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Treball Final de Grau

Determination of parameters of pharmaceutical interest in biological media

Determinació de paràmetres d'interès farmacèutic en medis Biològics

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Gener 2019

Universitat de Barcelona

B:KC Barcelona Knowledge Campus
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Vull dedicar agraïments

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REPORT

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1. SUMMARY

Prior to the introduction to the market of a pharmaceutical product, there is a whole process called drug discovery. This development includes four phases: i) R&D, ii) preclinical studies, iii) clinical studies in humans, iv) approval by competent organisms such as the European Medicines Agency (EMA) or the American Food and Drug Administration (FDA). During the R&D and pre-clinical studies different parameters are evaluated such as the absorption, distribution, metabolization and excretion of the drugs, the potential benefits and the mechanism of action, the best form and quantity of dosage, the adverse effects (AEs), the interaction with other co-administered substances, and the effectiveness of treatment. Due to ethically questionable experiments, the use of laboratory animals is currently reduced, therefore, predictive techniques as similar as possible to *in vivo* studies must be developed. A strategy to simulate the behaviour in animals and/or humans is to perform studies of solubility, protein interaction, etc. using media that resemble the best to fluids of living beings. Many studies reported in the scientific literature have been carried out using water as medium or simple buffers. Nowadays, mediums with compositions much more complex and much more similar to biological fluids such as blood or gastrointestinal fluids are being developed. In this work, a search about which biological media can be acquired from the different suppliers, their preparation and the possible interaction with the drugs will be carried out.

Keywords: Pre-clinical studies, Drugs, Biorelevant media, ADMET properties.

2. RESUM

Prèviament a la sortida al mercat dels fàrmacs existeix tot un procés anomenat *drug discovery*. Aquest desenvolupament inclou quatre fases: i) I+D, ii) estudis pre-clínics, iii) estudis clínics en humans, iv) aprovació per part dels organismes competents com ara l'Agència Europea del Medicament (EMA) o l'Agència Americana dels Aliments i dels Medicaments (FDA). Durant els estudis de I+D i pre-clínics s'avaluen diferents paràmetres com ara l'absorció, distribució, metabolització i excreció dels medicaments, els beneficis potencials i el mecanisme d'acció, la millor forma i quantitat de dosificació, els efectes adversos (AEs), l'interacció amb altres substàncies coadministrades i l'efectivitat del tractament. Degut a experiments èticament qüestionables, actualment es redueix l'ús d'animals de laboratori, per tant, s'han de desenvolupar tècniques predictives el més similars possibles als estudis *in vivo*. Una estratègia per simular el comportament en animals i/o humans és realitzar els estudis de solubilitat, interacció amb proteïnes, etc. fent servir medis que s'assemblin el màxim possible als fluids dels éssers vius. Molts estudis recollits a la bibliografia científica s'han portat a terme fent servir com a medi l'aigua o solucions tampó simples. Avui en dia, medis amb composicions molt més complexes i molt més similars als fluids biològics com ara la sang o els fluids gastrointestinals es troben en desenvolupament. En aquest treball es realitzarà una cerca sobre quins medis biològics es poden adquirir dels diferents proveïdors, la seva preparació i la possible interacció amb els fàrmacs.

Paraules clau: Estudis pre-clínics, Medicaments, Fluids biològics simulats, propietats ADMET.

3. INTRODUCTION

3.1. DRUG DEVELOPMENT

Developing a novel drug is an interdisciplinary process involving a multitude of competences from biologists, chemists, computer scientists, medical staff, statisticians, and regulatory experts. Taking a compound from bench to bedside requires up to 12 years at an average estimated cost exceeding 883M €, mostly for candidates that fail along the way[1].

According to the American Food and Drug Administration (FDA)[2], the drug development process consists of four steps: discovery, preclinical research, clinical studies (including post-marketing safety monitoring) and regulatory approval (Figure 1).

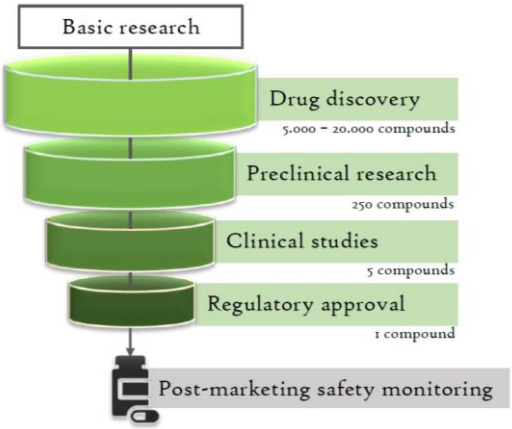

Figure 1. Drug development process scheme

3.1.1. Drug discovery

Target identification and validation

The first step in the development of a novel drug is to identify and validate a good target, which may be for example organs, proteins or genes. A good target needs to be efficacious, safe, meet clinical and commercial needs and be accessible by the drug molecule[3].

Hit identification and validation

Initial identification of compound candidates can come from different sources. Research can provide new knowledge into disease processes and discover new pathways for which drugs can be developed to intervene. Alternatively, companies conduct large scale trial and error-based programs to identify molecular compounds that may be of interest. This step identifies whether the molecule hits have the desired effect against the identified targets[4].

Lead identification and optimization

After several hit compounds are established, the process continues with the optimization of their pharmacodynamic and pharmacokinetic behaviour to make a lead candidate. Once these are selected, their optimization is aimed to maintain the desired properties while improving on possible deficiencies of their structures, with a view to produce a preclinical drug candidate.

This stage can be used to find out whether the drug metabolizes in the right area of the body, or whether there are any side effects that are cause for concern. For this process, the combination of specialists in computational chemistry, medical chemistry, drug metabolism, and other areas can provide unique insights[4].

Late lead identification and optimization

Before progression to preclinical tests, further pharmacological safety of a lead compound is assessed. If this stage is overlooked, problems in efficacy, pharmacokinetics, and safety are more likely to occur later in drug development[4].

3.1.2. Preclinical research

The process of developing a novel drug is time consuming and costly. To increase the chances of successfully completing a clinical trial leading to the approval of a new drug, the choice of appropriate preclinical models is essential[1].

Thousands of modifications of these lead compounds are synthesised and tested during preclinical activities. Once an optimised compound is identified, this investigational new drug (IND) becomes a candidate for clinical trials involving human subjects[1].

Nevertheless, merely a small fraction of INDs tested in clinical trials ends as a marketed product. Hence, there is a need for optimising current standard preclinical approaches to better

mimic the complexity of human disease mechanisms[1] and understand the translation from preclinical to clinical results[5].

Preclinical studies must comply with the guidelines dictated by Good Laboratory Practice (GLP) to ensure reliable results and are required by authorities such as the European Medicines Agency (EMA) [6] or the FDA before filing for approval as IND. Insights into the compound's dosing and toxicity levels are essential to determine whether it is justified and reasonably safe to proceed with clinical studies. In conclusion, identifying a safe, potent, and efficacious drug requires preclinical testing, which evaluates aspects of pharmacodynamics and pharmacokinetics[1].

Pharmacodynamics

Pharmacodynamics is the study of the effects of drugs on the body, including the mechanisms of drug action and the relationship between the concentrations of a drug at the site of action and its response. The interaction of a drug with specific receptors initiates a cascade of molecular, biochemical and physiologic events that trigger a pharmacodynamic response. Factors that influence the circulating drug concentration over time in the body are expected to influence the onset, duration, and intensity of the pharmacodynamic effect[7].

During drug discovery and development, pharmacodynamic studies assess the potency and efficacy of drug candidates, including safety aspects and adverse effects (AEs). Therefore, pharmacodynamics establishes the therapeutic index of a drug, describing the ratio of the dose causing toxicity and the dose provoking a therapeutic effect[1 7].

Pharmacokinetics

The essence of pharmacology is the relationship between the dose of a drug given to a patient and the resulting change in physiological state. Then, the concentration or dose of drug is the independent variable and the pharmacological effect returned by the therapeutic system is the dependent variable. The value of the dependent variable has meaning only if the value of the independent variable is correctly known[8].

Pharmacokinetics helps to determine the true value of the independent variable, seeking to answer where in the body does the drug go, how much of the drug reaches the target organ and how long does the drug stay in the body. Drugs can be effective only if enough amount is present at the target site, and they can be harmful if enough amount is present to produce toxic side effects[8].

Therefore, as a prerequisite to pharmacodynamics, pharmacokinetics examines the journey of drugs into the body toward their intended therapeutic target organ. A useful acronym to describe pharmacokinetics is ADMET (absorption, distribution, metabolization, elimination and toxicology). This generally describes the process of drug absorption into the body, distribution throughout the body, metabolism by enzymes in the body, elimination from the body and drug toxicity resulting from this process. It is useful to consider each of these steps, as together they summarize pharmacokinetics[8].

Pharmacokinetics studies are performed both during the drug discovery process and the preclinical studies. To obtain relevant results from preclinical studies are required appropriate preclinical models that are as comparable to the target population as possible. Typically, this involves a series of experiments using *in vitro* or *ex vivo*, *in vivo* and, more recently, also *in silico* models.

In vitro or ex vivo models

In vitro and *ex vivo* studies are a relatively fast, simple, and cost-efficient way of preclinical testing. A clear frontier between their definitions has not been agreed among the scientific literature, but the main difference relies on how directly the sample comes from a living organism and how long it can be functionally maintained outside it.

More accurately, *in vitro* studies include the experimentation on biorelevant simulated media, on single cell populations derived from an animal, but cultured and treated in artificial medium, and on cell components such as proteins or other biological macromolecules. The final requirements of *in vitro* testing system are self-sustainability and not limitation to time.

Ex vivo studies, otherwise, utilise cell, tissue and organ cultures taken out and maintained and treated outside of the animal body for short period of times. In most of the cases *ex vivo* experiments also use artificial media[9]. *Ex vivo* studies permit tight control and monitoring of experimental settings and often provide mechanistic evidence for the investigational compound's mode of action[1].

Nevertheless, most of the publications used in this work do not make a differentiation between these two models. Hence, this work comprehends *in vitro* and *ex vivo* models in the same *in vitro* category.

In vivo models

In vivo studies consider the complete organism utilising various animal models. Like studies in humans, animal testing is tightly regulated in most countries and permission from local ethical review boards is required. Naturally, controlling experimental settings is far more complicated for *in vivo* studies and, due to the complexity of the living organism, compounds may behave differently from what is expected based on results obtained in a test tube[1].

Typically, *in vivo* studies are performed in a rodent (e.g., mouse, guinea pig, hamster) and non-rodent model to comply with regulatory agencies (EMA or FDA) requirements. Mice, rats, and dogs are among the most frequently used animal models while testing in primates (e.g., monkeys, apes, etc.) is performed occasionally and typically for larger molecules[1].

In silico models

Progress in bioinformatics over the past decades has made *in silico* studies attractive so that they often precede or complement *in vitro* and *in vivo* studies. *In silico* models are based on computer simulations and provide information on how an investigational compound might behave in subsequent experiments. Apart from technological requirements, these computer simulations demand expert knowledge in biochemistry and molecular biology[1].

Merely between one or two out of five INDs tested in clinical trials eventually gains approval for clinical use. Nevertheless, preclinical research is indispensable to protect human subjects in clinical trials and an adequate and careful choice of model systems is vital[1].

3.1.3. Clinical studies

Clinical trials are conducted over different phases (Phase I-IV), starting from a small number of subjects and extending to large cohorts. Phase IV in clinical research is not included in this section, because it takes place after the regulatory review and approval, but in an independent one: *post-marketing safety monitoring*.

Phase I

The IND is administered to humans for the first time. In early studies, a small group of subjects, usually 10 to 15 individuals, receive a single, sub-therapeutic dose to obtain pharmacokinetic information without inducing pharmacological effects. The goal of these

exploratory studies is to investigate whether the drug candidate performs as expected based on preclinical studies.

If successful, further studies typically involving 20 to 50 healthy volunteers determine the drug's maximum tolerated dose (by increasing the treatment dose until dose-limiting toxicity is reached) and the drug's most common and serious AEs, as well as other pharmacodynamic and pharmacokinetic properties[1].

Phase II

Approximately 70% of drug candidates move to Phase II, in which therapeutic efficacy of the IND is assessed by a comparison of pre- and post- treatment status of patients and the response of patients receiving IND and a placebo drug. Based on the dose range determined in Phase I, dose response in several hundred patients and the drug's biological activity are evaluated.

Although researchers obtain indications about the drug's benefit, Phase II studies are not comprehensive enough to provide enough evidence. Nevertheless, these trials commonly determine the optimum dose regimen to be used in Phase III[1].

Phase III

About 33% of tested INDs transition into Phase III, having 100 to 500 patients and with the primary objective of confirming the therapeutic benefit of the IND as well as its safety and efficacy. Moreover, the use of different dosages and study populations and combination with other therapeutic agents are investigated to provide information regarding indications and contra-indications as well as dose range and AEs.

As Phase III studies include a larger cohort and have a longer duration than previous clinical studies, they can potentially reveal rare and long-term side effects. From 25% to 30% of INDs progress to the next phase.

3.1.4. Regulatory approval

If a drug developer has evidence from its early tests and preclinical and clinical research that a drug is safe and effective for its intended use, the company can file an application to market the drug. The EMA is responsible for the scientific evaluation of centralised marketing authorisation applications. Once granted by the European Commission, the centralised marketing authorisation is valid in all European Union Member States, Iceland, Norway and Liechtenstein[10].

3.1.5. Post-marketing safety monitoring

Even though clinical trials provide important information on a drug's efficacy and safety, it is impossible to have complete information about the safety of a drug at the time of approval[2]. Phase IV clinical studies are long-term and typically conducted after regulatory agency approval. They often involve more than 10.000 individuals of the relevant patient population and aim at gathering additional information on safety, efficacy, and new indications. In some cases, this might result in withdrawal of the drug from the market or restriction to specific uses[1].

3.2. *IN VITRO* PHARMACOKINETIC STUDIES

The common challenges of achieving optimal drug attributes include that early leads often have suboptimal physicochemical and pharmacokinetic properties. Understanding of drug properties such as pK_a, solubility, lipophilicity, drug-protein and drug-drug interactions, and stability is essential for drug development and compound design[5].

3.2.1. pK_a

The pK_a has a relevant importance because it describes the ionization of a drug in the studied medium. In fact, pK_a values are different depending on the media where the drug is tested, and the ionization state not only has an impact on solubility but also affects permeability, distribution or other pharmacokinetics properties, binding to transporters or enzymes, complexation, and partition into organic solvents. Therefore, many properties need to be considered and balanced when it comes to the selection of an optimal pK_a value for a drug candidate[5].

Most drugs have aqueous pK_a in the range from 2 to 11 and, while it is possible to estimate pK_a by computational methods, it is desirable to experimentally measure the value. Potentiometry and UV/Visible absorption spectrometry are the most widespread techniques for the pK_a determination, due to their accuracy and reproducibility. Also, techniques based on separation methods such as HPLC and capillary electrophoresis have been developed in the past decade[11].

3.2.2. Solubility

Solubility is one of the key attributes of a drug candidate, as it can significantly impact on *in vitro* profiling, *in vivo* exposure, and compound design. Low solubility is a major challenge for drug discovery, since up to 75% of drug candidates are classified as low soluble[5]. Solubility measurements are typically performed during the early stage of development. Aqueous solubility

of a given molecule depends on multiple factors, including solid-state properties (crystal packing, lattice energy), ionization (pH, pK_a), and solute/solvate interactions[5].

When referring to intermolecular interactions in the solid state, compounds must overcome crystal packing to dissolve in the solvent. The most thermodynamically stable form has the lowest energy stage and the lowest solubility. The melting point and heat of fusion can be reasonable indicators for lattice energy at early development. Disruption of the tight crystal packing through the molecule design can increase the solubility at the drug discovery stage[5].

The solubility of an ionisable compound can be described by the Henderson-Hasselbalch equation (Equation 1), where the solubility is a function of the intrinsic solubility (S_0), pH of the medium, and pK_a of the molecule[5].

$$logS = logS_0 + \log(1 + 10^{pH-pK_a}) \qquad \textit{Equation 1}$$

It highlights the importance of selecting a compound with a higher intrinsic solubility. It also indicates the benefit of having a stronger pK_a in chemical structure for solubilization purpose[5]. The shake-flask method is the reference solubility testing procedure, although there exist other methods such as the pH-metric one. The medium in which the drug is solved can be water, aqueous buffer, biorelevant media, etc.

3.2.3. Lipophilicity

Lipophilicity plays a significant role in drug discovery and compound design. The lipophilicity of a compound is described by a partition coefficient, log*P* (Equation 2), which is defined as the ratio of the concentration of the unionized compound at equilibrium between organic (most commonly octanolic) and aqueous phases.

$$logP_{o/w} = \left(\frac{[solute]^{unionized}_{octanol}}{[solute]^{unionized}_{water}}\right) \qquad \textit{Equation 2}$$

For compounds with ionizable groups, the distribution of species is impacted by pH. This leads to the definition of the distribution coefficient, log*D* (Equation 3), of a compound, which considers the dissociation of weak acids and bases[5].

$$logD_{o/w} = \left(\frac{[solute]^{unionized}_{octanol} + [solute]^{ionized}_{octanol}}{[solute]^{unionized}_{water} + [solute]^{ionized}_{water}}\right) \qquad \textit{Equation 3}$$

Lipophilicity not only impacts solubility but also influences membrane permeability, potency, and selectivity. Membrane permeability is a key determinant in pharmacokinetic behaviour of

drugs (ADMET) and especially of absorption, distribution and excretion. To reach the desired target, a drug must cross various cellular barriers and must have an optimal lipophilicity, because if the solute is too lipophilic it will remain trapped in the membrane. Besides, high lipophilicity often contributes to high metabolic turnover, low solubility, and poor oral absorption. Low lipophilicity can also negatively impact permeability and potency, resulting in low bioavailability and efficacy[5]. It is generally considered that compounds with a log*P* greater than 1 and lower than 4 (between 0 and 3 for log*D* for ionizable drugs) are more likely to have optimal properties as oral drugs[5].

There are several analytical methods to determine the partition coefficient of a substance, for example the classic shake-flask method (measures the distribution of the solute after equilibrium), the potentiometric method (determines lipophilicity pH profiles directly from the pK_a value and a single acid-base titration in a two-phase water-organic solvent system) and the chromatographic method (determines lipophilicity of a solute by correlating its retention time with similar compounds with known lipophilicity values), among others.

For a better approximation to biological environment drug lipophilicity, measures in a liposome-water partition system can be done. The determination of partition coefficients using liposomes as a lipid phase requires the sample to be equilibrated with a suspension of liposomes, followed by a separation procedure (ultrafiltration, centrifugation, equilibrium dialysis, etc.) before the sample is quantitated in the fraction free of the lipid component[12].

3.2.4. Drug-protein interactions

The binding to plasma proteins has a significant impact on most ADMET properties, particularly on distribution and metabolism. Low binding may result in excessive metabolism or toxicity, and very high binding may result in insufficient tissue distribution and low efficacy[13].

Drugs in plasma may associate with single or multiple proteins at single or multiple sites, each with a different strength of association and association kinetics. This often results in a complex binding profile that cannot be quantified solely by the magnitude of the unbound fraction at equilibrium[13]. The distribution behaviour of drug compounds may be classified using generalized protein binding profiles comprised of the ligand structure, proteins involved, binding affinity, and the binding kinetics. The consequences of binding are restrictive or permissive, depending on whether binding limits distribution into the target tissue from the circulatory system[13].

Traditionally, equilibrium values (e.g., percentage binding) for drug-protein binding have been measured, but such studies overlook the influence of kinetics. A rapid method of simultaneously determining kinetic rate constants and equilibrium constants from chromatographic profiles has been developed, based on the use of immobilized protein columns and HPLC. By measuring the chromatographic profiles (position and width) of a retained and an unretained compound, one can determine both the rate and equilibrium constants in an aqueous pH 7.4 environment at 37 °C[14].

3.2.5. Drug-drug interactions

Drug-drug interactions (DDIs) are one of the commonest causes of medication error, particularly in the elderly due to poly-therapy, which increases the complexity of therapeutic management inducing adverse drug reactions or reducing the clinical efficacy[15]. DDIs can be classified into two main groups: pharmacokinetic (involves ADMET properties) and pharmacodynamic (involves direct effect at receptor function, interference with a biological or physiological control process and additive/opposed pharmacological effect)[15]. Focusing on pharmacokinetics DDIs:

- Absorption interactions comprise formation of insoluble complexes (e.g. the decreased bioavailability of bisphosphonates when calcium is co-administered) and inhibition of transporters.
- Distribution interactions embrace the competition for transport protein binding sites (e.g. phenytoin and valproate compete for the same protein binding sites).
- Metabolic interactions include competition for the same enzymes and inhibition or induction of metabolic enzymes (e.g. the effect of carbamazepine, which increases the rate of warfarin and oral contraceptive metabolism).
- Finally, interactions influencing elimination cover competition for active transport and interference with solubility (e.g. increased ion trapping of salicylate in alkaline urine due to the use of acetazolamide).

There are different methods to evaluate DDIs, for example, the isothermal titration calorimetry (ITC) based on the direct measurement of heat either released or absorbed in molecular binding during gradual titration, or the fluorescence method, based on the variation of fluorescence parameters (including the wavelengths of maximal activation and emission, quantum yield, fluorescence lifetime, etc.) when a drug molecule binds to another one.

3.2.6. Stability

The primary goals of physical and chemical stability assessment are to clear up the development risk with respect to temperature, pH, light, humidity, and oxygen; guide compound design; and define the storage condition and shelf life[5].

To improve chemical stability via compound design is necessary to understand the rate, mechanisms, and pathways of degradation. The major routes of degradation are through hydrolysis, oxidation (and autoxidation) and photochemical means. The reactions are typically acid or base catalysed; therefore, pH is one of the drug stability key factors[5]. In addition, the use of excipients such as cosolvents, complexing agents and surfactants, or the optimization of concentration range and storage conditions (e.g. solutions, suspensions, oil/lipid or solid formulations) can maximize chemical stability.

3.2.7. Testing media

An important application of pharmacokinetics testing is to predict *in vivo* performance of drug dosage forms. However, purified water or the simple aqueous buffer solutions typically used for quality control testing do not represent all aspects of physiological conditions in the human body and usually offer, at best, empirical *a posteriori* correlation with *in vivo* data. Prediction of pharmacokinetic effects on drugs generally requires adequate simulation of the conditions in the targeted organs and those which the drug goes through.

For example, in Vertzoni *et al.* work[16], solubilities of the studied compounds are much higher in the colonic aspirates than would be predicted from plain TRIS-maleate buffers at the equivalent pH values. This observation suggests that bile acids, phosphatidylcholine, palmitic acid and, perhaps, peptides/proteins that are present in the ascending colon have significant solubilization effects. Consequently, these components are included in the corresponding simulated biological media to improve concordance with *in vivo* conditions.

Also, Kokubo *et al.*[17] proposed that the *in vivo* bone bioactivity of a material can be predicted from the apatite formation on its surface only in a simulated body fluid with ion concentrations nearly equal to those of human blood plasma. Since then, *in vivo* bone bioactivity of various types of materials are evaluated by apatite formation in biorelevant media.

4. OBJECTIVES

The aim of this project is a bibliographic research concerning the biorelevant media commercially and non-commercially available, their components, preparation and their possible interaction with drugs.

Additionally, the composition and physicochemical properties comparison between the commercially available products, the *in-situ* preparation biorelevant medias and the human biological ones has been set as an objective, to ensure the correct performance of the *in vitro* studies prior to the clinical phase of the drug development.

5. METHODS

The research has been done using scientific data bases as Scopus and Science Direct, ResearchGate, SciFinder, PubChem and PubMed, etc.

Science Direct has been of great help, and most of the searches using keywords as Drug Development, Simulated Biological Fluids, Human Intestinal Composition or Biorelevant Media have conducted to get pleasant results.

Research Gate has been also useful; although there were not as many published papers as in other data bases, information about Drug Discovery and Preclinical Studies keywords was provided. PubChem has been used principally to define chemical molecules that appear in this work, such as lecithin, TRIS base or taurocholic acid.

For short and concise definitions, searchers as Google or Ecosia have been used, focusing the selection of the results in expert web sites (for example, EMA's, FDA's, Encyclopaedia Britannica's, WebMD's or ChEBI's (Chemical Entities of Biological Interest) web sites).

For the bibliography citation the program Mendeley has been used.

6. RESULTS AND DISCUSSION

As introduced, the media typically used for quality control dissolution testing (such as buffer solutions, organic and aqueous solvents) do not represent all aspects of the physiological conditions of the most used routes of administration and do not allow correlation with *in vivo* data. The use of simulated biological fluids (also known as biorelevant media) can give a better understanding of the release mechanisms and possible *in vivo* behaviour of a product and enhance the predictive capability of the dissolution testing[18].

The most used routes of administration of drug substances are parental and oral route.

- Parenteral drug administration means any non-oral means of administration but is generally interpreted as relating to injecting directly into the body, bypassing the skin and mucous membranes. The common parenteral routes are intramuscular, intravenous, and subcutaneous and are normally associated with short-term effects. To evaluate the *in vitro* drug release from these dosage form, the dissolution medium should have ion concentrations almost equal to those of the human plasma[19].
- The oral route is the most common and convenient administration method for the systemic delivery of drugs. It affords high patient-acceptability, compliance, and ease of administration. Moreover, the cost of oral therapy is generally much lower than that of parenteral therapy. Nevertheless, the oral route is not without disadvantages, particularly with respect to labile drugs such as peptide- and oligonucleotide-based pharmaceuticals. Drug absorption may be affected by several physiological factors including volume and composition of gastrointestinal fluids, the pH and buffer capacity of these fluids, digestive enzymes, contraction patterns, and bacterial flora in the gut. In addition, the extent of drug absorption and bioavailability may be further affected by gastrointestinal transit, the presence of cellular transporters, and metabolic enzymes. Several of those factors are affected by intake of food[18].

Once both routes of administration are presented, a description of four human biological fluids that have a remarkable impact on them is done. These are the human plasma in relation with the parenteral route and the gastric, intestinal and colonic fluids for the oral route.

6.1. SIMULATED HUMAN PLASMA (SHP)

Plasma is the liquid portion of blood and the largest component of it, making up about 55% of its overall content[20]. Plasma serves as a transport medium for delivering nutrients to the cells of the various organs of the body and for transporting waste products derived from cellular metabolism to the kidneys, liver, and lungs for excretion. It is also a transport system for blood cells, and it plays a critical role in maintaining normal blood pressure. Plasma helps to distribute heat throughout the body and to maintain homeostasis, or biological stability, including acid-base balance in the blood and body[21].

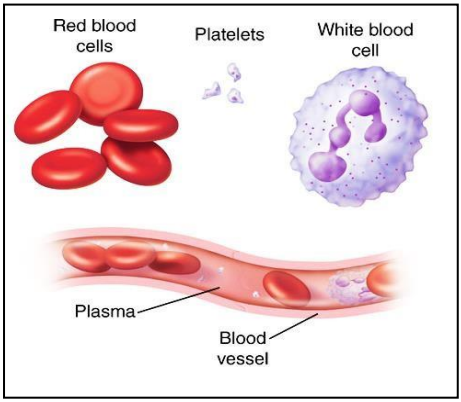

Figure 2. Blood cells and plasma representation.
(Image withdrawal from University of Rochester Medical Center Webpage[20])

Plasma is derived when all the blood cells - red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes) - are separated from whole blood (Figure 2). When isolated on its own, blood plasma is a light-yellow liquid[20], which is 90 to 92 percent water. Nevertheless, it contains critical solutes necessary for sustaining health and life[21].

The electrolytes and acid-base system found in the plasma are finely regulated. Small molecules are primarily responsible for the concentration of dissolved particles in the plasma. However, it is the concentration of much larger proteins (especially albumin) on either side of semipermeable membranes that creates crucial pressure gradients necessary to maintain the correct amount of water within the intravascular compartment and, therefore, to regulate the volume of circulating blood[21].

6.1.1. Biological composition

Important constituents of human plasma include electrolytes such as sodium, potassium, chloride, bicarbonate, magnesium, and calcium. In addition, there are trace amounts of other substances, including amino acids, vitamins, organic acids, pigments, enzymes, hormones and nitrogenous wastes (e.g., urea and creatinine)[21].

Serum albumin, a protein synthesized by the liver, constitutes approximately 60% of all plasma proteins. It is very important in maintaining osmotic pressure in the blood vessels; it is also an important carrier protein for a number of substances, including hormones. Other proteins called alpha and beta globulins transport lipids (lipoproteins) such as cholesterol as well as steroid hormones, sugar and iron[21]. At last, glycoproteins contain chains of sugars linked to them, which increase their stability, determine their shape in space, facilitate their interaction with other proteins and also the differentiation and development of cells[22].

On Table 1, the concentration of most relevant components is shown, aiming to help creating an idea about the diversity of this medium. On the *Comparison* section, Table 3 focuses on the concentration of the mineral salts and buffer solution in human plasma[17], with the resulting pH.

Table 1. Composition of human plasma[23].

Composition	**Concentration [g L^{-1}]**
Water	900
Mineral salts	9
Albumin proteins	40
Globulin proteins	32
Fibrinogen proteins	3
Lipids	6
Glucose	1
Urea and uric acid	2

6.1.2. Laboratory preparation

An acellular SHP that has inorganic ion concentrations like those of human extracellular fluid has been effectively simulated by T. Kokubo *et al.*[17] following a simple procedure:

1. Wash all the bottles and wares with 1M-HCl solution, neutral detergent, and ion-exchanged and distilled water, and then dry them.

2. Put 700 mL of ion-exchanged and distilled water into 1L polyethylene bottle and cover the bottle with a watch glass.
3. Stir the water in the bottle with a magnetic stirrer and adjust the temperature of the solution in the bottle at 36.5ºC with a water bath. Then, dissolve only the reagents of 1st to 8th order into the solution one by one in the order given in Table 2 (one after the former reagent was completely dissolved). Addition of the 9th reagent should be little by little in order to avoid local increase in pH of the solution.
4. Controlling the temperature at 36.5ºC, adjust pH of the solution at pH 7.40 by stirring the solution and titrating 1M-HCl solution (when the pH electrode is removed from the solution, add the water used for washing the electrode to the solution).
5. Transfer the pH-adjusted solution from the polyethylene bottle to a volumetric glass flask. Add the water used for washing the bottle to the solution in the flask.
6. Adjust the total volume of the solution to one litter by adding ion-exchanged and distilled water and shaking the flask at 20 ºC.
7. Transfer the solution from the flask to a polyethylene or polystyrene bottle and store it in a refrigerator at 5 to 10ºC. The SHP shall be used within 30 days after preparation.

Table 2. Regents for preparing SHP (pH 7.40, 1L)

Order	Reagent	Amount	Purity [%]
1	NaCl	8.035 g	99.5
2	$NaHCO_3$	0.355 g	99.5
3	KCl	0.225 g	99.5
4	$K_2HPO_4 \cdot 3H_2O$	0.231 g	99.0
5	$MgCl_2 \cdot 6H_2O$	0.311 g	98.0
6	1M-HCl	39 mL	-
7	$CaCl_2$	0.292 g	95.0
8	Na_2SO_4	0.072 g	99.0
9	TRIS	6.118 g	99.0
10	1M-HCl	0 to 0.5 mL	-

- *Note 1*: Since SHP is supersaturated with respect to apatite, an inappropriate preparation method can lead to its precipitation in the solution. Always make sure that the preparing solution is kept colourless and transparent and that there is no deposit on

the surface of the bottle. If any precipitation occurs, stop preparing SHP, abandon the solution, restart from washing the apparatus and prepare SHP again.

- *Note 2*: Stability of the solution must be examined. After 2-3 days, check whether the solution has any precipitation or not. If any precipitation is found, do not use the solution. Bottles in which precipitation occur must not be used for any further SHP, because some calcium phosphates would be adhered on their walls inside.

As result of the previous preparation, the final ion concentration and pH of the SHP is presented on Table 3.

6.1.3. Product suppliers

The commercial chemical brands Merck (Sigma-Aldrich), Biomol, R&D Chemicals, AG Scientific, mpbio,Thomas, Biorelevant and RICCA do not provide any SHP at date of december 2018.

6.1.4. Comparison

As not a single SHP has been found commercially available, the comparison aims to determine if the simulated plasma preparation is analogous to human plasma and, therefore, useful for *in vitro* studies.

Table 3. Composition and pH of human plasma and SHP.

Ion	Human plasma [mM]	SHP [mM]
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	103.0	147.8
HCO^{3-}	27.0	4.2
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5
TRIS	*N.A.*	50.5
pH	7.2 – 7.4	7.4

N.A.: Not Availiable

It can be seen from Table 3 that SHP is richer in Cl^- ion and poorer in HCO_3^- ion than human blood plasma. In 2003, Oyane *et al*. tried to correct this difference by preparing a revised SHP in which the concentrations of Cl^- and HCO_3^- ions were, decreased and increased respectively, to the levels of human blood plasma. However, calcium carbonate has a strong tendency to precipitate from this SHP, as it is supersaturated with respect not only to apatite, but also to calcite. In 2004, Takadama *et al.* proposed a newly improved SHP in which they decreased only the Cl^- ion concentration. This improved SHP was compared with the corrected SHP in its stability and the reproducibility and there was not any significant difference between them[17].

Concerning to the other molecules reported in the human plasma sush as proteins, lipids, glucose or urea, the search through the literature base data using keywords such as *Simulated human plasma* and *SHP preparation* among others, did not provide any paper describing a SHP which include them, maybe because of the difficulty in maintaining the stability after preparation. Also, it may be due to the fact that most of the studies found focus on the relation of the SHP with the bone bioactivity, normally through the formation of hydroxyapatite, and so they are not of principal interest.

6.2. SIMULATED GASTRIC FLUID (SGF)

The stomach is a muscular J-shaped pouchlike hollow organ that hangs inferior to the diaphragm in the upper left portion of the abdominal cavity and has a capacity of about 1 L or more (Figure 3).

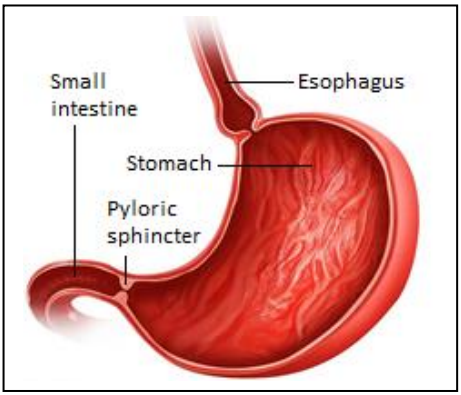

Figure 3. Stomach and its environment representation.

The stomach receives food from the oesophagus, secretes acid and enzymes, mixes the food with gastric juice, initiates protein digestion and carries on limited absorption. The stomach muscles contract periodically, churning food to enhance digestion. Finally, the pyloric sphincter (a muscular valve) opens to allow food to pass from the stomach to the small intestine[24].

6.2.1. Biological composition

To determine whether the prepared SGF is similar or not to the human gastric fluid (HGF), a wide study of its real composition and physicochemical properties has been done. Starting with the gastric fluid components, the amount of bile salts is very variable, because reflux from the duodenum into the stomach occurs sporadically[25]. Nevetheless, Rhodes *et al.*[26] found a pleasant bile salts average concentration in 10 healthy volunteers in the fasted state. Bile salts are natural surfactants that lower the surface tension of the intestinal fluids significantly and aggregate in aqueous solutions to form micelles[37].

The stomach secretes two enzymes important for nutrient digestion; pepsin and human gastric lipases (HGL). Pepsin is an endopeptidase that breaks down proteins into smaller peptides and amino acids (a protease), which can be readily absorbed by the small intestine. It is most active in environments between 37 °C and 42 °C, exhibits maximal activity at pH 2.0 and is inactive at pH 6.5 and above. Basal pepsin output is about 0.8 mg mL^{-1}. Therefore, assuming a resting volume of about 25 mL, ingestion of a glass of water (about 200 to 250 mL) brings the pepsin concentration down to about 0.08 mg mL^{-1}.

HGL is present in the fasted stomach, and its basal level is about 0.1 mg mL^{-1}. The lipolysis of lipids begins in the stomach catalysed by HGL, which is active and stable at acidic pH, so it has great importance in drug dissolution when ingesting a drug in a lipid-based drug delivery system. Finally, a large variation in protein content exists when comparison is done among different studies, and the major ions are sodium, calcium, potassium, and chloride[25].

Regarding now the physicochemical properties, the average pH of the stomach in the fasted state is reported to lie in the range 1.5 to 1.9[27]. The surface tension of gastric aspirates was measured in five healthy subjects by Pedersen *et al.*[27] The osmolarity of gastric juice in 36 gastric aspirates was reported by Lindahl *et al.* and Pedersen *et al.* reported values slightly above. All the named parameters values can be easily inquired in the Table 6 on the *Comparison* section.

6.2.2. Laboratory preparation

The SGF is a synthetic form of the gastric fluid in the stomach. Drugs made for dissolution and disintegration in the small intestine must not disintegrate or dissolve in the SGF. On the other hand, drugs designed to act in the stomach should dissolve in this synthetic solution. Both fasted state simulated gastric fluid (FaSSGF) and fed state simulated gastric fluid (FeSSGF) laboratory preparation data is presented.

FaSSGF

Before discussing about the solution preparation, it must be considered the volume needed to simulate the conditions in the stomach. In the fasted state, resting volumes have been estimated to be about 25 mL. However, when a tablet or capsule is administered, some fluid is usually co-administered. In pharmacokinetic studies, this volume is often in the 200 to 250 mL range. Assuming secretions at a rate of just under 1 mL min^{-1}, about 50 mL secretions are expected within 1 h, the longest period during which a fast disintegrating immediate release dosage form is expected to be totally emptied from the fasted stomach. Thus, a realistic volume to simulate the total fluid available in stomach would fall in the range of 250 to 300 mL[27].

Vertzoni *et al.* developed a SGF that more adequately reflects the physiological conditions of the fasted state. This medium contains pepsin and low amounts of sodium taurocholate (*Note 3*) and lecithin (*Note 4*). The use of sodium lauryl sulphate (SLS), the most used artificial surfactant in dissolution testing, is inconvenient because it hydrolyses in solutions having pH lower than 4, leading to inconsistent medium composition. Also, it interacts with gelatine at pH values lower than 5 making its use with gelatine capsule products problematic[27].

- *Note 3*: Sodium taurocholate (NaTC) is the sodium salt of taurocholic acid, a bile acid composed of cholic acid and taurine.[28] It is the chief ingredient of the bile of carnivorous animals. It is also often used to solubilize lipids and membrane proteins, as well as functioning as an activator during studies involving different types of lipases[29].
- *Note 4*: Lecithin is a generic term to designate any group of yellow-brownish fatty substances (mixtures of glycerophospholipids) occurring in animal and plant tissues, which are amphiphilic. It has low solubility in water but is an excellent emulsifier because its phospholipids can form either liposomes, bilayer sheets, micelles, or lamellar structures, depending on hydration and temperature. It is available from sources such as soybeans, eggs, milk, rapeseed, cottonseed and sunflower[30].

Unfortunately, Vertzoni *et al.* do not describe the preparation of the simulated media and only indicates the final concentrations and physicochemical properties of the 500 mL solution. Nevertheless, the standard procedure could be followed, dissolving the reagents in 475 mL of deionized water into a 500 mL volumetric flask, adjusting the pH using concentrated HCl and finally adjusting the volume to the mark.

FeSSGF

A major issue when trying to simulate the intragastric environment in the fed state is the composition change with time as digestion proceeds and emptying occurs. Media that simulates the initial composition after food intake include full-fat milk (3.5%), which simulates a light meal, and Ensure Plus, which has physicochemical properties similar to those of a standard meal[18].

One way of modelling composition changes is to develop "snapshot" media, each corresponding to a certain time after ingestion of the meal. The composition of each snapshot medium is calculated to reflect the properties of the gastric aspirates during the first 75 min (early), from 75 to 165 min (middle), and 165 min (late) after meal ingestion (Table 4)[18].

Table 4. Reagents compositions of FeSSGF snapshots.

Composition	**Early [mM]**	**Middle [mM]**	**Late [mM]**
NaCl	148	237.2	122.6
Acetic acid	-	17.12	-
Sodium acetate	-	29.75	-
Orthophosphoric acid	-	-	5.5
Sodium dihydrogen phosphate	-	-	32
Milk/Buffer	1:0	1:1	1:3
HCl	To pH adjust	To pH adjust	To pH adjust
Physicochemical properties			
Buffer capacity [mmol L^{-1} ΔpH^{-1}]	21.33	25	25
Osmolality [mOsm kg^{-1}]	559	400	300
pH	6.4	5	3

6.2.3. Product suppliers

Researching on SGF suppliers none for the fed state has been found, but one for the fasted state is available: Biorelevant (Table 5).

Table 5. General product data (SGF)

Product	Supplier	Powder weight	Solution volume	Price	Product Code
FaSSGF	Biorelevant	5.8 g	93 L	99.99 €	FFF01

It is important to emphasize the preparation method in Biorelevant's solution because the solid powder contains phospholipids and surfactants but, as indicated in their web page[31], needs to be dissolved in purified water, the addition of sodium chloride and the adjustment to the indicated pH with hydrochloric acid 1M. This same web side calculates the amounts of each component (FaSSGF and buffer solution) needed for a desired volume of solution. Furthermore, all the information about the procedure can be required to the brand and is sent to the purchaser by e-mail. Table 6 contains the concentration of Biorelevant's FaSSGF after preparation.

6.2.4. Comparison

To facilitate the comparison between the HGF, the laboratory preparation by Vertzoni *et al.* and the Biorelevant's product, the sodium concentration in the Vertzoni *et al.* solution (Table 6) has been deducted from the NaCl and NaTC ones, and the chloride concentration has been set as "to pH adjust", due to its dependence on the HCl used to simulate the biological pH.

Table 6. Composition and physicochemical properties of HGF and FaSSGFs.

Components	HGF [mM]	Vertzoni *et al.* [mM]	Biorelevant [mM]
Bile salts	0.08	0.08	0.08
Phospholipids	*N.A.*	0.02	0.02
Pepsin	0.8 mg mL^{-1}	0.1 mg mL^{-1}	-
Human Gastric Lipase	0.1 mg mL^{-1}	-	-
Protein Content	4.9 ± 1 g L^{-1}	-	-
Sodium	70	34.28	34
Chloride	100	To pH adjust	59
Calcium	0.6	-	-
Potassium	15	-	-
Physicochemical properties			
Surface Tension [mN m^{-1}]	36–51	42.6	*N.A.*
Osmolality [mOsm kg^{-1}]	191 ± 36	120.7 ± 2.5	*N.A.*
Buffer capacity [mmol L^{-1} ΔpH^{-1}]	14.3 ± 9.3	*N.A.*	*N.A.*
pH	1.5–1.9	1.6	1.6

Regarding the composition of the mediums, phospholipids (lecithin) are used (in combination with NaTC and pepsin) to achive a surface tension lowering effect, avoiding the use of the artificial surfactant SLS. NaTC is included in both preparations following the physiological bile salts data.

As can be observed, the presence of pepsin is the main difference between the laboratory preparation proposed and the Biorelevant's media. The addition of pepsin was neither done in the prevoius United States Pharmacopeia's (USP) SGFs, but Vertzoni *et al.* considered that it may improve dissolution characteristics.

According to Vertzoni *et al.*[27], HGL basal concentration is about 0.1 mg mL^{-1} and, taking into account the dilution effect and the fact that gastric lipase is active at pH values between 3 and 6, the presence of this enzyme is unlikely to be important to drug dissolution in the fasting state. Therefore, this enzyme is not included in the laboratory and suppliers preparations. Similary, the protein content is not reproduced, maybe due to the variability of HGF availiable data.

Concerning electrolytes, the sodium concentration in both simulations is half the concentration measured in HGF. Chloride depends on the pH adjustment in the Vertzoni *et al.* preparation but, due to its similarity with Biorelevant's simulated fluid, an aproximation to this last SGF can be done. Anyway, both SGF contain a lower concentration than HGF. In none of the reviewed papers[25,27,32] there is an explanation for this fact, although in Jantratid *et al*.[33] study, the amount of NaCl needed to adjust the medium to the physiologic osmolality is calculated on the basis of the freezing-point depression and refined experimentally. Finally, calcium and potassium are not included in the simulations reagents lists because of their minor presence and effect.

In the case of the physicochemical properties, Biorelevant's FaSSGF only provides the pH value, which in fact is comprised for the 1.5 to 1.9 interval value measured in HGF. Finally, in the Vertzoni *et al.* solution, the pH and the surface tension values coincide with the HGF ones and the buffer capacity of the FaSSGF is not mentioned in this study.

6.3. SIMULATED INTESTINAL FLUID (SIF)

The intestines are a long, continuous tube running from the stomach to the anus and include the small intestine, large intestine, and rectum. Most absorption of nutrients and water happen in the intestines. The SIF refers to the human small intestine fluids; the human large intestine fluids are described by the simulated colonic fluid, which will be studied further in this work.

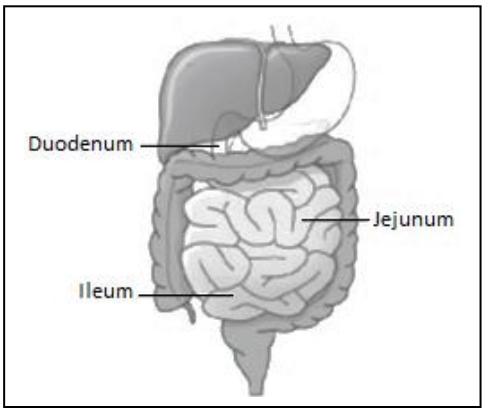

Figure 4. Small intestine division and near organs representation.

The small intestine is about 20 feet long and about an inch in diameter and is divided into the duodenum, jejunum, and ileum (Figure 4)[34]. The muscles of the small intestine mix food with digestive juices from the pancreas, liver and intestine to complete the breakdown of proteins, carbohydrates, and fats. Bacteria in the small intestine make some of the enzymes needed to digest carbohydrates. The walls of the small intestine absorb water and the digested nutrients into the bloodstream. Later, the small intestine pushes the mixture forward for further digestion using a movement called peristalsis[35].

6.3.1. Biological composition

The first step in simulating human intestinal fluids (HIF) is to summarize all relevant available data from the literature. Regarding the components, the three main bile salts species (about 70% to 75%) in the fasted-state HIF are NaTC, glycocholate, and glycochenodeoxycholate[37]. Next to the bile salts, the most important natural surfactants are the phospholipids, which are secreted with the bile into the duodenum[36]. The predominant phospholipids found in the human gut are phosphatidylcholine and especially its hydrolysis product lysophosphatidylcholine. In addition,

Persson *et al.*[37] reported other components that can also be found in fasted HIF, such as cholesterol and free fatty acids. Furthermore, comparing literature data on pancreatin levels, three sources reported lipase levels in the fed state[33]. Pancreatin is a mixture of several digestive enzymes produced by the exocrine cells of the pancreas and is composed of amylase, lipase and protease[38].

Finally, Lindahl *et al.*[32] evaluated the composition of fasted-state human jejunal fluid in 24 volunteers (12 males and 12 females) and found high concentrations of sodium and chloride and lower amounts of potassium and calcium[36].

About the physicochemical properties, the pH value in the duodenum was determined to be 6.5. Consequently, it is slightly lower than the pH value for the jejunum, where the pH was 6.8. An important consideration is the possibility of a change of the pH in the small intestine during the dissolution of a drug because of the low buffer capacity of HIF reported by Bergström *et al.*[39]. This could potentially have a dramatic effect on the solubility of acidic and basic compounds.

The surface tension of a medium has a key influence on the wetting behaviour of poorly soluble drugs and thus on their dissolution rate. This is influenced by the qualitative and quantitative composition of all amphiphilic structures in the medium and reflects the sum composition of different kinds of various bile salts, phospholipids, and their hydrolysis products (e.g., free fatty acids) and cholesterol[36]. The values of the reported parameters in this section and other properties, as osmolarity and ionic strenght, can be inquired in the Table 9 on the *Comparison* section.

6.3.2. Laboratory preparation

Again, before discussing about the solution preparation, is necessary to determine the volume needed to simulate the conditions in the small intestine. According to literature, to simulate fasted conditions in the small intestine, a volume of up to 200 mL would be appropriate. Volumes of up to one litter would be recommended for the fed-state small intestine[36]. In the proceeding pages, novel fasted and fed states SIFs preparations are presented, completed with a summary table to quickly examine reagents and physicochemical properties.

FaSSIF-V2

To update the simulation of fasted state conditions in HIF, only minor changes to previous used FaSSIF are necessary (Table 9, on *Comparison* section). The amount of lecithin is

decreased from 0.75 mM to 0.2 mM, the osmolality is somewhat lower in accordance with *in vivo* data and the pH of 6.5 is maintained, with substitution of phosphate buffer for maleate buffer[33]. To prepare 1 L of FaSSIF-V2, the same procedure than described later for the fed state can be followed, excluding steps 5 and 6, where glyceryl monooleate and sodium oleate are added.

FeSSIF-V2

The composition of the intestinal fluids changes over time in the fed state. Therefore, three "snapshot" media were developed to reflect conditions in the upper small intestine during the digestion process. Their compositions are indicated in Table 7.

NaTC was used to represent the bile salts due to its comparatively low pK_a value and hence good solubility at all pH values under consideration. To prepare 1L of medium this procedure was followed[33]:

1. Prepare 900 mL blank buffer using amounts of NaCl, maleic acid and NaOH calculated for 1 L of medium. Adjust the pH to the target pH.
2. Transfer 500 mL of this "blank" buffer into a 1 L round-bottom flask.
3. Dissolve NaTC in the blank buffer by continuous stirring, and add a freshly prepared solution of lecithin in dichloromethane (100 mg mL^{-1}). This produces an emulsion, resulting in a turbid product.
4. Drive off the dichloromethane, initially using a rotary evaporator and vacuum at approximately 40 °C for 15 min at 650 mbar. Decrease the pressure stepwise to the final pressure of 100 mbar, which is maintained for 15 min. This procedure results in a clear to slightly hazy, micellar solution, having no perceptible odour of dichloromethane.
5. Then, add a freshly prepared solution of glyceryl monooleate in dichloromethane (50 mg mL^{-1}) and perform a second evaporation step.
6. Next, add appropriate amounts of sodium oleate slowly into the micellar solution under continuous stirring.
7. Finally, adjust the volume to 1 L after the final pH adjustment using the "blank" buffer and deionized water.

Optionally, pancreatin can be incorporated into the fed state media right before the final pH adjustment. In this case, $CaCl_2$ (5 mM) is added to the micellar solution just before the pancreatin to facilitate lipolysis. The amount of pancreatin added is based on the lipase activity needed for digestion of lipid in the medium and in the dosage form to be tested (100 lipase USP units mL^{-1}).

A concentrated suspension of pancreatin is prepared by mixing pancreatin powder in deionized water to obtain a lipase activity of 10.000 U mL^{-1}. The suspension is then centrifuged at 5 °C for 15 min at 4.000 rpm, 20 mL of the supernatant are added, and the final volume of the medium is adjusted to 1 L[33].

Table 7. Composition and physicochemical properties of FeSSIF snapshots and FeSSIF-V2.

Composition	**Early FeSSIF [mM]**	**Middle FeSSIF [mM]**	**Late FeSSIF [mM]**	**FeSSIF-V2 [mM]**
Bile salts (NaTC)	10	7.5	4.5	10
Phospholipids (lecithin)	3	2	0.5	2
Glyceryl monooleate	6.5	5	1	5
Sodium oleate	40	30	0.8	0.8
Maleic acid	28.6	44	58.09	55.02
Sodium hydroxide	52.5	65.3	72	81.65
Sodium chloride	145.2	122.8	51	125.5
Physicochemical properties				
Osmolarity [mOsm kg^{-1}]	400 ± 10	390 ± 10	240 ± 10	390 ± 10
Buffer Capacity [mmol L^{-1} ΔpH^{-1}]	25	25	15	25
pH	6.5	5.8	5.4	5.8

Addition of pancreatin (and $CaCl_2$) is optional (see text).

6.3.3. Product suppliers

On the next Table 8 some data of different suppliers SIFs is shown. The same Product Code can be noticed for both Biorelevant FaSSIF and FeSSIF (also for the previous studied Biorelevant FaSSGF), because the three of them are prepared from the same product but dissolved in a different buffer solution.

Table 8. General product data (SIF)

Product	**Supplier**	**Powder weight**	**Solution volume**	**Price**	**Product Code**
FaSSIF	Biorelevant	5.8 g	2.5 L	99.99 €	FFF01
FaSSIF-V2	Biorelevant	4.6 g	2.5 L	99.99 €	V2FAS01
FeSSIF	Biorelevant	5.8 g	0.5 L	99.99 €	FFF01
FeSSIF-V2	Biorelevant	5.0 g	0.5 L	99.99 €	V2FES01
SIF-Test Solution	RICCA	-	1 L	92.39 €	R7109000-1A

RICCA's product consist on an aqueous solutions containing 0.68% (w/w) potassium dihydrogen phosphate, 0.06% (w/w) sodium hyroxide, and pancreatin at 1% (w/w). Regarding the studied literature[33,36,40] about the HIF biological composition, the presence of pancreatin is mentioned only for the fed state. Similary, pancreatin is only used for laboratory preparation on fed states[33]. As RICCA's medium does not differ between fasted and fed state, the use of pancreatin, the main enzyme responsible for lipid digestion, to assess the dissolution of lipid-based drugs is comprensible. Nevertheless, as only the fasted state for HIF ans SIFs is compared, RICCA's product will not be further studied.

Biorelevant's products data has been acquired from the *Composition* section of the web site[31]. Due to its improvement with respect to the previous versions, FaSSIF-V2 and FeSSIF-V2 have been selected for the present study.

It is needed to highlight that these two Biorelevant's products, FaSSIF-V2 and FeSSIF-V2, when prepared present the same composition than the ones with the same name described in the laboratory preparation earlier in this chapter, respectively. This could mean that both are based on the same literatutre reports, or that Biorelevant's products are based in Jantratid *et al.* studies[33]. One way or another, the values of FaSSIF-V2 have been merged in one column to avoid repetitions in the following *Comparison* section, and the composition of FeSSIF-V2 can be extracted from the prevoius Table 7.

6.3.4. Comparison

The first difference to be noticed in Table 9 is that fasted-HIF is divided in two columns (duodenum and jejunum) but FaSSIF-V2 is not. Therefore, as the simulated fluid tries to be representative of both small intestine parts, if values in duodenum and jejunum are different these are treated as an interval. For example, bile salts concentration in fasted HIF is from 3 to 3.3 mM.

Regarding the components of the mediums, for FaSSIF-V2 the phospholipids (lecithin) and bile salts (NaTC) concentrations are really suitable for the simulation of fasted HIF. These two components have a larger concentration and effect on the physicochemical properties than others like cholesterol and free fatty acids, so the last ones are not included. The same reasoning is done on calcium and potassium that are excluded from de recipe.

Sodium and chloride concentrations are (like in the studied FaSSGF) lower than the physiological ones. This could be due to the conversion of different salts to the chloride salt

investigated by Li *et al.*[41], which slowed dissolution due to a common ion effect observed at higher chloride ion concentrations.

Table 9. Composition and physicochemical properties of HIF and FaSSIF-V2.

Components	**Fasted-HIF [mM]**		**FaSSIF-V2 [mM]**
	Duodenum	**Jejunum**	
Bile salts	3.3	3	3
Phospholipids	0.26	0.19	0.2
Cholesterol	0.08		-
Free fatty acids	0.1		-
Sodium	142 ± 13		106
Chloride	126 ± 19		69
Calcium	5.4 ± 2.1		-
Potassium	0.5 ± 0.3		-
Maleic acid	*N.A.*		19
Physicochemical properties			
Surface tension [mN m^{-1}]	33.8		54.3
Ionic strength [mM]	0.139		*N.A.*
Osmolarity [mOsm Kg^{-1}]	197	264	180 ± 10
Buffer Capacity [mmol L^{-1} ΔpH^{-1}]	5.6 to 8.5	4	10
pH	6.5	6.8	6.5

Finally, to maintain the desired pH, maleic acid is used in FaSSIF-V2. With a pK_{a2} of 6.27 (the first pK_a is at 1.92 and therefore irrelevant for buffering at intestinal pH values), appropriate buffer capacities can be achieved over the required pH range of 5.4 to 6.5 covering both fasted and fed state media without exceeding the physiologically relevant osmolarity[33].

Concerning the physicochemical properties, the ionic strenght of FaSSIF-V2 is not availiable. The surface tension is higher in the simulated media than in fasted HIF, probably because the human fluid contents more molecules with capacity to act as surfactants than the ones studied or presented in the studies.

The osmolarity in duodenum and jejunum differs considerately, but the one in FaSSIF-V2 is very similar to the duodenum one. The buffer capacitty in the simulated media is higher than in the human fluids, but this difference is short enough to not interfere in pharmacokietic testings. The pH, otherwise, does not present rellevant differences.

6.4. SIMULATED COLONIC FLUID (SCOF)

The colon, also called large intestine, is about 5 feet long and about 3 inches in diameter. The ileum (last part of the small intestine) connects to the cecum (first part of the colon) in the lower right abdomen. The rest of the colon is divided into four parts: the ascending, transverse, descending and sigmoid colon (Figure 5)[42].

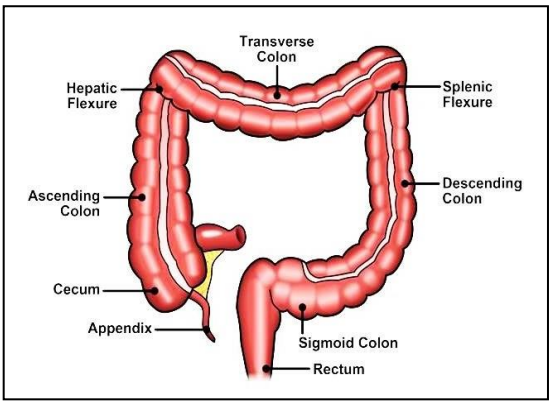

Figure 5. Colon representation and division

The colon removes water, salt, and some nutrients forming stool. Billions of bacteria coat the colon and its contents, living in a healthy balance with the body[42]. Colon-specific drug delivery usually focuses on the treatment of its local disorders, but it can also be used as an absorption site for the delivery of drugs to the systemic circulation. Although absorption from the colon is generally much lower than from the small intestine, systemic drug delivery via the colon has some advantages such as prolonged residence time, avoidance of hepatic first-pass effect, relatively low enzyme secretion, and low activity of proteolytic enzymes[18].

6.3.1. Biological composition

About 1 or 1.5 L of a nearly isotonic fluid enters the colon daily. Most of the fluid is absorbed, so the volume excreted in feces is only about 50 to 200 mL[18]. Total bile acid concentration in the fasted state is significantly lower than in the fed state. The same relation is followed by concentration values of phosphatidylcholine, cholesterol and long chain fatty acids (palmitic acid, linoleic acid and oleic acid) in the fasted and in the fed state[43].

Relevant carbohydrate and protein levels were determined in the HCoF, for both fasted and fed states. All seven short-chain fatty acids (SCFAs) were quantified but acetate dominated. Mean total levels in the fed state are not significantly different from total SCFA levels in the fasted state[43]. With regard to electrolytes, SCFAs stimulate absorption of sodium and water, whereas chloride is absorbed in exchange for bicarbonate (secreted to neutralize the organic acids produced)[18].

Concerning the physicochemical properties, average pH values are 7.8 and 6.0 in the fasted and in the fed state, respectively. The mean surface tension value is significantly lower in the fed state than that in the fasted state. In the fed state, osmolality is higher than that in the fasted state. When measured with HCl, mean buffer capacity increase from the fasted state to the fed state and, when measured with NaOH, the mean values for both are much lower[43].

The average values for physicochemical characteristics and composition of fluids of ascending colon of healthy adults are summarised in Table 11 for the fasted state and in Table 12 for the fed state.

6.4.2. Laboratory preparation

Recently, Vertzoni *et al.*[16] have developed media simulating the physicochemical characteristics of the ascending colon in the fasted and fed states.

FaSSCoF

To prepare 1L of medium the following procedure is used[16]:

1. Prepare a TRIS/maleate buffer solution by dissolving 5.5 g of TRIS and 8.8 g of maleic acid in water. Adjust the pH to 7.8 with about 240 mL of 0.5 M sodium hydroxide, and adjust the final volume to 1 L with water.
2. Transfer about 450 mL of this solution to a 1 L round-bottom flask, and add 0.113 g of bile salt extract.
3. Prepare two separate solutions by dissolving 0.222 g of phosphatidylcholine and 0.026 g of palmitic acid separately in 3 mL of dichloromethane each.
4. Transfer these two solutions (3 mL each) into the round-bottom flask. Evaporate the dichloromethane under vacuum at 40 °C until obtaining a clear solution having no perceptible odor of dichloromethane.

5. Adjust the volume of the solution to 1 L with TRIS/maleate buffer, add 3 g of bovine serum albumin and dissolve it by gentle agitation with a magnetic stirrer. The final solution is lightly turbid.

The resulting composition and physicochemical properties of this medium are listed in the Table 11 of the *Comparison* section.

FeSSCoF

To prepare 1L of medium the following procedure was used[16]:

1. Prepare a TRIS/maleate buffer solution by dissolving 3.7 g of TRIS and 3.5 g of maleic acid in water. Adjust the pH to 6.0 with about 33 mL of 0.5 M sodium hydroxide, and adjust the final volume to 1 L with water.
2. Transfer about 450 mL of this solution to a 1 L round-bottom flask, and add 0.451 g of bile salt extract.
3. Prepare two separate solutions by dissolving 0.370 g of phosphatidylcholine and 0.051 g of palmitic acid separately in 3 mL of dichloromethane each.
4. Transfer these two solutions (3 mL each) into the round-bottom flask. Evaporate the dichloromethane under vacuum at 40 °C until obtaining a clear solution having no perceptible odor of dichloromethane.
5. Adjust the volume of the solution to 1 L with TRIS/maleate buffer, add 2 g of sodium chloride, 14 g of glucose, and 3 g of bovine serum albumin and dissolve them by gentle agitation with a magnetic stirrer. The final solution is lightly turbid.

The resulting composition and physicochemical properties of this medium are listed in the Table 12 of the *Comparison* section.

6.4.3. Product suppliers

When researching on SCoF suppliers, only Biorelevant commercially prepares it. This brand provides both fast state and fed state SCoF. On the next Table 10 some basic information about these products is shown. Composition values are described on the *Comparison* section, in Table 11 for the fasted state and on Table 12 for the fed one.

Table 10. General product data (SCoF)

Product	Supplier	Powder weight	Solution volume	Price	Product Code
FaSSCoF	Biorelevant	3.4 g	10 L	179.99 €	COFAS01
FeSSCoF	Biorelevant	7.4 g	10 L	179.99 €	COFES01

The preparation of the Biorelevant's products requires, on one side the brand's powder and, on the other, the buffer components such as sodium hydroxide pellets, maleic acid, TRIS base and purified water.

6.4.4. Comparison

Concerning the SCoFs, both fasted (Table 11) and fed (Table 12) states for lab-prepared and supplied are included for comparison.

Table 11. Composition and physicochemical properties of fasted HCoF and FaSSCoFs.

Components	Fasted HCoF	Vertzoni *et al.* FaSSCoF	Biorelevant's FaSSCoF
Bile acids [μM]	115	150	150
Phospholipids [μM]	362	300	300
Cholesterol [μM]	594	-	-
Long chain fatty acids [μM]	120	100	100
Total carbohydrates [mg mL^{-1}]	*8.1*	-	-
Proteins [mg mL^{-1}]	*9.7*	3	-
Sodium hydroxide [mM]	*N.A.*	120	120
Maleate [mM]	*N.A.*	76	76
TRIS [mM]	*N.A.*	45	45
Physicochemical properties			
Surface tension [mN m^{-1}]	42.7	51.4	*N.A.*
Osmolarity [mOsm Kg^{-1}]	81	196	*N.A.*
Buffer Capacity [mmol L^{-1} ΔpH^{-1}]	21.4 / 10.3	26 / 16	*N.A.*
pH	7.8	7.8	7.8

As it can be observed, bile acids ans phospholipids concentrations are similar to those in fasted HCoF, as well as long chain fatty acids (yet Vertzoni *et al.*[16] use palmitic acid and Biorelevant uses oleate). Glucose is not included because it would have raised even more the osmolarity in FaSSGF, but it is added to the FeSSCoF to HCoF levels. Cholesterol is not included

in either FaSSCoF or FeSSCoF, based on earlier observations that bile salts and phospholipids promote cholesterol crystallization[16].

Surface tension values for the laboratory medias are comparable to those for the respectives HCoFs, and pH values in all SCoFs agree with the biological ones. Since the required pH and buffer capacity levels can not be achieved by using biorelevant buffer species (e.g. short chain fatty acids), TRIS/maleate buffer systems are used in FaSSCoF and FeSSCoF in both Vertzoni *et al.* and Biorelevant medias, and the pHs are adjusted with NaOH and HCl in the last one.

Table 12. Composition and physicochemical properties of fed HCoF and FeSSCoF.

Components	**Fed HCoF**	**Vertzoni *et al.* FeSSCoF**	**Biorelevant's FeSSCoF**
Bile acids [μM]	587	600	600
Phospholipids [μM]	539	500	500
Cholesterol [μM]	1502	-	-
Long chain fatty acids [μM]	225	200	200
Total carbohydrates [mg mL^{-1}]	14	14	-
Proteins [mg mL^{-1}]	6.9	3	-
Sodium hydroxide [mM]	*N.A.*	16.5	34
Sodium chloride [mM]	*N.A.*	34	-
Maleate [mM]	*N.A.*	30	30
TRIS [mM]	*N.A.*	31	31
Physicochemical properties			
Surface tension [mN m^{-1}]	39.2	50.4	*N.A.*
Osmolarity [mOsm Kg^{-1}]	227	207	*N.A.*
Buffer Capacity [mmol L^{-1} ΔpH^{-1}]	37.7 / 16.4	15 / 14	*N.A.*
pH	6.0	6.0	6.0

Total protein content in HCoF corresponds to the sum of peptides and proteins. Based on this analysis[43], a single protein (bovine serum albumin) at a concentration lower than the measured total protein content is incorporated in Vertzoni *et al.* FaSSCoF and FeSSCoF[16]. Finally, in both FaSSIFs a large amount of NaCl is added even exceeding the fasted HCoF osmolarity. In contrast, NaCl is added in Vertzoni *et al.* as to adjust it to the fed HCoF osmolarity.

6.5. DRUG PROPERTIES IN BIORELEVANT MEDIA

The Biopharmaceutics Classification System (BCS) is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability. According to the BCS, a drug substance is considered highly soluble when the highest dose strength is soluble in 250 ml water over a pH range 1 to 7.5. At the same time, a drug is considered highly permeable when the extent of absorption in humans is determined to be 90% of an administered dose, based on the mass balance or in comparison to an intravenous dose.

The BCS defines four major classes (Table 13). Class I drugs have the optimal properties for oral administration, most of the actual drugs are classified as Class II or Class III tough. According to Box *et al*.[44] studies, molecules in Class I and Class III tend to have solubilities higher than logS_0 of - 4, and molecules in Class I and Class II tend to have log*P* higher than 1.2.

Table 13. Biopharmaceutics classification system

Solubility	**Permeability**	
	High	Low
High	*Class I*	*Class III*
Low	*Class II*	*Class IV*

The BCS is based on aqueous solubility, but different and more accurated results and classification would be reached if solubility testing was done in biorelevant media. There has been an increasing interest in studying the properties of drugs in biorelevant media, and up to now the solubility has been the most considered one. Comparison studies between solubility values and drug behaviour in water and biorelevant media had been conducted, and they often show an increase of drug solubility in biorelevant media[45,46,47]. The different behaviour could be due to interactions of the drug with the components of the biorelevant media.

For example, in Table 14 a description of earlier studied FaSSIF and FeSSIF is shown. These fluids consist of bile salts, phospholipids, inorganic salts and buffers. Therefore, one or more of these components may be responsible for the drug ADMET properties variation. As commented earlier in this work, bile salts are natural surfactants that aggregate in aqueous solutions to form micelles. In the presence of phospholipids and their hydrolysis products as well as glycerol, free fatty acids, and cholesterol, they can form mixed micelles. These are more stable and have a

higher solubilization capacity than micelles that contain only bile salts. Poorly water-soluble compounds are often well solubilized in mixed micelles, the prevalent micellar species in HIF[36].

Table 14. Description of FaSSIF-V2 and FeSSIF-V2

Components	FaSSIF-V2 [mM]	FeSSIF-V2 [mM]
Bile salts	3	10
Phospholipids	0.2	2
Sodium	106	81.65
Chloride	69	125.5
Maleic acid	19	55.02
Glyceryl monooleate	-	5
Sodium oleate	-	0.8
pH	6.5	5.8

The critical micelle concentrations (CMCs) of bile salts are from 3.0 to 12.0 mM in aqueous medium. The CMC is defined as the concentration of the surfactant at which the surfactant starts to aggregate forming micelles, therefore, the presence of bile salts micelles can be expected in low quantities on FaSSIF and in higher amounts in FeSSIF (Table 14). Furthermore, in Zhou et al.[48] studies, the CMC value of phosphatidylcholin-sodium deoxycholate mixed micelles is approximately 0.31 mM, much less than the CMC of mono-component sodium deoxycholate solution (3 mM).

This results, considering that the PL and BS used are similar than the ones in SIFs but at different concentrations, lead to the conclusion that mixed micelles form at earlier concentrations than BS micelles. Taking into account the concentration of phospholipids in both media, the SIFs micelles can be assumed to exist in phospholipid-bile salts-mixed micelles (PL-BS-MMs) form. Moreover, the presence of fatty material (glyceryl monooleate and sodium oleate) in FeSSIF significantly decreases the surface tension, indicating a higher interaction with the surfactants[49]. In conclusion, the hydrophobic core of micelles can accommodate high concentration of hydrophobic drugs and, therefore, modify ADMET-related parameters. This solubilizing effect of PL-BS-MMs as additive components of biorelevent media can be expressed by the solubility ratio (SR: $S_{biorelevan\ mediat}$ / $S_{blank\ buffer}$)[45].

Further works on ADMET properties in biorelevant media must be carried out. For example, not many studies concern the variation of pK_a values between water and biorelevant media.

However, pK_a determination in biorelevant media is of outmost importance because its value is used for the evaluation of other ADMET properties such as solubility or lipophilicity. Nowadays, the pK_a value used is the one determined in water but, as stated before, the ionization in different media can significantly differ.

7. CONCLUSIONS

To improve the prediction of *in vivo* performance of pharmaceutical dosage forms, *in vitro* testing must simulate the best the biological environment. In this sense, the use of biorelevant media for *in vitro* testing in drug discovery and preclinical research is more adequate than more simple media like aqueous buffer solutions.

In this work, the most important simulated biological fluids have been described, initially with a research on the composition of the respective human biological fluids and their properties, succeeded by the preparation of biorelevant media in the laboratory from their reagents or form the commercially available products. Concerning these simulated biological fluids:

- An effective laboratory preparation of SHF has been described and compared to the human fluid. None supplier has been found.
- Two resembling FaSSGFs have been reported, one requiring a complete preparation and the other prepared from a commercial product. A laboratory preparation for three FeSSGF snapshots media has been also described.
- Two FaSSIF preparations have been reported, from the reagents and from a commercial product. Four lab-prepared FeSSIFs (1 global and 3 snapshots) have been detailed.
- Four SCoFs have been reported, two for the fasted state and two for the fed state. For the two different states, also two possible preparations, one complete and the prepared from a commercial product, have been recounted.

In addition, the fasted states SGFs, SIF and SCoFs and the fed states SCoFs have been stated as highly equivalent to the respective human fluids, and the differences between them have been highlighted and reasoned.

Finally, the influence of the medium to the ADMET drug properties has been discussed. The comparison is focused on the difference when performing the studies in traditional medium and in biorelevant media such as FaSSIF and FeSSIF. It has been shown that these properties are often influenced by the medium due to the interaction of its components with the drugs. In concrete, the formation of phospholipid-bile salts-mixed micelles in biorelevant media and the partition of the drug between the lipophilic interior of the mixed micelles and the external aqueous solution are related to the ADMET properties variations.

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9. ACRONYMS

EMA - European Medicines Agency
FDA - American Food and Drug Administration
ADMET - Absorption, Distribution, Metabolism, Excretion and Toxicity
IND - Investigational New Drug
GLP - Good Laboratory Practice
AEs - Adverse Effects
HPLC - High Performance Liquid Chromatography
UV - Ultraviolet
DDIs - Drug-Drug Interactions
ITC - Isothermal Titration Calorimetry
TRIS - Tris(hydroxymethyl)aminomethane
SHP - Simulated Human Plasma
SGF - Simulated Gastric Fluid
HGF - Human Gastric Fluid
HGL - Human Gastric Lipases
FaSSGF - Fasted State Simulated Gastric Fluid
FeSSGF - Fed State Simulated Gastric Fluid
SLS - Sodium Lauryl Sulphate
NaTC - Sodium Taurocholate
USP - United States Pharmacopeia
SIF - Simulated Intestinal Fluid
HIF - Human Intestinal Fluid
FaSSIF – Fasted State Simulated Intestinal Fluid
FeSSIF – Fed State Simulated Intestinal Fluid
SCoF - Simulated Colonic Fluid
SCAFs - Short Chain Fatty Acids
FaSSCoF – Fasted State Simulated Colonic Fluid
FeSSCoF – Fed State Simulated Colonic Fluid
BCS - Biopharmaceutics Classification System
CMC - Critical Micelle Concentration
PL-BS-MMs – Phospholipids-Bile Salts-Mixed Micelle