaches, a decrease in the number of clinical trials may even be achieved as well.

The work presented in this thesis is an initial phase of a more complete project, due to the complexity of this theme. It aimed to prove that these methods are an advantage for several products with distinct characteristics, real industry case studies, but leaves much to be investigated.

The next steps of this work intend to optimize the permeation methods to obtain more biorelevant results. It is intended to test new dialysis membranes with other characteristics, cell membrane models, and alternative artificial membranes, with different diameters and compositions. It is essential to investigate and optimize the issues identified as the limiting steps of the discussed methods to obtain a better correlation with *in vivo* data.

The quantification of the samples also needs to be improved, to be able to quantify directly without dilutions and avoid human errors. The development of normal phase HPLC methods would have added value. Optimized HPLC methods would speed up the whole development process of the biorelevant method, increasing the speed and the accuracy of the results, which would then be translated into immediate benefits for the product development.

The biomimetic method proposed in Chapter IV should be tested for a wide range of molecules and characteristics, testing several *in vivo* variables in a single experiment. This will allow not only in-depth knowledge of the behaviour of the drug product throughout the entire GIT simulated *in vitro* but also reduce the number of tests required for each formulation prototype.

Moreover, the data obtained with these methods may also serve as a basis for mathematical modeling and *in silico* experiments. This will strengthen the correlations, making the conclusions more robust and generating new data to be applied in new biorelevant models. The goal is common to all these methodologies, guide the development of the pharmaceutical product from the very early stage until it finally reaches the market.

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