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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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DEVELOPMENT OF A RESVERATROL SOLID DISPERSION WITH ENHANCED ORAL BIOAVAILABILITY BY THE LYOPHILIZATION METHOD

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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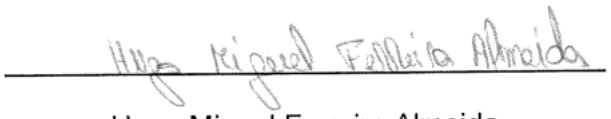
Declaração de Honra

Eu, Hugo Miguel Ferreira Almeida, declaro que a presente tese é da minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos), respeitam escrupulosamente as regras de atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.



Hugo Miguel Ferreira Almeida

Porto, 22/03/2024

List of Publications

Ao abrigo do disposto do nº 2, alínea a) do artigo 31º do Decreto-Lei nº 115/2013 de 7 de agosto, fazem parte integrante desta tese de doutoramento os seguintes trabalhos já publicados ou submetidos para publicação:

All authors of the articles were aware of their use in this thesis and did not raise any objections.

- **Almeida H**, Vieira ACF, Teixeira J, Gomes MJ, Barrocas P, Vasconcelos T. Sarmento B. Cell-Based Intestinal In Vitro Models for Drug Absorption Screening. In: Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays. 2022:1-22.

In this book chapter Amélia Catarina Fernandes Vieira, João Teixeira, Maria João Gomes, Pedro Barrocas and I, were responsible for the conception, bibliographic research and writing. Teófilo Vasconcelos was responsible for supervision and revising. Bruno Sarmento was responsible for supervision, revising and submitting the book chapter. This book chapter has not been included in another thesis.

- **Almeida H**, Ferreira B, Fernandes-Lopes C, Araújo F, Bonifácio MJ, Vasconcelos T, Sarmento B. Third Generation Solid Dispersion through Lyophilization Enhanced Oral Bioavailability of Resveratrol. In: ACS Pharmacology & Translational Science. 2024;7(3):888-898.

In this original paper, I was responsible for the conception, methodology, validation, data analysis, writing and execution. Bárbara Ferreira was responsible for methodology (cell culture), and early proof-reading. Carlos Fernandes-Lopes was responsible for methodology (*in vivo* samples analytical quantification) and early proof-reading, Francisca Araújo was responsible for methodology (rat handling*, in vivo* oral administration and blood sampling) and early proof-reading. Maria João Bonifácio was responsible for supervision and revising. Teófilo Vasconcelos was responsible for supervision and revising. Bruno Sarmento was responsible for supervision, revising and submitting the paper. This paper has not been included in another thesis.

- **Almeida H**, Teixeira N, Sarmento B, Vasconcelos T. Freeze-drying cycle optimization of an amorphous solid dispersion of resveratrol. In: European Journal of Pharmaceutical Sciences. 2024;200. Available online: 17 July 2024.

In this original paper, I was responsible for the conception, methodology, validation, data analysis, writing and execution. Natália Teixeira was responsible for methodology (SEM imaging), and early proof-reading. Teófilo Vasconcelos was responsible for supervision and revising. Bruno Sarmento was responsible for supervision, revising and submitting the paper. This paper has not been included in another thesis.

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Abstract

Most of the new chemical entities are low water-soluble drugs, poorly absorbed after oral administration. Currently one of the major challenges for the pharmaceutical industry is the development of strategies that improve the water solubility of drugs, and consequently their bioavailability.

The current study aimed to develop a drug delivery system of Resveratrol with enhanced oral bioavailability, manufactured by the lyophilization method. Later, this system was included into a tablet formulation for oral administration.

Resveratrol is a natural product that possesses very high antioxidant potential, exhibits antitumor activity, and is considered a potential candidate for prevention and treatment of several types of cancer. Anti-inflammatory, cardioprotective, vasorelaxant, phytoestrogenic and neuroprotective activities have also been reported. Nonetheless, Resveratrol application is still being a major challenge for pharmaceutical industry due to its poor solubility and bioavailability.

The drug delivery system produced improved Resveratrol solubility and bioavailability through the development of a third-generation solid dispersion prepared by the solvent evaporation method (Lyophilization). The experimental work was divided in several steps: selection and optimization of hydrophilic carrier content, selection and optimization of surfactant (metabolism and/or efflux transporter inhibitor) content, optimization of a robust and adequate lyophilization cycle, solid dispersion characterization, stability assessment, tablet formulation development, *in vitro* permeability studies, and finally *in vivo* pharmacokinetic studies.

The third-generation solid dispersion developed has the following composition: Resveratrol:Eudragit E PO (1:2)_Gelucire 44/14 16%. Resveratrol is the API, Eudragit E PO is the hydrophilic polymer. The ratio between API and polymer is 1:2 (w/w). Gelucire 44/14 is present in the formulation as surfactant and at 16% (w/w) of Resveratrol.

The obtained products were characterized for solid state, solubility, dissolution, particle size distribution, particle morphology, porosity, assay, and moisture content. The degree of amorphization in the product was conducted through differential scanning calorimetry (DSC), scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), polarized light microscopy (PLM), and X-ray powder diffraction (XRPD). Solid dispersion produced presented an enhanced solubility and dissolution rate related to the pure drug and physical mixture. The manufacturing process (Lyophilization), allowed to obtain a stable product under accelerated storage conditions with an adequate assay.

Solid dispersion produced was formulated into an oral solid dosage form, (tablet). Tablets including Resveratrol solid dispersion, presented a better dissolution performance compared to the ones manufactured with Resveratrol alone and physical mixture.

The *in vitro* studies were conducted in Caco-2 and Caco-2/HT29-MTX model cell lines with Resveratrol third-generation solid dispersion presenting enhanced intestinal permeability compared to physical mixture and solid dispersion without surfactant (second-generation solid dispersion).

Finally, the pharmacokinetic *in vivo* studies in rat, intended to confirm the achievement of the proposed objectives, allowed to obtain an improved oral bioavailability for Resveratrol in the third-generation solid dispersion compared to physical mixture and second-generation solid dispersion without the surfactant.

Resumo

A maioria das novas entidades químicas são medicamentos pouco solúveis em água e pouco absorvidos após administração oral. Atualmente um dos grandes desafios da indústria farmacêutica é o desenvolvimento de estratégias que melhoram a solubilidade em água dos medicamentos e consequentemente a sua biodisponibilidade. O presente estudo teve como objetivo desenvolver um sistema farmacêutico de libertação de Resveratrol com biodisponibilidade oral melhorada.

O Resveratrol é um produto natural que possui um elevado potencial antioxidante, apresenta atividade anti-tumoral, e é considerado um potencial candidato para a prevenção e tratamento de diversos tipos de cancro. Atividades anti-inflamatórias, cardioprotetoras, vasorelaxantes e fitoestrogénicas também foram relatadas. No entanto, a aplicação do Resveratrol ainda constitui um grande desafio para a indústria farmacêutica devido à sua fraca solubilidade e biodisponibilidade.

O sistema de libertação de fármacos produzido, melhorou a solubilidade e a biodisponibilidade do Resveratrol através do desenvolvimento de uma dispersão sólida de terceira-geração, preparada pelo método de evaporação do solvente (Liofilização). O trabalho experimental foi dividido em várias etapas: seleção e otimização do teor do polímero hidrófilo, seleção e otimização do teor do surfactante (inibidor do metabolismo e/ou dos mecanismos de efluxo), otimização de um ciclo de liofilização robusto e adequado, caraterização da dispersão sólida, avaliação da sua estabilidade, desenvolvimento de uma formulação em comprimido, estudos de permeabilidade *in vitro*, e finalmente estudos farmacocinéticos *in vivo*.

A dispersão sólida de terceira-geração desenvolvida possui a seguinte composição: Resveratrol:Eudragit E PO (1:2)_Gelucire 44/14 16%. O Resveratrol é a substância ativa, e o Eudragit E PO é o polímero hidrófilo, que se encontram numa proporção de 1:2 (m/m) na formulação. O Gelucire 44/14 está presente como surfactante, numa quantidade de 16% (m/m) em relação ao Resveratrol.

Os produtos desenvolvidos foram caracterizados quanto ao estado sólido, solubilidade, dissolução, distribuição do tamanho e morfologia das partículas, porosidade, doseamento do Resveratrol e teor em humidade. O grau de amorfização foi avaliado por calorimetria de varrimento diferencial (DSC), microscopia eletrónica (SEM), espetroscopia de infravermelho (FTIR), microscopia de luz polarizada (PLM) e difração de raio X (XRPD).

A dispersão sólida desenvolvida apresentou maior solubilidade e taxa de dissolução em comparação com o Resveratrol sozinho e com a mistura física. O processo de fabrico (Liofilização), permitiu obter um produto estável e com um teor adequado de Resveratrol em condições de estabilidade aceleradas.

A dispersão sólida desenvolvida foi formulada numa forma farmacêutica solida oral (comprimido). Os comprimidos contendo a dispersão sólida de Resveratrol desenvolvida apresentaram um melhor desempenho em termos de dissolução em comparação com os comprimidos contendo apenas Resveratrol sozinho ou a mistura física.

Os estudos de permeabilidade *in vitro* foram realizados em células Caco-2 e Caco-2/HT29-MTX, com a dispersão sólida de terceira-geração a apresentar uma maior permeabilidade do Resveratrol em comparação com a mistura física e com a dispersão sólida sem surfactante (dispersão sólida de segunda-geração).

Finalmente, os estudos farmacocinéticos *in vivo*, destinados a confirmar o cumprimento dos objetivos propostos neste trabalho, permitiram demonstrar uma biodisponibilidade oral melhorada para o Resveratrol na dispersão sólida de terceira-geração em comparação com a mistura física e com a dispersão sólida de segunda-geração sem o surfactante.

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Abbreviations

- ACN – Acetonitrile
- ANOVA – One way analysis of variance
- API – Active pharmaceutical ingredient
- ATR – Attenuated total reflection
- AUC – Area Under the Curve
- BCS – Biopharmaceutics Classification System
- CE – Circle equivalent diameter
- CRSD – Controlled release solid dispersions
- CYP – Cytochrome P450
- DAD – Diode array detector
- DCS – Developability Classification System
- DSC – Differential Scanning Calorimetry
- DMSO – Dimethyl sulfoxide
- DoE – Design of Experiments
- FDA – US Food and Drug Administration
- FTIR – Fourier transform infrared spectroscopy
- GI – Gastrointestinal tract
- HLB – Hydrophilic-lipophilic balance
- HME – Hot melt extrusion
- HPC – Hydroxypropyl cellulose
- HPLC – High performance liquid chromatography
- HPMC – Hydroxypropyl methyl cellulose
- HPMCAS – Hypromellose Acetate Succinate
- HPMCP – Hypromellose Phthalate
- HS circularity – High sensitivity circularity
- ICH – International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
- LC–MS/MS – Liquid chromatography with tandem mass spectroscopy
- LF – Lyophilized formulation
- MDH – Magnesium dihydroxide
- MW – Molecular weight
- NCs – Nanocrystals
- OM – Optical microscopy

- P_{app} – Apparent permeability
- PEG – Polyethylene glycol
- Pgp – P-glycoprotein
- PLM – Polarized light microscopy
- PM – Physical mixture
- PVP – Polyvinylpyrrolidone
- RES – Resveratrol
- RH – Relative humidity
- SD – Solid dispersion
- SDS – Sodium dodecyl sulphate
- SEDDS – Self-emulsifying drug delivery systems
- SEM – Scanning electron microscopy
- SIRT1 – Sirtuin-1
- SLAD – Solubility-limited absorbable dose
- SMEDDS – Self-micro emulsifying drug delivery systems
- SNEDDS – Self-nano emulsifying drug delivery systems
- SULT – Sulfotransferase
- TBA – *Tert*-butanol
- T_c – Critical collapse temperature
- TEER – Transepithelial Electrical Resistance
- T_g – Glass transition temperature
- T_g'– Glass transition temperature in the frozen state
- T_p – Target product temperature
- UGT – Uridine diphosphate-glucuronosyltransferase
- USP – United States Pharmacopoeia
- UV – Ultraviolet
- XRPD – X-ray powder diffraction

I. Introduction

Oral administration is the most popular route in the pharmaceutical industry, regarded as the simplest, and most effective form of drug administration. The success of this route is explained for being the safest, most convenient, and most economical method of drug delivery. Additionally, its ease of production, great stability, dosing accuracy and ease of administration, leads to increased compliance to therapy by patient (1).

However, this route also has drawbacks, such has the great variability passage time along the gastrointestinal tract, the liver first pass effect, and the fact that absorption is limited in the lower portion of the gastrointestinal tract (2, 3).

Recent combinatorial chemistry, computational molecular modelling, and high throughput screening in drug discovery have significantly increased the number of poorly soluble drugs. Drug solubility and bioavailability enhancement are the important challenges in the field of formulation of pharmaceuticals (3-5).

The absorption of drugs from the gastrointestinal tract can be limited by several factors with the most important contributors being poor aqueous solubility and/or poor intestinal permeability of the drug molecule (4). While delivering an active agent orally, it is mandatory that it must dissolve in gastric and/or intestinal fluids before it can reach systemic circulation through gastrointestinal membrane permeability. Hence, a drug with poor aqueous solubility will exhibit dissolution rate limited absorption, and a drug with poor membrane permeability will basically exhibit permeation rate limited absorption. The formulation challenge will focus on improving the oral bioavailability of the active agents by enhancing solubility and dissolution rate of poorly water-soluble drugs and increasing the permeability of poorly permeable drugs (6-9).

Traditional pharmaceutical processes may not be used for the development of dosage forms of poorly water-soluble drugs. New technologies are needed to improve their solubility, dissolution, and bioavailability. Several factors that influence the bioavailability of the drug include the gastric emptying rate, physiochemical properties of the drug, drug formulation type, enzymes induction/inhibition by other drugs/foods, circadian differences, transporters, diseased state or health of the gastrointestinal tract (6, 10). As the content of gastrointestinal tract is aqueous in nature, hence a drug with poor aqueous solubility possesses low saturation solubility, which is correlated with a low dissolution rate, resulting in a poor oral bioavailability. About 60% of drugs coming directly from synthetic origin have solubility below 0.1mg/mL (6, 10-16).

Therefore, one of the biggest challenges that the pharmaceutical industry currently faces, consists of developing strategies to improve the solubility of these drugs in water, thus improving their bioavailability.

1. Biopharmaceutical classification system of drugs

The therapeutic effect of drugs depends on their concentration at the site of action. Drug absorption into the systemic circulation is a prerequisite for all drugs reach the site of action, except those for topical application, or those that are administered intravenously. After oral administration and circulation in the gastrointestinal tract, many factors determine the fraction of drug that reaches the systemic circulation, i.e., its bioavailability (17).

Since only the dissolved drug can cross the gastrointestinal membrane, dissolution is one of those factors. However, drug metabolism in the intestinal lumen, the intestinal wall and the liver, may also reduce its bioavailability. In general, the rate of absorption is determined by the solubility/dissolution of the drug and its subsequent first pass effect and by transport through the intestinal membrane (permeability). These two aspects form the basis of the Biopharmaceutical Classification System (BCS), for immediate release solid oral dosage forms which is an integral part of the Food and Drug Administration (FDA) (17-19). This system divides drugs in 4 classes, depending on its aqueous solubility and gastrointestinal permeability. The aim is to predict the *in vivo* pharmacokinetic behaviour of drugs from measurements of their *in vitro* solubility and permeability. The Biopharmaceutical Classification System of drugs is presented in Figure 1.

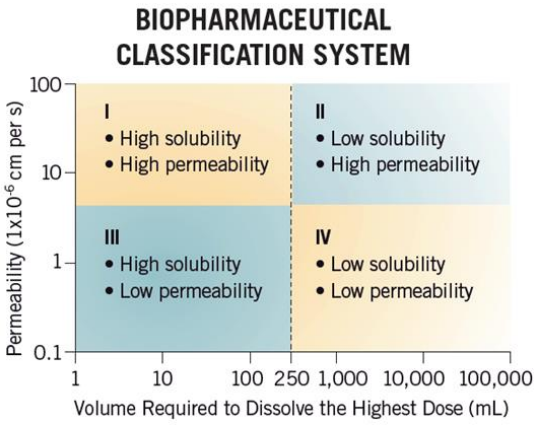


Figure 1: Biopharmaceutical Classification System (from (18)).

Drugs belonging to class I dissolve quicky in aqueous media and are rapidly transported across the intestinal membrane, not requiring strategies to increase its absorption. When drug release from formulation is slower than the gastric filling rate, good *in vitro*/*in vivo* correlation can be obtained. The absorption rate of class II drugs can be improved by accelerating dissolution. Class II drugs have a good *in vitro*/*in vivo* correlation, from the moment that the *in vivo* dissolution is equal to the *in vitro*. However, because the dissolution rate is critical for class II compounds, the *in vivo* absorption can be affected by several physiological fluctuations, such as the volume and pH of intestinal fluids, the presence of bile, salts, food, enzymes, bacteria, the mobility, and viscosity of the intestinal lumen. For class III drugs, absorption is limiting, and *in vitro* dissolution experiments cannot be used to predict *in vivo* uptake. Also, for class IV drugs, no *in vitro*/*in vivo* correlation can be expected. In this case the challenge is to try to improve the absorption rate, but also improve the *in vitro*/*in vivo* correlation. This will decrease variability between patients and improve bioavailability and predictability of pharmacokinetic parameters.(17-20)

Depending on the BCS drug classification, different strategies can be applied to increase or accelerate drug absorption. Increased permeability across the intestinal membrane, or increased amounts of dissolved drug which is in contact with the intestinal membrane. **Class I drugs** do not require a formulation strategy to increase absorption (17-19, 21).

The strategy for **class II drugs**, presenting dissolution limitations, but non-absorption, involves increasing the amount of drug molecules at the site of absorption. This is a good strategy if there are no permeability limitations. When, for example, the drug is transported through the membrane by passive diffusion, the flux across the membrane increases proportionally to the concentration of drug at the site of absorption. However, when the drug is transported by a carrier, this is not the case, because the transport capacity can limit the rate of absorption (17-23).

For **class III drugs**, passage through the membrane limits the rate of absorption. The strategy in this case is to increase the permeability of the membrane of absorption. The strategy depends on the mechanism of transport across the absorption membrane, i.e., transcellular, paracellular or matrix mediated (17-23).

In case of **class IV drugs**, both dissolution and permeability have to be improved. However, increasing dissolution is more effective than increasing permeability, because in practice, the amount of drug dissolved at the site of absorption varies by an order of 6-fold in magnitude (0.1 µg/l to 100 mg/l), while the permeability presents a very small

range of variation. In this way, the potential to enhance absorption by increasing drug concentration, passes by increasing the solubility, even if the permeability is later compromised (17-23).

2. Developability classification system

The Biopharmaceutical Classification System (BCS) correlates *in vitro* solubility and permeability with the potential *in vivo* performance of drugs. This system was originally used by US Food and Drug Administration (FDA), as the basis for biowaiver applications for immediate release formulations to reduce the need for additional *in vivo* studies for bioavailability and bioequivalence. Since the introduction of the BCS, the solubility landscape of market drugs continues to evolve. Approximately 40 to 75% of drugs in current development pipelines can be considered to have either poor bioavailability, nonlinear pharmacokinetics, or a significant food effect on drug bioavailability due to low solubility (17, 24).

In 2010, the Developability Classification System (DCS) was created. This system was proposed to tighten the gap between the Biopharmaceutical Classification System, which is aiming at guiding regulatory decisions about well-characterized drugs, and the need for early evaluation of drug candidates with respect to their suitability for oral delivery. The DCS applied solubility in fasted state simulated intestinal fluid to estimate intestinal solubility, assessed the compensatory nature of permeability and solubility during oral absorption and providing a way of estimating the critical particle size at which dissolution becomes rate-limiting to absorption. BCS class II was divided into two sub-categories: DCS class IIa and DCS class IIb (20, 24-26).

Another modification was the shift in dose/solubility ratio, resulting in a lower threshold for a molecule to be considered *soluble*. Consequently, the appearance of the DCS is different than the BCS, with the cut-off between DCS I and III and DCS IIa/b and IV appearing at a dose/solubility ratio of 500, as opposed to 250 in the BCS. The developability classification system of drugs is presented in Figure 2.

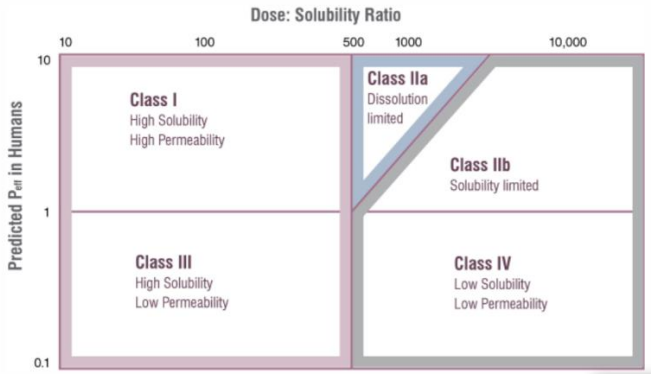


Figure 2: Developability Classification System (from (26)).

The line that separates DCS IIa and IIb, is the solubility-limited absorbable dose (SLAD) line. It uses a ratio of solubility/permeability to determine if an increase in dissolution rate will have a measurable impact on overall absorption. Molecules above the SLAD are dissolution rate limited (DCS class IIa). Theoretically, if the maximum solubility is reached faster, absorption of the molecule can be improved. In this case formulation development should focus on the dissolution rate. Molecules below SLAD line (DCS class IIb) have such a low solubility that independent how quick the drug gets into solution, there will be no impact on absorption. The crucial challenge for class IIb molecules is therefore solubility (24, 27).

The value of having two new classifications to guide development of solubility enhanced formulations is clear. A coarser understanding of the root cause of solubility limitations, whether driven by dissolution rate or intrinsic solubility, can be used to guide design of optimized formulations. Figure 3 provides a workflow for selecting some of the best formulation strategies based on the DCS class of the molecule (24, 26, 27).

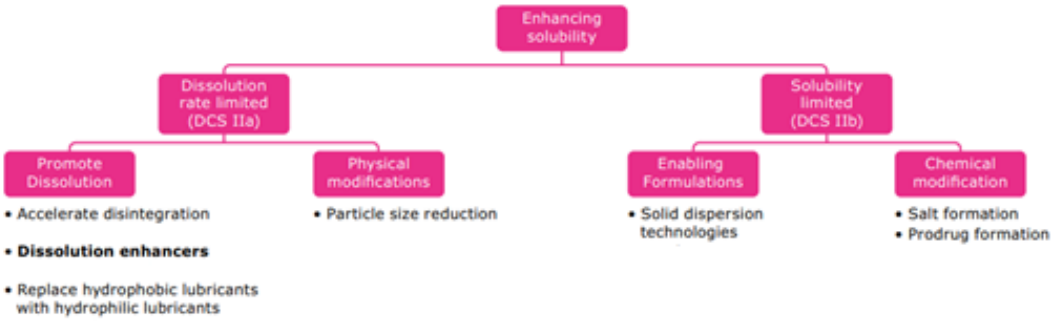


Figure 3: Formulation strategies for poorly soluble molecules (from (25)).

For dissolution rate-limited compounds, DCS IA, if the dissolution rate can be improved, a very simple and effective formulation can be developed. This could be achieved either by particle size reduction, accelerating disintegration, incorporating wetting agents or surfactants, or replacing hydrophobic excipients with hydrophilic excipients to reduce the retardation effect of excipients on the formulation. For molecules DCS IIb, solubility limited compounds, the formulation must address the intrinsic solubility. This could require modification of the chemical structure of the molecule or of the solid state of the molecule using solid dispersion technologies such as melting method, like hot melt extrusion (HME), or solvent evaporation methods such as spray drying, lyophilization or use of carriers such as mesoporous silica (24, 26-28).

3. Absorption vs Bioavailability

Although closely related, these 2 terms are essentially different as detailed in Table 1.

Table 1: Differences between absorption and bioavailability (adapted from (29)).

Absorption	Bioavailability
Strongly related to the dosage form	Related to the dosage form and to clearance
Sometimes related to the therapeutic effect	Highly related to the therapeutic effect
Dependent on the transport across the membrane	Dependent on absorption and blood clearance

Absorption refers to transport across the absorption membrane at the site of administration, while bioavailability is related to the concentration of the drug on the systemic bloodstream. When the drug concentration in plasma is measured as a function of time, the area under the curve represents the absolute bioavailability. Relative bioavailability, often referred in absorption studies, is calculated based on the comparison with the bioavailability after intravenous administration (29-31).

Bioavailability is affected by intestinal and hepatic blood clearance of the drug. When the clearance rate is linearly dependent on the blood concentration, absorption and bioavailability are proportionally correlated. However, when the clearance process occurs by active secretion or enzymatic metabolism, saturation may occur. In this case the intestinal and hepatic clearance depends on the blood concentration in a nonlinear

way, resulting in a nonlinear pharmacokinetics. When in this case, the drug absorption is increased to a certain extent, bioavailability may increase more than proportionately. Thus, a doubling absorption may result in more than the double bioavailability (29-31).

4. Low soluble drugs

The therapeutic effect of a drug depends on its bioavailability and solubility of its molecules. Solubility is one of the most important parameters to achieve the desired concentration of drug in the systemic circulation to obtain a pharmacological response. Currently only approximately 8% of the new drugs possess both high solubility and high permeability.

The solubility of a solute is the maximum amount of solute that can dissolve in a given amount of solvent or solution at a given temperature. In other words, solubility can also be defined as the ability of a substance to form a solution with another substance. The substance to be dissolved is called the solute, and the liquid in which the solute dissolves is called a solvent, which together form a solution. The solubility of a drug is a key parameter in preformulation studies (32-34). The substances can be classified according to their solubility as presented in Table 2.

Table 2: Solubility of substances (adapted from (33)).

Descriptive term	Approximate volume of solvent in millilitres per gram of solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10000
Practically insoluble	10000 and over

4.1. Solubilization process

The solubilization process involves breaking inter-ionic or inter-molecular bonds in the solute, and separation of solvent molecules to provide space to the solute in the solvent (Figures 4 and 5), and interaction between the solvent and the solute molecule or ion (Figure 6) (32).

Figure 4: Opening of spaces in the solvent.

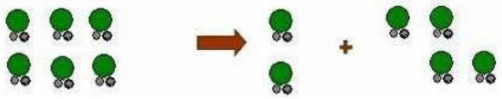


Figure 5: Separation of solid molecules.

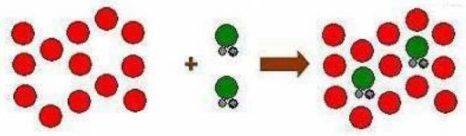


Figure 6: Interaction between solid molecules and solvent.

4.2. Factors affecting solubility

The **size of the solid particles** influences the solubility. As the particle gets smaller, the surface area/volume ratio increases. A large surface area allows for greater interaction with the solvent (32, 35).

Temperature affects solubility. If the dissolution process absorbs energy, the solubility increases as the temperature increases. If the dissolution process releases energy, solubility increases with increasing temperature. Generally, an increase in dissolution temperature increases the solubility of a solid solute. For gases, solubility decreases, as the solution temperature increases (32, 35).

In the case of gaseous solutes, an increase in **pressure**, increases solubility, and a decrease in pressure, decreases solubility. For liquid and solid solutes, changes in pressure have virtually no effect on solubility (32, 35).

Molecular size affects solubility. The larger the molecule, or the larger its molecular weight, the less soluble the substance. Large molecules are more difficult to envelop by solvent molecules (32, 35).

The **polarity** of solute and solvent molecules will affect solubility. Generally non-polar solute molecules dissolve in non-polar solvents, and polar solute molecules dissolve in polar solvents. The polar solute molecules have a positive and negative terminal. If the solvent molecule is also polar the positive terminals of solvent molecules will attract the negative terminals of solute molecules. This is a type of inter-molecular force, known as dipole-dipole interaction. All molecules also possess a type of inter-molecular force that is much weaker than the other forces, called the London dispersion force, in which the cations of the solute atoms attract the electrons of the atoms of the solvent molecules. This allows the non-polar solvent a chance to solvate the solute molecules.

The ability of a substance to crystallize into more than one crystalline form is called **polymorphism**. It is possible that all crystals can crystallize in different forms or polymorphs. If the change from one polymorph to another is reversible, the process is called enantiotropic. If the system is monotropic, there is a transition point above the melting point of both polymorphs. They cannot convert into one another without going through a phase transition. Polymorphs can vary in melting point. As the melting point of the solid is related with its solubility, then the polymorphs will have different solubilities. Generally, the range of solubility differences between different polymorphs is only 2-3 degrees, due to relatively small differences in free energy (32, 35-37).

5. Improve the solubility of low soluble drugs

There are several technologies that have been successfully employed to enhance the solubility, dissolution, and bioavailability of poorly water-soluble drugs. Particle size reduction by micronization or nanosuspensions; lipid-based delivery systems; inclusion complexes; and solid dispersions by spray drying, lyophilization or hot melt extrusion are some of the technologies employed to overcome drug solubility issues (38-41).

5.1. Physical modifications

5.1.1. Particle size reduction

Particle size reduction techniques decrease the particle size to the micron range thus increasing substantially the exposed surface area of a determined amount of powder. The **micronized powder** can then be further engineered, and its particle size further decreased to the nanosized range, when the surface area increases sharply. Milled drug particles oftentimes are cohesive and exhibit poor flow properties, largely due to their higher surface energies. Apart from size, milling also alters the structure roughness and shape of particles. Furthermore, milled particles are rarely isometric or spherical in shape (10, 38, 42, 43).

Nanosuspensions are colloidal dispersions of pure drug nanoparticles, which are stabilized by surfactants. The advantages offered by nanosuspensions are the increased dissolution rates due to the large, exposed surface area (40, 44).

Recrystallization of poorly soluble materials using liquid solvents and antisolvents has also been successfully employed to reduce the size of the particle.

Sonocrystallization uses ultrasonic force, characterized by a frequency range of 20-100 kHz to induce crystallization. Improves not only the nucleation rate, but it has also effect in controlling and reducing the drug particle size (40, 45).

Supercritical fluids are fluids whose temperature and pressure are greater than the critical temperature (Tc) and critical pressure (Tp). These are highly compressible, allowing moderate changes in pressure, to greatly alter the density and mass transport characteristics of the fluid, which determines its solvent power. Once dissolved by supercritical fluids, drug particles can recrystallize with very small particle sizes (40, 46).

Spray drying is a drying method commonly used to dry a liquid sprayed by a current of hot gas. This is usually air, but sensitive materials as pharmaceuticals, and solvents such as ethanol, require on oxygen-free drying gas, being nitrogen normally used. The liquid flow rate varies according to the material to be dried, and can be a solution, a colloid, or a suspension. This drying process is a fast one-step process and eliminates additional processing (40).

5.1.2. Crystal habit modification

Polymorphism is the ability of an element or compound to crystallize into more than one crystalline form. Different drug polymorphs are chemically identical, but have different physical properties, including solubility, melting point, density, texture, stability, etc. Polymorphs can be classified as enantiotropic or monotropic based on their thermodynamic properties. Once characterized in one of these categories, is researched the existence of metastable forms of the crystal. These are associated with greater energy, and consequently greater solubility. Similarly, the amorphous form of the drug is always more suitable than the crystalline form, due to a higher associated energy and an increased surface area (8, 40, 45).

Generally, the anhydrous form of a drug has greater solubility than the hydrated form. This is because the hydrated forms are already in interaction with the water, thus having less free energy for crystals rupture, in comparison with anhydrous forms possessing a higher thermodynamic energy state, for additional interaction with water (8, 40, 45).

Some drugs may exist in an amorphous form, without an internal crystalline structure. These drugs represent the highest energy state and can be considered as overcooled liquids. They have greater aqueous solubility than crystalline forms because require less energy to transfer a molecule to the solvent (8, 40, 45).

The dissolution order of different solid forms of a drug is from higher to lower, amorphous form, metastable polymorph, and stable polymorph.

Melting followed by rapid cooling or recrystallization from different solvents can produce metastable forms of a drug (8, 40, 45).

5.1.3. Drug dispersion in polymers

The term **solid dispersions** refer to the dispersion of one or more active substances in an inert polymer in the solid state, often prepared by the melting method, solvent evaporation method or melting/evaporation solvent method. Preparation techniques include extrusion, spray drying, rapid precipitation by freeze-drying/lyophilization and the use of supercritical fluids. Some of the most common hydrophilic polymers in solid dispersions include Polyvinylpyrrolidone (PVP), Polyethylene glycol (PEG) or Hydroxypropyl methylcellulose (HPMC). Surfactants, such as Tween 80, Sodium lauryl

sulfate (SDS), Sodium docusate, Myrj-52, Pluronic-F68, etc, can also be used in the formation of solid dispersions (47, 48).

5.1.4. Complexation

Complexation is the association between two or more molecules to form an “unbound” structure, with a well-defined stoichiometry. Complexation relies on relatively weak forces such as London dispersion forces, hydrogen bounds and hydrophobic interactions.

Inclusion complexes are formed by the insertion of a non-polar or through the non-polar region of one molecule (guest), into the cavity of another molecule, or group of molecules (host). The cavity of the host molecule must be big enough to accommodate the guest, and small enough to eliminate the water, so that the total contact between the water and the non-polar regions of the host and guest is reduced (49).

The most frequently used hosts are cyclodextrins. These consist of glucose monomers, with a donut or ring-shaped rearrangement. There are 3 natural cyclodextrins, α-cyclodextrins, β-cyclodextrins and γ-cyclodextrins.

Complexation with cyclodextrins can be used to improve the solubility of drugs. Inclusion in cyclodextrins is a molecular phenomenon in which a host molecule (or part of it) interacts with the cavity of a host cyclodextrin molecule, to become trapped and form a stable association. The internal surface of the cavity is hydrophobic, and the outside is hydrophilic, due to the arrangement of its hydroxyl groups in the molecule. The cavities size, as well as possible chemical modifications, determine the affinity of the various cyclodextrins for the various molecules (49-51).

Drug-cyclodextrin inclusion complexes can be prepared by coprecipitate, spray drying and lyophilization methods. Limiting factors to be considered are drug loading, dosage form, design, and manufacturing process scale-up (49-51).

5.1.5. Solubilization by surfactants

Surfactants are molecules with distinct polar and non-polar regions. Most surfactants consist of a hydrocarbon segment attached to a polar group. When small non-polar molecules are added they can accumulate in the hydrophobic core of micelles.

This solubilization process is very important in biological and industrial processes. The presence of surfactants can reduce surface tension and increase drug solubility in the organic solvent (52).

A **microemulsion** is a 4-component system, consisting of an external phase, an internal phase surfactant and co-surfactant. The addition of the surfactant, which is predominantly soluble in the internal phase, unlike the co-surfactant, results in the formation of an optically clean emulsion, isotropic and thermodynamically stable. It is called a microemulsion because the internal or scattered phase is less than 0.1 µm in diameter. The formation of the microemulsion is spontaneous and does not involve the addition of external energy. Surfactant and co-surfactant alternate between them, forming a mixed film at the interface, contributing for the stability of microemulsions (53, 54).

5.1.6. Lipid based approach

Lipid based formulations have been evaluated to enhance the solubility and bioavailability of poorly water-soluble drugs for several years. A critical early step is to establish the solubility range of the drug in various lipids. Once formed, the drug/lipid mixture can sustain drug concentrations in targeted environments. Lipid formulations produce complex structures comprising micelles, micro or nanostructures, which are directly responsible for delivering the drug. Most of the lipid-based formulations are filled into soft or hard gelatine capsules. Lipid based formulations are generally liquids but may also be solid or semi solid at room temperature when high melting lipids are used or when lipids are adsorbed onto a carrier. The excipients employed to form lipid-based formulations are generally from three categories – lipids, surfactants and co-solvents. Self-emulsifying drug delivery systems (SEDDS), SMEDDS (micro) and SNEDDS (nano), were explored in many studies as an efficient approach for improving the dissolution rate and bioavailability of poorly water-soluble drugs. One of the greatest advantages of incorporating poorly soluble drugs into such formulations is their spontaneous emulsification and formation of an emulsion, microemulsion or nanoemulsion in aqueous media. On the other hand, they are more complex to formulate due to many variables that must be considered (53-55).

5.2. Chemical modifications

For ionizable organic solutes, system pH modification may be the most simple and effective way to increase the aqueous solubility. Under appropriate conditions, the solubility of ionizable drugs can increase exponentially by adjusting the pH of the solution. For a drug to be efficiently solubilized by a pH value control, it must be a weak acid with a low pKa, or a weak base with a high pKa. As well as the lack of effect of heat on the solubility of non-polar substances, there is a small effect of pH on non-ionizable substances. Non-ionizable hydrophobic substances, may have improved solubility by changing the dielectric constant of the solvent, more due to the use of co-solvents, than by controlling the pH of the solvent. The use of saline forms is a well-known technique to improve the dissolution profile of a substance. Salt formation is the most effective and common method to increase solubility and dissolution rate of acidic and alkaline drugs (56, 57).

5.3. Nanotechnology approach

Nanotechnology refers to the study and use of materials and structures at the nanoscale of approximately 100 nanometers or less. For many new low-soluble chemical entities, improving oral bioavailability by micronization is not enough because the micronized product tends to agglomerate, which leads to an effective decrease in the surface area for dissolution.

Nanocrystallization is defined as a process of decreasing the size of drug particles to a size between 1-1000 nanometers. This can be applied to any drug. There are two different methods for nanocrystals production: “bottom-up” and “top-down” development.

The “top-down” methods (grinding and high-pressure homogenization), start the grinding at a macroscopic level, i.e., from a powder that is micronized. In “bottom-up” methods, (precipitation and cryo-vacuum), nanomaterials are chemically composed starting from atomic and molecular components (58, 59).

6. Solid dispersions

6.1. Definition, classification, and general considerations

The term solid dispersion (SD), refers to a group of solid products, consisting of at least two different components, usually a hydrophilic matrix/carrier and a hydrophobic drug. The matrix can be crystalline or amorphous. The drug can be molecularly dispersed in amorphous particles (clusters), or in crystalline particles (8, 16, 60, 61).

Solid dispersions are one of the most successful strategies to improve drug release of poorly water-soluble drugs as described by Vasconcelos et al. (16). They enhance the oral absorption of poorly water-soluble drugs by attaining and sustaining a supersaturated concentration of drug in the gastrointestinal fluid. Formulation of poorly water-soluble compounds as solid dispersions might lead to particle size reduction, improved wettability, reduced agglomeration, changeability in the physical state of the drug molecules and possibly a dispersion in the molecular level, according to the physical state of the solid dispersion. Solid dispersions may be prepared by melting, solvent evaporation, and combined melting/solvent evaporation methods. Solid dispersions change the physical state of the drug from crystalline to amorphous. Normally, the amorphous version of a compound is more soluble in water, more hygroscopic, present improved bioavailability and is thermodynamic instable compared to its crystalline form. Instability due to moisture and temperature are some of the drawbacks of solid dispersions, that may cause drug return to the crystalline state and dissolution decrease with aging. Hot melt extrusion, spray drying and freeze-drying/lyophilization are common methods to prepare solid dispersions (8, 16, 60, 61).

Based on its physical state and molecular arrangement of both API and carrier, six different types of solid dispersions can be distinguished as presented in Table 3 (8, 15, 60, 62-64).

Table 3: Classification of solid dispersions based on its physical state and molecular arrangement (adapted from (60)).

Type of solid dispersion		Matrix*	Drug**	Observations	Nº of phases
I	**Eutectic mixture**	C	C	First type of solid dispersions	2
II	**Amorphous precipitations in crystalline matrix**	C	A	Rarely found	2
III	**Solid solutions**				
	Continuous solid solutions	C	M	The components are miscible in all proportions. Never prepared or reported	1
	Discontinuous solid solutions	C	M	Partially miscible; 2 phases (although the drug is molecularly scattered)	2
	Substitutional solid solutions	C	M	Molecular diameter of the drug (solute) differs less than 15% from the diameter of the matrix (solvent). In this case, the drug and the matrix are substituents. They can be continuous or discontinuous: 2 phases (despite drug molecularly dispersed)	1 or 2
	Interstitial solid solutions	C	M	Diameter of drug molecules (solute) less than 59% of the diameter of matrix molecules (solvent) Usually limited and discontinuous miscibility	2
IV	Vitreous suspension	A	C	Particle size of the dispersed phase dependent on the rate of cooling/evaporation Obtained after drug crystallization in an amorphous matrix	2
V	Vitreous suspension	A	A	Particle size of the dispersed phase dependent on the rate of cooling/evaporation. This type of solid dispersions is very frequent	2
VI	Vitreous solution	A	M	Requires solid miscibility/solubility. Complexes formation after fast cooling/evaporation during preparation. There are many examples, especially with PVP	1

* A: matrix in the amorphous state; C: Matrix in the crystalline state

** A: drug dispersed in the matrix as an amorphous cluster; C: drug dispersed as crystalline particles in the matrix; M: drug molecularly dispersed through the matrix

In several studies, solid dispersions designation is based on the preparation method. However, given that different methods may result in the same subtypes, or similar preparation methods may result in different subtypes, it can be argued that solid dispersions should be preferably named according to their molecular arrangement. Furthermore, it is not the preparation method, but the molecular arrangement, that determines solid dispersions properties. Accelerated dissolution of hydrophobic compounds using molecular dispersions are commonly referred, but the mechanisms that are the basis for that improved behaviour are rarely discussed (47, 48, 53).

Formulation of poorly water-soluble compounds as solid dispersions might lead to particle size reduction, improved wetting, reduced agglomeration, changeability in the physical state of the drug molecules and possibly a dispersion in the molecular level, according to the physical state of the solid dispersion. The most important reason for this fact, is the lack of knowledge about the hydrophobic drug incorporation into the matrix, despite numerous efforts to clarify it. Knowing whether the drug is present in a crystalline form, as amorphous nanoparticles, or molecularly dispersed through the matrix is rarely discussed. These 3 situations result in different drug concentrations in the dissolution interface, not have been clarified yet, how this affects the dissolution behaviour of solid dispersions. Secondarily, physical, and chemical stability of the incorporated drug or matrix, depends on the mode of incorporation. For example, if drug molecules are in the form of amorphous nanoparticles, crystallization only needs a rotational rearrangement. On the other hand, for a molecularly dispersed drug, it is necessary translational diffusion before crystallization can occur by rotational rearrangements. The physical state of the matrix is also important for the chemical stability of the drug. Matrix crystallinity influences rotational rearrangements and translational effects of the drug, necessary for the degradation reactions. Finally, the influence of drug loading and preparation method on the dissolution behaviour and stability of solid dispersions, can only be predicted and understood, when the relation between these characteristics and the incorporation mode is known (8, 16, 47, 48, 53, 60, 65).

6.2. First-generation solid dispersions

The first solid dispersions appeared in 1961 with Sekiguchi and Obi (66, 67), having found that the formation of eutectic mixtures improved the release rate of the drug and consequently the bioavailability of poorly water-soluble drugs. The small particle size and

the greater wettability of the drug were the main reasons for the increase in the bioavailability of the solid dispersions that used urea as matrix.

Later, Levy (68), and Kaning (69), developed solid dispersions that contained mannitol as matrix, preparing solid solutions through molecular dispersions. The improvements were attributed to faster dissolution of the matrix, releasing microcrystals or drug particles. These solid dispersions designated first generation solid dispersions were prepared using crystalline matrices such as urea and sugars. They had the disadvantage of forming crystalline solid dispersions, which were thermodynamically more stable and did not release drug as quickly as amorphous ones (8, 32, 53, 70).

6.3. Second-generation solid dispersions

At the end of the 1960s, the second-generation of solid dispersions appeared. These contained amorphous rather than crystalline matrices, in which drugs are molecularly dispersed in an irregular fashion (71, 72).

Polymeric matrices are the most successful because they allow the formation of amorphous solid dispersions. They are divided into fully synthetic polymers and synthetic polymers based on natural products. Among the first is povidone (PVP), polyethylene glycols (PEG) and polymethacrylates. The seconds are mainly cellulose derivates, such as hydroxypropyl methylcellulose (HPMC), ethyl cellulose, hydroxypropyl cellulose, or starch derivates such as cyclodextrins (73-75).

Amorphous solid dispersions can be classified based on the interaction between drug and matrix, in solid solutions, solid suspensions or a mixture of both (76).

In amorphous solutions, the drug and the solute are completely miscible and soluble, originating a homogeneous interaction between them. These systems have a high interaction energy, which results in the formation of a true solution.

The use of polymers in the preparation of a true solid solution allows the creation of an amorphous product in which the crystalline drug is dissolved. This type of amorphous solid dispersion is homogeneous at the molecular level, and as such, only one phase is present (73, 76).

The formation of amorphous solid dispersions occurs when the drug has limited solubility in the matrix or a very high melting point. The obtained dispersion does not show a homogeneous molecular structure, being composed by two phases. When the drug is

simultaneously dissolved and suspended in the matrix, a heterogeneous structure with mixed properties of solution and amorphous solid suspension is obtained (16, 74, 75).

In second-generation solid dispersions, the drug is in its saturated state, due to the forced dissolution in the matrix. These systems make it possible to reduce the particle size at an almost molecular level, solubilize or co-dissolve the drug, improve its wettability and dispersibility, and produce amorphous forms of the drug and the matrix (64, 77).

6.4. Third-generation solid dispersions

The third-generation solid dispersions emerged with the realization of an improvement in the dissolution profile, in cases where the matrix has surface activity or self-emulsifying properties. These contain a surfactant matrix, or a mixture of amorphous polymers and surfactants as carriers. With this approach, the aim is to reach the maximum bioavailability for poorly water-soluble drugs and stabilize the solid dispersion, preventing drug recrystallization (16, 62, 64, 76).

The use of surfactants such as inulin, Compitrol® 888 ATO, Gelucire® 44/14, and Poloxamer® 407, demonstrated efficacy in producing high polymorph purity and in the improvement of bioavailability. The inclusion of surfactants in the formulation containing a polymeric matrix can help prevent precipitation or protect a fine crystalline precipitate from agglomeration into much larger hydrophobic particles (10, 16, 62, 64, 76, 78-80).

6.5. Fourth-generation solid dispersions

Different from traditional solid dispersion for improving the dissolution rates of poorly water-soluble drugs, the upgraded fourth-generation solid dispersion was developed to furnish a drug sustained-release profile (70, 81). These types of dispersions can be referred as controlled release solid dispersions (CRSD). It contains poorly water-soluble drug with a short biological half-life (70, 73, 82).

The carriers used are either water-soluble carriers or insoluble water carriers. Solubility enhancement and extended drug release in a controlled manner are the two targets in controlled-release solid dispersion. The water-soluble carriers used in CRSD are ethyl cellulose, Eudragit RS, Eudragit RL, HPC, etc (70, 73, 76, 82, 83).

6.6. Solid dispersions advantages vs other strategies of solubility enhancement

Water solubility changes through chemical or formulation approaches, have allowed an improvement in the bioavailability of drugs (39, 84, 85).

Chemical approaches include the formation of salts and the incorporation of polar or ionizable groups in the drug structure, resulting in the formation of a prodrug. Salt formation can only be applied to drugs that are weak acidic or alkaline and not for neutral drugs. Furthermore, sometimes the salt formation does not allow to obtain better bioavailability, due to its conversion in acidic or alkaline forms (86, 87).

This type of approach has the great disadvantage of the need to carry out clinical trials, given that are considered new chemical entities (11).

Solid dispersions seem to present a better approach to increase the solubility of drugs than those techniques, given their greater ease of production and applicability. Solid dispersions are more accepted than solubilization products, since they give rise to solid oral dosage forms, instead of liquid forms, as is usually the case with solubilization products (86, 87).

Grinding and micronization are techniques commonly used to reduce the particle size, and enhance solubility, based on a surface area increase (10, 38, 40). Solid dispersions are more efficient than these particle size reduction techniques, as they allow a size reduction of particles that stays at 2-5 µm, which is often not enough to greatly improve drug solubility or drug release in the small intestine, and consequently improve bioavailability.

Furthermore, powders with such a small particle size have poor mechanical properties, i.e., poor flowability and high adhesion, being extremely difficult to handle (43, 86, 87).

6.7. Solid dispersions advantages

Solid dispersions present amorphous particles with reduced particle size, improved wettability, and great porosity.

In solid dispersions, after matrix dissolution, the drug is molecularly dispersed in the dissolution medium. They allow the formation of a **large surface area**, resulting in an increased dissolution rate and consequently increased bioavailability (8, 88, 89).

Solid dispersions **improve wettability** characteristics, increasing thus the solubility of drugs (86). The matrices can influence drug dissolution profile by direct dissolution or by its co-solvent effect (10, 90). The inclusion of **surfactants** in the third-generation of solid dispersions reinforced the importance of this property (91, 92).

Particles in solid dispersions present **higher porosity**. The increase in porosity also depends on the matrix properties. Solid dispersions containing linear polymers produce larger and wider particles than those with reticular polymers, thus present a higher dissolution rate (89).

Crystalline drugs that are poorly soluble in water, when in the **amorphous state**, present higher solubility (93). Improved drug delivery can usually be achieved using the drug in the amorphous state, because no energy is required to break the crystalline arrangement during the dissolution process. In solid dispersions, drugs are found in super-saturated solutions, after system dissolution, being speculated that if drugs precipitate, it is in a polymorphous metastable form with higher solubility than the most stable crystalline form (8, 86, 92).

6.8. Solid dispersions disadvantages

Solid dispersions are not very common in commercial products, mainly because there is a possibility that during the process (**mechanical stress**), or storage (**temperature** and **humidity**), the **amorphous state of the drug becomes crystalline**. The effect of humidity on the stability of amorphous solid dispersions during storage can increase the drug mobility and promote its crystallization. Furthermore, most polymers used in solid dispersions can absorb moisture, which can result in phase separation, crystals growth, conversion of the amorphous to the crystalline state, or conversion of a metastable crystalline form into a more stable structure during storage. This may result in a solubility and dissolution rate decrease. The exploration of solid dispersions great potential requires their solubilization in the solid state, as well during their *in vivo* performance (92, 94, 95).

Another disadvantage is scale-up difficulties to higher scales, namely industrial commercial production.

6.9. Physical stability of amorphous solid dispersions

The dissolution behaviour of solid dispersions must remain unchanged during storage, maintaining its physical state and molecular structure. For optimal amorphous solid dispersion stability, molecular mobility should be as low as possible, however partially or totally amorphous solid dispersions are thermodynamically unstable. Figure 7 shows the physical changes that can occur in solid dispersions.

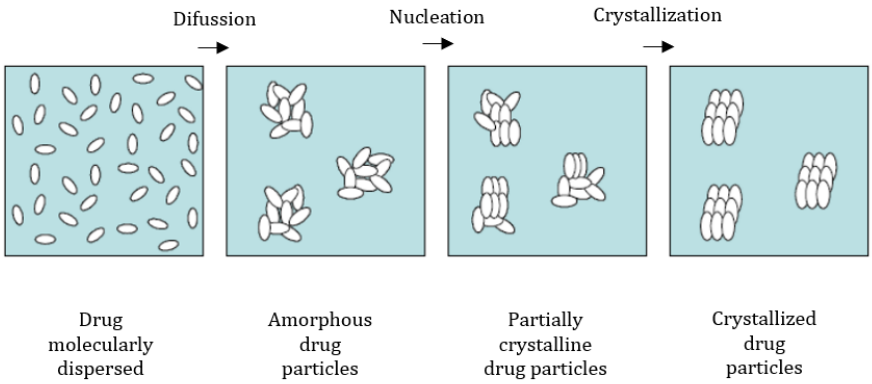


Figure 7: Physical changes in solid dispersions resulting in crystallization (from (96)).

Materials can exist in different states. The crystalline state and liquid state above melting temperature are thermodynamically stable. Amorphous materials are thermodynamically unstable and have a natural tendency to crystallize, due to the lower energy of the crystalline state than the amorphous state. However, amorphous material can be kinetically stable, which implies that the equilibrium state (crystalline), is not reached during the time of the experiment, or shelf life of the product. The kinetic stability of the amorphous material depends on the physical state of the material. Two physical states can be defined for the amorphous material: The glassy state and the amorphous state. The semi-crystalline solids have crystalline and amorphous regions. Depending on the temperature, these amorphous regions can be in the glassy or in the rubber state. The temperature at which transition occurs in amorphous regions between the glassy and rubber states is called the glass transition temperature (T_g) (97, 98).

By matrix addition, usually with a higher glass transition temperature (T_g), the T_g of the solid dispersion is increased compared to the T_g of the drug alone. In this way the molecular mobility of the drug was reduced. It becomes obvious that the T_g of the matrix should be as high as possible, to obtain a solid dispersion with a high T_g and

consequently low molecular mobility. Related with this, the plasticizing effect of the water absorbed in the solid dispersion must also be considered. Many matrices are hygroscopic, and water will be homogeneously distributed throughout the solid dispersion. Matrix T_g may decrease to values below the storage temperature and the material is prone to devitrification. Plasticizing capacity of water is enormous, due to its low T_g (135K). As conclusion, the T_g of a homogeneous solid dispersion determines its stability (97-100).

6.10. Solid dispersions preparation methods

The selected method for each case depends on factors such as the hydrophilicity/hydrophobicity balance of the drug, drug load, and molecular weight. There is no such thing as an ideal method, therefore try different methods might be the best approach (101).

6.10.1. Melting method

The first solid dispersions created for pharmaceutical use were prepared by the fusion method. Sekiguchi and Obi (66, 67), used this method. Sulfathiazole and urea were melted together and then cooled in an ice bath. The resulting solid mass was subsequently calibrated to reduce particle size. The cooling leads to supersaturation but due to solidification, the dispersed drug becomes trapped in the matrix. A variation of this method consists of suspending the drug in a previous melted matrix, instead of both being fused, thus reducing the process temperature. Although often applied this method has serious limitations (62, 66, 67).

First disadvantage is the fact that these methods can only be applied when the drug and matrix are compatible and well miscible at the heating temperature. When drug and matrix are incompatible, two liquid phases or a suspension can be observed in the heated mixture, and this results in an inhomogeneous solid dispersion. This can be prevented using surfactants.

Secondly, a problem can occur during cooling down when the drug-matrix miscibility changes. In this case, phase separation can occur. Indeed, it has been observed that when the mixture is cooled slowly, drug crystallization occurs, while rapid cooling originates amorphous solid dispersions.

Third, drug and/or matrix degradation can occur during heating to the target temperature.

To overcome these limitations of the fusion method, several modifications were introduced to the original method, thus emerging the hot melt extrusion method (HME), or the melt agglomeration method.

6.10.1.1. Hot melt extrusion method (HME)

It is essentially the same as the melting method, except for intensive mixing of the components that is induced in the extruder. When compared to melting in a vessel, product stability and dissolution are similar, but HME allows shaping the heated drug/matrix mixture into implants, ophthalmic inserts, and oral solid dosage forms (10, 92, 102).

HME has been shown to molecularly disperse poorly water-soluble drugs in a polymer carrier, increasing dissolution rate and bioavailability. HME is a thermo-mechanical processing technique by which the drug is intimately or molecularly mixed with excipient carriers. The processing temperature is generally greater than melting or glass transition temperature to facilitate the formation of homogeneous dispersions or solutions of the drug in the carrier system. An important requisite for the formation of solid dispersions by the HME method is the miscibility of the drug and the carrier in the molten form. Another important requisite is the thermal stability of both the drug and the carrier. HME involves the application of heat, pressure, and agitation through an extrusion channel to mix the drug and carrier together, and subsequently forcing them out through a die. HME enhances solubility by producing a stable, amorphous solid dispersion, increased energy form through a combination of the process and the chemical properties of the excipient. The resulting product, called the extrudate, can be milled, blended with a few other ingredients (e.g., diluent, glidant, lubricant) and can be compressed into a tablet or encapsulated into a capsule (10, 16, 91, 102-105).

As in traditional melting processes, miscibility of the drug and matrix can be an issue. Solubility parameters are assessed to predict solid state miscibility and select the proper die for melt extrusion. High mixing strengths, resulting in high local temperatures in the extruder, can be problematic for heat sensitive materials. However, compared to the melting method, this technique offers the possibility of continuous production, which makes it suitable for large scale production. Furthermore, the product is easier to handle,

because at the exit of the extruder, the form can be adapted to the next processing step without calibration (103, 104).

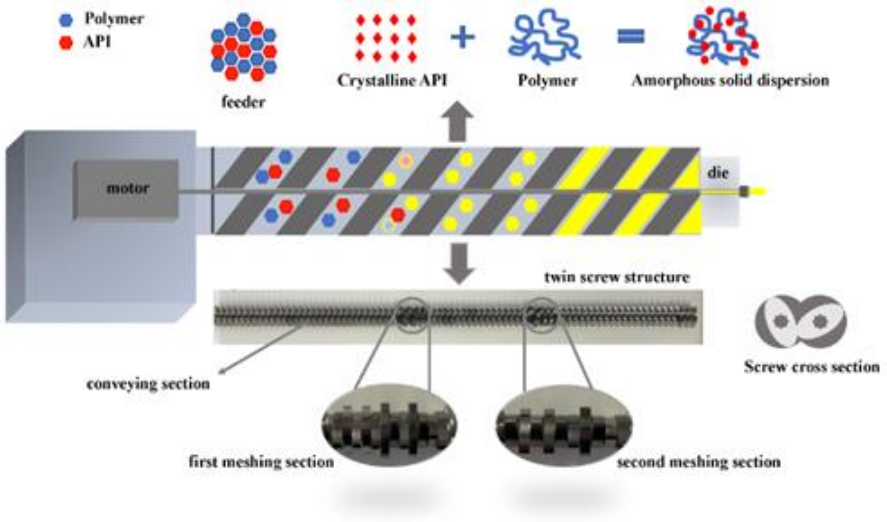


Figure 8: Schematic representation of a Hot Melt Extruder (from (106)).

6.10.1.2. Melt agglomeration method

Allows the preparation of solid dispersions in conventional high-speed mixers. In this process, the molten matrix containing the drug is added to the heated excipients (103, 104).

6.10.2. Solvent evaporation method

This method consists in dissolving the drug and the matrix in a common solvent, and then evaporate the solvent under vacuum to produce a solid solution. An important prerequisite for producing a solid dispersion by this method is that the drug and matrix are sufficiently soluble in the solvent (75, 93).

The first step in the solvent evaporation method is the preparation of a solution containing the matrix and the drug. The second step involves removing the solvents resulting in the formation of a solid dispersion. Mixing at a molecular level is preferable, as this leads to optimal dissolution properties. This method presents two challenges (85).

The first consists of mixing the drug and the matrix in a solution, which becomes difficult when they differ significantly in polarity. Several strategies have been applied to dissolve the lipophilic drug and the hydrophilic matrix together in a solution. Low drug

concentrations are used to dissolve the drug and the matrix in water, but this requires the evaporation of large amounts of solvent making the process costly and impractical. Solubilizers such as cyclodextrins or surfactants such as Tween 80, substantially increase the aqueous solubility of the drug. Chloroform and dichloromethane have been used to dissolve drug and PVP as matrix simultaneously. These solvents are also used in other preparation methods. However, according to the ICH guidelines these solvents belong to class I, which compromises the most toxic solvents. Therefore, the use of these solvents is unacceptable and impractical because the quantities of residual solvents present in solid dispersions after drying must be below limits present in the guidelines. The last strategy, for the dissolution of the drug and the matrix is the use of solvent mixtures. Water and ethanol, or dichloromethane and ethanol have been used for this purpose. However, the dissolution of the drug and the matrix in these mixtures is not always possible at the intended concentrations or ratio (16, 42, 85, 86, 107, 108).

The second challenge in the solvent evaporation method is to prevent phases separation, i.e., crystallization of drug and matrix during solvent removal. Drying at high temperatures speeds up the process and reduces the time available for phase separation. On the other hand, at high temperatures, molecular mobility of drug and matrix remains high, favouring phase separation and consequently crystallization (108).

Vacuum drying and moderate heat is often used to dry the solutions (85, 107). Sometimes solvent evaporation is accelerated using a rotavapor (109). Then the solid dispersion formed, is often stored in a desiccator under vacuum to remove residual moisture.

6.10.2.1. Spray-drying method

A spray-dried dispersion is an amorphous molecular dispersion of a drug in a polymer matrix created by dissolving drug and polymer in an organic solvent and then spray drying the solution. Solution is dispersed as fine particles in a stream of hot air. Due to the large specific surface area offered by the droplets, the solvent quickly evaporates, and solid dispersion is formed within seconds, which can be fast enough to avoid phase separation. Additionally, solid dispersions prepared by spray drying, consist of particles whose size can be modelled, through changing the size of the drops, to meet the necessary requirements to further processing or application. The spray-drying technique usually produces drug in the amorphous state however, the drug might have been

partially crystallized during processing. For spray drying, the solubility of API in the solvent is crucial to ensure a readily scalable and viable process (92, 94, 110).

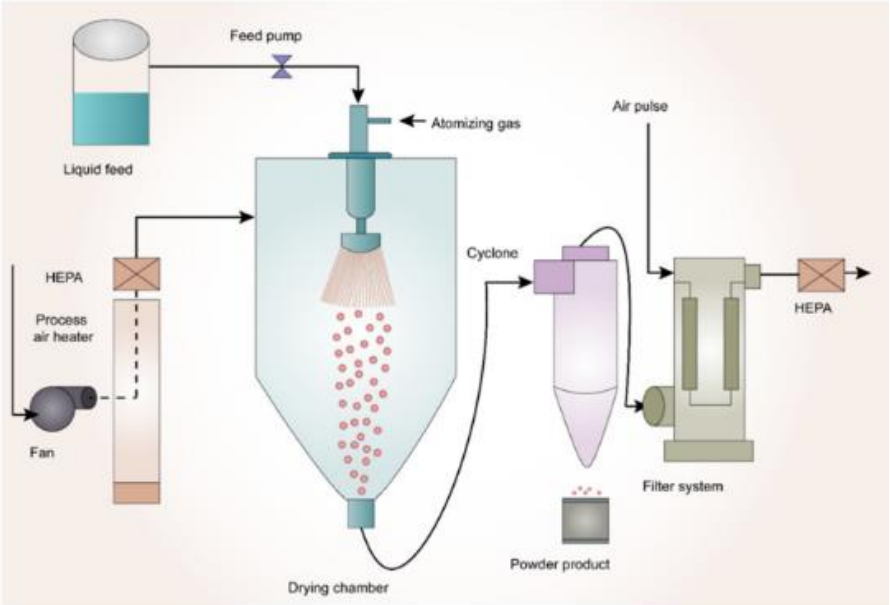


Figure 9: Schematic representation of a Spray-Dryer (from (111)).

6.10.2.2. Lyophilization or freeze-drying method

Lyophilization or freeze-drying is a process in which water an organic solvent, or a co-solvent system is frozen, followed by its removal from the sample, initially by sublimation (primary drying) and then by desorption (secondary drying). The principle of the lyophilization technique is drying carried out at low temperature under conditions involving removal the solvent system by sublimation. In this technique, the material is initially frozen and then reducing pressure of the system and giving the necessary heat allows the sublimation process. Here, the drug is physically entrapped in a water/solvent soluble matrix which is then freeze-dried to give a product that is highly porous and has a large surface area (16, 107, 112).

The fundamental process involves freezing the product which provides the necessary condition for low temperature drying. After freezing, the product is placed under vacuum. This enables the frozen solvent in the product to vaporize without passing through the liquid phase, a process known as sublimation. Heat is then applied to frozen product to accelerate sublimation. Low temperature condenser plates remove the vaporizing solvent from the vacuum chamber by converting it back to a solid. The resulting product

has a very large surface area thus promoting rapid dissolution of the dried product (113, 114).

An important advantage of lyophilization is elimination of the adverse effect of heat on the pharmaceutical product as APIs are not exposed to elevated temperatures. However, the most important advantage is that the risk of phase separation is minimized as soon as the solution is vitrified. The removal of water also enhances product stability in a dry state. Water insoluble drugs are generally preferred because incomplete freezing or collapse may occur with water soluble drugs at low eutectic freezing temperature. Low solubility drugs are weakly bonded to the solvent and are hence more easily converted into the freeze-dried form. Freeze-dried products have shorter dissolution time compared to other solid products (16, 112-116).

Although it is mentioned in the literature, that it is a promising technique to incorporate drug in stabilizing matrices, its use has been little explored. One of the reasons could be the low freezing temperature of the most organic solvents. Obviously, sublimation during lyophilization is only possible when the solvent remains frozen. To obtain a lyophilization process with an acceptable duration, the vapour pressure of the solvent must be high enough (107, 109).

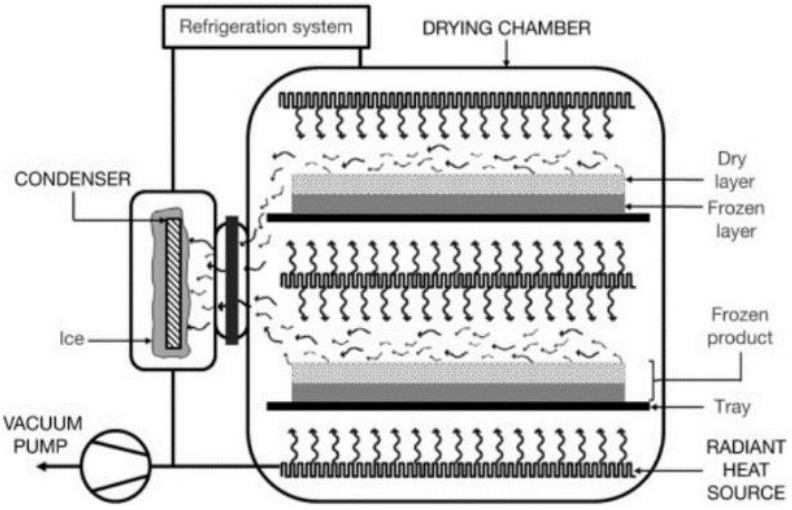


Figure 10: Schematic representation of a freeze-dryer (from (117)).

6.10.2.3. Spray-freeze-drying method

An even more promising technique is spray-freeze-drying. The solvent is vaporized in liquid nitrogen or cold dry air, and the frozen drops are subsequently lyophilized. The large surface area, and the direct contact with the cooling agent, results in even faster

vitrification, thus reducing the risk of phase separation to minimal. Additionally, spray-freeze-drying offers the potential to model the size of particles to make them suitable for further processing or applications such as pulmonary or nasal administration (107-109).

These techniques present problems such as negative effect of solvents on the environment, and high production costs due to the need for special solutions for solvents removal (10, 108).

6.10.3. Supercritical Fluid method

Supercritical fluids have both liquid and gas characteristics. Materials exhibit liquid-like solvent characteristics and gas-like viscosity, diffusivity, and thermal conductivity under supercritical conditions. While the solvent properties are advantageous for drug/polymer solubilization, the gas-like properties considerably improve the fluids mass transport characteristics (118). This strategy is most used with supercritical carbon dioxide (CO_2) as a drug and polymer solvent or as an antisolvent. When supercritical CO_2 is used as a solvent, the matrix and the drug are dissolved and sprayed into an expansion vessel under reduced pressure, and particles are immediately formed. The adiabatic expansion of the mixture results in a rapid cooling. This approach enables the creation of drug particles with a much smaller particle size (119). Current supercritical fluid approaches allowed to generate nanoparticles from 5-2000 nm. This process is considered environmentally friendly because it does not require the use of organic solvents and the small amount of residual CO_2 trapped inside the polymer causes no risk to patients. The capacity of CO_2 to plasticize and swell polymers can also be exploited. However, the application of this technique is very limited, given the very low solubility of most pharmaceutical compounds in carbon dioxide. Thus, the scale-up of this process, to a kilogram scale will be impractical (39, 42, 108, 110, 119, 120).

6.10.4. Coprecipitation method

Matrix is weighed and dissolved in water, while drug is dissolved in an organic solvent. The aqueous carrier solution is then added to the organic drug solution after complete dissolution. Solvents are then removed. The dispersion is crushed, sieved, and dried using a pestle and a mortar. This process can be performed as presented in Figure 11 (82, 121).

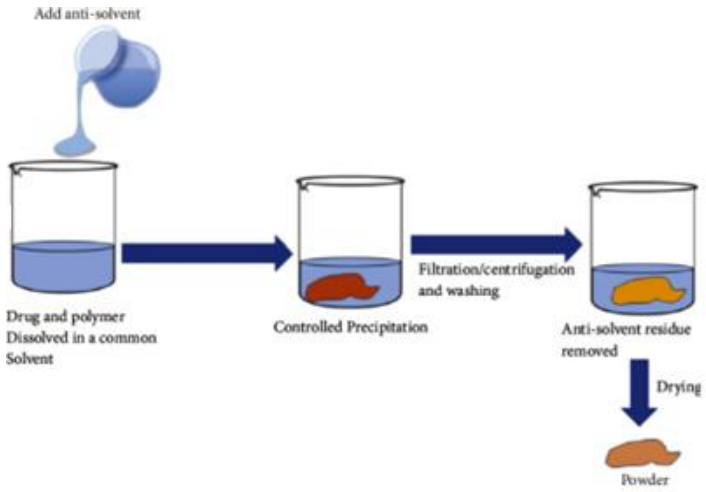


Figure 11: Coprecipitation method to produce solid dispersions (from (82)).

6.10.5. Kneading method

In an adequate equipment (high-shear mixer) drug and carrier are wetted with a solvent and kneaded for a period of time (122). In this method, the liquid (water or hydroalcoholic mixture), is added dropwise while the drug and polymer are mixed, involved by the liquid, and triturated. This results in the formation of a slurry and particle size reduction, which increases drug bioavailability due to the kneading action. The mixture is then dried and sieved to obtain a controlled and consistent particle size (123, 124).

6.10.6. Electrospinning method

This technique combines solid dispersion technology with nanotechnology, exposing a liquid stream of a drug/polymer solution to a voltage between 5 and 30 kV. Fibres of submicron diameter arise when electrical forces exceed the surface tension of the drug/polymer at an air contact (125). The generated fibres can be collected on a screen to make a woven fabric, or they can be gathered on a spinning mandrel, as the solvent evaporates. Because it is the simplest and cheapest technology for preparing nanofibers and controlling the release of medicines, it has enormous potential. In the future this approach could be used to make solid dispersions (122, 126).

6.11. Challenges in solid dispersions

Amorphous solid dispersions, which have a higher free energy and greater chemical and thermodynamic activity compared to crystalline polymorphs, provide faster dissolution rates and higher apparent solubility. However, the most common concern of this products is the lack of thermodynamic stability (8).

Critical parameters, namely temperature, moisture, and pressure, could jeopardize physical stability of amorphous solid dispersions. Some can be easily plasticized with water resulting in low T_g. Typically, plasticization enhances the molecular mobility, leading to gelling or crystallization of the amorphous form. Temperature and pressure naturally enhance molecular mobility and crystallization rate of amorphous drugs. Crystallization of amorphous drugs could also occur during the transit through the gastrointestinal tract (GI) (8, 82).

One of the approaches typically used to overcome physical stability, is to formulate the drugs with pharmaceutically acceptable polymers to form stable amorphous solid dispersions. The ability to form intermolecular interactions (hydrogen bonding, ionic interaction, or dipole-dipole interactions), is one of the most important criteria in the formation of amorphous molecular dispersions. Improved physical stability can be attributed to the reduction of molecular mobility of the drug molecules and/or by nucleation inhibition and crystal growth, through preferential drug-polymer interactions (127).

Pure amorphous drugs are not commonly developed as commercial dosage forms. They are formulated in combination with excipients to stabilize the amorphous form during storage as well as to prevent crystallization during *in vivo* dissolution in the GI. The high kinetic solubility of the amorphous form can drop to the equilibrium of the crystalline form if devitrification is induced by the dissolution medium. Therefore, appropriate carriers that can be used as stabilizers of the amorphous form of the drug are needed for the formulation. The dissolved carrier can also influence the supersaturated drug solution that is formed following dissolution. Some carriers, solubilize the released drug, while others stabilize the supersaturated drug solution (70, 76, 82, 128).

Carriers play a major part in the formulation of solid dispersions. They can be hydrophilic, hydrophobic or water swellable. Depending on their characteristics they can be used as release enhancers or release retardants (76). Also, the dissolution characteristics of drug

molecules are dependent on the nature of carriers (70, 128, 129). A carrier must meet the following criteria to be appropriate to enhance the dissolution rate of a drug:

- Freely water-soluble with intrinsic quick dissolving capabilities
- Non-toxic and pharmacology inert
- The melting process must be heat stable and should have a low melting point
- It must be soluble in a wide range of solvents
- It should be able to preferably increase the aqueous solubility of the drug
- It should be chemically compatible with the drug and should not form a firmly bound complex with it

Amorphous solid dispersions stabilized by polymers can be classified as:

- **Solid solution**

  If an amorphous drug is miscible with the polymer, the system is known as an amorphous solid solution or molecular dispersion, presenting one T_g value. Physical stability is expected to be drug concentration dependent.

- **Solid suspension**

  If an amorphous drug is dispersed in the polymer matrix at the particle level, it is known as an amorphous solid suspension, presenting two separate T_g values of the drug and the polymer. Physical stability depends on immobilization and isolation of the amorphous particles in a rigid polymer matrix (8, 70, 82, 128, 129).

Polymers play a crucial role in solid dispersions by:

- attaining and maintaining supersaturation
- preventing drug from nucleation and crystallization
- modulating the hygroscopicity of the amorphous drug
- providing mechanical rigidity due to increase T_g of a given matrix

Polymer selection is very important as it influences manufacturing, bioavailability, and stability of the solid dispersion. Initial assessment of potential excipients should be based on basic polymers physicochemical properties such as T_g, hygroscopicity, solid solution and solubilization capacity, etc (8, 128, 130).

6.11.1. Glass transition temperature (T_g)

Glass transition temperature is the temperature at which the material is converted from a “rubbery” to a “glassy” state. Generally, polymers with a high molecular weight have a high glass transition temperature (e.g., cellulose ethers). Polymer T_g can be lowered by using a plasticizer, that should be perfectly mixed with the polymer at a molecular level. The polymer selected should have a high enough T_g to reduce molecular mobility and thus decrease drug crystallization propensity, while still having acceptable processability attributes (70, 82, 130).

6.11.2. Molecular mobility

Molecular mobility is related to macromolecular properties like viscosity. It is generally quantified in terms of mean relaxation time, and it determines physical stability and reactivity. It is also viewed as antiplasticizing of the drug by the polymer. Miscible amorphous solid dispersions will typically have a higher T_g compared to the amorphous API due to the antiplasticizing effect of a high T_g polymer in the formulation. Additionally, certain specific chemical drug/polymer interactions can also limit the molecular mobility of the drug in the amorphous state, stabilizing the system. Polymer content and its molecular weight are key factors in restricting molecular mobility of amorphous drugs (130-132).

6.11.3. Polymer molecular weight

Molecular weight of the polymer is directly related with its T_g, i.e., a polymer with high molecular weight has a high T_g, thus preferable to be used as a stabilizing carrier. Additionally, the molecular weight of the polymer is also directly related to its intrinsic viscosity. A high molecular weight polymer forms a high viscosity diffusion boundary layer evolving the amorphous solid dispersion particles, resulting in drug diffusion-controlled release. On the other hand, a low molecular weight polymer dissolves rapidly, releasing the drug as a single entity (8, 132-134).

6.11.4. Drug/Polymer ratio

Drug/polymer ratio in the solid dispersion is based on the polymer impact on drug’s physical state. The maximum content of the polymer that can be used is conditioned by

its ability to formulate the solid dispersion into a dosage form of administrable size. High drug loading may lead to crystallization within the dispersion. The polymer also prevents fusion/nucleation of amorphous drug particles under compaction. Thus, drug/polymer ratio has to be optimized to formulate an amorphous solid dispersion into a suitable dosage form (8, 129, 132, 134).

6.11.5. Solubility parameters

Solubility parameters are used to predict drug/polymer miscibility. Systems with similar soluble parameter values are likely to be miscible due to the energetics of interactions within one component are similar to those in another component. As a result, the overall energy necessary to facilitate components mixing is small because the energy required to break the interactions within like molecules is equally compensated for the energy released by interactions between unlike molecules (8, 129, 132, 134).

6.11.6. Solid solution capacity

Solid solution capacity is the maximum quantity of a drug that can be completely dissolved into a polymer. Generally, this solid solution capacity is conditioned by drug lipophilicity, solubility parameters, presence of hydrogen bounds, as well as presence of amide structures that can act as hydrogen bound acceptors. For instance, polymers with amide structure such as polyvinyllactam polymers (e.g., Kollidon® VA 64) present better solid solution capacity than polymers with other structures (8, 129, 132, 134).

6.11.7. Solubilization capacity

Solubilization capacity is the solubilization effect of polymers on drug in an aqueous solution. If the polymer can retain the drug in supersaturation state in the GI, it will significantly enhance bioavailability. Amphiphilic polymers such as Soluplus® present better solubilization capacity due to their ability to create micellar structures. On the other hand, most of ionic polymers such as methacrylate copolymers can create complexes with the drug and thus increase its solubility (8, 129, 132, 134).

6.11.8. Hygroscopicity

Moisture influences the T_g of solid dispersions, acting as a plasticizer by increasing the free volume of the product, enhancing structural mobility, and thereby decreasing T_g. Moisture can also accelerate chemical degradation and crystallization (8, 130).

Enteric polymers such as Hydroxypropyl methylcellulose Acetate Succinate (HPMCAS), and acrylate polymers such as Eugragit® are somehow less hygroscopic than the water-soluble polymers like povidone or copovidone, thus providing better stability to the amorphous form (135). Additionally due to the hydrophobicity and low hygroscopicity the ionic polymers also allow an advantage with respect to water immiscibility (pH-dependent solubility). As these polymers are water insoluble, they can absorb water without dissolving and consequently polymer/drug interactions may be preserved to ensure stability of the amorphous form (8, 136).

6.11.9. Chemical reactivity

Amorphous forms tend to degrade at a higher rate than crystalline forms, likely due to an increased surface area and higher molecular mobility which reduces the activation energy for solid state chemical reactions. Additionally, higher hygroscopicity of amorphous forms may enhance the degradation rate by plasticization. Chemical reactivity of amorphous systems, can be reduced by the addition of high molecular weight polymers to the formulation, resulting in a high level of positional specificity between reacting components. Differently, the enhanced chemical reactivity of amorphous systems can be overcome using appropriate packaging and storage conditions (130, 132).

6.12. Excipients in solid dispersions

The excipients used in solid dispersions can be classified as presented in Figure 12.

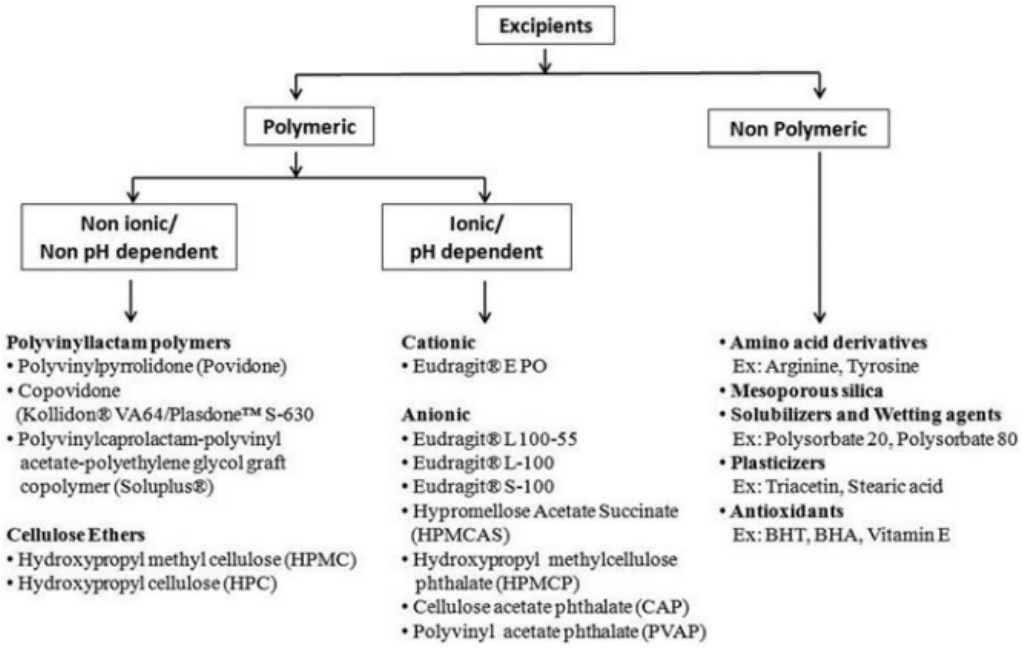


Figure 12: Classification of excipients used in solid dispersions (from (134)).

Polymeric excipients are the primary whereas the non-polymeric are the auxiliary excipients.

6.12.1. Non-ionic/Non pH dependent polymers

6.12.1.1. Polyvinyllactam polymers

These are typically synthesized using vinylpyrrolidone as a monomer. This is polymerized to the homopolymer polyvinylpyrrolidone (povidone; PVP) or copolymerized with vinyl acetate to copovidone. More recently vinylcaprolactam (Soluplus®) was added to this group of excipients (70, 128, 134).

- Polyvinylpyrrolidone (Povidone)

The different grades of povidone are predominantly based on their molecular weight (137). They have good solubility in water and organic solvents with medium lipophilicity

and can interact with both hydrophilic and lipophilic drugs. Due to their hydrophilic nature, they have been the most commonly used as precipitation inhibitors (138).

- Copovidone (Kollidon® VA 64 / Plasdone™ 630)

Kollidon® VA 64 is a vinylpyrrolidone-vinyl acetate copolymer soluble in water and alcohols. This polymer is amorphous in nature, it has good processability and is used for solid dispersions manufacturing using either HME or spray-drying. Kaletra® is an example of a copovidone-based solid dispersion on the market (70, 134).

- Polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (Soluplus®)

This polymer is amphiphilic in nature, providing high solid solution and solubilization capacity. It is an excellent solution to produce solid dispersions using either HME or spray-drying. Soluplus® -based solid dispersions showed promising results when used with model drugs like itroconazole, fenofibrate and carbazepine, etc. Sempera® is an example of a Soluplus® -based solid dispersions on the market (70, 134).

6.12.1.2. Cellulose ethers polymers

These polymers are hydrophilic in nature. The most commonly used polymers of this family are HPMC and hydroxypropyl cellulose (HPC).

- Hydroxypropyl methyl cellulose (HPMC)

Hydroxypropyl methyl cellulose (HPMC), has been shown to be a good stabilizer for amorphous tacrolimus by maintaining a kinetic supersaturation over a much longer time compared to PVP and PEG (139). Certican®, Nivadil®, Crestor®, Prograf®, and Sporanox® are examples of HPMC-based solid dispersions on the market (134).

- Hydroxypropyl cellulose (HPC)

HPC has excellent thermoplastic properties low-melt viscosity and fast melt-flow properties. Low molecular weight grades such as Klucel™ LF or ELF are typically

processed at lower temperatures (120ºC) and are commonly used in immediate release applications. On the other hand, high molecular weight grade (HF) is processed at high temperature (200ºC) and is used for controlled release applications (70, 82, 134).

In Table 4 are presented the physicochemical properties of the non-ionic polymers commonly used in solid dispersions.

Table 4: Physicochemical properties of non-ionic polymers commonly used in solid dispersions (adapted from (134)).

Polymer	Chemical category and classification	Glass transition temperature (T_g)	Molecular weight (MW)	Solubility	Hygroscopicity	Solubility parameter	Degradation temperature	Technologies
Polyvinylpyrrolidone (Povidone)	Polyvinyllactam polymers; Non-ionic	150-180ºC	50 000 g/mol	Water soluble	High	19.4	175ºC	HME
Copovidone (Kollidon® VA 64 / Plasdone™ 630)	Polyvinyllactam polymers; Non-ionic	101ºC	45 000 – 70 000 g/mol	Water soluble	High	21.2	230ºC	HME Spray-drying Fluid bed spray-drying
Polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (Soluplus®)	Polyvinyllactam polymers; Non-ionic	± 70ºC	118 000 g/mol	Water soluble	High	19.4	250ºC	HME
Hypromellose (HPMC)	Cellulose ethers polymers; Non-ionic	± 190ºC	85 000 – 150 000 g/mol	Water soluble	High	23.7	190ºC	HME Spray-drying Fluid bed spray-drying
Hydroxypropyl cellulose (HPC)	Cellulose ethers polymers; Non-ionic	0ºC	40 000 – 115 000 g/mol	Water soluble	High	26.4	220ºC	HME Spray-drying

6.12.2. Ionic/pH dependent polymers

6.12.2.1. Cationic polymers

- Eudragit® E PO

Eudragit® E PO is a cationic copolymer composed of dimethylaminoethyl methacrylate, butyl methacrylate, and methyl methacrylate. This polymer gets ionized and solubilized at pH below 5.5. It is swellable and permeable at higher alkaline pH conditions. Although it has a low T_g, due to its cationic nature, this polymer has the capability to form a complex with anionic drugs, thus stabilizing the amorphous drug in the matrix due to strong intermolecular drug/polymer interactions. Due to these unique properties, it is commonly used as an excipient in solid dispersions as well as in taste-masking applications using both HME and spray-drying technologies. For example, Eudragit® E PO with cationic tertiary amine groups demonstrated to form a complex with anionic drugs like ibuprofen and masking the taste of its bitterness. High drug load (> 35%), with 10% talc using an extrusion process gave good results in terms of taste masking (140). In another study, bioavailability of fenofibrate solid dispersion manufactured using HME with Eudragit® E PO was significantly compared to conventional formulations (141).

6.12.2.2. Anionic polymers

- Eudragit® L 100-55

Eudragit® L 100-55 is an anionic polymer based on methacrylic acid and ethyl acrylate, that starts dissolving at pH 5.5. It has a strong intermolecular interaction with cationic drugs (134).

- Eudragit® L 100

Eudragit® L 100 is fully ionized at pH 6.8. This polymer is commonly used for enteric functional coating as well for controlled release delivery systems. Eudragit® L 100 being an ionic polymer, dissolves by exchanging ions with the alkaline phosphate buffer ions, thus surface erosion is the main mechanism of dissolution. Additionally, the steady and fast hydration of the polymer is accelerated by the ion water-absorbing capacity of the drug (134, 142).

- Eudragit® S 100

Eudragit® S 100 ionizes at pH 7.0 and is primarily used as an excipient for colonic drug delivery. However, this polymer has recently gained importance in the formulation of amorphous solid dispersions (70, 128, 134).

- Hypromellose Acetate Succinate (HPMCAS)

HPMCAS is commonly used in solid dispersions due to its desirable melt viscosity. It is soluble in organic solvents and insoluble in water and acidic media (pH < 5.5), but it dissolves at pH higher than 5.5. The selection of the appropriate grade of HPMCAS polymer is key in terms of solubilization and crystallization inhibition. There are three available chemical grades (MF, AF and LF) based on the succinyl to acetate ratios, each of which has two physical grades with different particle size. The LF and LG grades are soluble at pH ≥ 5.5, MF and MG at pH ≥ 6.0, and HF and HG at pH ≥ 6.80. Higher succinyl to acetate ratio leads to higher hydrophilicity compared to lower ratios, which are more hydrophobic in nature. With higher melting temperature drugs, lower succinyl to acetate ratio produce better solubilization. HPMCAS is an excellent candidate for solid dispersion technology due to its high T_g in the un-ionized state, high solubility in organic solvents and low hygroscopicity. Additionally, due to its amphiphilic nature, it has the capability to interact with the hydrophilic and hydrophobic pockets of the drug molecules. Moreover, its low adsorption of water molecules enhances the physical stability of the solid dispersions (134, 143-145).

- Hypromellose Acetate Phthalate (HPMCP)

Hydroxypropyl methyl cellulose phthalate (HPMCP) is a phthalic ester of HPMC. Two types with different solubility (HP-50 and HP-55) are available. There is also HP-55S, with higher molecular weight, higher film strength, and higher resistance to simulated gastric fluid compared to the regular grades (48, 146, 147).

- Cellulose acetate phthalate (CAP)

Cellulose acetate phthalate (CAP) is a partial acetate ester of cellulose. One carboxyl group of the phthalic acid is esterified with the cellulose acetate. This polymer contains about 20% of acetyl groups and about 35% of phthalyl groups. In the acid form, is soluble in organic solvents and insoluble in water (148).

- Polyvinyl acetate phthalate (PVAP)

Polyvinyl acetate phthalate (PVAP) is a vinyl acetate polymer that is partially hydrolysed and then esterified with phthalic acid. Its promising ability as solid dispersion polymer is due to a high T_g and tendency for hydrogen bound donating and accepting ability (149).

In Table 5 are presented the physicochemical properties of the ionic polymers commonly used in solid dispersions.

Table 5: Physicochemical properties of ionic polymers commonly used in solid dispersions (adapted from (134)).

Polymer	Chemical category and classification	Glass transition temperature (T_g)	Molecular weight (MW)	Solubility	Hygroscopicity	Solubility parameter	Degradation temperature	Technologies
Poly(butylmethacrylate-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate 1:2:1 (Eudragit® E PO)	Methacrylate copolymers; Cationic	± 48ºC	± 47 000 g/mol	Soluble in gastric fluid up to 5.0	Low	19.6	200ºC	HME Spray-drying Solvent-controlled precipitation
Poly(methacrylic acid-co-ethylacrylate) 1:1 (Eudragit® L100-55)	Methacrylate copolymers; Anionic	± 110ºC	± 320 000 g/mol	Above pH 5.5	Low	22.5	300ºC	HME Solvent-controlled precipitation
Eudragit® L100	Methacrylate copolymers; Anionic	> 150ºC	± 125 000 g/mol	Above pH 6.0	Low	22.9	380ºC	HME Solvent-controlled precipitation
Eudragit® S100	Methacrylate copolymers; Anionic	> 130ºC	± 125 000 g/mol	Above pH 7.0	Low	---	380ºC	HME Spray-drying Solvent-controlled precipitation
Hypromellose acetate succinate (HPMCAS)	Cellulose ethers polymers; Anionic	± 130ºC	± 50 000 g/mol	Above pH 5.0	Low	29.1	200ºC	HME Spray-drying Solvent-controlled precipitation

Polymer	Chemical category and classification	Glass transition temperature (T_g)	Molecular weight (MW)	Solubility	Hygroscopicity	Solubility parameter	Degradation temperature	Technologies
								Fluid bed spraying
Hydroxypropyl methyl cellulose phthalate (HPMCP)	Cellulose ethers polymers; Anionic	137ºC (HP-50) 133ºC (HP-55)	80 000 – 130 000 g/mol	Above pH 5.0	Hygroscopic	28	185ºC	HME Spray drying
Cellulose acetate phthalate (CAP)	Cellulose ethers polymers; Anionic	175ºC	± 2534.12 g/mol	In acid form, soluble in organic solvents and insoluble in water	Hygroscopic	27	200ºC	Ultra-rapid freezing
Polyvinyl acetate phthalate (PVAP) **(Opaseal®, Sureteric®)**	Polyvinyllactam polymers; Anionic	42.5ºC	47 000 – 60 700 g/mol	Soluble in methanol and ethanol; <1% soluble in isopropanol and acetone and insoluble in chloroform and methylene chloride	Non-hygroscopic	25	150ºC	Spray drying

6.12.3. Non-polymeric excipients

6.12.3.1. Amino acid derivates

The polymer high T_g, does not always prevent amorphous solid dispersion from crystallization. Thus, very few drugs are commercially available on the market due to the drug physical instability in solid solutions. A strategy normally used in formulation (co-amorphous drugs), utilizes low molecular weight polymers together with the amorphous drug. They protect the amorphous drugs by strong specific molecular interactions, which are better than the higher T_g effect of solid solutions. This concept was used by Lobmann et al. with the low molecular weight amino acids (e.g., phenylalanine, arginine, tyrosine, and tryptophan) as the polymer excipients for the co-amorphous drug formulations. The low molecular weight of the amino acids results in lower fraction of the excipient in the formulation (8, 134, 150).

6.12.3.2. Mesoporous silica

These excipients have very high specific surface area and small pore size. Surface adsorption of drug molecules to the mesoporous silica not only enhances dissolution but also prevents crystallization of amorphous drug. Due to the relatively finite space available to the amorphous molecules, the probability to align with their crystalline counterparts is low to negligible, resulting in amorphous stabilization of the drug. Mesoporous silica could be a viable option for drugs that are less miscible with the established polymers (70, 134, 151).

6.12.3.3. Solubilizers and wetting agents

These types of excipients are commonly used as solubilizers and emulsifying agents in solid dispersions. Their primary objective is to improve the apparent aqueous solubility and bioavailability of the drug. As with polymers, solubility in organic solvents is an important aspect when preparing solid dispersions from solutions in solvents. In the case of HME surfactants can have a plasticizing effect, which allows processing at lower temperatures. Some of the commonly used surfactants Polysorbate 20, Polysorbate 80, Polyethylene glycol succinate, Polyoxyl 40, Gelucire® 44/14, hydrogenated castor oil, Labrasol®, etc (70, 134, 152).

6.12.3.4. Plasticizers

When using HME the use of polymeric carriers generally requires the incorporation of a plasticizer into the formulation to facilitate the processing conditions of certain high molecular weight polymers or to improve the physical and mechanical properties of the final product. With the inclusion of plasticizers (usually small molecules) in the polymers, the free volume between the polymer chains is increased, resulting in higher molecular motion, which is referred as the plasticization effect. The selection of the most suitable plasticizer depends on factors such as plasticizer/polymer compatibility and plasticizer stability (153). Plasticizers help in lowering the processing temperatures needed for production and improving the stability profile of the drug and/or of the polymeric carrier (8, 154-156). However, the impact of plasticizer on long-term stability of the solid dispersion and maintenance of supersaturation kinetics of the amorphous drug needs to be assessed carefully. Triacetin and stearic acid are some of the excipients used as plasticizers (70, 157, 158).

6.12.3.5. Antioxidants

Antioxidants are most effective in stabilizing drug formulations prone to oxidize. Antioxidants are classified as preventive or chain-breaking based upon their mechanism. Preventive antioxidants include materials that act to prevent initiation of free radical chain reactions. Reducing agents such as ascorbic acid, can interfere with autoxidation in a preventive way since they preferentially undergo oxidation, protecting drugs, polymers, and other excipients. Chelating agents like edetate disodium (EDTA) and citric acid are another type of preventive antioxidants that decrease the extent of free radicals formation by forming a stable complex with metal ions that catalyse these reduction reactions.

Hindered phenols and aromatic amines are the two main types of chain-breaking antioxidants, that inhibit free radical chain reactions. Commonly used antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and vitamin E are hindered phenols. Due to the O-H bonds of phenols and the N-H bonds or aromatic amines are very week, the oxidation extent is generally with the antioxidant than with the polymer (8, 70, 134, 159).

6.13. Characterization of solid dispersions

The methods that have been used for the characterization of solid dispersions are presented in Table 6.

Table 6: Methods used for the characterization of solid dispersions.

Characteristics	Methods used	Purpose
Physical state	Differential scanning calorimetry Powder X-ray diffraction Hot stage microscopy Humidity stage microscopy	Find out sample physical state Crystallinity and degree of crystallinity, of the drug, polymer, and solid dispersion
Surface microscopy	Scanning electron microscopy Hot stage microscopy Polarized light optical microscopy Particle morphology	To examine microscopy and crystallinity
Structure elucidation	Solid state nuclear magnetic resonance spectroscopy Fourier transform infrared spectroscopy	To investigate bound between drug and carrier (e.g., hydrogen bound)
Drug/carrier interactions	Differential scanning calorimetry Nuclear magnetic resonance spectroscopy Fourier transform infrared spectroscopy	To investigate physical and chemical interactions between drug and polymer
Solubility/dissolution rate	Dissolution studies Dynamic solubility studies	To study extent and rate of drug release
Stability	Differential scanning calorimetry Nuclear magnetic resonance spectroscopy Fourier transform infrared spectroscopy	To investigate physical and chemical interactions between drug and polymer during its manufacturing and storage

The most important methods are thermoanalytical methods, X-ray diffraction, Fourier transform infrared spectroscopy (FTIR), and measurement the extent (solubility) and release rate (dissolution) of the drug (8, 70). Additionally, these methods can be used to differentiate between solid solutions (molecularly dispersed drug), solid dispersions (drug is only partly molecular dispersed) and physical mixtures of drug and polymer. Due to the complexity of these forms, it is frequently difficult to distinguish between molecularly dispersed and not molecularly dispersed systems and different analytical methods may present noncoherent results. It is generally assumed that dispersions in which no crystallinity can be detected are molecularly dispersed and the absence of crystallinity is use as a criterium to differentiate between solid solutions and solid suspensions. In this method test and reference samples are heated in such a way that temperature of both is kept identical. If an energy requiring transition phase occurs in the test sample, extra heat is applied to keep its temperature climbing equal to the reference. The extra heat required allows to quantify the energy of phase transition. Exothermic transitions, such as conversion from one polymorph to a more stable one, can also be detected. The absence of a melting peak in the thermogram of a solid dispersion indicates that the drug is present in an amorphous form. The degree of crystallinity can be calculated in systems in which the drug is partly amorphous and partly crystalline, due to the quantitative nature of the method. However, crystallinities of under 2% cannot generally be detected by DSC (39, 70, 76, 134, 160, 161)

The principle behind X-ray diffraction is that when an X-ray beam crosses the sample, interference bands can be detected. The angle at which can be detected depends on the wavelength applied and the geometry of the sample with respect to periodicities in the structure. Crystallinity is reflected by a characteristic fingerprint region in the diffraction pattern. Due to the specificity of the fingerprint, crystallinity in the drug can be separately identified from crystallinity in the carrier. Therefore, it is possible with this technique to differentiate between solid solutions (drug is amorphous), and solid suspension (drug is partly in the crystalline form), regardless of whether the carrier is amorphous or crystalline. However, crystallinities of under ± 10% cannot generally be detected by this method (8, 70, 76, 162).

Fourier transform infrared spectroscopy (FTIR), can detect structural changes in bonding between functional groups, that occur with modifications in the solid state. As not all peaks in the FTIR spectrum are sensitive to crystalline changes, it is possible to differentiate between those that are sensitive to changes in crystallinity and those that are not (8, 70, 76, 163).

As enhanced solubility, dissolution rate and consequently bioavailability are the ultimate goals of solid dispersions formation, the extent and rate of drug release in several physiologically relevant pH buffers is also examined. Together with other physicochemical data, drug release experiments provide strong evidence for the formation of a molecularly dispersed or nearly molecularly dispersed system. Well-developed release methods will show whether drug solubility and dissolution rate has been enhanced, and whether the resulting supersaturated solution is stable or tends to precipitate quickly. Results comparison with those for pure drug and physical mixture can help to indicate the mechanism by which the carrier improves solubility and dissolution: via solubilization and wetting effects which could be evidenced by the physical mixture of the components, or by the formation of a solid dispersion or solid solution (8, 70, 76).

6.14. Application of solid dispersions in pharmaceutical dosage forms

The development of pharmaceutical oral dosage forms that use solid dispersions represent a great challenge. Essentially, steps such as mixing, sieving and granulation, compression can affect the properties of the solid dispersions. Many dissolution studies are conducted with powders or sieved solid dispersions rather than tablets, due to tablet disintegration difficulties. Many matrices become serous and adherent or even melt during compression.

The two most common carriers, PEG and PVP, present good binding properties. Sometimes capping occurs, caused by the elastic properties of the completely dried amorphous materials. In the formulation of tablets using solid dispersions, selection of excipients with a high tensile strength and adequate disintegration and dissolution properties is vital to obtain a robust and stable dosage form with the desired performance (130, 164).

6.15. Market products using amorphous solid dispersions

Polymer and manufacturing process selection are key factor in the development of amorphous solid dispersions. An overview of market products using amorphous solid dispersion is presented in Table 7 (82, 134).

Table 7: Market products using amorphous solid dispersions.

Trade name	Drug	Carrier	Technology	Company
Grispeg®	Griseofulvin	PEG	Melt, the exact process is unknown	Pendinal pharm
Cesamet®	Nabilone	PVP	Unknown process	Eli Lilly
Sporanox®	Itroconazole	HPMC, PEG 20000	Spray layering	Jansen
Rezulin®	Troglitazone	PVP	HME	Pfizer
Keletra®	Lopinavir	PVP	HME	Abbott
Certican®	Everolimus	HPMC	Melt or spray drying	Novartis
Fenoglide®	Fenofibrate	PEG	Unknown process	Life cycle pharma
Nivadil®	Nivaldipine	HPC/HPMC	Solvent method	Fujisawa pharmaceuticals
Nimotop®	Nimodipine	PEG	Unknown process	Bayer
Ibuprofen®	Ibuprofen	PEG, HPMC, PVP	HME	Soliqs
Prograf®	Tacrolimus	HPMC	Wet granulation	Fujisawa pharmaceuticals
Cymbalta®	Duloxetine	HPMC AS	Unknown process	Eli Lilly
Noxafil®	Posaconazole	HPMC AS	HME	Merck
Intelence®	Etravirine	HPMC	Spray drying	Tibotec
Isoptin SRE-240®	Verapamil	Various	HME	Soliqs
Crestor®	Rosuvastatin	HPMC	Solvent evaporation	AstraZeneca
Zortress®	Everolimus	HPMC	Spray drying	Novartis
Onmel®	Itroconazole	HPMC	HME	Stiefel
Kalydeco®	Ivacaftor	HPMC AS	Spray drying	Vertex

7. Lyophilization (Freeze-drying)

7.1. Principles of lyophilization

The lyophilization process consists of three stages: freezing, primary drying, and secondary drying.

7.1.1. Freezing

Since freeze-drying is a change in state from the solid phase to the gaseous phase, material to be freeze-dried must first be adequately frozen. The method of freezing and the final temperature of the frozen product can affect the ability to successfully freeze dry the material. Rapid cooling results in small ice crystals, useful in preserving structures to be examined microscopically, but resulting in a product that is more difficult to freeze dry. Slower cooling results in larger ice crystals and less restrictive channels in the matrix during the drying process. Most products that are subjected to lyophilization consist primarily of water, the solvent, and the materials dissolved or suspended in the water, the solute. Most samples that are to be freeze-dried are eutectics (crystalline), which are a mixture of substances that freeze at lower temperatures than the surrounding water. When the aqueous suspension is cooled, changes occur in the solute concentrations of the product matrix. And as cooling proceeds, the water is separated from the solutes as it changes to ice, creating more concentrated areas of solute. These pockets of concentrated materials have a lower freezing temperature than the water. Although a product may appear to be frozen because of all the ice present, it is not completely frozen until all the solute in the suspension is frozen. The mixture of various concentration of solutes with the solvent constitutes the eutectic of the suspension. Only when all the eutectic mixture is frozen is the suspension properly frozen. This is called the eutectic temperature. It is very important in freeze-drying to freeze the product to below the eutectic temperature before beginning the freeze-drying process. Small pockets of unfrozen material remaining in the product expand and compromise the structural stability of the freeze-dried product. The second type of frozen product is a suspension that undergoes glass formation during the freezing process (amorphous). Instead of forming eutectics, the entire suspension becomes increasingly viscous as the temperature is lowered. Finally, the product freezes at the glass transition point forming a vitreous solid (165-168).

A typical profile of the product temperature during freezing in presented in Figure 13.

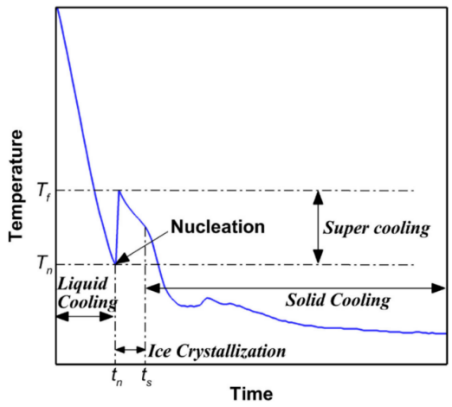


Figure 13: Typical product temperature profile during lyophilization (from (167)).

The product is cooled till the nucleation temperature (T_n) is reached and nucleation begins. Due to the latent heat generated by nucleation, the product temperature increases to the freezing temperature (T_f). Beyond this point, the ice crystallization occurs while heat is released till the entire sample is frozen. The temperature decreases continuously during the crystallization process and during the final step of solid cooling (167).

The degree of supercooling affects the number and size of the ice crystals formed as well as the porosity of the freeze-dried product. When ice nucleation occurs at a high degree of supercooling (i.e. a lower temperature), a large quantity of fine ice crystals is initially formed. On the other hand, when ice nucleation occurs close to the thermodynamic freezing point (i.e. a higher temperature), a smaller quantity of coarser ice crystals is formed. A higher ice nucleation temperature reduces the primary drying time due to a larger average pore diameter in the cake/product, leading to more efficient removal of sublimed water/solvent vapor (165, 166, 169).

7.1.1.1. Primary drying

After freezing the product, conditions must be established in which ice can be removed from the frozen product via sublimation, resulting in a dry, structurally intact product. This requires very careful control of the two parameters, temperature, and pressure, involved in the freeze-drying system. The rate of sublimation of ice from a frozen product depends upon the difference in vapor pressure of the product compared to the vapor pressure of the condenser. Molecules migrate from the higher-pressure sample to a lower pressure

area. Since vapor pressure is related to temperature, it is necessary that the product temperature is warmer than the condenser temperature. It is extremely important that the temperature at which a product is freeze dried is balanced between the temperature that maintains the frozen integrity of the product and the temperature that maximizes the vapor pressure of the product. This balance is key to optimum drying. The typical phase diagram shown in Figure 14 illustrates this point. Most products are frozen well below their eutectic (T_{eu}) or glass transition in the frozen state temperature ($T_{g'}$; Point A), and then the temperature is raised to just below this critical temperature (T_c; Point B) and they are subjected to a reduced pressure. At this point the drying process is started (165, 166).

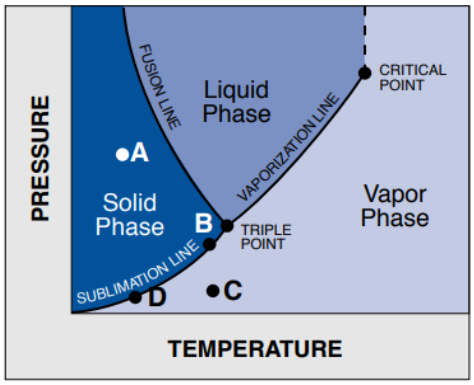


Figure 14: Typical phase diagram of water (from (165)).

Conditions must be created to encourage the free flow of water/solvent molecules from the product. Therefore, a vacuum pump is an essential component of a freeze-drying system and is used to lower the pressure of the environment around the product (to Point C). The other essential component is a condenser to collect the moisture that leaves the frozen product. This condenses out all condensable gases (i.e; the water/solvent molecules), and the vacuum pump removes all non-condensable gases. The molecules have a natural affinity to move toward the condenser because its vapor pressure is lower than that of the product. Therefore, the condenser temperature (Point D) must be significantly lower than the product temperature (165, 166, 168).

The target product temperature during primary drying (T_p), should always be several degrees below critical or collapse temperature (T_c), to obtain a dry product with acceptable appearance (168).

A third component essential in a freeze-drying system is energy. Energy is supplied in the form of heat. Therefore, with all other conditions being adequate, heat must be

applied to the product to encourage the removal of water in the form of vapor from the frozen product. The heat must be very carefully controlled, as applying more heat than the evaporative cooling in the system can remove, warms the product above its eutectic or collapse temperature. Heat can be applied by several means. One method is to apply heat directly through a thermal conductor shelf such as is used in tray drying. Another method is to use ambient heat as in manifold drying (165, 166).

7.1.2. Secondary drying

After primary freeze-drying is complete, and all ice has sublimed, bound moisture is still present in the product. The product appears dry, but the residual moisture content may be as high as 5-20% depending on the formulation. Continued drying is necessary at the warmer temperature to reduce the residual moisture content to optimum values. This process is called isothermal desorption as the bound water is desorbed from the product. Secondary drying is normally continued at a product temperature higher than ambient but compatible with the sensitivity of the product. All other conditions, such as pressure and condenser temperature, remain the same. Because the process is desorptive, the vacuum should be as low as possible (no elevated pressure) and the condenser temperature as cold as can be attained. Secondary drying is usually carried out for approximately 1/3 to 1/2 the time required for primary drying (165, 166, 168).

7.2. Lyophilization process cycle

A typical freeze-drying cycle without the secondary drying stage is presented in Figure 15.

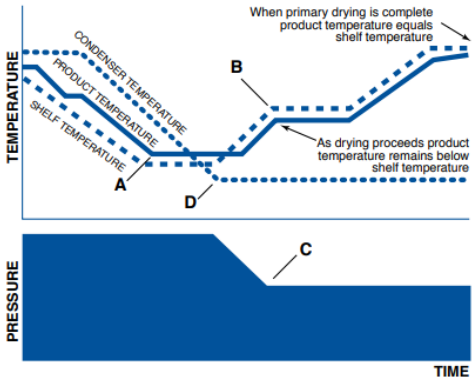


Figure 15: Typical freeze-drying cycle (no secondary drying phase) (from (165)).

The product is first cooled to below its eutectic temperature (Point A). The condenser is cooled to a temperature approximately 20°C cooler than the product temperature, generally around -50 to -105°C. The product should be freeze dried at a temperature slightly lower than its eutectic or collapse temperature (Point B) since the colder the product, the longer the time required to complete primary drying, and the colder the condenser temperature required to adequately freeze dry the product. After the product is adequately frozen and the condenser temperature achieved, the system is evacuated using a vacuum pump (Point C). At this point, primary drying of the product begins and continues until the entire frozen matrix appears dry. Heat input to the product may be achieved by several means such as increasing the shelf temperature in the case of tray drying or using a liquid bath for manifold drying. While the condenser and vacuum pump create the conditions for allowing sublimation to occur, heat input is really the driving force behind the whole process. Care must be taken to prevent the pressure within the system from exceeding the ice vapor pressure of the product or melting of the sample may occur. The volume and configuration of the solution or suspension to be freeze dried often determines how the material is freeze-dried. For example, the greater the ratio of the surface area to the volume of the suspension, the faster drying occurs. This is because there is greater area for the water molecules to leave the product compared to the distance they have to travel to reach the surface of the frozen matrix. Drying occurs from the top of the product and initially the removal of water molecules is efficient. However, as the drying front moves down through the product, drying becomes more and more difficult. The water molecules must now travel through the dried portions of the product which impedes their progress. As the drying front moves farther and farther down the matrix, the application of heat to the product becomes more important. The vacuum system is very important during freeze-drying because the pressure must be maintained at a low level to ensure adequate water vapor flow from the product to the collector (165-168).

7.3. Lyophilization methods

Three methods of freeze-drying are commonly used: (1) manifold drying, (2) batch drying, and (3) bulk drying. Each method has a specific purpose, and the method used depends on the product and the final configuration desired.

7.3.1. Manifold method

In the manifold method, flasks, ampules, or vials are individually attached to the ports of a manifold or drying chamber. The product is frozen in a freezer or by direct submersion in a low temperature bath. The frozen product is quickly attached to the drying chamber or manifold to prevent warming. The vacuum must be created in the product container quickly, and the low temperature of the product is maintained by evaporative cooling. This procedure can only be used for relatively small volumes and products with high eutectic and collapse temperatures. A manifold equipment is presented in Figure 16 (165).

Figure 16: Manifold equipment.

7.4.1. Batch method

In batch drying, large numbers of similar sized vessels containing like products are placed together in a tray dryer (Figure 17). The product is usually frozen on the shelf of the tray dryer. Precise control of the product temperature and the amount of heat applied to the product during drying can be maintained. Generally, all vials in the batch are treated alike during the drying process, although some variation in the system can occur. Slight differences in heat input from the shelf can be experienced in different areas. Vials located in the front portion of the shelf may be radiantly heated through the clear door. These slight variations can result in small differences in residual moisture. Batch drying allows closure of all vials in a lot at the same time, under the same atmospheric conditions. The vials can be stoppered in a vacuum, or after backfilling with inert gas. Stoppering of all vials at the same time ensures a uniform environment in each vial and uniform product stability during storage. Batch drying is used to prepare large numbers of ampules or vials of one product (165).

7.4.2. Bulk method

Bulk drying is generally carried out in a tray dryer like batch drying (Figure 17). However, the product is poured into a bulk pan and dried as a single unit. The heat transfer from the shelf to the trays is via all three modes: gas conduction, contact conduction, and radiation. Bulk freeze-drying is traditionally done in open stainless-steel trays, which have several drawbacks. One issue is the contamination of the equipment due to product blowout, for high sublimation rate processes, resulting in loss of material and clean-up problems. Also, the trays become warped with repeated use, resulting in poor and variable heat transfer from the shelf to the tray. If thermocouples are used to determine the end point of primary drying, they might indicate that primary drying is done in the local region around the sensor, but there could still be regions of ice in the tray resulting in product melt back if the cycle is progressed into secondary drying. In general, aluminium trays, because of their relatively higher thermal conductivity, are preferred over stainless-steel trays. However, the drug cannot be exposed to aluminium and hence laminated pans, with stainless steel inside and aluminium outside, would be required. A general concern with freeze-drying in trays is the inability to seal the container inside the freeze-dryer. For hygroscopic material, this deficiency could be a serious issue as the product could pick up moisture when exposed to room conditions before transferring into a fully sealed container system for long-term storage (165).

Figure 17: Tray freeze-dryer.

7.5. Freeze-drying process development and scale-up issues

The theory of heat and mass transfer during freeze-drying is well established and several process development and scale-up issues are well addressed in the literature.

The product may fail to meet the desired specifications if fixed cycle time from a lab scale dryer is used on a pilot or production scale dryer. Thus, the freeze-drying cycle needs to be adjusted by changes in shelf temperature or chamber pressure when the process is scaled-up from laboratory to pilot and ultimately to production scale. The process should be designed based on sound principles of heat and mass transfer rather than a trial-and-error approach. The basic physics is well understood, and this understanding should be effectively used in Quality by Design programs (165, 166, 168).

7.5.1. Effect of equipment load on process cycle design

The dryer load condition is an important process variable during freeze-drying process development and scale-up. A full dryer load is defined as vials occupying essentially all available shelf surface area. Under partial load conditions, one may never notice the potential for dryer overload (*i.e.*, choked flow or condenser overload) during a high heat and mass flux freeze-drying process. Thus, under partial load, the process runs well, but under full load condition dryer overload occurs with loss of chamber pressure control. Partial load may also result in the shelf surface temperature running slightly higher than under full load condition. In addition, as the load on a given shelf decreases, a greater fraction of the vials become edge vials (*i.e.*, vials experiencing higher heat transfer from the walls to the door of the dryer). As a result, the overall heat transfer to the batch increases (*i.e.*, mean vial heat transfer coefficient increases, product temperature increases, and drying time decreases). Lastly, there could be a load condition where the number of vials on the shelf is not sufficient to maintain the molar flux of water much higher than the molar flux of nitrogen, and the gas composition in the chamber changes from 100% water vapor to a significant level of nitrogen. The gas composition in the chamber is important since the heat transfer via gas conduction depends on the thermal conductivity of the gas. If the gas composition were 100% nitrogen, the overall vial heat transfer coefficient decreases (by roughly 30%) in most applications, product temperature decreases and hence drying time increases (15, 165, 166, 170).

The effect of load condition on critical process parameters, such as product temperature and drying time, on a lab scale, pilot scale, and a clinical scale dryer has been recently

investigated. In going from full load to partial load condition, drying time decreased and product temperature increased. The radiation effect was reported to be the dominant cause of these trends as the fraction of edge vials increased at lower load conditions. Thus, the freeze-drying cycle may need to be adjusted (*i.e.*, shelf temperature or chamber pressure is changed) to achieve the same product temperature profile and drying times under different load conditions (111, 165, 166, 170).

8. Resveratrol (RES)

Resveratrol (3,5,4'-trihydroxy-trans-stilbene), has been detected in many plant species, especially grapes, berries, peanuts, and was found in discrete amounts in red wines. It is a phytoalexin that acts against pathogens, including bacteria and fungi. As a natural food ingredient, numerous studies have demonstrated that Resveratrol possesses a very high antioxidant potential. It is currently being developed by several companies and universities as anti-cancer (prostate, breast and colorectal), (171), anti-inflammatory (172), blood sugar-lowering agent (diabetes type II and impaired glucose tolerance (173), cardioprotective (coronary artery disease, atherosclerosis, hypertension, and oxidative stress), (174, 175), and neuroprotective (Alzheimer's disease and ischemic stroke), (176) among others.

8.1. Chemistry of Resveratrol

Resveratrol (RES), is a stilbenoid polyphenol, possessing two phenol rings linked to each other by an ethylene bridge. Resveratrol is found in nature as both *cis* and *trans* isomers (Figure 18), although the *trans* form is believed to be the most abundant and biologically active form (177, 178). Resveratrol is a highly photosensitive compound susceptible to UV-induced isomerization, as more than 80% of the *trans* form is converted to *cis* isomer if exposed to light for 1h (179).

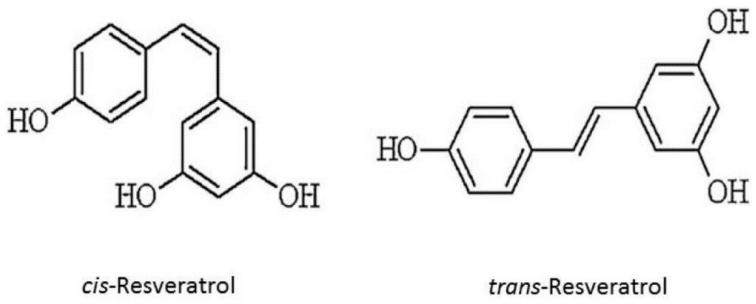


Figure 18: Resveratrol chemical structure.

8.2. History of Resveratrol

Presented as “The Red Wine Molecule” beginning around 2003, Resveratrol gained fame after it was demonstrated to improve longevity in obese male C57BL/6Nia mice (180, 181). After over two decades of high impact and highly publicized *in vivo* research (182, 183), the research surrounding it maintains a controversial enigmatic status as its potential clinical utility remains uncertain. The current evidence supports that Resveratrol primarily acts via direct and indirect activation of the histone deacetylase Sirtuin-1 (SIRT1), in laboratory models (184). SIRT1 activation, governs a complex array of signalling cascades, related to apoptosis, autophagy, and insulin sensitivity. Early research which evaluated the bioavailability of Resveratrol in humans suggested that its bioavailability was rather limited (185). Though cell culture models using high concentrations of Resveratrol, often 10 μM–50 μM or greater, have demonstrated much potential (186), there has been substantial concern that the concentrations used *in vitro* and in animal models are not reasonably attainable *in vivo*. Early research which evaluated the bioavailability of Resveratrol in humans suggests that its bioavailability is rather limited (1.5%) (185-188).

The enthusiasm surrounding Resveratrol was sufficient to lead to the development of human clinical trials in the absence of a full understanding of its mechanism of action or optimal dosage protocols to attain sufficient concentrations in humans. Much of the human literature has yielded promising results consistent with data from laboratory animal models, however, conflicting findings between human and animal also have arisen, which have fuelled healthy scepticism regarding Resveratrol ultimate clinical potential (189).

There are many challenges to overcome in developing Resveratrol into effective therapeutic agents. One of the major issues surrounding Resveratrol future may be

achieving adequate bioavailability at tolerable doses. There are several mechanisms to explore which can be employed to potentially enhance the delivery of Resveratrol to achieve therapeutic range (190).

8.3. Resveratrol pharmacokinetics

Resveratrol shows peculiar pharmacokinetic characteristics that limit its use. In mammals, Resveratrol is extensively metabolized and rapidly eliminated and therefore it shows a poor bioavailability (185). After oral administration, Resveratrol is absorbed at a relatively high rate through the small intestine (191). The small and non-polar character of *trans*-Resveratrol may allow for its absorption across the membranes by passive diffusion, yet there are evidences that Resveratrol is mostly transported across the intestinal epithelium cell via ATP-dependent binding cassette (ABC) transporters (192).

Inside the enterocytes of the small intestine and hepatocytes of the liver, the glucuronide and sulfate conjugation of *trans*-Resveratrol to the major metabolites is extensive. This conjugation to sulfates and glucuronides increases Resveratrol's aqueous solubility and reduces flux across membranes preventing non-polar molecules from interacting with essential macromolecules and allows for excretion by the kidneys via urine (193, 194). Five different metabolites were detected in the urine: Resveratrol monosulfate, two isomeric forms of Resveratrol monoglucuronide, monosulfate dihydro-Resveratrol, and monoglucuronide dihydro-Resveratrol (195). It has been reported that the majority of plasma Resveratrol metabolites are RES-3-*O*-sulfate, RES-4′-*O*-glucuronide, and RES-3-*O*-glucuronide, all with very little bioactivity, even if RES-3-*O*-sulfate possesses estrogen receptor α-preferential antagonistic activity (196). Moreover, extremely rapid sulfation by the intestine/liver appears to be the rate-limiting step in Resveratrol bioavailability (193, 194). It has been demonstrated that both sulfates and glucuronides can be converted to Resveratrol in target tissues such as liver (197). In addition, Resveratrol metabolites undergo enterohepatic recirculation, which allows its deconjugation in the small intestine and reabsorption (198).

Although Resveratrol is quickly metabolized, oral administration is the preferred and only viable route, except for topical application. It is known that plasma concentration of the unchanged Resveratrol depends on the dose ingested. Several preclinical studies aimed to determine the appropriate Resveratrol oral dosage and bioavailability in humans (199). It has been demonstrated that oral dose of 25 mg of Resveratrol resulted in plasma

concentration for unchanged Resveratrol in the range of 1 to 5 ng/mL (194). Administration of higher doses (up to 5 g) led to the increase of unchanged Resveratrol up to 530 ng/mL, indicating how after a high Resveratrol dose only a low amount of the unchanged Resveratrol is present in the plasma (200). Even if Resveratrol seems to be well tolerated and safe, administration of higher oral doses does not allow to improve therapeutic effects but, instead, may be the cause of the side effects observed at the dose of 1 g/kg (body weight) including diarrhoea, nausea, and abdominal pain (199). The main obstacle that must be overcome to consider Resveratrol as a therapeutic agent is its low bioavailability. Strategies to increase bioavailability from oral delivery of Resveratrol are generally focused on increasing the rate of Resveratrol absorption into the enterocytes and decreasing intracellular metabolism (201).

8.4. Methodologies to improve Resveratrol oral bioavailability

Resveratrol is a weak acid consisting of two phenols joined together by a double bond. Due to the deprotonation of the hydroxyl groups present in the compound, Resveratrol has three acidic dissociation constants ($pK_{a1,2,3}$ = 8.8, 9.8 and 11.4) (202). The Biopharmaceutical Classification System (BCS), as defined by Amidon et al. (20), classifies Resveratrol as class II drug characterized by low water solubility (< 60 µg/mL in the pH range of 1.2-6.8) and high intestinal membrane permeability. It has a partition coefficient of 3.1 (203). Earlier studies demonstrated solid state of Resveratrol to be stable regardless of changing different temperature, humidity and light conditions (204). However, the solubility in other solvents and stability at various pH is not fully understood. Since Resveratrol has a limited dissolution rate in the aqueous environment, a small increase in solubility may significantly enhance its bioavailability (205).

The greatest C_{max} and AUC of free *trans*-Resveratrol have been observed following the largest dosages, generally 5 g per day (206, 207). These doses demonstrate that a C_{max} of approximately 4 µM is attainable in human plasma, and this may be sufficient to achieve some of the physiological benefits demonstrated in laboratory models from *trans*-Resveratrol alone (208). Theoretically, greater dosages could be used to maximize Resveratrol exposure, but larger dosages of Resveratrol may also be associated with poor tolerance (206, 207). Thus, considerable effort has been put forth to increase the ratio between free *trans*-Resveratrol concentration and dosage administered.

In this regard, a delivery system that can facilitate rapid absorption of a large amount of Resveratrol, could effectively increase its plasma concentration.

To improve Resveratrol poor bioavailability, various methodological approaches have been developed lately. These include several delivery systems such as the Resveratrol encapsulation in lipid nanocarriers or liposomes, emulsions, micelles, insertion into polymeric nanoparticles, solid dispersions, and nanocrystals (Figure 19) (209).

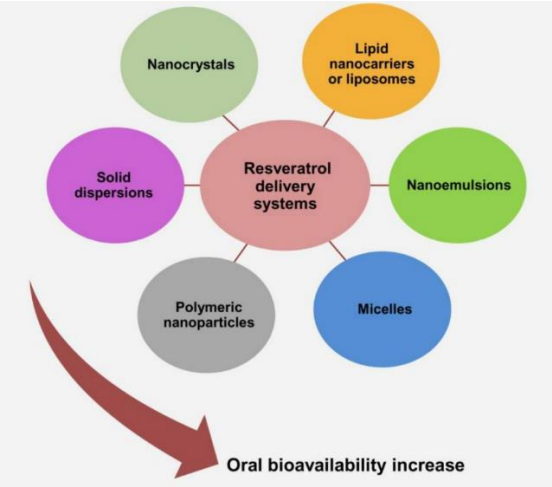


Figure 19: Resveratrol drug delivery systems to improve its oral bioavailability (from (209)).

Protecting Resveratrol from rapid metabolization in the gastrointestinal tract and liver is one general mechanism which can increase bioavailability. Given that CYP, UGT, and SULT are the key enzymes which conjugate Resveratrol, any intervention which decreases their rate of reaction on *trans*-Resveratrol should increase concentration of the parent compound (210).

8.4.1. Lipid nanocarriers and lipossomes

Nanoencapsulation of specific compounds in lipid nanocarriers or liposomes represents a good strategy to significantly increase aqueous solubility and chemical stability. It has been demonstrated that lipid nanoparticles act as a vehicle to enhance the oral bioavailability and therapeutic potential of Resveratrol (211).

Solid lipid nanoparticles and nanostructured lipid carriers have been found to protect the incorporated Resveratrol from rapid metabolism, to increase its physical stability and to allow a controlled release after uptake (211).

8.4.2. Nanoemulsions

Different oil/water (O/W) nanoemulsion-based delivery systems have been developed to optimize the bioavailability of encapsulated Resveratrol for potential oral administration. Sessa et al. demonstrated the ability of subcellular size nanoemulsions, produced by high pressure homogenization (HPH), to protect Resveratrol from chemical degradation, preventing oxidation reactions that would lead to conversion into *cis*-form (212, 213).

8.4.3. Micelles

It has been reported that micellar solutions of bile acids can solubilize poorly soluble organic compounds, improving their resorption (214). Atanacković et al. examined the ability of different bile acids micellar solutions to make Resveratrol more soluble. In addition, they evaluated cell membrane toxicity degree by measuring the hemolytic potential. Results from this study showed that among the tested bile acids micellar solutions, those containing 3,7,12-triketocholic acids displayed the smallest membranolytical potential and solubilized Resveratrol more effectively (215).

8.4.4. Polymeric nanoparticles

Among various nanoparticles-based formulations prepared to enhance Resveratrol delivery some involve encapsulation into biodegradable polymeric nanoparticles (216). Poly(lactic-co-glycolic acid) (PLGA) is used as the hydrophobic portion of polymeric nanoparticles for a variety of biomedical delivery systems and has the advantage of being biodegradable and biocompatible. Recently, it has been demonstrated that RES-PLGA-Nanoparticles represent a stable drug delivery method because it is characterized by small particle size, high capsulation efficiency, well-controlled drug release, enhanced chemical stability, water solubility, and bioactivity (217).

8.4.5. Solid dispersion

It has been reported how Resveratrol solubility can also be enhanced by preparing a solid dispersion of Resveratrol on magnesium dihydroxide (RES@MDH). Spogli et al., using the dissolution test in simulated gastric environment, demonstrated how RES@MDH had higher solubility compared to Resveratrol alone. In addition, the oral

administration of 50 mg/kg of Resveratrol from RES@MDH in rabbits determined a 3-fold increase in Resveratrol bioavailability (218). Similar results were obtained by incorporation of grape peel extract (GPE) into a solid dispersion delivery system or dripping pill (DP) (GPEDP) oral delivery system and testing effects on rats. Pharmacokinetic profile analyses displayed increased Resveratrol bioavailability more than 10-fold, suggesting how the drug product oral delivery system can represent a valid device for clinical applications (219).

8.4.6. Nanocrystals

Singh et al., using the probe sonication method, demonstrated how drug nanonization can represent a valid approach in preparing nanocrystals (NCs) with physical and chemical stability, higher dissolution profile, and then *in vivo*-enhanced oral bioavailability compared to Resveratrol (220).

8.5. Advantages and limitations of the different methodologies

Different strategies to increase Resveratrol solubility have been developed. *In vitro* studies demonstrated that the increase in Resveratrol solubility determines a partial saturation of its metabolism with consequent improvement of its bioavailability (221). Growing evidence revealed that encapsulation of Resveratrol into solid lipid nanoparticles, liposomes, emulsions, or micelles, or insertion into polymeric nanoparticles improves Resveratrol absorption and stability. However, the lipid-based formulations showed several disadvantages. For instance, many have low solvent capacity, unless the active components are highly lipophilic, and low stability of active components loaded in liposomal and nanoparticle systems. Furthermore, drug encapsulation in a lipid matrix reduces the drug load capacity and, at the same time, increases its amount to achieve the therapeutic results desired (222).

Concerning to the use of nanoparticles as carriers, they have several drawbacks including the ability to cross biological membranes, such as the blood–brain barrier, and any modification has to be carefully evaluated because it could reduce their half-life due to the response of immune system in the liver and spleen (223).

Drug nanonization represents a very promising strategy that is able to improve the solubility and dissolution rate of insoluble drugs in water, physical and chemical stability,

compatibility in oral forms of dosage and, finally, *in vivo* oral bioavailability (220). Additionally, nanocrystals (NCs) present the advantages of simplest composition, lower manufacturing cost, and excipient side effects (224). However, due to the extremely high free surface energies, NCs undergo agglomeration; for this reason, they require the use of stabilizers that can be adsorbed on the particle surface to generate repulsive forces leading to the steric or electrostatic stabilization of formulation.

Solid dispersion drug delivery systems are attractive as alternative solubilization methods because manipulating the carriers and powder properties of the active components can improve the solubility, dissolution, and even *in vivo* absorption (8). The bioavailability can be also improved by controlling molecular weight, carrier composition or crystalline status, and powder porosity of the active component. However, the therapeutic application of the solid dispersions is limited by the thermodynamic instability of the molecules in the amorphous state which leads to the drug uncontrolled crystallization during storage (16).

9. Solid dispersions by the lyophilization method – a strategy to enhance bioavailability of Resveratrol

Freeze-drying or lyophilization is the process by which the solvent is removed from a frozen solution by sublimation. The freeze-drying process may be divided in three phases: freezing, primary drying (sublimation), and secondary drying (desorption) (16, 113, 114). Most market products are lyophilized with aqueous solutions, where water is removed from the solid phase, firstly by sublimation. However, some hydrophobic and insoluble drugs, such as Resveratrol, cannot be freeze-dried adequately with water-based formulations, so pure organic solvent or organic co-solvent + water formulations have also been investigated in recent years (113).

The main advantages of using non-aqueous solvents are increased drug solubility, great acceleration of the sublimation rates, increased chemical stability of the pre-dried bulk solution, increased chemical stability of the dried product, and facilitate manufacture of bulk solution by increasing drug wettability and solubility in solution. On the other hand, potential disadvantages concern the necessity of using a freeze-dryer equipped with a lower temperature condenser, safe handling of flammable components with equipment, toxicity and control of residual solvents and overall costs benefits (113, 114, 225).

An exhaustive review concerning freeze-drying with various organic co-solvents was published by Teargarden et al. (225) and by Vessot and Andrieu. (226). These authors compiled different published studies on these topics and listed some case studies with these formulations, especially those using *tert*-butanol (TBA) and water mixtures or pure TBA as API solvent.

Daoussi et al. (227) reported sublimation kinetics data of formulations with an API using organic co-solvent + water mixtures. They observed that the use of organic solvents like TBA considerably reduced the sublimation times by about 10-fold. With the aim of explaining these data, Bogdani et al. (228) investigated some thermodynamic properties of these systems in a frozen state. The sublimation enthalpies with organic systems were much lower than the corresponding values observed with formulations using pure water. Composition at 90% (w/w) of TBA the equilibrium vapour pressure values in the solid state were about twice as high as the data obtained with pure water. Additionally, they observed that the formulation itself (organic co-solvent concentration) and the freezing conditions, namely, the nucleation temperature and the freezing rates, had a key role in controlling the morphology as well as stability of the final freeze-dried product.

Moreover Cui et al. (11) used TBA + water formulations to prepare dehydrated liposomes by freeze-drying. The authors also observed that the addition of TBA co-solvent to water-based solutions significantly enhanced the sublimation kinetics, leading to short freeze-drying cycles. Elgindy et al. (229) studied the freeze-drying of flutamide with TBA + water mixtures to increase the solubility and to improve some important physicochemical properties (amorphization, dissolution rate, surface area, etc.) impaction this drug delivery.

The use of organic solvents can be challenging at first, but for the lyophilization of low soluble drugs is necessary. Many organic solvents have a low freezing point, often well below that of the condenser surface temperature. A list of some of the solvents which have been tested in freeze-drying in presented in Table 8.

Table 8: Properties of organic solvents evaluated in freeze-drying (adapted from(225)).

Solvent	Solubility in water (%[a])	Vapor pressure (mmHg at 20ºC)	Freezing point (ºC)	Boiling point (ºC)
***tert*-Butanol**	100	26.8	24.0	82
Ethanol	100	41.0	-114	78.5
***n*-Propanol**	100	14.5	-127	97.1
***n*-Butanol**	7.7	5.6	-90	117.5
Isopropanol	100	31.0	-89.5	81
Ethyl acetate	8.7	64.7	-84	77.1
Dimethyl carbonate	9.5	72	2	90
Acetonitrile	100	69.8	-48	80.1
Dicloromethane	1.3	343.9	-97	40
Methyl ethyl ketone	27.0	76.2	-87	79.6
Methyl isobutyl ketone	2.0	5.1	-80	117
Acetone	100	160.5	-84	56.2
1-Pentanol	2.7	1.8	-78	138
Methyl acetate	25	148.7	-98	57
Methanol	100	87.9	-98	65
Carbon tetrachloride	0.08	78.9	-23	76
Dimethyl sulfoxide	100	0.5	18.4	189
Hexafluoroacetone	100	5.0[b]	-129	-26
Chlorobutanol	0.8	---	97	167
Dimethyl sulfone	100	---	107	248
Acetic acid	100	11.6	16.2	118.5
Cyclohexane	0.008	66.4	6.5	81

[a] 100% = miscible
[b] 25ºC

The low freezing temperature and triple point of the organic solvents makes it difficult to evacuate the system fast enough and to maintain a low enough pressure to avoid solvent melting, even at ultimate vacuum. Even -105ºC condensers can fail at trapping these types of solvents. Instead, the organic solvents liquify in the condenser or leave the system through the pump as vapour and damaging it (226).

Tert-butanol (TBA) a class 3 solvent with low toxicity, high vapor pressure, high melting point (± 24ºC) and acceptable by FDA, is an excellent choice as a freeze-drying medium.

Organic co-solvent systems have been evaluated for their potential and increasing use for freeze-drying of solutions to produce stabilized powders of marketed pharmaceutical products. The formulations most often investigated include TBA + water mixtures. These organic co-solvent systems present many interesting advantages: high freezing temperatures, very short sublimation time, low sublimation enthalpies, high equilibrium vapor/solid pressure values, and low toxicity (225, 226).

It has been proved that optimum freeze-drying cycles are established by simultaneously considering the impact of formulation variables, especially the TBA content, and classical freeze-drying variables during the freezing step (nucleation temperatures, freezing rates), and the sublimation step (shelf temperature, total pressure), to maximize the drying rates and to minimize the residual solvent levels while preserving the main quality attributes of the freeze-dried powder (225, 226).

9.1. Solution preparation

The first step in the manufacture of almost all freeze-dried products is to be able to create a more or less homogeneous solution containing the API and excipients. Because APIs and/or excipients are hydrophobic and difficult to dissolve, the use of organic co-solvents can greatly facilitate the wetting of the hydrophobic substance, decrease the time to achieve a solution or uniform dispersion, and decrease the amount of solvent needed to be removed during the drying process. Several examples of increased drug solubility with TBA have been listed by Teargarden and Baker (225) and Daoussi et al. (230).

Another important challenge concerns drug stability in solution during the manufacturing steps prior lyophilization, (e.g., solution filtration, vial filling, etc.). Teargarden and Baker (225), presented some data for different types of drugs showing that the presence of TBA can have a significant positive effect on the chemical solution stability by decreasing the reaction kinetics.

9.2. Impact on the freeze-drying process

9.2.1. Effect on freezing

The first step of a freeze-drying process is solution/homogeneous suspension freezing, during which the mixed ice and solvent crystals separate from a cryo-concentrated phase containing API and excipients. The cooling process, which involves complex supercooling, nucleation, and crystal growth, is maintained to transform all formulation components is a frozen solid. This freezing step results in a complex mixture of crystalline, amorphous or metastable phases thar are already affected by the concentration of TBA and other additives, as demonstrated by Seager et al. (231). The solvent type and its concentration affect the phases' structure and the morphology of the frozen material (crystallinity or amorphous degrees). In some cases, the use of organic solvents can result in drug precipitation due to some solvent vaporization. In the case of high melting point solvents, like TBA, the solvent crystallizes between the ice matrix as the temperature is decreased and the presence of TBA alters the crystal habit of the regular ice matrix (230-233).

9.2.2. Nucleation temperature

Nucleation temperature, which is a non-deterministic parameter is the more relevant parameter that determines the ice crystal structure (size and shape of the crystals), and the primary drying times (234, 235). The initial concentration of TBA is an important factor that governs the nucleation phenomena (230-233).

9.2.3. Solvent crystal morphology

Another reason that explains the great advantages of using organic formulations relies on the differences in frozen sample morphologies observed with organic solvents due to different nucleation and crystal growth mechanisms compared with water-based-formulations. Solvent crystal morphology of frozen formulations prepared with TBA + water mixtures revealed unexpected results compared with the results reported in the literature for water-based formulations. It was observed that mean crystal sizes increased significantly with the freezing rates, and degree of supercooling decreased as the freezing rate increased (230-233). However, in purely aqueous systems the degrees of supercooling increased when the cooling rates increased, leading to smaller crystal

sizes when the freezing rates increased (234, 235). TBA concentration and freezing rate had a strong influence on crystal morphology as well on some quality factors related with the freeze-dried cake morphology and on the operation costs of the whole process (sublimation and desorption times).

9.3. Thermodynamic properties

9.3.1. Phase diagram

With amorphous or glassy systems, the sublimation step must be carried out below the critical collapse temperature (T_c), which is generally a few degrees above the glass transition temperature of the product in the frozen state ($T_{g'}$). If the product is sublimated above this T_c, the solid phase collapses. Therefore, a state diagram, which is greatly characteristic of the formulation composition, represents the basic thermodynamic data necessary for setting up the optimal freeze-drying cycle. A phase diagram of TBA + water system has been published by Kasraian and DeLuca (236) as presented in Figure 20.

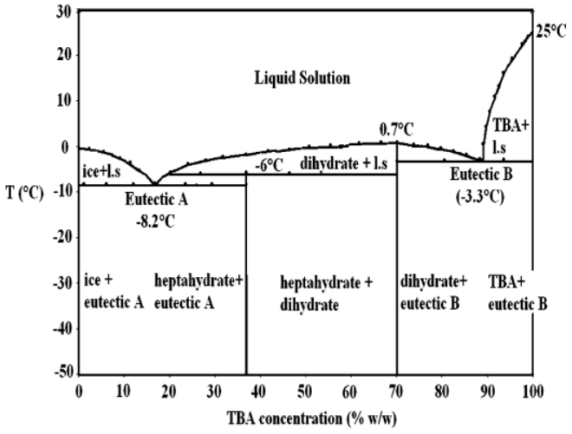


Figure 20: Phase diagram of TBA + water system plotted on TBA fraction. (from (226)).

This system presents an eutectic point B, at -3.3ºC and for a TBA concentration of 90% (w/w). Below this eutectic temperature, solid phases of pure TBA and TBA dehydrate coexist. Another eutectic point at high water concentration exists at -8.2ºC with a TBA concentration near 15% (w/w).

9.3.2. Equilibrium vapor pressure

Vapor pressure is important for set up and validation of physical models of freeze-drying processes. Data demonstrated that for pure TBA the equilibrium solid/vapor pressure values were equal to the values of a pure ice-water vapor system, which seems quite surprising due to the usual high volatility of organic compounds at liquid/vapor equilibrium. For the eutectic composition at 90% (w/w) TBA, the equilibrium frozen solid/vapor pressure values were about twice as high as the vapor pressure of pure ice (Figure 21).

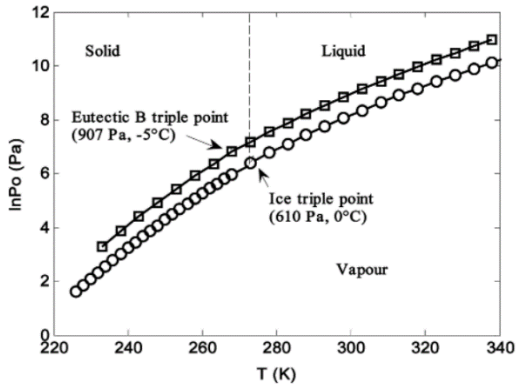


Figure 21: Equilibrium vapor pressure vs Temperature for the 90% (w/w) + 10% (w/w) water system and for pure water (from (228)).

This eutectic mixture corresponds to a positive azeotrope composition in the gas/liquid equilibrium state, which could physically explain these high equilibrium vapor pressure values at this composition. Thus, this composition presents the most favourable thermodynamic data to increase the sublimation rate in the case of a sublimation process controlled by the mass transfer resistance of the solvent vapor through the dry layer (227).

9.3.4. Sublimation enthalpies

Equilibrium solid/vapor pressure is not the only thermodynamic parameter that directly affects the sublimation rate. The enthalpy of sublimation also directly influences this, especially when heat transfer is the limiting mechanism (230, 234). The values obtained by Bogdani et al. (228) for pure TBA were 3.8 times lower than the corresponding value for pure ice. A significantly lower value for the latent heat of sublimation was also observed for the eutectic mixture at 90% (w/w) TBA + 10 % (w/w) water. Thus, these low

values of sublimation represent the main factor that explains the very high increase in sublimation rate observed when using TBA-based formulations.

Table 9: Experimental sublimation enthalpies and melting temperatures for water, 90% (w/w) TBA+10% (w/w) water and pure TBA formulations (adapted from (230-233)).

	Melting temperature (ºC)	ΔH_{sub} (kJ/kg)	ΔH_{sub_ice} (kJ/kg)/ $\Delta H_{sub\ \_TBA}$
Water	0	2809	1
Pure TBA	25	732	3.8
90% TBA	5	1027	2.7

9.3.5. Sublimation kinetics

During sublimation, the mass transfer of the solvent vapor from the sublimation front up to the condenser has to face three mass transfer resistances that can be considered additive. The first is the dried layer resistance, which increases with the dry layer thickness and is the most important (170, 237), secondly the resistance of the vial stopper (often negligible and not existent in case of bulk lyophilization in trays) and finally the chamber resistance, that depends on the vapor flow regime and chamber geometry.

The mass transfer in the porous dried layer takes place via two main mechanisms. By viscous flow under a total pressure gradient according to Fick's law, which generally occurs at moderate chamber total pressure or by Knudsen diffusion, in the case of very low total gas pressures when the mean free pass of solvent vapor molecules becomes higher than the mean pore diameter. Molecular diffusion in Knudsen regime generally occurs at low shelf temperature and very low total gas pressures, that correspond to the optimum sublimation conditions for thermosensible drugs (170, 227, 237). In all regimes, the morphology of the pores of the dried layer (permeability, mean pore diameter, pore surface area, etc), has an important impact on the intensity of the solvent vapor flux through the dried layer. This morphology is similar to the morphology of the crystals of the frozen solvent, that is mainly fixed at the nucleation and crystal growth steps.

Daoussi et al. demonstrated that the use of mixtures highly concentrated with organic co-solvent (TBA) resulted in very short sublimation times (± 3 h), about 10 times shorter than the values observed with pure aqueous-based formulations under the same operating conditions used by Hottot et al. (238, 239).

9.3.6. Residual TBA and water contents

Residual solvents that are too high generally have a negative impact on the storage of the freeze-dried drug and preservation of its therapeutical properties. In the case of freeze-drying with organic co-solvent formulations, these constrains can be more severe due to the possible toxicity of the solvent residues (225, 240).

9.4. Pharmaceutical products or drugs freeze-dried from co-solvent systems

Non-aqueous co-solvent systems are increasingly studied and practically used in the freeze-drying of drugs and pharmaceutical products. The reasons for this increasing interest in potential industrial applications with commercial products include increased solubility (hydrophobic products), great increase in sublimation rates and consequently decrease in sublimation times, decrease in operating costs, increase in pre-dried (solution) and freeze-dried product stability (225, 228).

TBA + water formulations have been the most well-evaluated and widely used systems in the manufacture of commercial pharmaceutical products. Their main advantages include the easy freezing in standard freeze dryers due to TBA high freezing temperature, high equilibrium solid/vapor pressure and low sublimation enthalpy values, low residual solvent contents, and low toxicity of residual TBA (225, 226, 228).

With regards to freeze-drying cycle optimization, both formulation and operating process parameters must be considered together, for example through design space.

Table 10 contain a list of examples of a few drugs preparation from co-solvent systems that have been evaluated.

Table 10: Examples of drug preparations freeze-dried from co-solvents (adapted from (225)).

Drug	Co-solvent system	Reference
Alprostadil (CAVERJECT® S. Po.)	20% v/v *tert*-butanol/water	Teagarden et al., 1998
Aplidine	40% v/v *tert*-butanol/water	Nuijen et al., 2000
Amoxicilin sodium	20% v/v *tert*-butanol/water	Tico Grau et al., 1988
Gentamicin sulfate	*tert*-butanol/water	Baldi et al., 1994
N-Cyclodexyl-N-methyl-4-(2-*oxo*-1,2,3,5-tetrahydroimidazo-[2,1-*b*] quinazolin-7-yl)oxybutyramide with ascorbic acid	50% v/v *tert*-butanol/water	Benjamin and Visor, 1989
Cyclohexane-1,2-diamine Pt(II) complex	*tert*-butanol	Tanno et al., 1990
Annamycin	*tert*-butanol/dimethyl sulfoxide/water	Zou et al., 1999
Caphalotin sodium	5% w/w isopropyl alcohol/water	Koyama et al., 1988
Caphalotin sodium	4% ethanol, 4% methanol or 4% acetone/water	Cise and Roy, 1981
Prednisolone acetate/polyglycolic acid	Carbon tetrachloride/hexafluoroacetone sesquihydrate	DeLuca et al., 1989
Gabexate mesylate	Ethanol/water	Kamijo et al., 1987
Piraubidin hydrochloride	Ethanol/water	Kaneko et al., 1993
Progesterone, coronene, fluasterone, phenytoin	Chlorobutanol hemihydrate/Dimethyl sulfone	Tesconi et al., 1999
Fructose-1,6-diphosphate	*tert*-butanol/water	Sullivan and Marangos, 1998
Poly(lactide-co-glycolide)	Acetic acid	Meredith et al., 1996
Dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol	Cyclohexane	Felgner and Eppstein, 1991
Vecuroniumbromide	Acetonitrile	Jansen, 1997
Bovine pancreatic trypsin inhibitor	Dimethyl sulfoxide/1% water	Desai and Klibanov, 1995

10. Dissolution assessment of poorly soluble drugs

Test the dissolution of poorly water-soluble drugs in immediate release oral solid dosage forms presents numerous challenges. These include the development and validation of the method used ensuring its discriminatory power and that potentiates an *in vitro*/*in vivo* correlation. The purpose of dissolution testing varies during the life cycle of a drug (241).

10.1. Importance of the *in vitro* dissolution testing

The characterization of the drug release mechanism, by establishing an *in vitro* dissolution method, to measure product performance, is particularly important for poorly water-soluble drugs. Historically, the dissolution tests have been an important tool during the different stages of drug development, as well as for its manufacture and commercialization. During drug development, dissolution tests are primarily used to help develop and evaluate new formulations, through drug release rate assessment from the dosage form and allowing to establish *in vitro*/*in vivo* correlations. For the commercialized products, the dissolution test is used primarily to confirm manufacturing and product performance consistency, assuring its quality during shelf-life, as well as supportive data for post-approval changes and assessment of the eventual need of bioequivalence studies (22).

A dissolution test measures the release rate of a drug. The aim is to develop a discriminatory method that is sensitive to variables that affect its dissolution rate. Such variables may include API characteristics (e.g., particle size, crystalline form, density), composition of the finished product (e.g., strength, type and excipients quantity in the formulation, etc), manufacturing process of the finished product (e.g., different equipment), and storage conditions (e.g., temperature, humidity) (22, 241).

10.2. Dissolution mechanism

Dissolution test method determine the cumulative amount of drug that “goes” (dissolves), into the solution medium as a function of time. As presented in Figure 22, drug dissolution from a dosage form involves at least two consecutive steps: drug or solute release from

the dosage form matrix (disintegration), followed by dissolution of the drug in the dissolution medium (242).

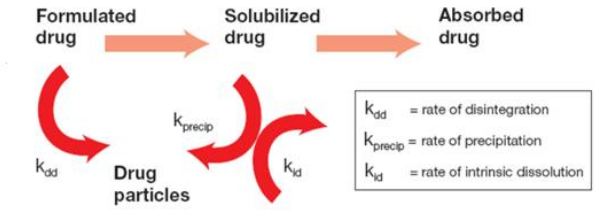


Figure 22: Drug dissolution process from the dosage form (from (242)).

The dissolution rate generally depends on the slower of the two steps. The relative difference between different rates, should be carefully considered when selecting the dissolution media. The cohesive properties of the formulated drug play a key role in the first step of dissolution. For solid dosage forms, these properties include disintegration and erosion (20, 22, 242-245).

If the first dissolution step is rate limiting, then dissolution rate is considered controlled by disintegration. A careful evaluation of the intrinsic dissolution rate and its effect on several aspects of the formulation (e.g., release profiles of pre-compressed granules, impact of the compression force applied, porosity and lubrication) allow to assess the relative contribution of the disintegration step to the global dissolution of the drug. In the second step of dissolution – solubilization of the drug particles – the physical (e.g., amorphous, polymorph, particle size), and chemical properties of the drug, (e.g., salt, free acid, free base), play an important role. If this step is dissolution rate limiting, then the dissolution rate is intrinsically controlled by the dissolution process. This is the case for most drugs under investigation and pharmaceutical development (20, 22, 242-245).

10.3. Dissolution medium

Dissolution medium selection depends on the purpose of the dissolution test. For batch-to-batch quality control, the selection is partly based on the solubility data and strength range of the product, to assure that “sink conditions” are achieved. By definition “sink conditions” is the volume of medium at least 3 times greater than necessary to form a saturated solution of the drug. One medium in which “sink conditions” cannot be

obtained, may be justifiable, if prove to be more discriminatory, or provide data which otherwise can only be obtained with the addition of surfactants. However, when the dissolution test, aims to assess the biopharmaceutical properties of the dosage form, it is more important that faithfully simulates the environment in the gastrointestinal tract, than necessarily product sink conditions. The dissolution properties of oral dosage formulations must be firstly assessed, using medium within the physiological pH range of 1.2 to 6.8, (1.2 to 7.5 for modified release formulations), because poorly soluble drugs include those with adequate aqueous solubility, either at acidic pH (e.g., amines), or neutral (e.g., organic acids). During method development it may be useful to measure the pH of the dissolution medium before and after the test, to verify if pH changes occurred (243, 244, 246).

10.4. Dissolution equipment

The physicochemical properties of the drug, (e.g., solubility and stability), as well as the formulation concept (e.g., immediate release, controlled release, delayed release) play a major role in dissolution equipment selection, especially in the case of poorly soluble drugs. The dissolution test should be conducted in an equipment that demonstrates suitability as described in the United States Pharmacopoeia (USP). The basket method (USP apparatus I) is regularly used for oral solid dosage forms such as capsules or tablets, with an agitation speed of 50 to 100 rpm, however, speeds up to 150 rpm can be used. The paddle method (USP apparatus II) is also used for oral solid dosage forms such as tablets or capsules but frequently at 50 or 75 rpm. Both methods can use volumes of medium that range from 500 to 1000 mL with larger vessels. For very potent, low dosage drugs, the use of 100 to 250 mL vessels should be considered (243, 244, 246).

10.5. *In vivo*/*In vitro* correlations

An important aspect of dissolution tests for poorly soluble drugs is the possibility to establish a method that allows an *in vitro*/*in vivo* correlation. These types of correlations should be explored for pharmaceutical forms of sustained or controlled release, where the formulation technology controls the release rate of the drug. For immediate release pharmaceutical forms, of BCS class II, (low solubility; high permeability), it may be

possible to establish an *in vitro*/*in vivo* correlation, if dissolution rate of drug controls the absorption (247).

II. Project Aim

1. Background

Resveratrol (3,5,4′-trihydroxystilbene), a non-flavonoid polyphenolic, is present in grapes, berries and other plants. Resveratrol (RES) intake from dietary supplements and red wine has been shown to have several therapeutic properties (248). Most of the clinical trials involving RES have focused on cancer (prostate, breast, and colorectal) (171), neurological disorders (Alzheimer's disease and ischemic stroke) (176), cardiovascular diseases (coronary artery disease, atherosclerosis, hypertension, and oxidative stress) (174, 175), diabetes (type 2 and impaired glucose tolerance), and non-alcoholic fatty liver disease (173).

Despite these advantages, based on the Biopharmaceutical Classification System, RES is a class II compound with low solubility and high permeability (20). Additionally, RES instability (conversion of *trans*-resveratrol to *cis*-resveratrol, chemical degradation by alkalizers, and ultraviolet light), and rapid metabolism in the liver, contribute to limit the efficacy of its therapeutic benefits (126, 203-205).

Several strategies have been explored to overcome RES low bioavailability, including nanotechnology, cyclodextrin inclusion complexes, micro-encapsulation and co-crystal, being one of the most promising and efficient techniques for RES solubility enhancement the formation of a solid dispersion (SD) (209, 211-215, 218, 219). Third-generation SDs are easier to prepare compared to chemical approaches and improve drug performance by drug-hydrophilic carrier-surfactant enhanced solubility and stability, via drug amorphization, increasing particle wettability and porosity and by reducing particle size (218, 219). Based on the above the development of a RES third-generation SD was the strategy pursued to improve its bioavailability.

Project activities were conducted at Pharmaceutical Sciences, Analytical Sciences, Medicinal Chemistry and Pharmacology Laboratories from Research and Development Department; Bial–Portela & Cª S.A., at Quality Control Laboratory; Bial–Portela & Cª S.A., and at Nanomedicines & Translational Drug Delivery Laboratory from i3S – Instituto de Investigação e Inovação em Saúde of Oporto University.

2. Objectives

The main objective of this project was to develop a new formulation and optimize the manufacturing process of a RES third-generation SD with enhanced oral bioavailability by solvent evaporation method (Lyophilization/Freeze-drying).

The specific objectives were:

- excipients selection and formulation optimization to convert RES to the amorphous state
- solid dispersion preparation by lyophilization method and lyophilization cycle optimization through critical parameters assessment for each phase (freezing, primary drying and secondary drying)
- solid dispersion solubility and dissolution rate evaluation
- solid dispersion solid state characterization
- solid dispersion stability assessment
- tablet formulation development and characterization
- permeability studies in *in vitro* intestinal models
- *in vivo* pharmacokinetic assessment in rat model

Initial studies focused on parameters that influence the performance and manufacturing process of the drug delivery system: third-generation SD formulation components qualitative and quantitative selection; manufacturing process development and optimization.

Final formulation was expected to allow an improved solubility, dissolution, and be physicochemical stable under adequate storage conditions. A robust and reproducible manufacturing process allowing to obtain a product with the adequate quality attributes was also aimed. Finally, improved *in vitro* and *in vivo* bioavailability for the third-generation SD was expected.

III. Resveratrol Solid Dispersion Development

1. Materials and methods

1.1. Reagents

Resveratrol was provided by Bial–Portela & Cª S.A., and was manufactured by Abatra Technology, China. Acetic acid R, Acetonitrile (ACN), Dimethyl sulfoxide, Glacial acetic acid R, Hydrochloric acid R at 37%, Polyethylene glycol **(**PEG 10000), Potassium dihydrogen phosphate monohydrate R, Sodium acetate trihydrate R, Sodium dodecyl sulfate (SDS), Sodium hydroxide R and *Tert*-butanol (TBA) were acquired from Merck, Germany. Cationic methacrylate copolymers (Eudragit® E100) and (Eudragit® E PO) were obtained from Evonik, Germany. Cellactose® 80 and Lactose 200 were purchased from Meggle, Germany. Cetrimide was purchased from Glentham Life Sciences, Germany. Compitrol 888 ATO, Gelucire® 44/14 and Labrasol were obtained from Gattefossé, France. Copovidone (Plasdone S-630) was purchased from Ashland, USA. Crospovidone, Kolliphor RH 40, Poloxamer 407, Polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft co-polymer (Soluplus) and Polyvinylpyrrolidone (Povidone K30) were purchased from Basf, Germany. Docusate sodium was purchased from Solvay, China. Dulbecco's Modified Eagle medium (DMEM), Hank's balanced salt solution (HBBS), Heat inactivated fetal bovine serum (FBS), L-glutamine, Non-essential amino acids (NEAA), Penicillin (10000 IU/mL), Streptomycin (10 mg/mL) and Trypsine-EDTA were obtained from HyClone; USA. Hydroxypropyl cellulose Low Viscosity (HPC SL; MW: 806.9) was obtained from Nippon Soda, Japan. Hydroxypropyl methyl cellulose (HPMC; MW: 1261.4) was purchased from Colorcon, UK. Hypromellose Acetate Succinate (HPMC AS-MG) was purchased from Ashland, USA. Mannitol was purchased from Cargil SRL, Italy. Stearic acid was obtained from Brenntag, USA. Tween 80 was purchased from Croda, UK. Water R was obtained from a Milli-Q® water system. All other reagents used were of analytical grade.

1.2. Equipment

- Lyophilizer SP Scientific – Advantage Pro EL lyophilizer; (Virtis, USA)
- HPLC Waters®, 2695 Separations Module; 2998 Photodiode Array Detector; (Massachusetts, USA)
- LC-MS/MS Triple Quadrupole G6470A; Agilent®; (California, USA)
- Dissolution tester Teledyne Hanson Vision® G2 Elite 8™; (Chatsworth, USA)

- Analytical balance Sartorius®; (Göttingen, Germany)
- Precision balance Sartorius® MSU1203S-100-DE; (Göttingen, Germany)
- Precision balance Mettler® Toledo XS1003S; (Greifensee, Switzerland)
- Mettler® Toledo HE 73 moisture analyzer; (Greifensee, Switzerland)
- Morphologi 4 Malvern® Panalytical; (Worcestershire, England)
- Calorimeter Q2000 – TA Instruments® ; (Delaware, USA)
- Fourier transform infrared spectrometer Bruker® Tensor 27; (Karlsruhe, Germany)
- Mycromeritics AccuPyc® 1330 Pycnometer 2.01, Model: 133/00000/00w/ V2.01 software; (USA)
- Overhead stirrer RW 20 IKA®; (Wilmington, USA)
- D/tex Ultra2 X-ray powder diffractometer; Rigaku®-Miniflex 600; (Tokyo, Japan)
- Scanning electron microscope Hitachi® TM400 Plus; (Tokyo, Japan)
- Refrigerated centrifuge Heraeus® Labofuge 400R; (Hanau; Germany)
- Heating bath Buchi® B-300 Base; (Switzerland)
- Polarizing photomicroscope Motic® BA410E; (China)
- Heating magnetic stirrer AREX; VELP® Scientifica; (Italy)
- Digital vortex mixer; VELP® Scientifica; (Italy)
- Single stroke tablet press; Romaco® Kilian Styl'One Evo; (Cologne, Germany)
- Disintegration tester Erweka® ZT 320; (Langen, Germany)
- Hardness tester Erweka® TBH 425; (Langen, Germany)
- Motor drive mixer Erweka® AR 403; (Langen, Germany)
- Culture flasks and Transwell® plates; (Corning Inc., USA)
- Orbital shaking incubator KS 4000 IC, IKA®; (Staufen, Germany)
- EVOM$^{2®}$ epithelial voltammeter (WPI), with chopstick electrodes; World Precision Instruments; (Sarasota, FL, USA)
- Pipettes, Gilson; (France)
- Lyophilization glass vials – 7 mL
- Glass vials – 4 mL
- Volumetric flasks
- 0.45 µm filters
- 500 µm sieve
- Pestle and mortar
- Graduated glass cylinder – 100 mL

- HPLC vials; Waters
- Aluminium trays

1.3. Lyophilization method

Lyophilization was performed in a SP Scientific – Advantage Pro EL lyophilizer (Virtis, USA). Initially, a lyophilization cycle (Table 11), with a conservative approach for freezing, primary drying and secondary drying stages was used. Cool to -5ºC without ice formation and hold for some time (60 min.), normally results in improved homogeneity of crystallization, both intra and inter vial. A slow cooling rate (0.5ºC/min.) and an annealing step (heat from -50ºC to -5ºC and hold for 120 min.), allows the formation of larger ice crystals, and facilitates solvent removal during primary drying. The target product temperature (T_p) was established experimentally at -10ºC, well below critical collapse temperature (T_c). Chamber pressure was set at 75 mTorr, a common value in lyophilization applications. In secondary drying a low terminal temperature (20ºC) was selected (168). After formulation selection, a design of experiments (DoE) was performed, considering several factors in the distinct phases of the process, to optimize (shorten) the lyophilization cycle.

In batch drying method, 7 mL lyophilization vials were filled with 2 mL solution. In bulk drying method, solution was poured into an aluminium tray and dried as a single unit.

Table 11: Lyophilization cycle used during formulation development.

Thermal Treatment								
Freeze				Drying				
Step	Shelf (ºC)	R*/H**	Time (min.)	Step	Shelf (ºC)	R/H	Time (min.)	Pressure (mTorr)
1	-5	R	30	1	-50	H	30	75
2	-5	H	60	2	-10	R	300	75
3	-50	R	90	3	-10	H	800	75
4	-50	H	120	4	5	R	180	75
5	-5	R	90	5	5	H	180	75
6	-5	H	120	6	20	R	180	75
7	-50	R	90	7	20	H	480	75
8	-50	H	120	Storage	20	---	---	500

*Ramp / **Hold

1.4. Assay and solubility methods: High-performance liquid chromatography-diode array detection (HPLC-DAD)

The content of RES in SDs assay, in solubility and dissolution evaluation, and in *in vitro* permeability study was assessed by high-performance liquid chromatography (HPLC). The method presented in Table 12, was validated according to the ICH guidelines.

Table 12: Chromatographic system (HPLC-DAD).

Chromatographic conditions	A liquid chromatograph equipped with a variable wavelength detector and integrator (Waters®)
Column	Symmetry Shield RP18 3.5 µm, 4.6 mm x 150 mm, (Waters®)
Mobile phase	Isocratic: ACN*:Water** (45:55) (v/v)
Run time	10 minutes
Retention time	± 4.5 minutes
Injection volume	10 µL
Column temperature	25ºC
Flow	0.8 mL/min.
Detector	308 nm

*Acetonitrile HPLC grade
**MilliQPlus ultra-pure water, filtered through a 0.45 µm membrane

1.4.1. Standard solution for assay, solubility, and *in vitro* permeability

The standard solution for assay, solubility and *in vitro* permeability was prepared by weighing accurately 20 mg of RES into a 200 mL volumetric flask, filled up with mobile phase and sonicated for 15 minutes.
[RES] = 100 µg/mL

1.4.2. Sample solution for assay method

The mass of SD, equivalent to 20 mg of RES was weighed and transferred into a 200 mL volumetric flask, filled up with mobile phase and sonicated for 15 minutes. Solution was filtered through a 0.45 µm membrane.

[RES] = 100 µg/mL

1.4.3. Sample solution for solubility method

An excess amount of RES, physical mixture (PM) or solid dispersion (SD) was added to a 4 mL vial. 2.5 mL of buffer was added to each vial and strongly agitated in a vortex mixer for 30 seconds to facilitate appropriate mixing. Samples were then maintained under magnetic stirring at room temperature (15-25ºC) for 24 hours. Each sample was prepared in triplicate. After stirring, suspensions were filtered through a 0.45 µm filter and analysed by HPLC.

1.5. Buffer solutions

The following aqueous solvent buffers with physiological relevant pH values were used in different formulation assessment phases:

- 0.1N HCl; pH 1.2
- Acetate buffer; pH 4.5
- Phosphate buffer; pH 6.8

1.5.1. 0.1N HCl; pH 1.2

Dissolve 8.3 mL of hydrochloric acid R at 37% in 1000 mL of water R.

1.5.2. Acetate buffer; pH 4.5

Dissolve 2.99 g of sodium acetate trihydrate R and 1.66 mL of glacial acetic acid R in 1000 mL of water R. If needed, adjust pH with acetic acid R or sodium hydroxide R 1M solution.

1.5.3. Phosphate buffer; pH 6.8

Dissolve 6.81 g of potassium dihydrogen phosphate monohydrate R and 22 mL of sodium hydroxide R 1M solution in 1000 mL of water R. If needed, adjust pH with hydrochloric acid R at 37% or sodium hydroxide R 1M solution.

1.6. Assay method (*In vivo* pharmacokinetics): High-performance liquid chromatography-mass spectometry (LC-MS/MS)

RES quantification was conducted in a LC-MS/MS Triple Quadrupole G6470A from Agilent® (California, USA). The analytical method was validated according to ICH guidelines.

Table 13: Chromatographic system (LC-MS/MS).

Chromatographic conditions	LC-MS/MS Triple Quadrupole G6470A from Agilent® (California, USA)		
Acquisition	MassHunter workstation data acquisition version B.08.00, from Agilent®		
Analysis	MassHunter workstation software for quantitative analysis version B.07.01, from Agilent®		
Column	Acquity CORTECS T3 column (2.7µm, 100×2.1 mm), from Waters®		
Mobile phase	(A) 0.1% formic acid in water (B) 0.1% formic acid in acetonitrile		
Gradient	Time (min.)	A (%)	B (%)
	0.50	80.0	20.0
	3.00	20.0	80.0
	4.00	20.0	80.0
	4.10	80.0	20.0
Run time	6 minutes		
Retention time	± 4.5 minutes		
Injection volume	2 µL		
Column temperature	40ºC		
Autosampler temperature	4ºC		
Flow	0.3 mL/min.		
MS settings	Compounds	Resveratrol	Naringenin (IS)
	Precursor ion	229,0	271,3
	Product ion (m/z)	106,9	152,9
	Dwell	200	200
	Fragmentation	166	166

Collision energy (mV)	20	25
Polarity	Positive	Positive

1.6.1. Sample preparation

The desired serial concentrations of spiking solutions were achieved by diluting stock solution of analyte with DMSO. 2.5 µL of spiking solutions (1; 2.5; 5; 10; 50; 100; 200 and 500 ng/mL) were added to 47.5 µL of the blank male SD Rat plasma to achieve calibration standards of 1-500 ng/mL (1; 2.5; 5; 10; 50; 100; 200 and 500 ng/mL) in a total volume of 50 µL. Six quality control samples at 2.5 ng/mL, 10 ng/mL, 200 ng/mL for plasma were prepared independently of those used for the calibration curves. These quality control samples were prepared on the day of analysis in the same way as calibration standards. To 50 µL standards, 50 µL quality control samples and 50 µL unknown samples, 100 µL of internal standard work solution, consisting of acetonitrile with 0.1% formic acid with naringenin, were added for protein precipitating. Eppendorf tubes were sealed, vortexed and centrifuged for 10 min. at 20000 x g (4ºC). Afterwards, samples were transferred to LC-MS/MS vials and aliquots injected into the LC-MS/MS system.

1.7. Dissolution

Dissolution study of RES, SD, PM powders and tablet formulations was performed using a Teledyne Hanson Vision® G2 Elite 8™ dissolution tester apparatus 2 (Teledyne Hanson, Chatsworth, USA), with paddle rotation speed of 100 rpm at 37±0.5ºC in 500 mL of pH 1.2 and pH 6.8 buffer solutions. Samples equivalent to 200 mg of RES were added to the equipment vessels; [RES = 400 µg/mL]. 5 mL samples were withdrawn at 5, 10, 15, 30, 60, 180 and 360 minutes. Samples were centrifuged at 3500 rpm for 5 minutes, and the supernatant was analysed by HPLC. The high-performance liquid chromatography method used is presented in Table 12. Each powder sample was prepared in triplicate and tablet samples in quadruplicate.

The standard solution was prepared by weighing accurately 20 mg of RES into a 50 mL volumetric flask, filled up with mobile phase and sonicated for 15 minutes.

[RES] = 400 µg/mL

1.8. Moisture content

Approximately 1-1.5 g of SD samples were analysed in a Mettler® Toledo HE 73 moisture analyser. Samples were heated at 105ºC until constant weight.

1.9. Differential scanning calorimetry (DSC)

Thermal analysis was performed in a TA Instruments® Q200 calorimeter (TA Instruments, USA). Approximately 2-10 mg samples (RES, PM and SD), were packed into a 100 µL aluminum pan with a lid. Each sample was heated from 25ºC to 280/300ºC at a heating rate of 5ºC/min. under nitrogen atmosphere (50 mL/min.).

1.10. Fourier transform infrared spectroscopy (FTIR)

The presence of molecular interactions between RES and hydrophilic carrier was determined with a Bruker® Tensor 27 Fourier transform infrared spectrometer (Karlsruhe, Germany). The attenuated total reflection (ATR) technique was used to obtain the spectrum from each sample. Before each measurement, the ATR crystal was carefully cleaned with isopropanol. During the measurement, the sample was in contact with the universal diamond ATR top-plate. For each sample, the spectrum representing an average of 16 scans was recorded in the range of 4000-400 cm^{-1} with a 4 cm^{-1} resolution. Data obtained was analysed with Spectragryph 1.2 software (Informer Technologies, Inc.; Dominica).

1.11. X-ray powder diffraction (XRPD)

The molecular conversion of RES from crystalline to amorphous state was assessed by XRPD. RES, PM and SD samples were measured using a D/tex Ultra2 X-ray powder diffractometer (Rigaku®-Miniflex 600; Tokyo, Japan). A Cu Kα radiation was used at 40 KV and 15 mA. The samples were scanned in the reflection mode from 3º to 40º at a scan speed of 5.00º/min. and a scanning step size of 0.02º.

1.12. Morphology surface observation

1.12.1. Optical microscopy (OM), and polarized light microscopy (PLM)

The shape, size and crystallinity of the particles was observed using optical and polarized light microscopy analysis respectively. A Motic® BA410E polarizing photomicroscope (Motic®, China) was used for analysing the samples. Briefly, the powdered sample was spread out evenly on a glass slide to avoid clumping. The excess powder was dusted off. The slide was then placed onto the microscope stage and observed under a 400X magnification. Presence of birefringence, a property observed in crystalline substances was considered as an indication of the presence of crystallinity. Images were captured using Moticam 3+ digital camera (Motic®, China) under light using dark background conditions. Captured images were analysed using Motic® Image Plus 3.0 software (Motic®, China).

1.12.2. Morphology and particle size distribution (PSD)

Automated morphological imaging was carried out using a Morphologi 4 Malvern® Panalytical (Worcestershire, England). This was used to investigate particles size and shape. Samples were prepared by dry powder dispersion using compressed air. Particle imaging was captured by scanning the samples underneath the microscope optics at 10X and 50X magnification. A total of 20 000 particles were analysed for RES and PM samples and 47 598 particles for the third-generation SD sample.

1.12.3. Scanning electron microscopy (SEM)

The surface morphology of samples was examined using a Hitachi® TM400 Plus scanning electron microscope (Tokyo, Japan). A small amount of sample was sprinkled onto a double sided electrically conductive adhesive tape mounted on an aluminium stub, coated with platinum, and then examined under vacuum using an ion sputter to provide electrical conductivity. Images were acquired at a beam acceleration voltage of 15 KV under a 500X magnification.

1.13. Porosity

Bulk density of RES, PM and third-generation SD powders was determined using a 100 mL graduated glass cylinder. Powders were allowed to flow to the empty cylinder previously tared. The bulk volume occupied by the powder and filled cylinder weight were registered. Samples were analysed in triplicate. True density was determined by helium pycnometry using a Mycromeritics Accupyc® 1330 2.01. A total of 10 readings were performed for each sample.

1.14. Statistical analysis

For solubility, dissolution and *in vitro* permeability determinations, triplicates or quadruplicates of formulations were analysed with Microsoft® Excel 2016. Student's T-Test was used between pairs of experiments using a two-tailed distribution with two-sample equal variance. Pairs were considered statistically different with *p* values below 0.05.

For lyophilization process optimization, DoE software Modde® 11, (Sartorius, Göttingen, Germany), was used, with the application of a 2-level full factorial design, with no replicates for the freezing phase and a D-optimal design, with 1 replicate run for the primary drying step. Moddle® 11 software used one-way analysis of variance (ANOVA) to analyse the data. The level of significance was set at probabilities of $p < 0.05$ for the regression, and $p > 0.05$ for the lack-of-fit.

For *in vivo* pharmacokinetics, Student's T-Test for pairs of samples and one-way analysis of variance for all tests (ANOVA) with unpaired and Bonferroni post-hoc test (GraphPadPrism, GraphPad® software Inc., CA, USA) were used to analyse the data, respectively. The level of significance was set at probabilities of $p < 0.05$.

2. Physical mixtures

2.1. Preparation of physical mixtures

Physical mixtures (PMs) equivalent to each formulation (used as control), were prepared by simple mixing of components using a spatula, in a vessel, or a mortar and pestle.

3. Selection and optimization of hydrophilic carrier content

The objective was to select a hydrophilic polymer able to disperse RES at a molecular level and consequently induce its amorphization and solubility improvement.
Through solvent screening, an appropriate solvent mixture (water/organic solvent), able to completely solubilize both RES and hydrophilic carrier and be adequate for lyophilization, was selected.
The following hydrophilic polymers were initially tested:

- **Non-ionic polymers**
  - Polyethylene glycol **(**PEG 10000)
  - Polyvinylpyrrolidone (Povidone K30)
  - Copovidone (Plasdone S-630)
  - Polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft co-polymer (Soluplus)
  - Hydroxypropyl methyl cellulose (HPMC; MW: 1261.4)
  - Hydroxypropyl cellulose Low Viscosity (HPC SL; MW: 806.9)
- **Cationic polymers**
  - Cationic methacrylate copolymer (Eudragit E100)
- **Anionic polymers**
  - Hypromellose Acetate Succinate (HPMC AS-MG)

RES is practically insoluble in water and, therefore, cannot be adequately freeze-dried with water-based formulations. The two main advantages of using non-aqueous solvents are increased drug solubility and great acceleration of the sublimation rates. On the other hand, disadvantages concern the necessity of using a freeze-dryer equipped with a low temperature condenser, safe handling of flammable components with equipment and rigorous control of residual solvents due to their possible toxicity (240, 249).

Due to the low freezing point of organic solvents, the condenser temperature might not be low enough to completely capture the solvents. Even -105°C condensers can fail at trapping these types of solvents. Instead, the organic solvents may liquify in the condenser or leave the system through the pump as vapor (250).

The low freezing temperature and triple point of the organic solvents makes it difficult to evacuate the system fast enough and to maintain a low enough pressure to avoid solvent melting, even at ultimate vacuum (240, 249-251).

Tert-butanol (TBA) a class 3 solvent with low toxicity, high vapour pressure, low melting point and accepted by FDA, was selected due to be an excellent freeze-drying medium.

Organic co-solvent systems have been evaluated for their potential and increasing use for freeze-drying of solutions to produce stabilized powders of marketed pharmaceutical products. The formulations most often investigated include *tert*-butanol (TBA) + water mixtures. These organic co-solvent systems present many interesting advantages: high freezing temperatures, very short sublimation time, low sublimation enthalpies, high equilibrium vapor/solid pressure values, and low toxicity (240, 250).

3.1. Hydrophilic carrier selection

3.1.1. RES:Polymer (1:1) in TBA/Water (70:30)

Percentage of solid content in solution was 2% (1% RES + 1% polymer)

3.1.2. RES:Polymer:Mannitol (1:1:8) in TBA/Water (50:50)

Percentage of solid content in solution was 5% (0.5% RES + 0.5% polymer + 4% mannitol)

Mannitol was added to the formulation as a bulking agent to prevent wafers from collapsing.

3.2. Hydrophilic carrier content optimization

The aim was to test Eudragit E PO at different ratios with RES and decrease mannitol content without compromising wafers appearance and mechanical strength. Eudragit E PO was solubilized in acetate buffer pH 4.5. HCl 0.1N solution was added to facilitate polymer solubilization due to pH increase. RES was solubilized in TBA. Both solutions were combined under agitation with an overhead stirrer RW 20 IKA® (Wilmington, USA) until complete dissolution.

3.2.1. RES:Eudragit E PO:Mannitol (1:1:2) in TBA/Acetate buffer pH 4.5 (50:50)

Percentage of solid content in solution was 2% (0.5% RES + 0.5% Eudragit E PO + 1% mannitol)

3.2.2. RES:Eudragit E PO:Mannitol (1:2:2) in TBA/Acetate buffer pH 4.5 (50:50)

Percentage of solid content in solution was 2.5% (0.5% RES + 1% Eudragit E PO + 1% mannitol)

3.2.3. RES:Eudragit E PO:Mannitol (1:4:2) in TBA/Acetate buffer pH 4.5 (50:50)

Percentage of solid content in solution was 3.5% (0.5% RES + 2% Eudragit E PO + 1% mannitol)

3.2.4. RES:Eudragit E PO:Mannitol (1:8:2) in TBA/Acetate buffer pH 4.5 (50:50)

Percentage of solid content in solution was 5.5% (0.5% RES + 4% Eudragit E PO + 1% mannitol)

4. Selection and optimization of surfactant content

The following surfactants were tested at 4% (w/w) of RES:

- Compitrol 888 ATO
- Docusate sodium
- Gelucire 44/14
- Poloxamer 407
- Tween 80
- Labrasol
- Cetrimide
- Sodium dodecyl sulfate (SDS)
- Kolliphor RH 40

4.1. Surfactant selection

4.1.1. RES:Eudragit E PO:Mannitol (1:1:2)_Surfactant at 4% in TBA/Acetate buffer pH 4.5 (75:25)

Percentage of solid content in solution was ± 2.7% (0.67% RES + 0.67% Eudragit E PO + 1.33% mannitol + 0.027% surfactant)

The surfactant with better solubility results was selected.

4.2. Surfactant content optimization

Lyophilized formulations (LFs) with Gelucire 44/14 at different concentrations, 1%, 8% and 16% (w/w) of RES, were produced and compared to previous one produced with 4% (w/w), to assess the impact of surfactant content in RES solubility. The concentration with better solubility performance was selected.

5. Bulking agent

At the beginning of formulation development wafers collapsed during freeze-drying and mannitol was selected as bulking agent, since is a commonly used excipient as lyoprotectant in freeze-drying. Bulking agents, such as mannitol and lactose, are utilized in lyophilized formulations to provide structure to the lyophilized cake, preventing collapse. Nevertheless, it is important to understand that bulking agents can be dangerous for lyophilized products. For example, when using mannitol, it is essential to ensure that it is fully crystallized. If mannitol crystallizes post-lyophilization, it can release the water associated with it back into the cake, potentially accelerating destabilization of the product (252, 253).

The presence of a bulking agent will also increase the mass of the SD formulation with impact in the size of the final oral solid dosage form. Based on the above, it was assessed if formulation selected (now, including the surfactant) presents sufficient mechanical strength and good wafers appearance when removing mannitol as bulking agent. Formulation with lactose as alternative to mannitol was also tested (254).

5.1. Bulking agent selection

The following formulations were tested:

- **RES:Eudragit E PO:Mannitol (1:1:2)_Gelucire 44/14 at 16% in TBA/Acetate buffer pH 4.5 (75:25)**
- **RES:Eudragit E PO:Lactose (1:1:2)_Gelucire 44/14 at 16% in TBA/Acetate buffer pH 4.5 (75:25)**
- **RES:Eudragit E PO (1:1)_Gelucire 44/14 at 16% in TBA/Acetate buffer pH 4.5 (75:25)**

6. Results and discussion

6.1. Hydrophilic carrier selection

6.1.1. RES:Polymer (1:1) in TBA/Water (70:30)

When Kollidon k30 previously dissolved in water was added to RES dissolved in TBA, precipitation occurred. For this reason, Kollidon k30 was abandoned as a possible choice for hydrophilic polymer selection.

After lyophilization, intact wafers with good appearance were obtained with HPMC alone. Wafers collapsed during freeze-drying with all other polymers tested. Probably, solid content was too low (2% w/v) in those formulations, to produce a wafer with good mechanical strength. It was decided to add mannitol at 4% (w/v) as bulking agent. Mannitol as a commonly used lyoprotectant prevents structural collapse during freeze-drying and enhances mechanical properties. The percentage of water in the co-solvent system was increased to allow solubilization of mannitol. Therefore, the content of RES decreased to 0.5% (w/v) due to reduction in the percentage of TBA in the co-solvent system.

6.1.1.1. Assay

Table 14: Formulation assay (hydrophilic carrier selection - phase 1). (mean ± SD, *n*=2).

Formulation	RES (%) ± SD
RES:HPMC (1:1)	101.7 ± 0.37

6.1.2. RES:Polymer:Mannitol (1:1:8) in TBA/Water (50:50)

After lyophilization intact wafers with good appearance were obtained for all polymers tested.

6.1.2.1. Assay

Table 15: Formulations assay (hydrophilic carrier selection - phase 2). (mean ± SD, *n*=2).

Formulation	RES (%) ± SD
RES:Eudragit E100:Mannitol (1:1:8)	101.1 ± 0.01
RES:HPC SL:Mannitol (1:1:8)	103.4 ± 0.08
RES:Plasdone S630:Mannitol (1:1:8)	106.4 ± 0.14
RES:HPMC AS-MG:Mannitol (1:1:8)	93.4 ± 0.29
RES:Soluplus:Mannitol (1:1:8)	106.0 ± 0.28
RES:PEG 10000:Mannitol (1:1:8)	111.0 ± 0.23

Assay results between 90-110% were obtained for all lyophilized formulations.

7.1.2.2. Solubility

Solubility for RES, PMs, and successful LFs from phase 1 and 2, was assessed after 24 hours stirring at room temperature, using the method described above. Determination was performed in triplicate samples in aqueous solvents with pH values 1.2, 4.5 and 6.8.

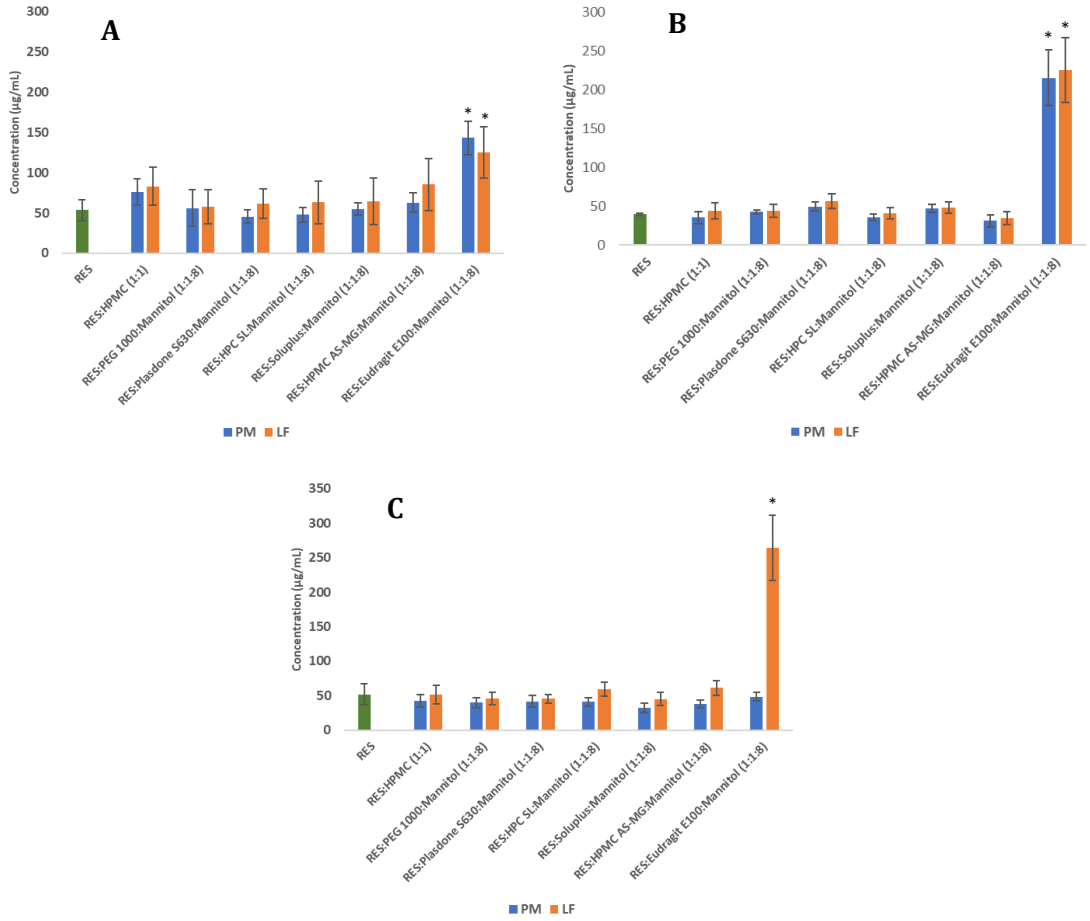


Figure 23: Solubility after 24 hours under magnetic stirring at room temperature in pH 1.2 – A, pH 4.5 – B and pH 6.8 – C, (high throughput polymer screening). (mean±SD, *n*=3), **p* < 0.05 comparing with pure RES.

RES was very low soluble in all aqueous solvents tested after 24 hours stirring at room temperature. RES solubility was 53.4 µg/mL (pH 1.2), 39.8 µg/mL (pH 4.5) and 52.0 µg/mL (pH 6.8) respectively. Eudragit E100 presented the highest increase in solubility, and this was observed both for PM and LF at pH 1.2 and 4.5. At pH 6.8, solubility for the PM was similar to RES alone, while for LF solubility was in average 264.5 µg/mL. Eudragit E100 being a cationic polymer precipitates above pH 5.5, explaining the low solubility of RES obtained in the PM at pH 6.8.

7.1.2.3. Differential Scanning Calorimetry (DSC)

RES, excipients, PMs and LFs were assessed by DSC using the method described above.

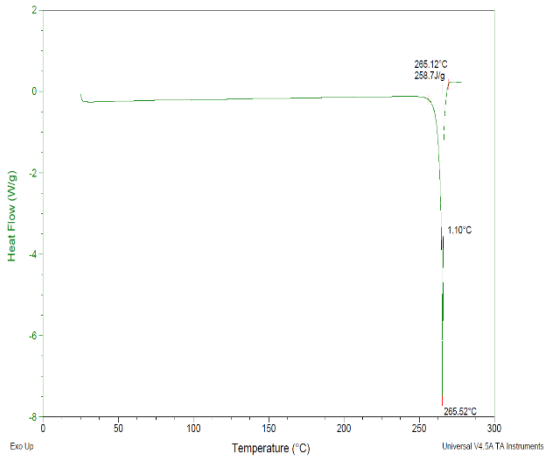


Figure 24: RES thermogram.

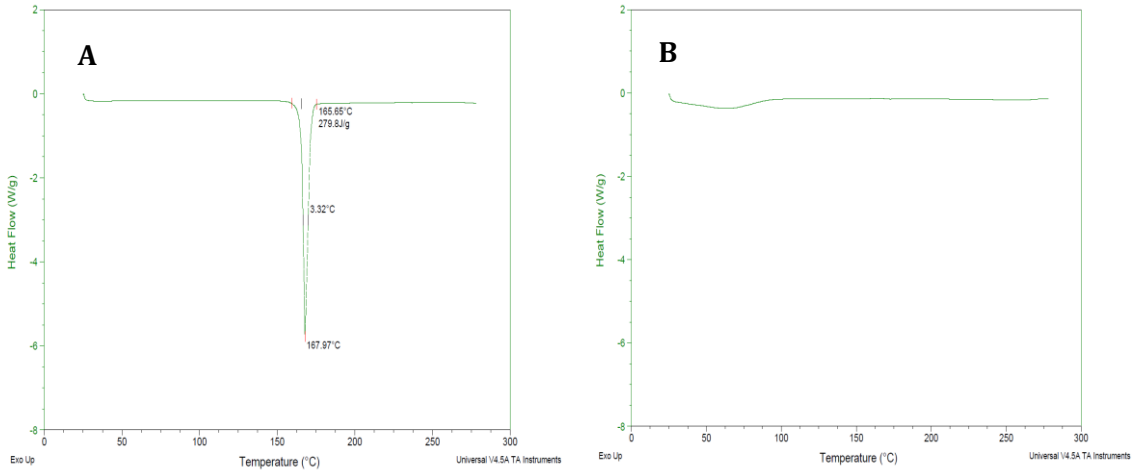


Figure 25: Mannitol – A and HPMC – B thermograms.

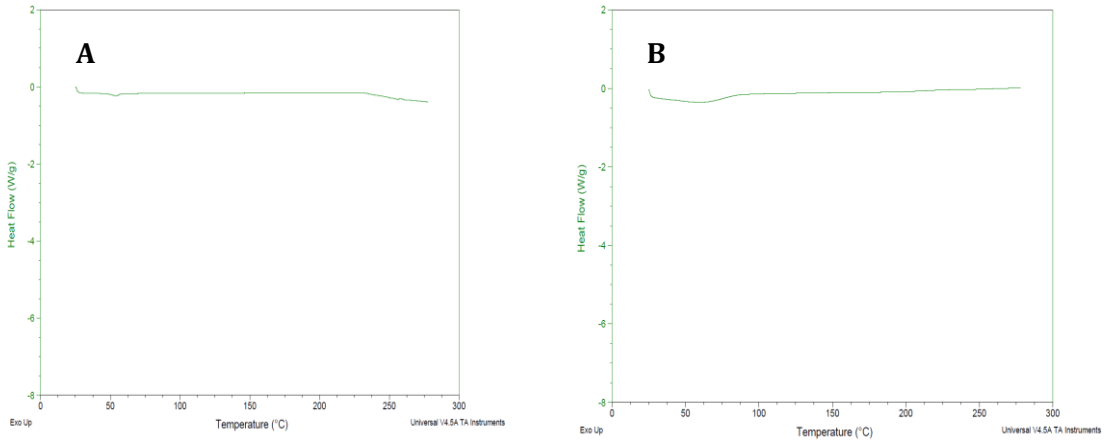


Figure 26: Eudragit E100 – A and HPC SL – B thermograms.

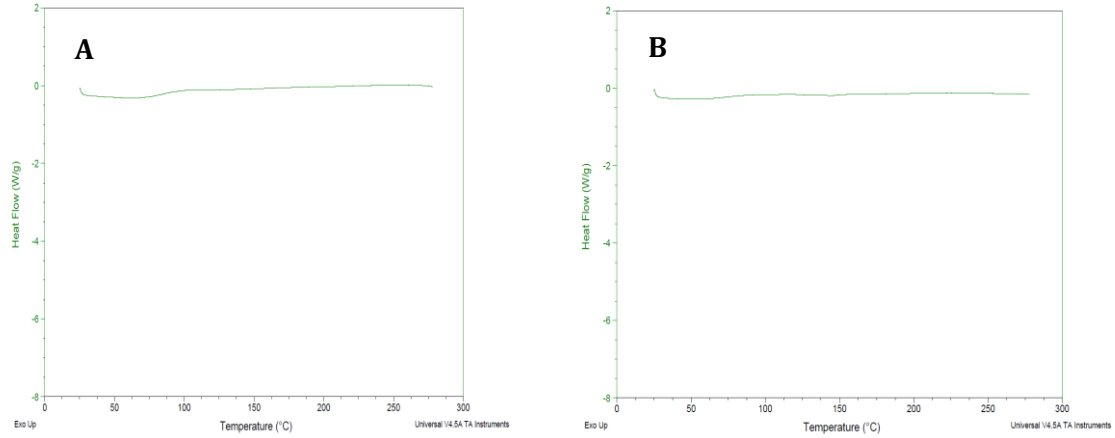


Figure 27: Plasdone S630 – A and HPMC AS-MG – B thermograms.

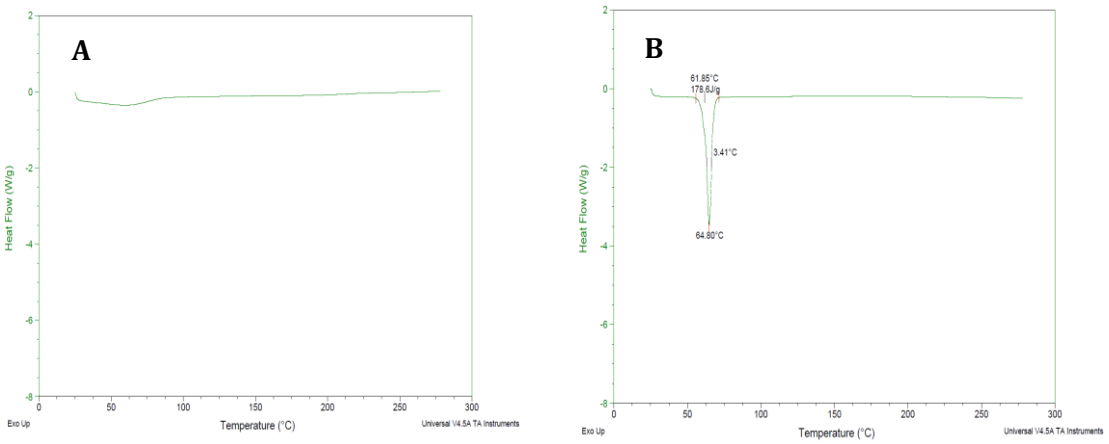


Figure 28: Soluplus – A and PEG 10000 – B thermograms.

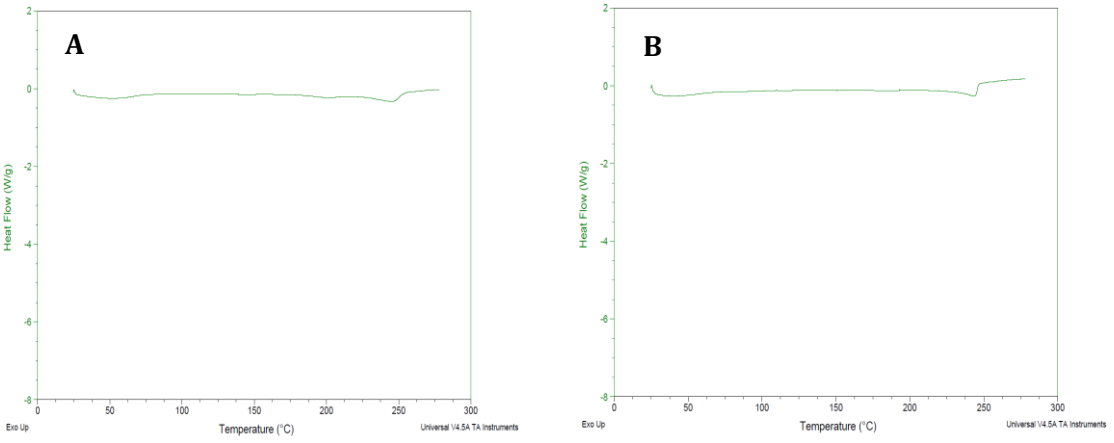


Figure 29: RES:HPMC (1:1) PM – A and LF – B thermograms.

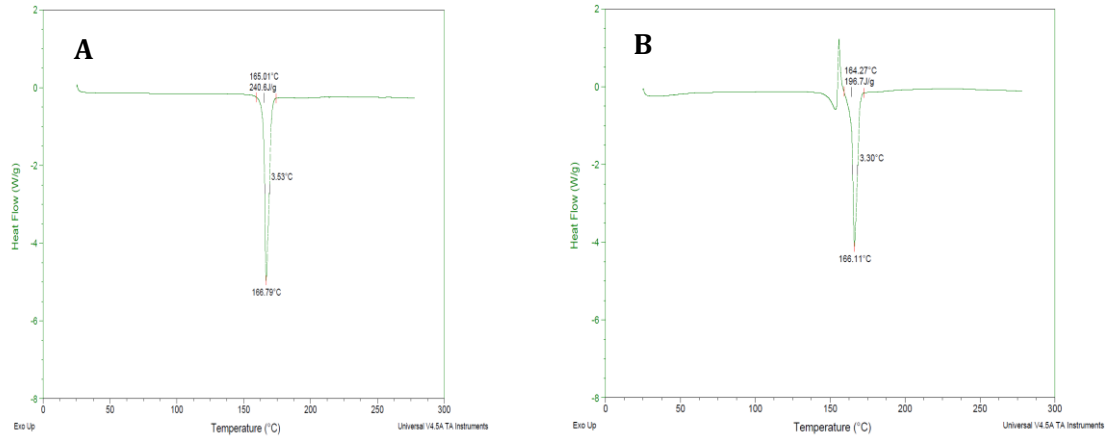


Figure 30: RES:Eudragit E100:Mannitol (1:1:8) PM – A and LF – B thermograms.

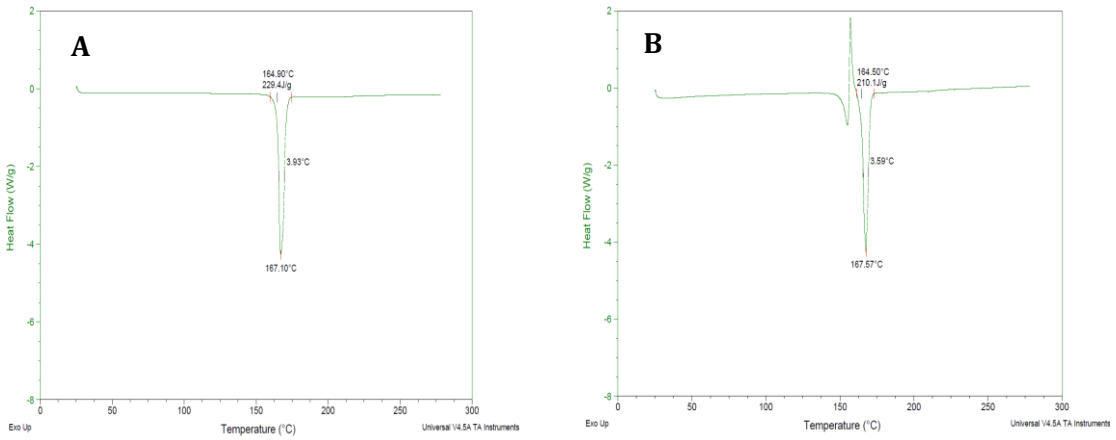


Figure 31: RES:HPC SL:Mannitol (1:1:8) PM – A and LF – B thermograms.

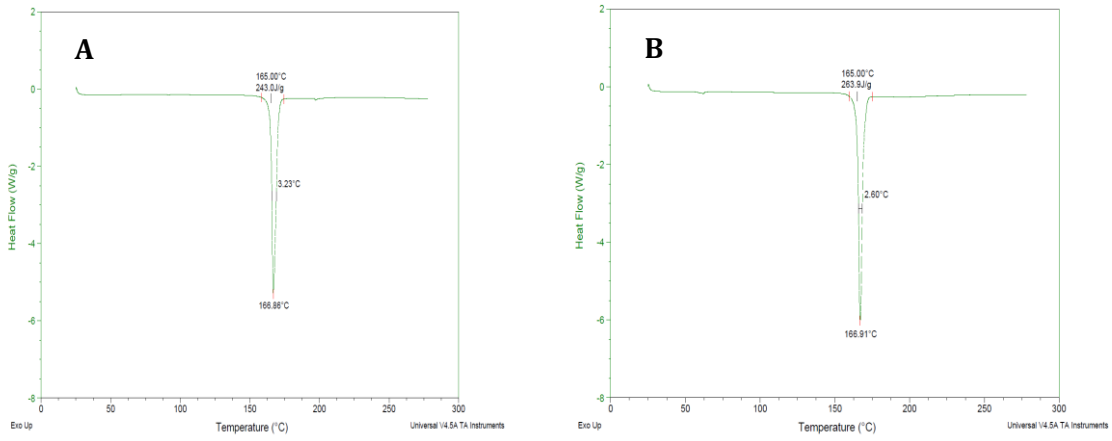


Figure 32: RES:Plasdone S630:Mannitol (1:1:8) PM – A and LF – B thermograms.

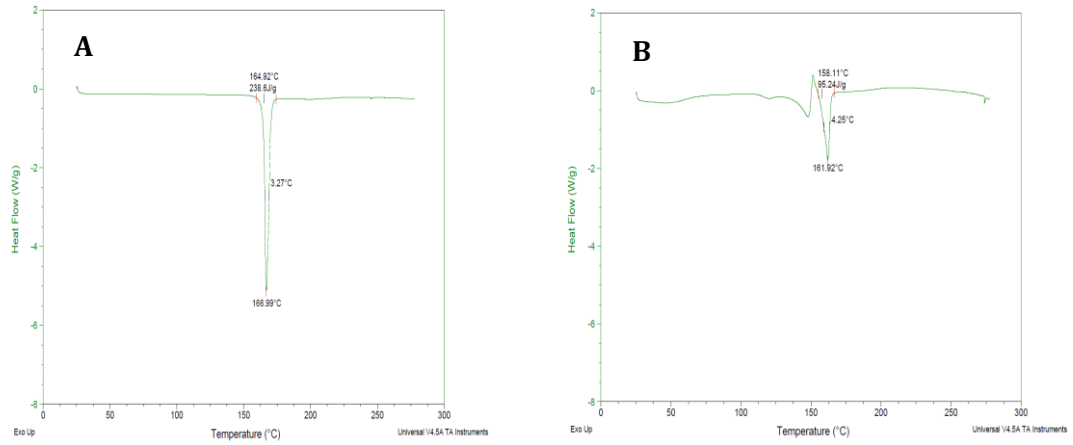


Figure 33: RES:HPMC AS-MG:Mannitol (1:1:8) PM – A and LF – B thermograms.

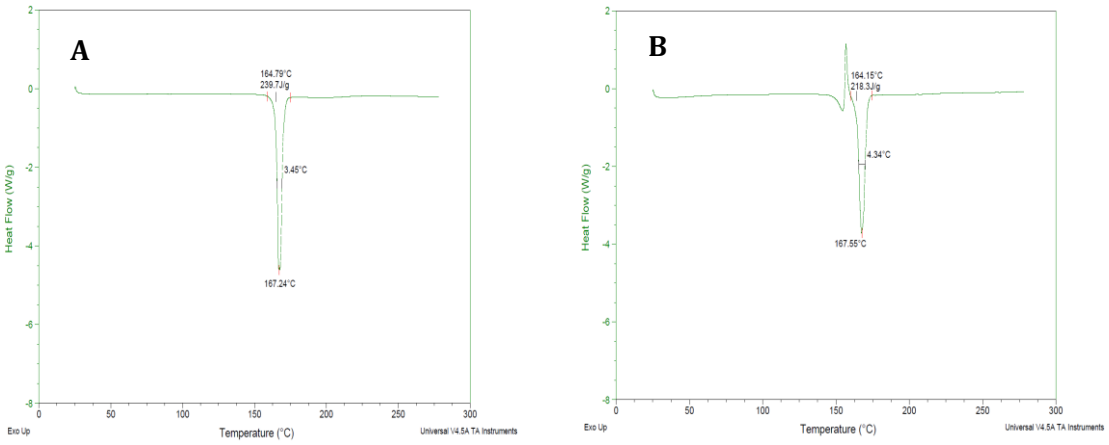


Figure 34: RES:Soluplus:Mannitol (1:1:8) PM – A and LF – B thermograms.

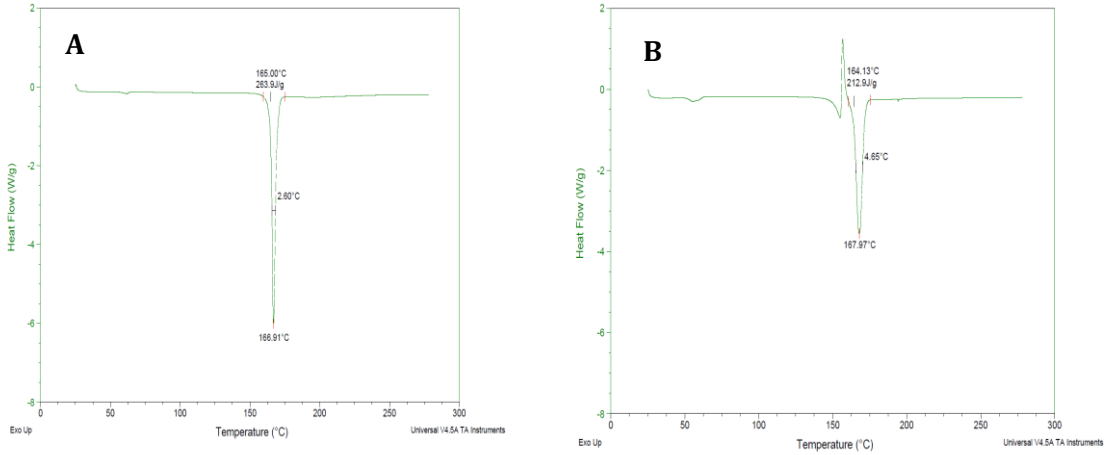


Figure 35: RES:PEG 10000:Mannitol (1:1:8) PM – A and LF – B thermograms.

Differential scanning calorimetry analysis allows evaluating possible molecular interaction between RES and the hydrophilic polymer, and consequent changes in the solid state. Based on the thermal events observed, greater or lesser solubility for the active pharmaceutical ingredient can be predicted. The lower the enthalpy value of a substance, the more amorphous it will be, therefore higher solubility and dissolution rate are expected (255).

The RES thermogram presents a single and sharp endothermic peak at 265.52ºC, that corresponds to its melting point with an enthalpy value of 258.7 J/g (Figure 24). Mannitol used as bulking agent is crystalline and melts at 169.97ºC (Figure 25 - A). All polymers are amorphous, except PEG 10000 which is crystalline and melts at around 65ºC (Figure 28 - B).

The formulation with HPMC presents a small endothermic event close to the melting temperature of RES (PM and LF). In the remaining PMs and LFs, the endothermic peak corresponding to the melting of RES has disappeared, while the melting peak of mannitol remains in both. The absence of RES melting peak may be due to its solubilization in the polymer and mannitol when they begin to melt or due to its molecular dispersion in the polymer. When comparing the PM and SD thermograms for each formulation, an exothermic event can be observed just before mannitol melting in the LFs, except in the formulation with Plasdone S630, which may indicate molecular interaction in the SD formulations.

Eudragit E100, a cationic copolymer, presented the best solubility results for RES but is difficult to micronize and dissolve. Another cationic methacrylate copolymer (Eudragit E PO) in the form of a dry powder, was further used to overcome the difficulties observed with Eudragit E100. This polymer was tested in the next phase.

6.2. Hydrophilic carrier optimization

After lyophilization intact wafers with good appearance were obtained for all formulations.

6.2.1. Solubility

Solubility for PMs and LFs was assessed after 24 hours stirring at room temperature. Determination was performed in triplicate samples at pH 6.8 as presented in Figure 36.

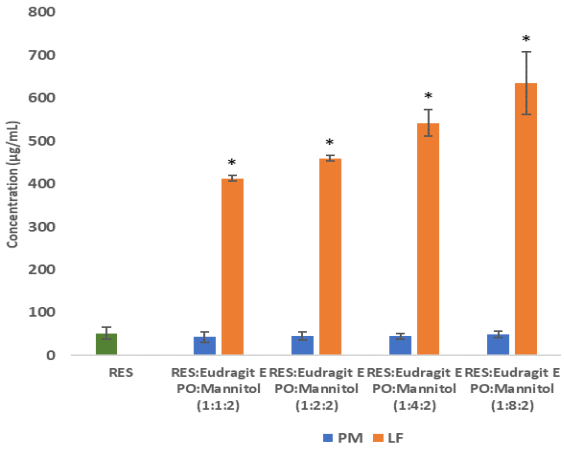


Figure 36: Solubility after 24 hours under magnetic stirring at room temperature in pH 6.8 (Eudragit E PO used at different ratios in formulation). (mean ± SD, *n*=3), $^{*}p < 0.05$ comparing with pure RES and PMs.

Eudragit E PO is a nonhygroscopic hydrophilic cationic polymer consisting of methyl methacrylate, N-N-dimethylaminoethyl methacrylate and butyl methacrylate monomers (1:2:1), possesses tertiary amines that ionize at the acidic pH to make the polymer highly soluble in fluids when pH is below 5 (256). A huge increase in solubility was observed for all LFs. At pH 6.8 in all PMs the solubility results are similar to the solubility of RES alone, also confirming the results obtained with Eudragit E100. All four LFs presented RES solubility values above 400 µg/mL at this pH.

6.2.2. Differential Scanning Calorimetry (DSC)

PMs and LFs were assessed by DSC using the method described above.

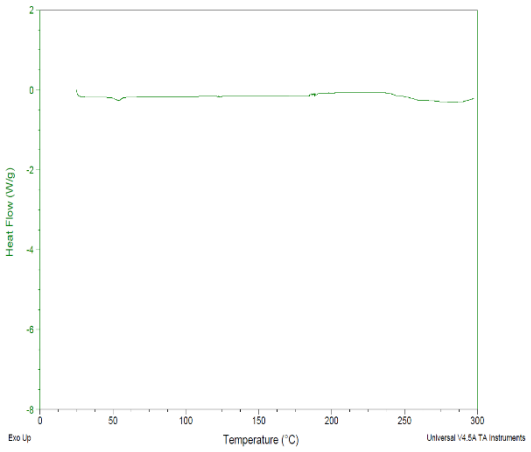


Figure 37: Eudragit E PO thermogram.

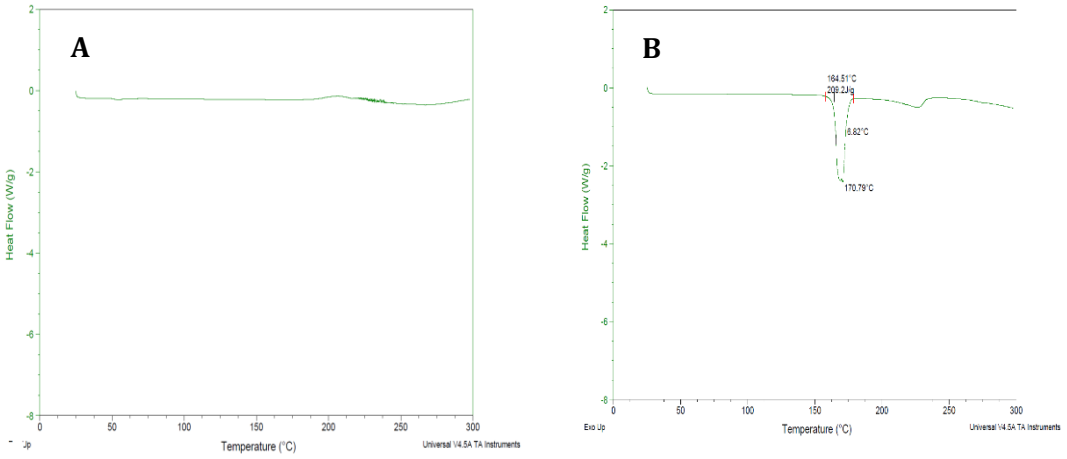


Figure 38: RES:Eudragit E PO (1:1) PM – A and RES:Mannitol (1:2) PM – B thermograms.

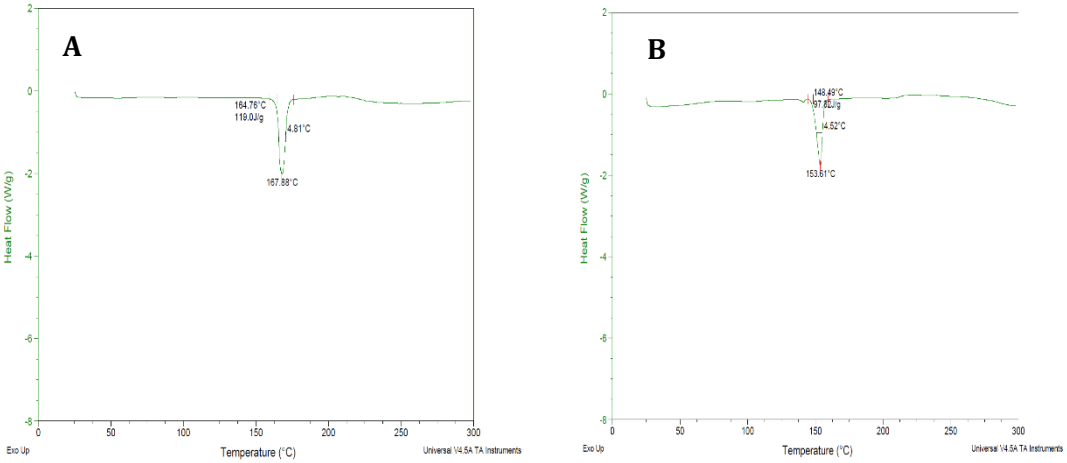


Figure 39: RES:Eudragit E PO:Mannitol (1:1:2) PM – A and LF – B thermograms.

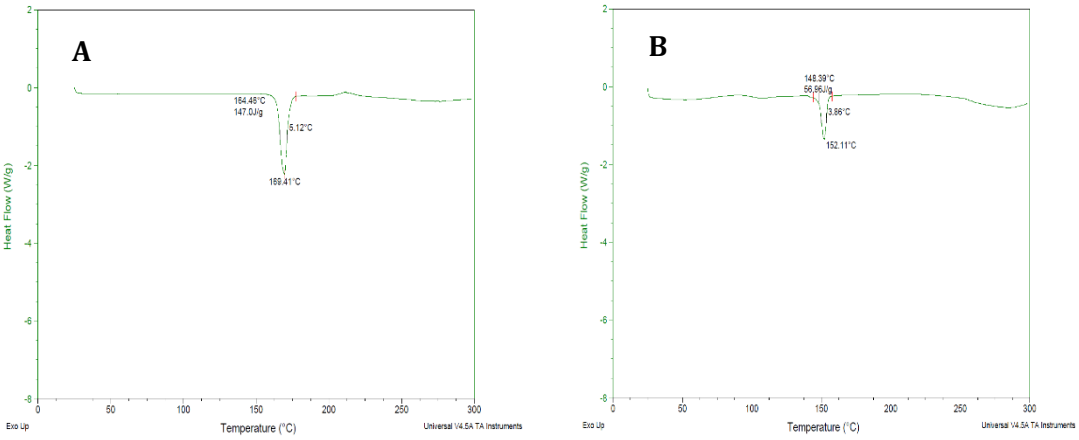


Figure 40: RES:Eudragit E PO:Mannitol (1:2:2) PM – A and LF – B thermograms.

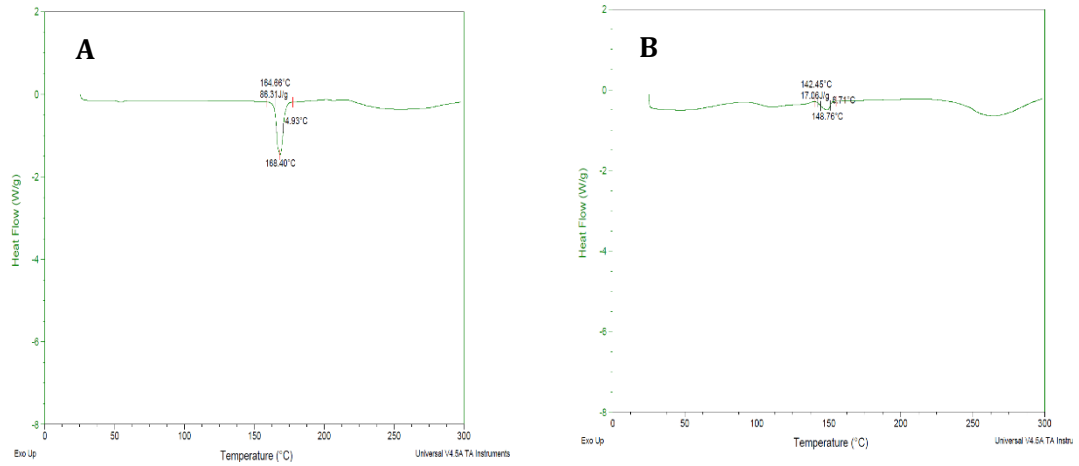


Figure 41: RES:Eudragit E PO:Mannitol (1:4:2) PM – A and LF – B thermograms.

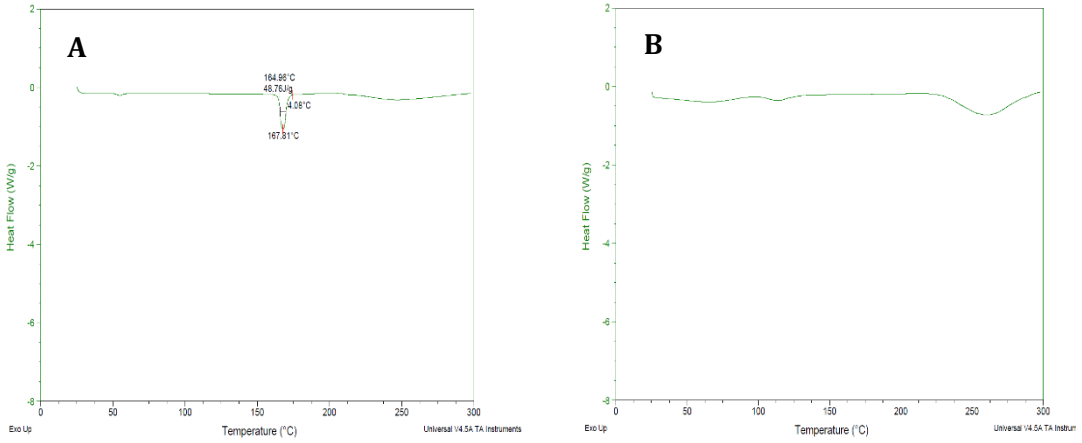


Figure 42: RES:Eudragit E PO:Mannitol (1:8:2) PM – A and LF – B thermograms.

Eudragit E PO is amorphous (Figure 37). The thermograms of RES with Eudragit E PO in the lowest ratio tested (1:1) do not present the melting peak of RES (Figure 38 - A). This may suggest improved solubility for RES in both the PM and the LF, which was confirmed in solubility assessment except for the PM at pH 6.8 (explained above). RES endothermic peak disappeared in all PMs and LFs thermograms, while the melting peak of mannitol decreased in formulations with a higher polymer ratio due to its lower percentage in the formulation (dilution effect). In Figures 39, 40 and 41, mannitol melting peak in the LFs, moved to a lower temperature suggesting a different interaction between its components compared to PMs.

Based on the results obtained, RES:Eudragit E PO:Mannitol (1:1:2) was the selected formulation. Wafers with good appearance and mechanical strength were obtained with this formulation. Although an increase in the polymer portion yields higher solubility

results, the mass of the SD in a future final solid dosage form, may be very high. It was expected that the increase in solubility presented by RES:Eudragit E PO:Mannitol at (1:1:2) ratio would allow an increase in bioavailability and consequently a reduction in the dose of RES administered.

The aim for the next phase was the selection of a surfactant to stabilize the SD and improve solubility allowing an enhanced bioavailability of RES.

6.3. Surfactant selection

After lyophilization intact wafers with good appearance were obtained for all surfactants tested.

6.3.1. Differential Scanning Calorimetry (DSC)

LFs were assessed by DSC using the method described above.

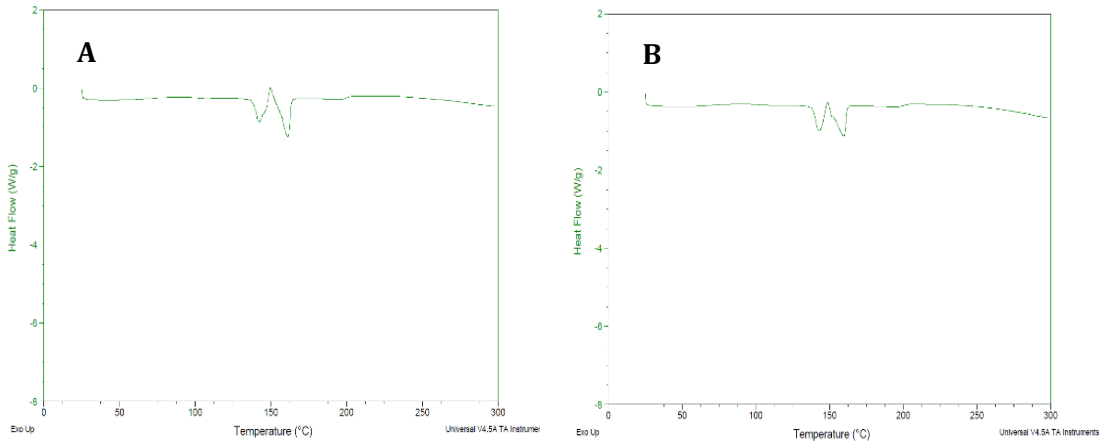


Figure 43: RES:Eudragit E PO:Mannitol (1:1:2)_Compitrol ATO 4% – A and RES:Eudragit E PO:Mannitol (1:1:2)_Docusate sodium 4% – B thermograms.

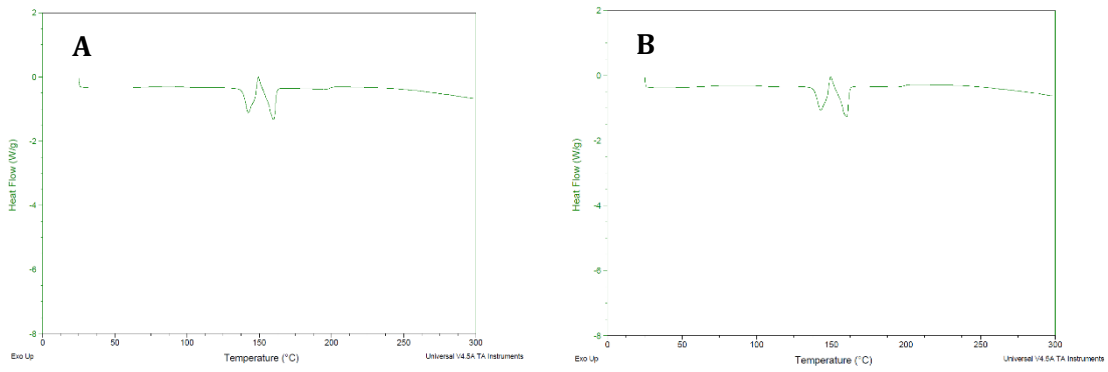


Figure 44: RES:Eudragit E PO:Mannitol (1:1:2)_Gelucire 44/14 4% – A and RES:Eudragit E PO:Mannitol (1:1:2)_Poloxamer 407 4% – B thermograms.

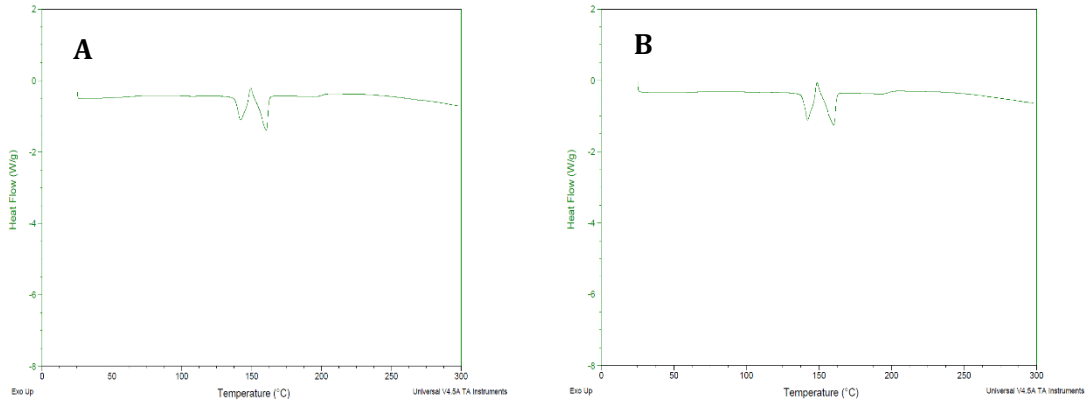


Figure 45: RES:Eudragit E PO:Mannitol (1:1:2)_Tween 80 4% – A and RES:Eudragit E PO:Mannitol (1:1:2)_Labrasol 4% – B thermograms.

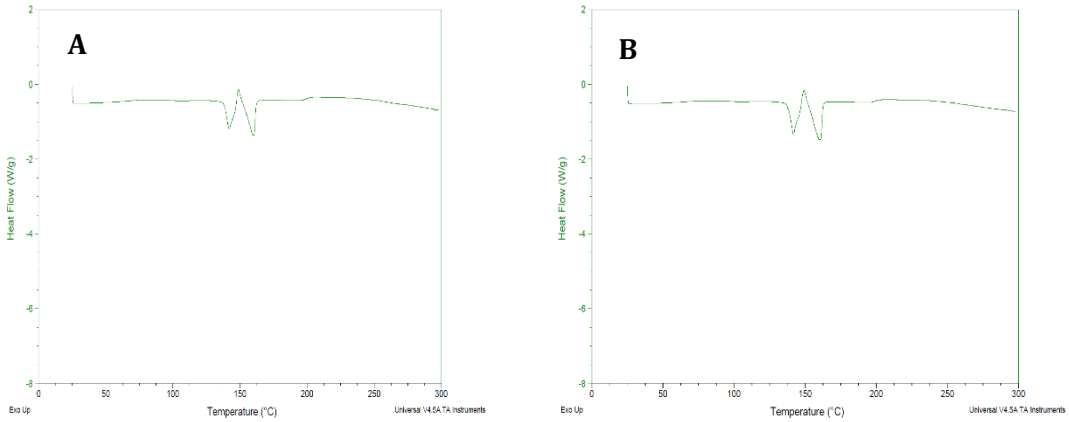


Figure 46: RES:Eudragit E PO:Mannitol (1:1:2)_Cetrimide 4% – A and RES:Eudragit E PO:Mannitol (1:1:2)_SDS 4% – B thermograms.

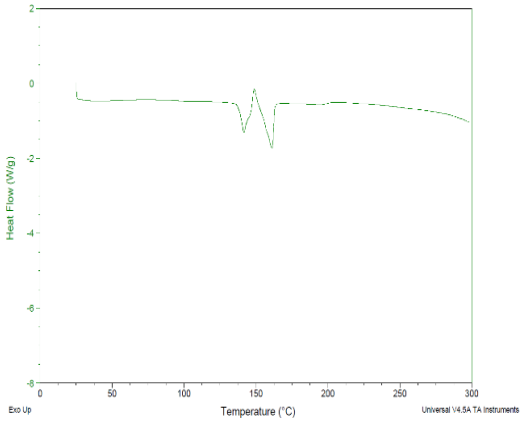


Figure 47: RES:Eudragit E PO:Mannitol (1:1:2)_Kolliphor RH 40 4% thermogram.

Similar thermograms were obtained for all LFs with the absence of RES endothermic peak. Two endothermic events at around 150ºC were present in all thermograms that correspond to mannitol melting.

LF stored in amber glass bottles were submitted to a stress study and placed during 1 month at ICH accelerated conditions (40ºC/75%RH). Samples were tested for assay and solubility at pH 1.2 at T0 and T1 month.

6.3.2. Assay

Table 16: Formulations assay (surfactant selection) at T0 and T1 (40ºC/75% RH). (mean ± SD, *n*=2).

Formulation	RES (%) ± SD	
	T0	T1 (40ºC/75% RH)
RES:Eudragit E PO:Mannitol (1:1:2)_Compitrol ATO 4%	94.6 ± 6.05	94.2 ± 1.15
RES:Eudragit E PO:Mannitol (1:1:2)_Docusate sodium 4%	95.5 ± 0.22	95.3 ± 0.27
RES:Eudragit E PO:Mannitol (1:1:2)_Gelucire 44/14 4%	96.4 ± 0.36	96.0 ± 0.05
RES:Eudragit E PO:Mannitol (1:1:2)_Poloxamer 407 4%	96.5 ± 0.46	96.2 ± 0.02
RES:Eudragit E PO:Mannitol (1:1:2)_Tween 80 4%	99.1 ± 2.59	95.8 ± 0.40
RES:Eudragit E PO:Mannitol (1:1:2)_Labrasol 4%	97.2 ± 0.18	94.5 ± 1.78
RES:Eudragit E PO:Mannitol (1:1:2)_Cetrimide 4%	97.0 ± 0.09	96.1 ± 0.39
RES:Eudragit E PO:Mannitol (1:1:2)_SDS 4%	88.7 ± 0.88	88.1 ± 0.05
RES:Eudragit E PO:Mannitol (1:1:2)_Kolliphor RH 40 4%	83.2 ± 3.93	87.4 ± 0.83

RES assay results did not decrease for all formulations tested after 1 month at 40ºC/75% RH, revealing no signs of apparent chemical degradation. The formulations containing SDS and Kolliphor RH 40 presented an assay value of less than 90% even at T0.

6.3.3. Solubility

Solubility for LFs was assessed after 24 hours stirring at room temperature. Determination was performed in triplicate samples in aqueous solvent with pH value 1.2, at T0 and after 1 month at 40ºC/75% RH (Figure 48).

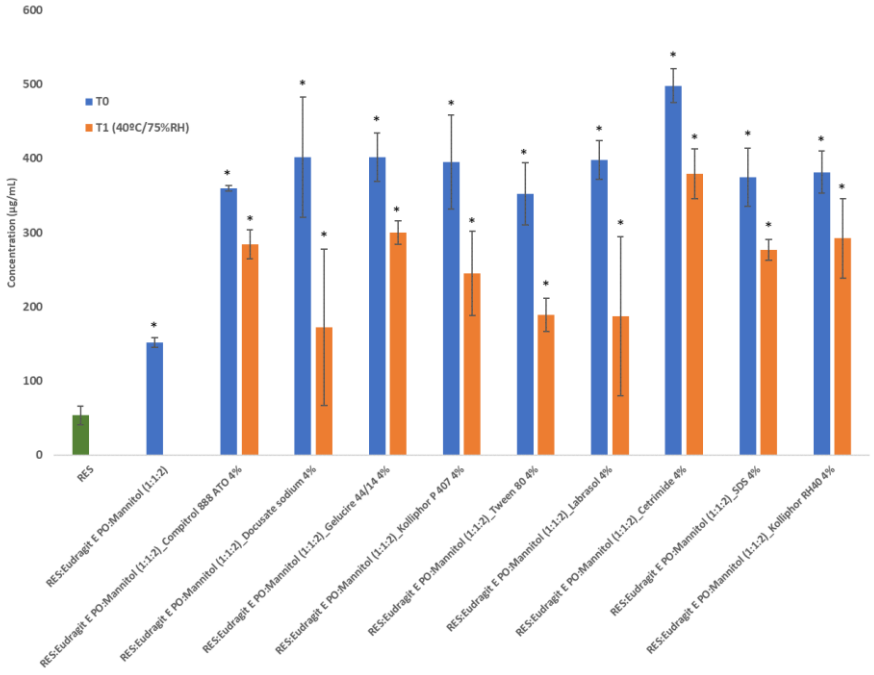


Figure 48: Solubility after 24 hours under magnetic stirring at room temperature in pH 1.2 at T0 and T1 month at 40ºC/75% RH, (high throughput surfactant screening). (mean±SD, *n*=3), **p* < 0.05 comparing with pure RES.

At T0 at least a 2-fold increase in solubility was observed for all LFs with surfactant compared to the formulation without surfactant (151.3 µg/mL). The lowest increase in RES solubility was observed with Tween 80 (352.4 µg/mL), while higher average values were obtained with cetrimide (498.2 µg/mL), docusate sodium (401.8 µg/mL) and Gelucire 44/14 (401.7 µg/mL).

After 1 month at 40ºC/75% RH, a decrease in RES solubility was observed for all LFs, with the greatest average reduction observed with docusate sodium (401.8 µg/mL to 172.4 µg/mL). The formulations with cetrimide and Gelucire 44/14 were among those that showed the smallest decrease in solubility and the only ones that maintained average values above 300 µg/mL, (Gelucire 44/14 – 300.1 µg/mL; cetrimide – 379.1 µg/mL).

Although cetrimide presented slightly higher solubility results compared to Gelucire 44/14, this was the surfactant selected, also considering its effect on presystemic drug metabolism inhibition and interference in P-gp mediated efflux, that contribute to an improved RES bioavailability (257-259). Gelucire 44/14 is also a non-ionic surfactant with a less irritant effect than a cationic surfactant such as cetrimide (260).

Gelucire 44/14 is a non-ionic water dispersible surfactant obtained by an alcoholysis reaction between coconut oil and polyethylene glycol-32 (PEG-32) under controlled

conditions. It consists of glycerides and PEG esters of fatty acids of varying chain length. Gelucire 44/14 melting begins as low as 10ºC, meaning that at 20-25ºC a fraction is already liquid. This is why the product is described as “semi-solid” and is supplied in block form. Its main physicochemical properties are presented in Table 17 below (261).

Table 17: Main physicochemical properties of Gelucire 44/14.

Drop point (Mettler, ºC)	45.0 ± 2.5
pH (10% in purified water)	4.4 ± 0.7
Relative density	1.023 at 50ºC
Calculated / pratical HLB	14 / 11
Critical micellar concentration (g/L, 25ºC)	0.07 ± 0.05
Solubility in ethanol 96º	Soluble
Solubility in chloroform, methylene chloride	Freely soluble
Solubility in n-hexane	Insoluble

The digestion of Gelucire 44/14 has been investigated by Fernandez et al. (262), using an *in vitro* set-up with enzymes that simulate the gastric and duodenal phases. During the gastric phase the glycerides fraction is digested. Triglycerides are rapidly and almost completely digested into diglycerides, monoglycerides and free fatty acids. Diglycerides are partially digested into monoglycerides and fatty acids. During the intestinal phase, PEG esters are partially digested releasing free fatty acids and free PEG. The digestion of lipids stimulates the secretion of bile salts, phospholipids, and cholesterol by the gallbladder. These amphiphilic compounds associate with the components of Gelucire 44/14 digestion and self-assemble into different colloidal structures: multi-lamellar, vesicles, mixed micelles and micelles. These structures have variable solubilizing capacities and contribute to maintaining the drug in solubilized state throughout the on-going digestion process. Ultimately, the fatty acids, monoglycerides and drug partition out of the mixed micelles and are absorbed in the enterocyte (263).

6.4. Surfactant optimization

6.4.1. Differential Scanning Calorimetry (DSC)

Gelucire 44/14 was assessed by DSC using the method described above.

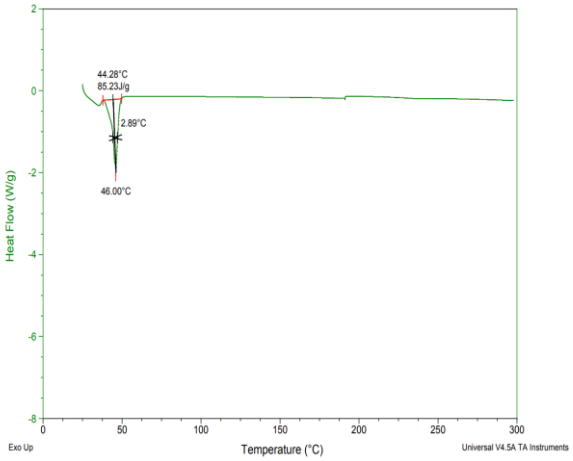


Figure 49: Gelucire 44/14 thermogram.

Gelucire 44/14 presents an endothermic event at 46ºC.

6.4.2. Assay

Table 18: Formulations assay (surfactant optimization). (mean ± SD, *n*=2).

Formulation	RES (%) ± SD
RES:Eudragit E PO:Mannitol (1:1:2)_Gelucire 44/14 1%	96.6 ± 0.26
RES:Eudragit E PO:Mannitol (1:1:2)_Gelucire 44/14 4%	96.4 ± 0.36
RES:Eudragit E PO:Mannitol (1:1:2)_Gelucire 44/14 8%	94.9 ± 0.52
RES:Eudragit E PO:Mannitol (1:1:2)_Gelucire 44/14 16%	96.1 ± 0.55

Assay results between 90-110% were obtained for all LFs.

6.4.3. Solubility

Solubility for LFs was assessed after 24 hours stirring at room temperature. Determination was performed in triplicate samples in aqueous solvent with pH value 1.2.

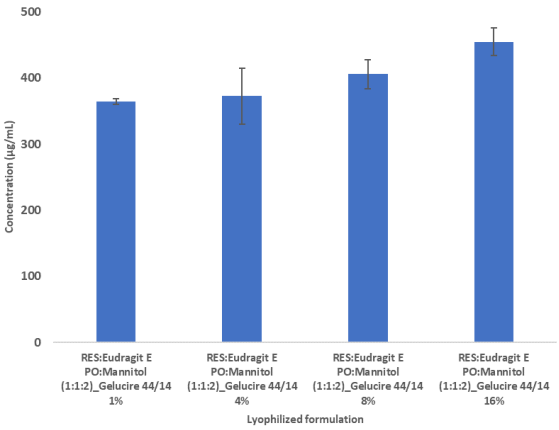


Figure 50: Solubility after 24 hours under magnetic stirring at room temperature in pH 1.2 (Gelucire 44/14 at 1%, 4%, 8% and 16% w/w of RES). (mean ± SD, *n*=3).

RES solubility slightly increased with the percentage of surfactant. Gelucire 44/14 at 16% (w/w) of RES was selected for the formulation, also considering that a greater amount can potentiate its effect on presystemic drug metabolism inhibition and interference in P-gp mediated efflux, that contribute to an improved RES bioavailability (257-259).

6.5. Bulking agent selection

After lyophilization intact wafers with good appearance were obtained for all formulations tested as presented in Figure 51.

Figure 51: RES:Eudragit E PO:Mannitol (1:1:2)_Gelucire 44/14 16% (*1*); RES:Eudragit E PO:Lactose (1:1:2)_Gelucire 44/14 16% (*2*); RES:Eudragit E PO (1:1)_Gelucire 44/14 16% (*3*) lyophilized wafers.

6.5.1. Assay

Table 19: Formulations assay (bulking agent selection). (mean ± SD, *n*=2).

Formulation	RES (%) ± SD
RES:Eudragit E PO:Mannitol (1:1:2)_Gelucire 44/14 16%	96.1 ± 0.55
RES:Eudragit E PO:Lactose (1:1:2)_Gelucire 44/14 16%	98.2 ± 3.88
RES:Eudragit E PO (1:1)_Gelucire 44/14 16%	91.3 ± 0.16

Assay results between 90-110% were obtained for all LFs.

6.5.2. Solubility

Solubility for LFs was assessed after 24 hours stirring at room temperature. Determination was performed in triplicate samples in aqueous solvents with pH value 1.2, 4.5 and 6.8.

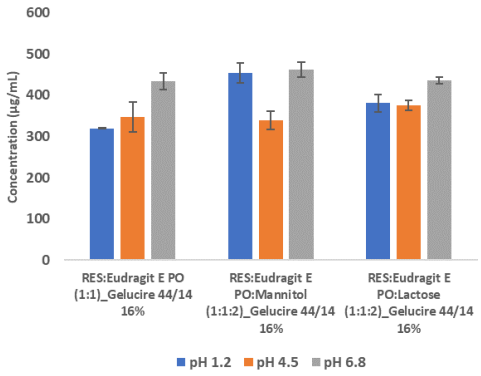


Figure 52: Solubility after 24 hours under magnetic stirring at room temperature in pH 1.2, pH 4.5 and pH 6.8. (mean±SD, *n*=3).

Solubility was similar in all aqueous media for formulations without bulking agent and lactose or mannitol as bulking agents.

Intact wafers with good appearance and adequate mechanical strength were obtained in the formulation without bulking agent, suggesting that there is no need for lyoprotectant.

7. Conclusion

Considering the above, RES:Eudragit E PO (1:2)_Gelucire 44/14 16% was the formulation selected to be tested and produced by the bulk method. The removal of lyoprotectant allowed to increase the percentage of Eudragit E PO in the formulation without increasing the final mass of the LF, which could be too high to develop an oral solid dosage form with favourable patient compliance. The bulk lyophilization method was performed in aluminium trays allowing the production of larger quantities of SD for inclusion in oral solid dosage forms.

IV. Lyophilization process optimization

1. Objectives

To optimize the lyophilization cycle by the bulk method, several factors were investigated at freezing, primary and secondary drying phases based on a DoE approach. Several key aspects that affect process performance and product quality were considered as presented below:

- Freezing – maximize crystal size and crystallization to increase drying rates (165, 166).
- Thickness of product – water vapor molecules experience resistance as they exit from the dried portion of the product. Thinner samples yield less resistance to vapor flow and lead to faster drying (165, 166).
- Critical collapse temperature (T_c) – this is the most important piece of information for cycle optimization. The ability to run primary drying at higher product temperatures greatly reduces drying time by creating a larger pressure differential between the vapor pressure over ice in the product and the pressure at the condenser. Each 1ºC increase in product temperature can decrease primary drying time by 13% (165, 166).

2. Freezing optimization

Freezing determines the porosity and surface topology. Different freezing cycles were tested to optimize the freezing process. Therefore, target temperature, cooling rate, and intermediate thermal procedures were adjusted to improve process, product characteristics and performance. Formulation was frozen to a temperature below its glass transition temperature in the frozen sate (T_g'). The parameters tested to optimize the freezing stage are presented in Table 20.

Table 20: Parameters tested for freezing optimization.

Input parameter	Values tested	Output parameter
Solution height in the tray (cm)	1 and 2	***Freeze-dried product appearance***
Cooling rate (ºC/min.)	0.5 and 1	

Supercooling temperature (ºC)	-30 and -40	***Sublimation rate (g/h/cm²)***
Supercooling time (h)	1 and 2	

3. Drying optimization

The network of ice crystals formed during freezing was sublimed to produce a highly porous structure. The lyophilization tray was placed under deep vacuum and dried at a temperature below the collapse temperature. Several drying cycles were tested to improve the sublimation rate and obtain a bulk with good appearance (not cracked or molten), uniform colour, adequate porosity and stable under storage conditions.

3.1. Primary drying optimization

Primary drying is a slow process conducted at cooler temperatures, safely below the product's critical collapse temperature at a reduced pressure. Sublimation requires heat energy to drive the phase change process from solid to gas. After pre-freezing the product, conditions must be established in which ice can be removed from the frozen product, resulting in a dry, structurally intact product. To improve process and product characteristics, the two parameters involved (temperature and pressure) in the freeze-drying system were tested and adjusted (264, 265). The parameters tested to optimize the primary drying phase are presented in Table 21.

Table 21: Parameters tested for primary drying optimization.

Input parameter	**Values tested**	**Output parameter**
Chamber pressure (mTorr)	75, 150 and 200	***Freeze-dried product appearance***
Target shelf temperature (ºC)	10, 20, 30 and 40	***Sublimation rate (g/h/cm²)***
		Freeze-dried product moisture (%)

3.2. Secondary drying optimization

After primary drying is complete, and all ice has sublimed, bound moisture is still present in the product. The product appears dry, but the residual moisture content may be as high as 5-20%. This is removed during secondary drying at a warmer temperature to reduce the residual moisture content to optimum values. This process is called isothermal desorption as the bound water/solvent is desorbed from the product (166, 168). The parameters tested to optimize the secondary drying phase are presented in Table 22.

Table 22: Parameters tested for secondary drying optimization.

Input parameter	Values tested	Output parameter
Target temperature (ºC)	40	***Freeze-dried product appearance***
Time at target temperature (h)	6, 12 and 18	***Freeze-dried product moisture (%)***

4. Bulk lyophilization

Each formulation solution used to test different cycle conditions was poured into an aluminium tray and submitted to bulk drying. It was decided to use a 1:2 (w/w) drug/polymer ratio in the formulation to increase molecular interactions, stability in solid dispersion, and to further improve RES solubility. The following formulation was selected and produced to assess the influence of the different factors on the lyophilization process and product characteristics.

RES:Eudragit E PO (1:2)_Gelucire 44/14 16%

in TBA/Acetate buffer pH 4.5 (75:25)

The sublimation rate was determined gravimetrically. A laboratory analytical balance, (Sartorius® MSU1203S-100-DE, Germany) was used to weigh the tray with the solution before and after lyophilization. Product moisture was determined using a thermogravimetric balance at 105ºC (Mettler® Toledo). Primary drying time begins when

the shelf temperature attains the desired shelf set point temperature for primary drying. The exact drying time for calculating the sublimation rate was defined as the time at which the shelf temperature reached the desired set point temperature.

5. Lyophilization cycle optimization results and discussion

5.1. Freezing assessment

The freezing time depends on fill volume. Supercooling temperature, and supercooling time should allow complete solution freezing. It is very important in freeze-drying to pre-freeze the product to below the T_g' before beginning the freeze-drying process. Small pockets of unfrozen material remaining in the product may expand and compromise the structural stability of the freeze-dried product (169). Another important aspect is the control of the crystal's growth during freezing. Rapid cooling origins smaller particles and more crystals, but resulting in a product that is, more difficult to freeze-dry, and consequently in increased drying time. Slower cooling results in large ice crystals and less restrictive channels in the matrix during the drying process which improves the speed of the freeze-drying process (167, 168).

Primary drying conditions were fixed in all cycles tested. Set point shelf temperature for primary drying was fixed at -10ºC. A 1ºC/min. was selected as shelf temperature increase rate from supercooling temperature to shelf set point temperature. A hold time of 30 min. at -5ºC during freezing was fixed, to potentiate crystals growth, reduce product's resistance to vapor flow and shorten the primary drying time. The chamber pressure set point was 75 mTorr.

In cycles where solution height in the tray was 2 cm, the freeze-dried product presented melted spots at the bottom. This means that freezing time was not sufficient to completely freeze the solution. Also in these cycles, primary drying time was significantly longer compared with cycles where solution height was 1 cm, due to greater resistance to vapour flow from the thicker dry mass at the top. This is evident from the lower sublimation rates in cycles with a 2 cm filling height. As the objective was to shorten the duration of the process, the increase in freezing and drying time, which would be necessary to obtain a successful product with a filling height of 2 cm, was discarded as a viable option, despite the greater quantity of product obtained per cycle. The freezing process was then optimized using a tray filling height of 1 cm.

The sublimation process was stopped when the product temperature reached shelf temperature. At the end of primary drying, there is no ice in the product, so its temperature increases to shelf temperature. This is considered the end point of primary drying.

Using the DoE software Modde® 11, (Sartorius, Göttingen, Germany), a 2-level full factorial design, without replicates was established, (experiences 3 to 10). The program cycles tested to assess freezing conditions are shown in Figure 53 and Table 23 below.

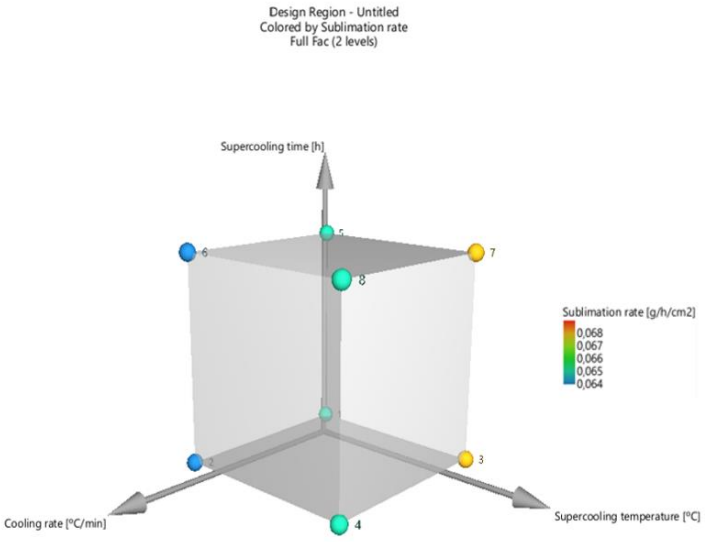


Figure 53: Design region for freezing optimization.

Table 23: Lyophilization program cycles - freezing optimization.

Cycle run	Filling height (cm)	Cooling rate (ºC/min.)	Supercooling temperature (ºC)	Supercooling time (h)	Primary drying time (h)	Sublimation rate (g/h/cm²)
1*	2	0.5	-40	2	28.6	0.059
2*	2	1	-30	1	38.6	0.045
3	1	1	-40	2	13.1	0.064
4	1	0.5	-40	2	13.1	0.064
5	1	1	-30	2	13.0	0.065
6	1	0.5	-30	1	12.5	0.068
7	1	1	-30	1	13.1	0.065
8	1	1	-40	1	12.9	0.064

9	1	0.5	-40	1	12.6	0.065
10	1	0.5	-30	2	12.4	0.068

* Melted spots in the freeze-dried product

An average primary drying time of 12.8 ± 2.3 h was obtained, and a very small variation in sublimation rate was observed with an average value of 0.065 ± 0.002 g/h/cm^2. Based on ANOVA, a $p < 0.05$ for the regression was obtained, meaning statistical significance and validity for the mathematical model selected. Contour plot for sublimation rate (Figure 54), demonstrated that within the range tested for the 3 parameters assessed there was a very low variation in response.

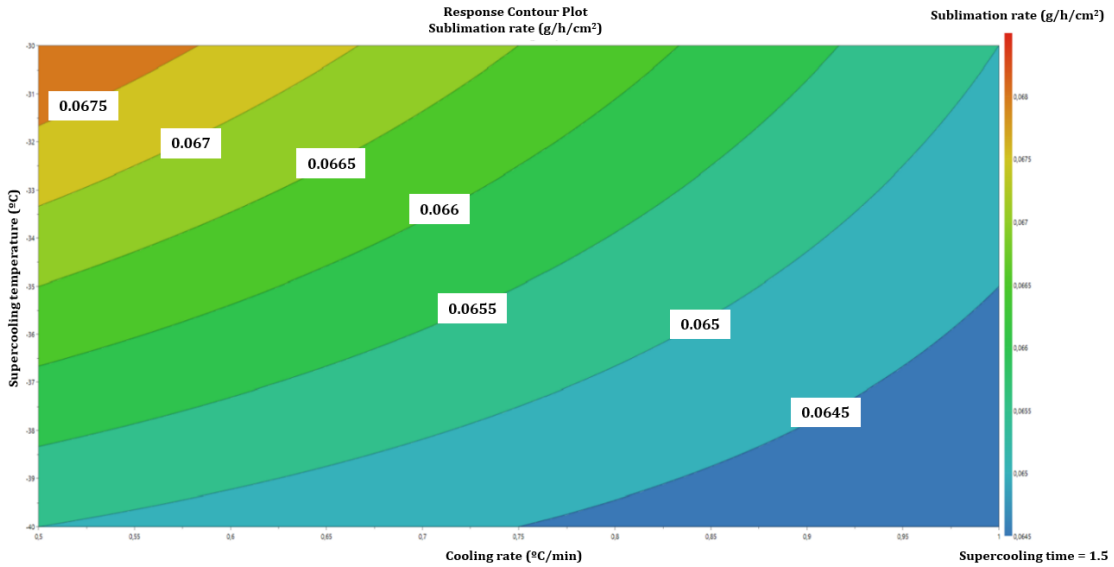


Figure 54: Sublimation rate contour plot - freezing optimization.

Cycle nº 3 conditions were selected, nevertheless freezing process demonstrated to be robust within the design space tested. A cooling rate of 1ºC/min. is a good compromise, avoiding some possible phase separation during freezing with slow cooling, and allows the formation of relatively large ice crystals, which facilitates sublimation. The degree of supercooling is the temperature difference between the thermodynamic or equilibrium ice formation temperature and the actual temperature at which ice begins to form, which is usually 10 to 25ºC lower (165, 167). A target supercooling temperature of -40ºC was selected, despite similar results obtained for -30ºC. The supercooling time of 2 hours was also selected despite there being no statistically significant differences with 1 hour

of supercooling. The lyophilization cycle obtained for the selected conditions is presented in Figure 55.

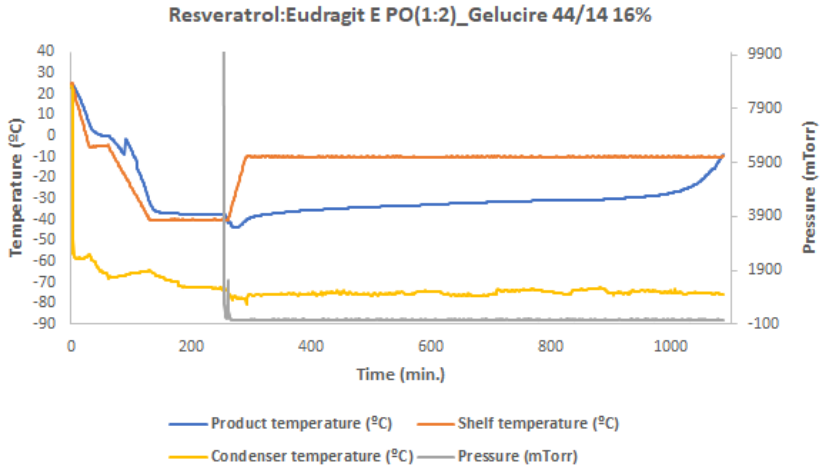


Figure 55: Lyophilization cycle - freezing optimization.

5.2. Primary drying assessment

As primary drying generally consumes the largest fraction of the freeze-drying cycle time, optimization of this phase in crucial. To optimize primary drying it is important to dry at a target shelf temperature as high as possible, but below critical temperature (T_c) to avoid product collapse (165). T_c is generally a few degrees above glass transition temperature of the product in the frozen state (T_g'). If the product is sublimated above this T_c, the solid phase collapses. It is crucial that the temperature at which a product is freeze dried is balanced between the temperature that maintains the frozen integrity of the product and the temperature that maximizes the vapor pressure of the product. This balance is key to optimum drying (166). Although the product is frozen below its T_g', warming during the freeze-drying process can affect the structure of the frozen matrix at the boundary of the drying front. This results in a collapse of the structural matrix (236).

The strategy in this phase is to choose the optimum product temperature (T_p) and bring the product to this temperature quickly. Attempts to determine glass transition temperature of the product in the frozen state (T_g') by DSC were unsuccessful. Therefore, the strategy was to test cycles with 10, 20, 30 and 40ºC as target shelf temperatures for primary drying and see if T_c was not reached. The T_p must always be several degrees below T_c to obtain a dry product with an acceptable appearance.

The chamber pressure impacts heat and mass transfer and must be well below the ice vapour pressure at the target product temperature to allow a high sublimation rate. The lowest chamber pressure provides the highest rate of ice sublimation, however, too low pressure may cause product contamination with pump oil and produce great heterogeneity in heat transfer. Typically the chamber pressure varies from 50 to 200 mTorr (165, 170).

Using DoE software Modde® 11, (Sartorius, Göttingen, Germany), a D-optimal design, with 1 replicate run was set. The program cycles tested to evaluate primary drying conditions are presented in Table 24 below.

Table 24: Lyofilization program cycles - primary drying optimization.

Cycle run	Chamber pressure (mTorr)	Target shelf temperature (ºC)	Primary drying time (h)	Sublimation rate (g/h/cm^2)	Product moisture (%)
1	200	40	6.2	0.134	6.7
2	75	40	7.4	0.113	7.2
3	200	10	6.7	0.126	7.8
4*	150	30	7.6	0.111	7.9
5	150	20	8.2	0.101	8.9
6	75	10	8.0	0.103	9.0
7	200	20	7.5	0.113	8.1
8*	150	30	7.2	0.113	7.7
9	75	20	7.6	0.109	8.2
10	150	40	7.0	0.115	6.6
11	200	30	7.2	0.115	7.2
12	150	10	7.2	0.115	8.7
13	75	30	7.9	0.104	8.8

* Cycle replicates

An average primary drying time of 7.4 ± 0.5 h was obtained, and a very small variation in sublimation rate was observed with an average value of 0.113 ± 0.009 g/h/cm^2. The moisture content of the freeze-dried product was on average 7.9 ± 0.8 %.

Based on ANOVA, for regression (experiments with different conditions) a $p < 0.05$ was obtained for sublimation rate and product moisture, and a $p > 0.05$ for lack-of-fit (replicated experiments) in both responses, meaning that selected mathematical model was statistically valid. The contour plots for sublimation rate and product moisture, demonstrated that within the range tested for the 2 parameters assessed there was a very low variation in responses.

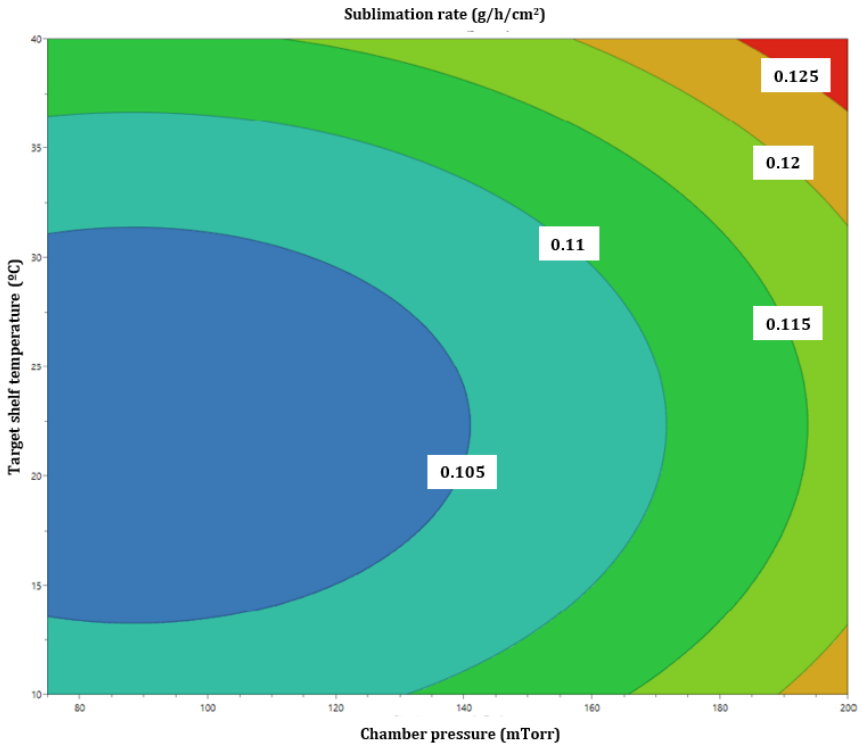


Figure 56: Sublimation rate contour plot - primary drying optimization.

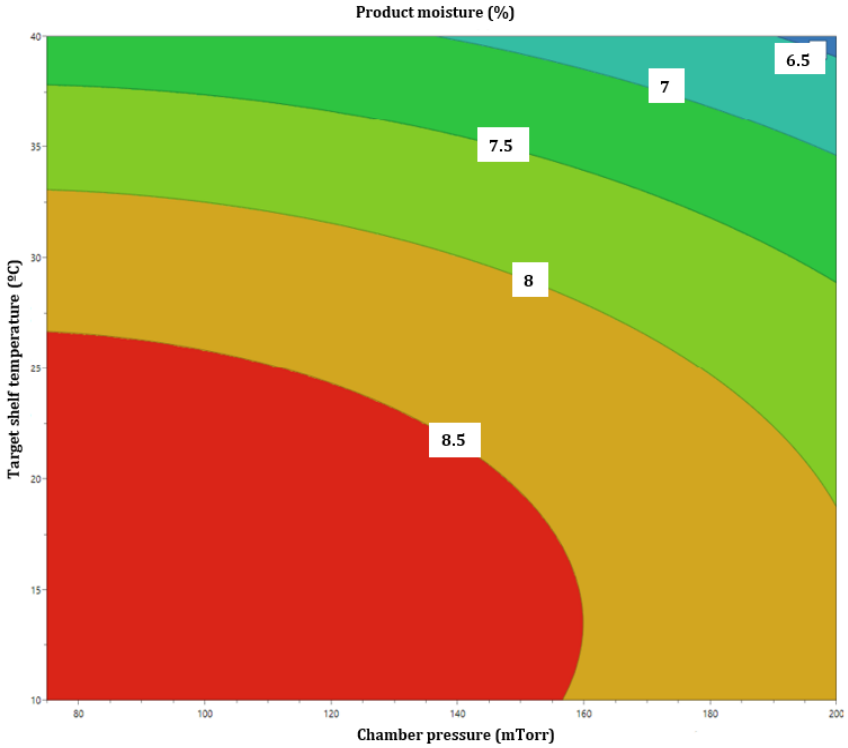


Figure 57: Product moisture contour plot - primary drying optimization.

Cycle nº 2 conditions were selected, nevertheless primary drying process demonstrated to be robust within the design space tested with small variations in the sublimation rate. Although a variation range of around 2.3% in product moisture was obtained, less relevance was given to this response at the end of primary drying, as different additional drying times were tested in the optimization of the secondary drying phase. A target shelf temperature of 40ºC and a chamber pressure of 75 mTorr were adequate to allow a shorter primary drying period without compromising product integrity. The lyophilization cycle obtained for the selected conditions is presented in Figure 58.

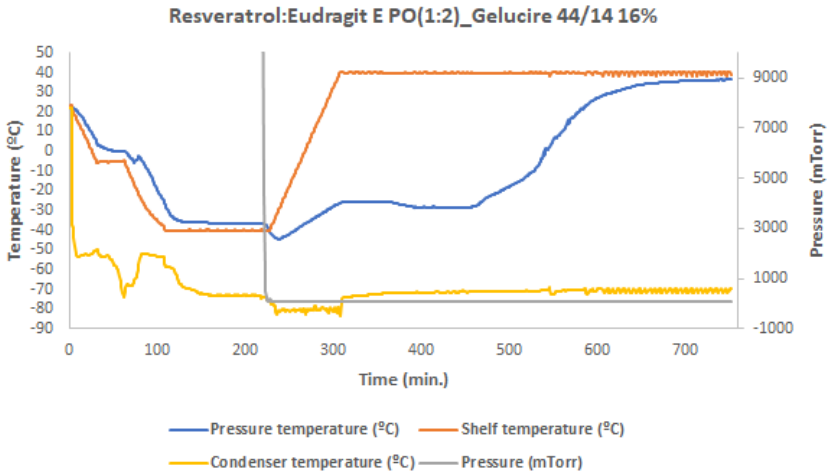


Figure 58: Lyophilization cycle - primary drying optimization.

5.3. Secondary drying assessment

Usually, secondary drying is performed at a much higher temperature than that used for primary drying so that desorption of solvents can occur at a practical rate, however in our case primary drying was already performed at 40ºC (165, 166). Higher temperatures could cause damage to the product and crystallization of RES. The strategy was to test whether prolonging the product's residence time at 40ºC would reduce residual moisture. The program cycles tested to assess secondary drying conditions are presented in Table 25.

Table 25: Lyophilization program cycles - secondary drying optimization.

Cycle run	Target shelf temperature (ºC)	Time at 40ºC (h)	Product moisture (%)
1	40	12	4.4
2	40	18	3.8
3	40	6	6.0

The lowest moisture content (3.8%) of the final product was obtained with 18 h of drying at 40ºC, and considering the stability of the product, this was the lyophilization cycle selected.

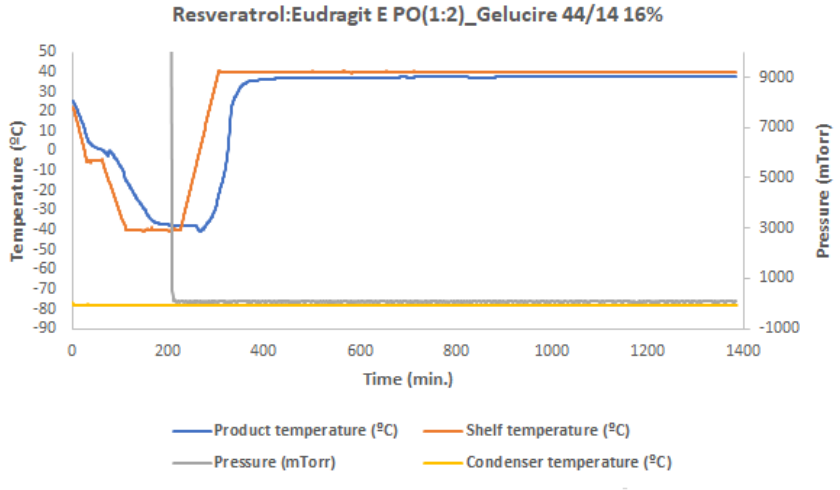


Figure 59: Lyophilization cycle - secondary drying optimization.

6. Conclusion

The cycle presented in Table 26 with a duration of approximately 23 hours was selected as the most optimized in terms of process performance, considering that the reduction of time and energy costs is highly important.

Table 26: Bulk lyophilization cycle selected.

Thermal Treatment								
Freeze				Drying				
Step	Shelf (ºC)	R*/H**	Time (min.)	Step	Shelf (ºC)	R/H	Time (min.)	Pressure (mTorr)
1	-5	R	30	1	40	R	80	75
2	-5	H	30	2	40	H	1080	75
3	-40	R	35	Storage	20	---	---	500
4	-40	H	120	---	---	---	---	---

* Ramp
** Hold

This cycle allowed to obtain a solid dispersion layer with good appearance, not cracked, collapsed, or melted as presented in Figure 60.

Figure 60: RES:Eudragit E PO (1:2)_Gelucire 44/14 16% SD.

V. Solid dispersions characterization

1. Objectives

Eudragit E PO and Gelucire 44/14 at 16% (w/w) of RES were selected, as hydrophilic polymer and surfactant respectively. The formulation was manufactured using the previously selected and optimized cycle (Table 26). RES:Eudragit E PO was used in a 1:2 ratio. A formulation without surfactant (second-generation SD), was also produced to assess whether its presence has any influence on *in vitro* permeability and *in vivo* pharmacokinetics. This formulation was also characterized for some parameters.

2. Bulk drying formulations

Each solution was poured into an aluminium tray and submitted to bulk drying. The following formulations were produced.

- **RES:Eudragit E PO (1:2)_Gelucire 44/14 16% in TBA/Acetate buffer pH 4.5 (75:25) – Third-generation SD**
- **RES:Eudragit E PO (1:2) in TBA/Acetate buffer pH 4.5 (75:25) – Second-generation SD**

RES SD with Eudragit E PO and Gelucire 44/14 (third-generation SD; Figure 60) and with Eudragit E PO alone (second-generation SD), were prepared by bulk lyophilization. This method allowed to obtain an uniform powder layer with good appearance, not cracked, collapsed, or melted.

Formulations were assessed for RES content (assay), moisture content, solubility in buffers with physiological relevant pH values (1.2, 4.5 and 6.8), dissolution performance in pH 1.2 and 6.8 buffers, particle size distribution (PSD), and porosity.

The physical state of RES, PMs and LFs/SDs was characterized by differential scanning calorimetry (DSC), X-ray powder diffraction (XRPD), Fourier transform infrared spectroscopy (FTIR), optical microscopy (OM), polarized light microscopy (PLM), particle morphology and scanning electron microscopy (SEM).

3. Results and discussion

3.1. Bulk drying lyophilization

After lyophilization an uniform powder layer with good appearance was obtained for both formulations tested.

The lyophilized formulation with Gelucire 44/14 stored in amber glass bottle was submitted to a stress study and placed for 1 month in ICH accelerated conditions (40ºC/75%RH).

3.1.1. Assay

Table 27: Third–generation SD assay. (*n*=1).

Formulation	RES (%)	
	T0	T1 (40ºC/75% RH)
RES:Eudragit E PO (1:2)_ Gelucire 44/14 16%	91.4	94.9

Assay values above 90% were obtained at T0 and after 1 month at 40ºC/75% RH, revealing no signs of apparent chemical degradation.

3.1.2. Moisture content

The moisture content value obtained by loss on drying was 4.7%. Adding the assay with the loss on drying value, a mass balance above 95% was obtained.

3.1.3. Solubility

Solubility for LFs and PM of formulation with Gelucire 44/14 was assessed after 24 hours stirring at room temperature and compared to RES solubility. Determination was performed in triplicate samples in aqueous solvents with pH value 1.2, 4.5 and 6.8 (Figure 61 – A).

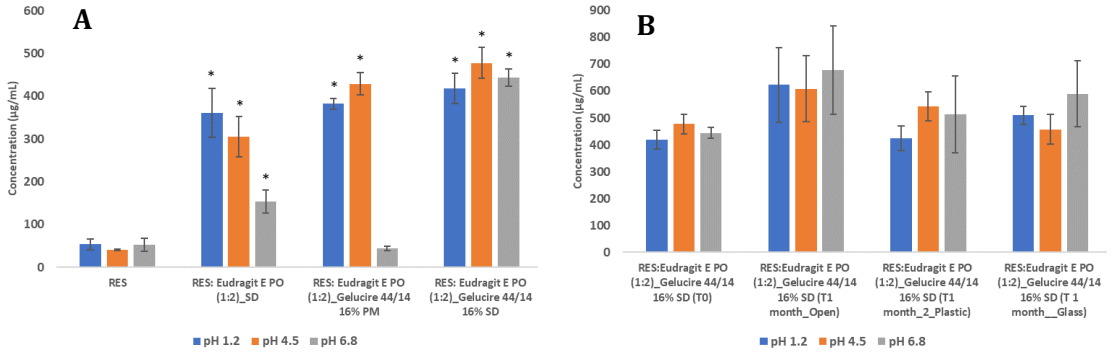


Figure 61: Solubility after 24 hours under magnetic stirring at room temperature in pH 1.2, 4.5 and 6.8 at T0 – A, and after 1 month at 40ºC/75% RH – B. (mean±SD, *n*=3), **p* < 0.05 comparing with pure RES.

The LF with Gelucire 44/14 (third-generation SD) presented greater solubility than formulation with only Eudragit E PO (second-generation SD) in all tested buffers. At pH 1.2, 4.5 and 6.8, third-generation SD, presented more than 7-fold increase in solubility compared to RES alone. Third-generation SD presented an 8, 12, and 8-fold increase of RES solubilized compared to pure RES at pH 1.2, 4.5 and 6.8 respectively (Figure 61 - A). At pH 6.8, a 10-fold increase in solubility compared to the PM was observed. Eudragit E PO being a cationic polymer precipitates above pH 5.5, explaining the lower solubility of RES obtained in the PM at pH 6.8 (Figure 61 - A). This is also evidence of a chemical interaction in the SD compared to the PM.

The solubility of the third-generation SD without packaging and packaged in plastic and amber glass bottles was also assessed after 1 month stored at 40ºC/75% RH (Figure 61 - B). These results indicate that the third-generation SD appears to be stable.

3.1.4. Dissolution

In vitro dissolution assessment in pH buffers 1.2 and 6.8 was performed for RES, and RES:Eudragit E PO (1:2)_Gelucire 44/14 16 % (PM and SD powders). SD was sieved through a 500 µm mesh prior dissolution. RES content in each vessel was 400 µg/mL. Due to its low density, each powder sample was placed inside a cloth bag with a mesh large enough to allow the powder to be released, whilst preventing it from floating on the liquid surface. A sinker was also placed inside the bag. Determination was performed in triplicate samples.

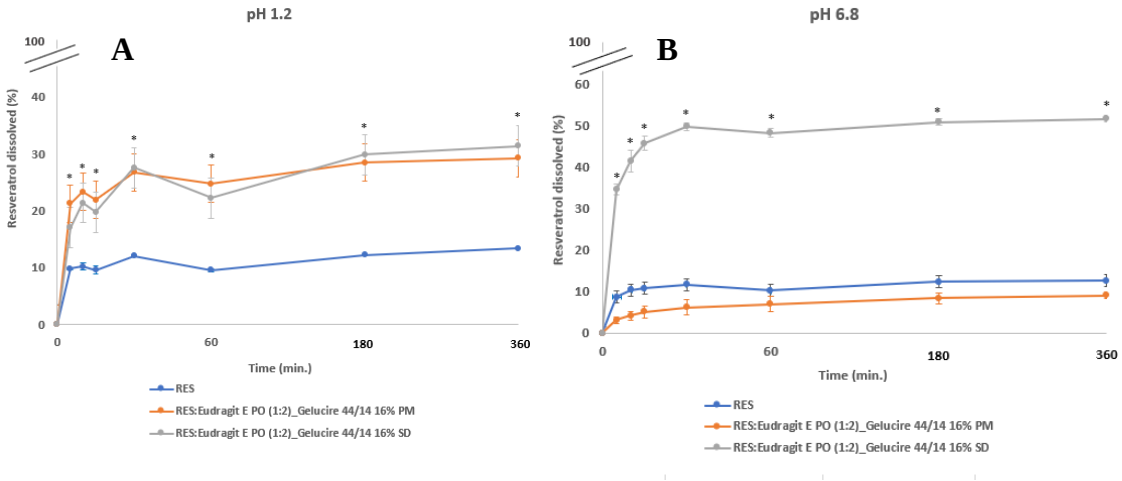


Figure 62: Dissolution profile of RES, PM and SD powders, at 37ºC in pH 1.2 – A, and pH 6.8 – B. RES = 400 µg/mL, in each sample. (mean±SD, *n*=3), **p* < 0.05 comparing PM and SD with pure RES in pH 1.2 and comparing SD with RES and PM in pH 6.8.

At pH 1.2, as expected, RES presented the lowest rate and extent of dissolution, due to its low solubility at acidic pH. Under these acidic conditions, as observed for solubility assessment, third-generation SD and its PM exhibited a similar dissolution rate with more than 2-fold increase in RES dissolved compared to RES alone after 30 minutes (Figure 62 – A).

Furthermore, in line with results obtained for solubility assessment at pH 6.8, the increase in RES dissolution for the third-generation SD was clear, compared to PM and RES alone at pH 6.8, with almost 5-fold increase compared to RES alone after 30 minutes (Figure 62 – A).

It is also worth highlighting the very low standard deviation values obtained between samples, even at earlier time points.

RES conversion from the crystalline to the amorphous state, particle size reduction, weakening of aggregation and agglomeration, solubilizing effect of the polymer and wettability improvement are some mechanisms that can explain the dissolution and solubility improvement in the third-generation SD compared to PM and pure RES.

3.1.5. Porosity

Porosity of RES, PM and SD was calculated using the following formula, and results are presented in Figure 63.

$$\boldsymbol{Porosity}\ (\%) = \left[\mathbf{1} - \frac{\boldsymbol{bulk\ density}}{\boldsymbol{true\ density}}\right] \boldsymbol{X}\ \mathbf{100}$$

Equation 1 – Powder porosity (%).

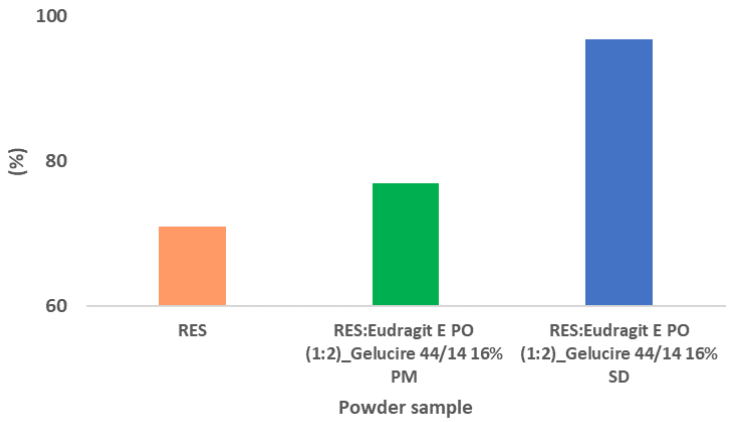


Figure 63: Porosity of RES, PM and SD powders.

It was noticed an increase of around 20% in porosity from RES alone to third-generation SD. Greater surface area also contributes to improved RES solubility in the SD.

3.1.6. Differential Scanning Calorimetry (DSC), X-ray powder diffraction (XRPD) and Fourier transform infrared spectroscopy (FTIR)

RES, RES:Eudragit E PO (1:2)_Gelucire 44/14 16% PM and RES:Eudragit E PO (1:2)_Gelucire 44/14 16% SD/LF powder samples were assessed by DSC, XRPD and FTIR as presented in Figure 64.

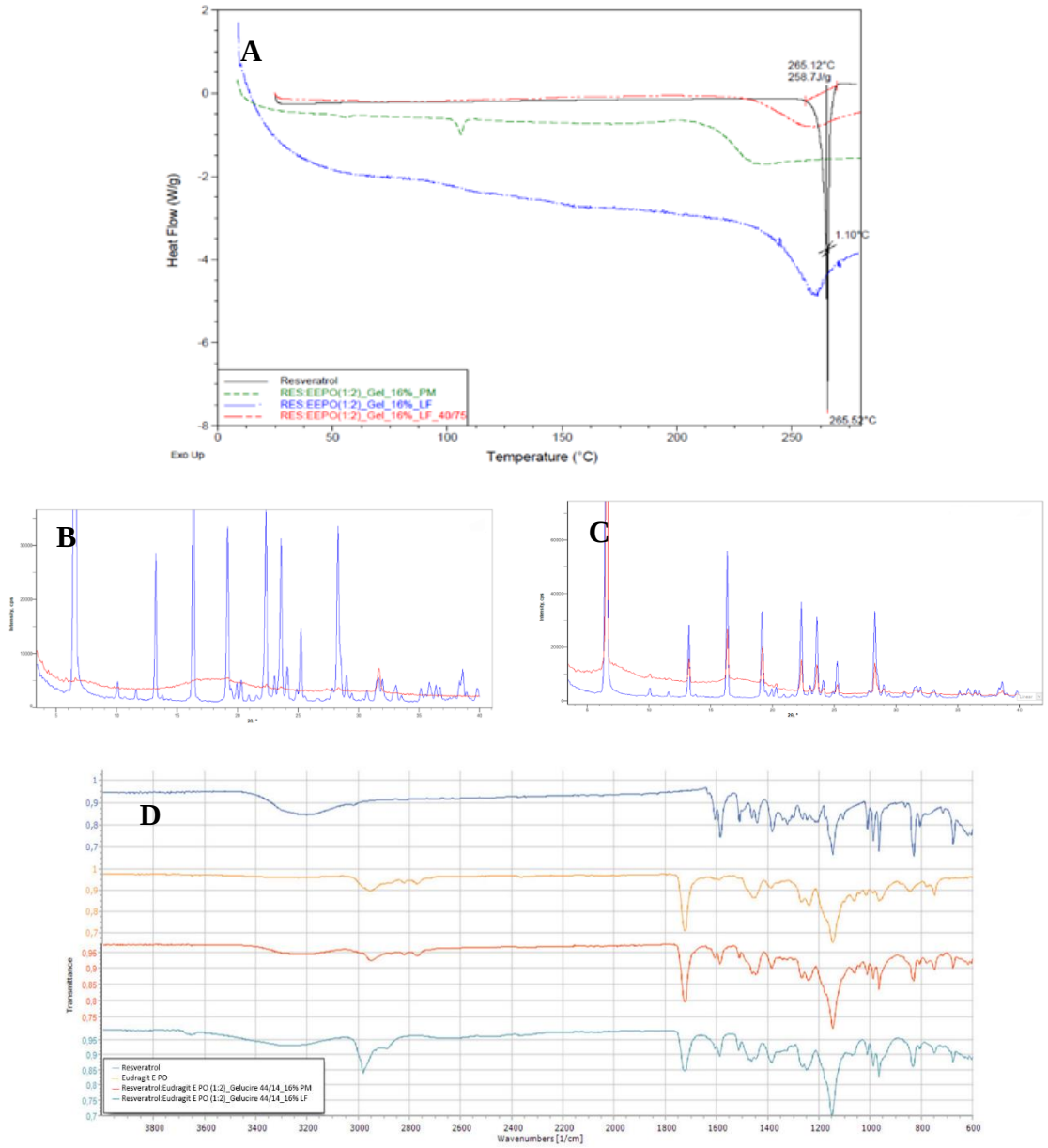


Figure 64: RES and RES:Eudragit E PO (1:2)_Gelucire 44/14 16% thermograms (PM and LF at T0 and T1 month at 40ºC/75% RH) – A. XRPD patterns of RES (blue) and RES:Eudragit E PO (1:2)_Gelucire 44/14 16% SD (red) – B. XRPD patterns of RES (blue) and RES:Eudragit E PO (1:2)_Gelucire 44/14 16% PM (red) – C. FTIR spectra for RES, Eudragit E PO and RES:Eudragit E PO (1:2)_Gelucire 44/14 16% (PM and SD/LF) – D.

Differential scanning calorimetry analysis allows evaluating possible molecular interaction between RES, hydrophilic polymer and surfactant, and consequent changes in the solid state. Based on the thermal events observed, greater or lesser solubility for the active pharmaceutical ingredient can be predicted. The lower the enthalpy value of a substance, the more amorphous it will be, therefore greater solubility and dissolution rate are expected.

The RES thermogram shows a single and sharp endothermic peak at 265.52ºC, which corresponds to its melting point with an enthalpy value of 258.7 J/g, (Figure 64 – A). Eudragit E PO is amorphous, (Figure 37), and Gelucire 44/14 presents an endothermic event at 46ºC, (Figure 49). In SD/LF thermogram at T0, a small endothermic event can be observed around the melting temperature of RES, which did not increase after 1 month at 40ºC/75%, (Figure 64 – A). The pronounced reduction in the endothermic event compared to RES alone, and the fact that it did not increase during stress study, can indicate amorphization and improved solubility for RES in the LF, which was confirmed in solubility assessment.

RES alone showed characteristic sharp diffraction peaks at 2θ, which highlights its crystallinity, (Figure 64 – B and C, in blue). In RES:Eudragit E PO (1:2)_Gelucire 44/14 16% PM, (Figure 64 – C, in red), there was no conversion of RES to the amorphous state, indicated by the presence of characteristic RES peaks. However these peaks were not detected for RES:Eudragit E PO (1:2)_Gelucire 44/14 16% SD, (Figure 64 – B, in red), suggesting conversion of RES from the crystalline to the amorphous state, despite the presence of small peak at around 31-32º, which do not correspond to any peak in the RES alone nor in PM XRPD patterns. Its origin can be possible explained by some sodium remnants from solvent in the formulation that was not completely removed.

RES showed a broad peak at 3203 cm^{-1}, that was assigned to the phenolic hydroxyl group stretch. Three sharp peaks at 1584, 1510 and 1461 cm^{-1}, correspond to the aromatic skeleton vibration. RES exists in *cis*- and *trans*-form, having *trans*-RES higher biological activity. The peak at 964.5 cm^{-1} was attributed to the out-plane vibration of the double-bond carbon of *trans*-RES (Figure 64 – D). Eudragit E PO presents a peak at 1723 cm^{-1} that was attributed to the carboxyl group (Figure 64 – D). In both the PM and SD spectra, the broad peak attributed to the phenolic hydroxyl group of RES decreased intensity or slightly shifted (Figure 64 – D). This may be attributed to the dilution effect of the polymer in the PM and/or additionally to the establishment of hydrogen bonds with the carboxyl group of the polymer. Moreover, the Eudragit E PO peaks at 2820 cm^{-1} and 2769 cm^{-1} corresponding to the non-ionized dimethylamino groups nearly disappeared in the SD/LF, suggesting the formation of acid-base interaction between the acidic phenol hydroxyls of RES and dimethylamino groups of Eudragit E PO.

3.1.7. Optical microscopy (OM) and polarized light microscopy (PLM)

Optical and polarized light microscopy pictures of RES, Eudragit E PO, and RES:Eudragit E PO (1:2)_Gelucire 44/14 16% PM and SD are presented below. Images were captured with a magnification of 400X.

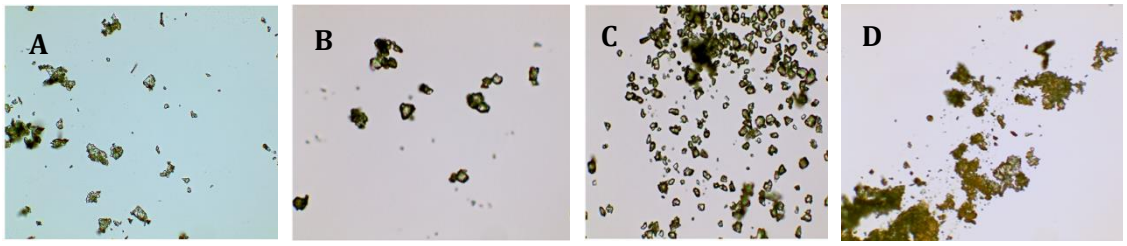


Figure 65: Optical microscopy of RES – A, Eudragit E PO – B, RES:Eudragit E PO (1:2)_Gelucire 44/14 PM – C, and RES:Eudragit E PO (1:2)_Gelucire 44/14 SD – D.

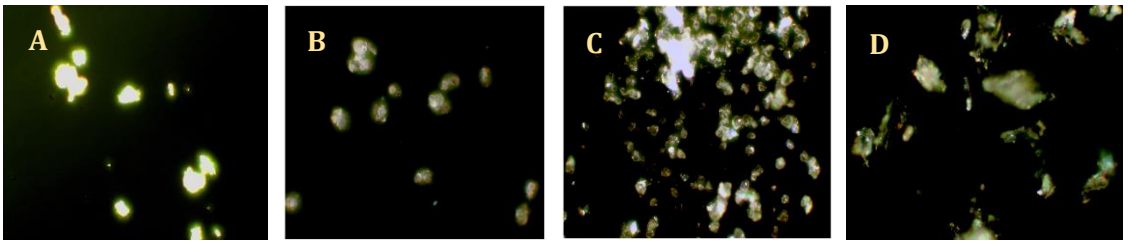


Figure 66: Polarized light microscopy of RES – A, Eudragit E PO – B, RES:Eudragit E PO (1:2)_Gelucire 44/14 PM – C, and RES:Eudragit E PO (1:2)_Gelucire 44/14 SD – D.

An amorphous sample does not exhibit birefringence under polarized light unlike a crystalline sample. The crystallinity of pure RES is evidenced by the birefringence of its particles in PLM (Figure 66 – A). The birefringence in PM is also clear (Figure 66 – C), compared to faded particles in SD (Figure 66 – D), suggesting amorphization of RES in the SD.

3.1.8. Morphology and particle size distribution (PSD)

Morphological imaging applies automated static imaging analysis technique to provide a detailed description of the morphological properties of the particles. Images of particles captured with a magnification of 10X and 50X are presented below.

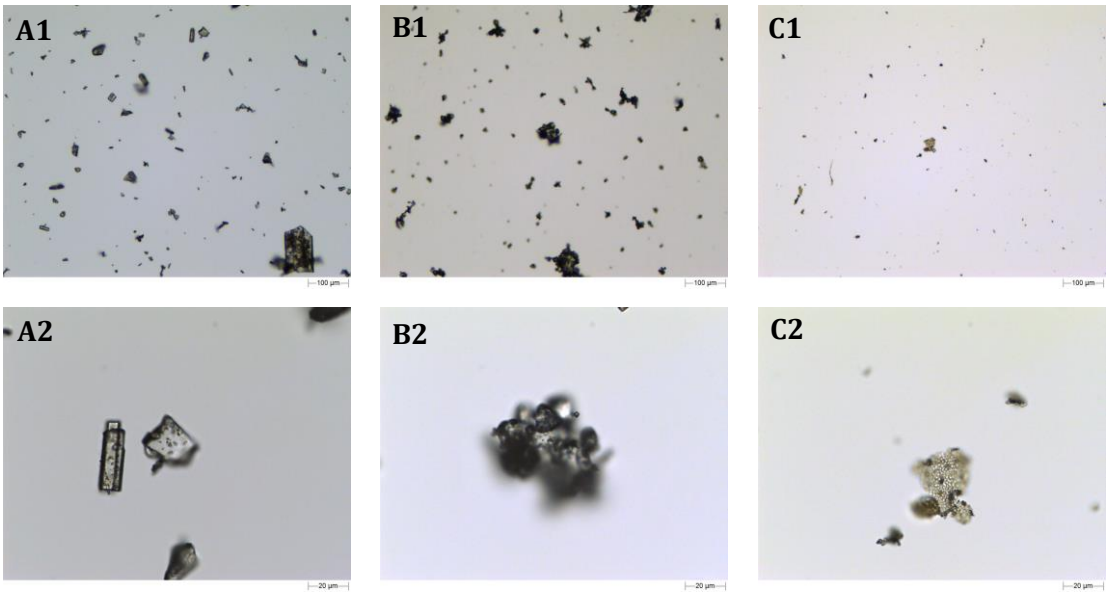
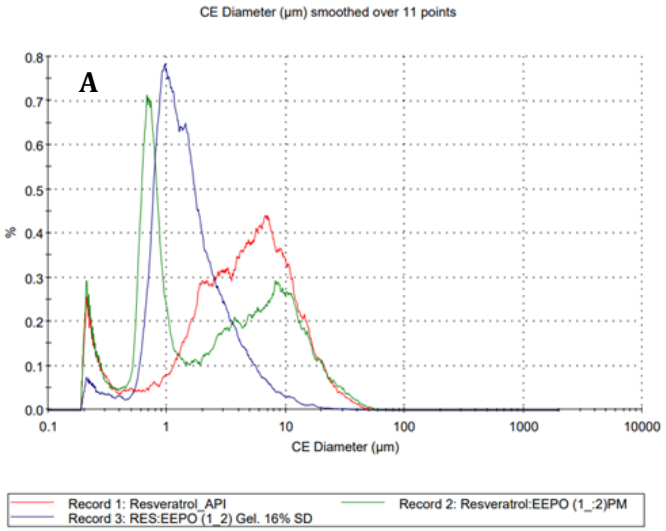


Figure 67: Morphological imaging of RES (A1 - 10X; A2 - 50X), RES:Eudragit E PO (1:2)_Gelucire 44/14 16% PM (B1 - 10X; B2 - 50X) and RES:Eudragit E PO (1:2)_Gelucire 44/14 SD (C1 - 10X; C2 - 50X).

The circle equivalent (CE) diameter, defined as the diameter of a circle with the same area as the 2D image of the particles was assessed, and results are presented in Figure 68.



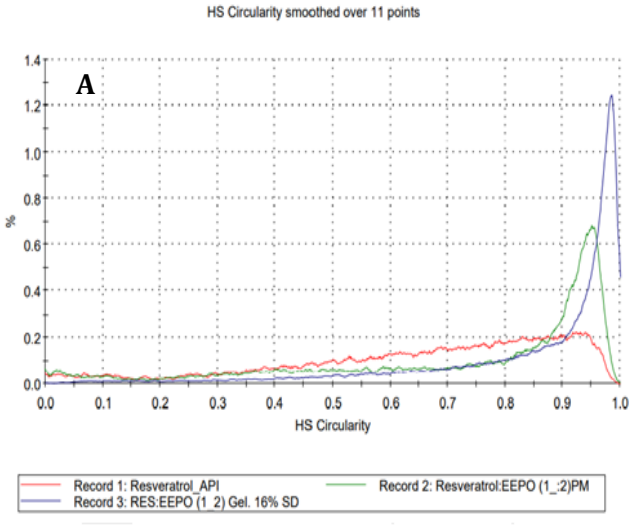
B

Sample	CE Diameter – Number distribution (µm)				Particles
	Mean	D[n, 0.1]	D[n, 0.5]	D[n, 0.9]	counted
RES	6.15	0.60	4.44	13.37	20 000
RES:E E PO (1:2)_Gel. 16% PM	5.17	0.50	2.08	13.41	20 000
RES:E E PO (1:2)_Gel. 16% SD	2.01	0.74	1.32	3.80	47 598

Figure 68: CE diameter plot – A and particle size distribution parameters – B, determined by Morphologi G4.

The preparation of a third-generation SD by the lyophilization method was able to origin particles with more than a 3-fold decrease in mean particle size, D_{50}, and D_{90}, compared to RES alone.

The particles high sensitivity circularity (HS circularity) defined as HS circularity = 4πA/P 2, where A is the particle area and P its perimeter, was also determined, and results are presented in Figure 69.



B

Sample	HS Circularity				Particles counted
	Mean	D[n, 0.1]	D[n, 0.5]	D[n, 0.9]	
RES	0.652	0.293	0.703	0.922	20 000
RES:E E PO (1:2)_Gel. 16% PM	0.727	0.300	0.852	0.957	20 000
RES:E E PO (1:2)_Gel. 16% SD	0.832	0.531	0.932	0.987	47 598

Figure 69: HS circularity plot – A and distribution parameters – B, determined by Morphologi G4

A circularity value of 1 shows a perfect circle whilst values approaching 0 indicate progressively elongated shapes. The third-generation SD prepared by lyophilization presented slightly more spherical particles in average compared to RES, with very close values for D_{90}.

3.1.9. Scanning electron microscopy (SEM)

SEM pictures of RES and RES:Eudragit E PO (1:2)_Gelucire 44/14 16% PM and SD are presented below. Images were captured with a magnification of 500X.

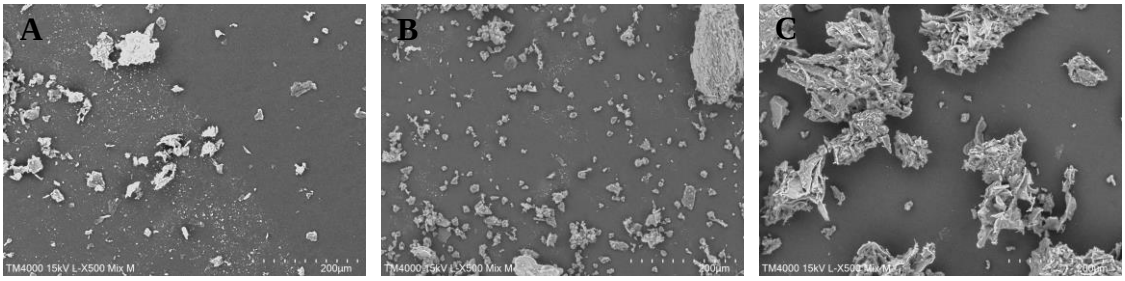


Figure 70: SEM of RES – A, RES:Eudragit E PO (1:2)_Gelucire 44/14 16% PM – B, and RES:Eudragit E PO (1:2)_Gelucire 44/14 16% SD – C.

RES particles tend to form agglomerates (Figure 70 – A). The SEM micrographs of PM revealed particles with smooth contours, (Figure 70 – B), while the SD particles are more porous with rough contours (Figure 70 – C). Compared to visible RES isolated particles in PM, the drug disappeared in the SD, suggesting that it can have been converted to the amorphous state dispersed in the polymer (Figure 70 – C).

4. Conclusions

An amorphous SD prepared by the bulk lyophilization method was obtained. RES in the LF appeared amorphous and chemically interacted with Eudragit E PO. The drug:polymer ratio in the formulation was 1:2 (w/w). Gelucire 44/14 at 16% (w/w) of RES was the surfactant selected due to good results in improving RES solubility, but also considering its metabolism effects and P-gp inhibition (257-259). The selected formulation exhibited enhanced solubility and dissolution compared to PM and RES alone. Solid state characterization, drug:polymer interactions, porosity, particle size distribution and morphology studies also corroborate the performance of RES third-generation SD.

VI. Tablet formulation development

1. Objectives

With the aim of formulating the third-generation SD into a solid oral pharmaceutical form, a tablet formulation was developed and characterized for assay, tablet hardness, disintegration and dissolution.

2. Formulation development

Based on market research on oral solid pharmaceutical formulations of RES, which can range approximately between 50 to 600 mg strengths, it was decided that the target dosage strength of the tablet to be developed should be 100 mg. The enhancement in RES solubility achieved by the SD could allow a greater amount of RES to be absorbed in the human body and consequently improve its therapeutic effects.

The bulk density of the SD is extremely low, (60 µg/mL), and consequently the powder did not flow. To overcome this problem a diluent with good flowability and compressibility was selected, (Cellactose® 80). Cellactose® 80 is a co-processed spray-dried excipient comprising of 75% alpha-lactose and 25% powdered cellulose. Lactose and cellulose are well established excipients and are frequently used as diluent/binder in oral dosage formulations (266). The final blend to be compressed must have a good flowability to allow uniform and robust tableting. The dimensions, weight and shape of the tablet were also considered. The length, width, and height of the tablet should allow for a balanced and robust dosage form with adequate mechanical properties. An individual tablet weighing 750 mg, 19X10 mm (dimensions), was considered adequate to allow patient compliance.

An immediate release tablet should disintegrate within a reasonable period, usually less than 30 minutes. This will allow it to completely disintegrate in the stomach, dissolve and become available to be absorbed in the intestine. For this purpose, a super disintegrant was selected for the formulation, (crospovidone). Crospovidone is a water-insoluble disintegrant and dissolution agent used in tablets prepared by direct compression or wet and dry granulation methods. It rapidly exhibits high capillary activity and pronounced hydration capacity, with little tendency to form gels (266). Stearic acid is widely used in oral dosage formulations and was selected as lubricant to prevent tablet adhesion to the punches and matrix (266).

Due to stability issues of SDs, an appropriate manufacturing process was also selected, to avoid moisture, and excessive mechanical stress, which could compromise the SD, and consequently cause the conversion of RES from the amorphous to the crystalline state. A dry granulation process was selected, to increase particle size, obtain adequate flowability and improve particles compressibility.

To compare the dissolution performance of the tablet formulation containing the SD, a tablet formulation containing the PM and a tablet formulation containing pure RES were also manufactured.

3. Tablet formulations

3.1. Tablets qualitative and quantitative composition

The following formulations were manufactured.

Table 28: Composition of RES tablets.

Components	Unitary (mg)	% (w/w)	Function
RES	100.00	13.33	API
Cellactose 80	567.50	75.67	Diluent/Binder
Crospovidone	75.00	10.00	Disintegrant
Stearic acid	7.50	1.00	Lubricant
Total	**750.00**	**100.00**	

Table 29: Composition of SD and PM tablets.

Components	Unitary (mg)	% (w/w)	Function
RES	100.00	13.33	API
Eudragit E PO	200.00	26.67	Polymer
Gelucire 44/14	16.00	2.13	Surfactant
Cellactose 80	351.50	46.87	Diluent/Binder
Crospovidone	75.00	10.00	Disintegrant
Stearic acid	7.50	1.00	Lubricant
Total	**750.00**	**100.00**	

3.2. Manufacturing process

RES, PM, and SD were initially manually sieved trough a 500 µm mesh.

RES, PM, or SD was mixed with Cellactose 80 and crospovidone in a laboratorial scale biconic blender coupled to a Erweka 403 powerful motor drive unit at 21 rpm for 30 minutes. Half of the stearic acid was added to the blend and mixed at 21 rpm for 3 minutes.

Each mixture was pre-compacted, gently pulverized with a pestle and mortar, manually sieved trough a 500 µm mesh, mixed with the other half of the stearic acid at 21 rpm for 3 minutes and finally compressed. Compaction and tabletting were performed on a R&D Romaco® Kilian Styl'One Evo single-stroke multilayer tablet press.

4. Results and discussion

4.1. Tablets appearance

Tablets obtained from each formulation are presented in Figures 71.

Figure 71: Pure RES 100 mg – A, PM RES 100 mg – B and SD RES 100 mg – C, tablets.

Off-white tablets were obtained for the 3 formulations. Some small dark spots were observed on the PM tablets, which correspond to Gelucire 44/14 alone and not incorporated into the drug:polymer complex as observed in SD.

4.2. Tablets hardness

Tablets were tested for hardness using an Erweka® Tablet Hardness Tester TBH 425 Series.

Table 30: Tablet hardness. (mean±SD, *n*=3).

Formulation	Average hardness (N) ± SD
RES	43.7 ± 1.5
RES:Eudragit E PO (1:2)_Gelucire 44/14_16% PM	73.3 ± 18.6
RES:Eudragit E PO (1:2)_Gelucire 44/14_16% SD	102.3 ± 7.2

The SD tablets presented a 1.4 and 2.3-fold increase in average hardness values compared to the PM and RES tablets respectively.

4.3. Tablets disintegration

Tablets were tested for disintegration in water at 37ºC, using an Erweka® ZT 320 Series Disintegration Tester.

Table 31: Tablet disintegration. (*n*=6).

Formulation	Disintegration time
RES	20 seconds
RES:Eudragit E PO (1:2)_Gelucire 44/14_16% PM	5 minutes
RES:Eudragit E PO (1:2)_Gelucire 44/14_16% SD	12 minutes

All tablet formulations disintegrated in less than 30 minutes as intended. RES tablets disintegrated in less than 30 seconds and SD tablets in 12 minutes.

4.4. Tablets dissolution

Tablets were tested for dissolution in phosphate buffer pH 6.8 using a Vision® G2 Elite 8 dissolution tester. The RES content in each vessel was 400 µg/mL, (2 tablets in 500 mL of dissolution media). Sinkers were used to prevent the tablets from floating on the liquid surface.

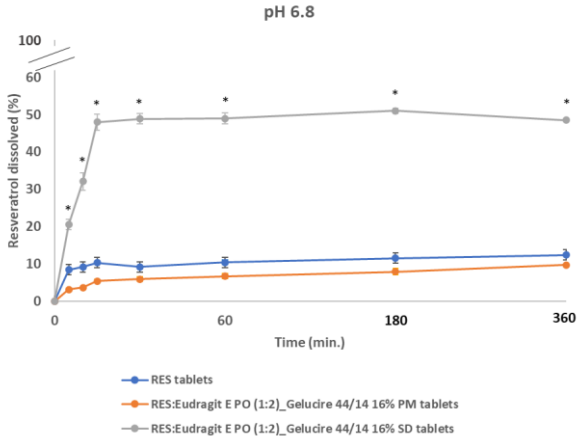


Figure 72: Dissolution profile of RES, PM and SD tablets, at 37ºC in pH 6.8. RES = 400 µg/mL, in each vessel. (mean±SD, *n*=4), **p* < 0.05 comparing SD tablets with pure RES and PM tablets.

RES SD tablets presented a clear increase in RES dissolution compared to pure RES and PM tablets, which was in line with the dissolution results obtained with the powders at this pH. Although SD tablets needed more time to completely disintegrate compared to pure RES and PM tablets, this was not a limitation for the dissolution of RES.

5. Conclusion

SD tablets presented higher average hardness values, essential for tablet integrity during the subsequent stages of the manufacturing process (packaging), distribution chain (transportation, storage, dispensing), and patient use (de-blistering or de-bottling).

The third-generation SD tablets also exhibited, an improved dissolution rate, with a 4.6 and 8.7-fold increase in RES dissolved at pH 6.8 after 15 minutes (Figure 72), compared to pure RES and PM tablets respectively, despite their longer disintegration time. Additionally, in Figure 62 – A, an improvement in dissolution of RES powder at pH 1.2 (stomach pH) from the SD compared to pure RES is also shown. This allows in SD a greater amount of RES to be solubilized and available for absorption in the intestine.

In vitro studies in cell-based models for intestinal permeability testing, and *in vivo* pharmacokinetics studies in rat model presented in the next chapters aimed to assess whether the third-generation SD formulation was able to achieve an improvement in the oral bioavailability of RES.

VII. Formulation Biological Assesssment

1. *In vitro* intestinal permeability

1.1. Objective

To assess intestinal permeability of RES an *in vitro* study was conducted in Caco-2 monoculture and Caco-2/HT29-MTX dual co-culture cell models.

The following formulations were tested in both models:

- **RES:Eudragit E PO (1:2) SD – Second-generation SD**
- **RES:Eudragit E PO (1:2)_Gelucire 44/14_16%_PM**
- **RES:Eudragit E PO (1:2)_Gelucire 44/14_16%_SD – Third-generation SD**

1.2. Introduction

Caco-2 cells, are from a human colorectal carcinoma, and represent a well-characterized cell culture system that displays enterocyte-like morphology and functionality, comparable to the small intestine, when differentiated by using certain culture conditions. In addition to enterocytes, the second most abundant type of cells in the intestine are mucus-producing goblet cells. These can be modelled using HT29-MTX cells, a sub-strain of the colorectal carcinoma-derived cell line HT29 and which can differentiate into goblet cell-like cells during cultivation. Both cell lines can be co-cultured to obtain a more physiological model of the small intestine, whereby the goblet cells provide a mucus layer, which turns it thicker than the regular Caco-2 layer. The introduction of HT29-MTX cells on the well-established Caco-2 model had significant repercussions on barrier tightness, resulting in a decrease of tight junction proteins expression. Furthermore, the expression of P-gp efflux transporter was found to be downregulated in the co-culture model, in contrast with the Caco-2 monoculture, where P-gp is overexpressed. This is consistent with the downregulation of P-gp observed *in vivo* (27), meaning that HT29-MTX addition significantly improves Caco-2 model toward *in vivo* similarities (267).

1.3. Cell culture

The Caco-2 and HT29-MTX cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both Caco-2 cell line and co-culture Caco-

2/HT29-MTX (9:1), were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin. Both models were sub-cultured once a week using 0.25% Trypsin-EDTA (0,25%/0,05%), to detach the cells from the flasks and seeded at a density of 0.5 x 106 cells per 75 cm^2 flasks. The culture medium was replaced every other day. Cells were maintained at 37ºC, 5% CO_2 and 95% relative humidity.

For the permeability experiments, $1x10^5$ cells/cm^2 of Caco-2 and Caco-2/HT29-MTX (9:1) were seeded in 12-Transwell® cell culture inserts and were allowed to grow and differentiate for 21 days at 37ºC in a carbogen (95% O_2, 5% CO_2) atmosphere, with the culture medium replacement every other day (268). After that time, medium was carefully removed from the apical and basolateral compartments and the inserts were gently washed twice with phosphate buffered saline (PBS) (pH 7.4, 37ºC). Then, 0.5 and 1.5 mL of HBSS were added to the apical and basolateral part of the Transwell®, respectively, and allowed to equilibrate for 30 min. inside the incubator. Afterwards, the media from the apical compartment was removed and 0.5 mL of each sample previously dissolved in DMSO with a RES theoretical concentration of 50 µg/mL in HBSS was added. The test samples were placed directly in the apical compartment without removing the media. Triplicate samples and an insert without the addition of sample was used as a control in both models. Plates were placed inside an orbital shaking incubator (KS 4000 IC, IKA®, Staufen, Germany) at 100 rpm and 37ºC. Aliquots (200 µL) were withdrawn from the basolateral chamber at pre-determined times (5, 15, 30, 45, 60, 90, 120, and 180 min.) and immediately replaced with HBSS. At the end, an aliquot from the apical compartment was collected (268). Before, during, and at the end of the permeability experiments, the Transepithelial Electrical Resistance (TEER) was measured using an EVOM²® epithelial voltammeter (WPI), with chopstick electrodes (World Precision Instruments, Sarasota, FL, USA) to monitor the formation, confluence, and integrity of the cell monolayers. The concentration of RES in the samples was determined by HPLC-ultraviolet (UV) analysis.

RES apparent permeability (Papp) was calculated using the formula below:

$$P_{app} = \frac{dQ/dt}{A \cdot C_0}$$

Equation 2 – Apparent permeability.

Where Papp is the apparent permeability (cm/s); dQ/dt, (μM/s): is the rate of permeation across the monolayer obtained from the angular coefficient of the curve of the amount of drug transported versus time; V, (cm^3): is the acceptor chamber volume, which in this case corresponds to 1.5 cm^3 (basolateral chamber); A, (cm^2): is the insert membrane growth area (equal to 1.1 cm^2 for a 12 well plate); and C_0 (μM) is the initial concentration of RES in the apical compartment (268).

1.4. Results and discussion

The permeability results for the formulations tested in both models are presented in Figure 73.

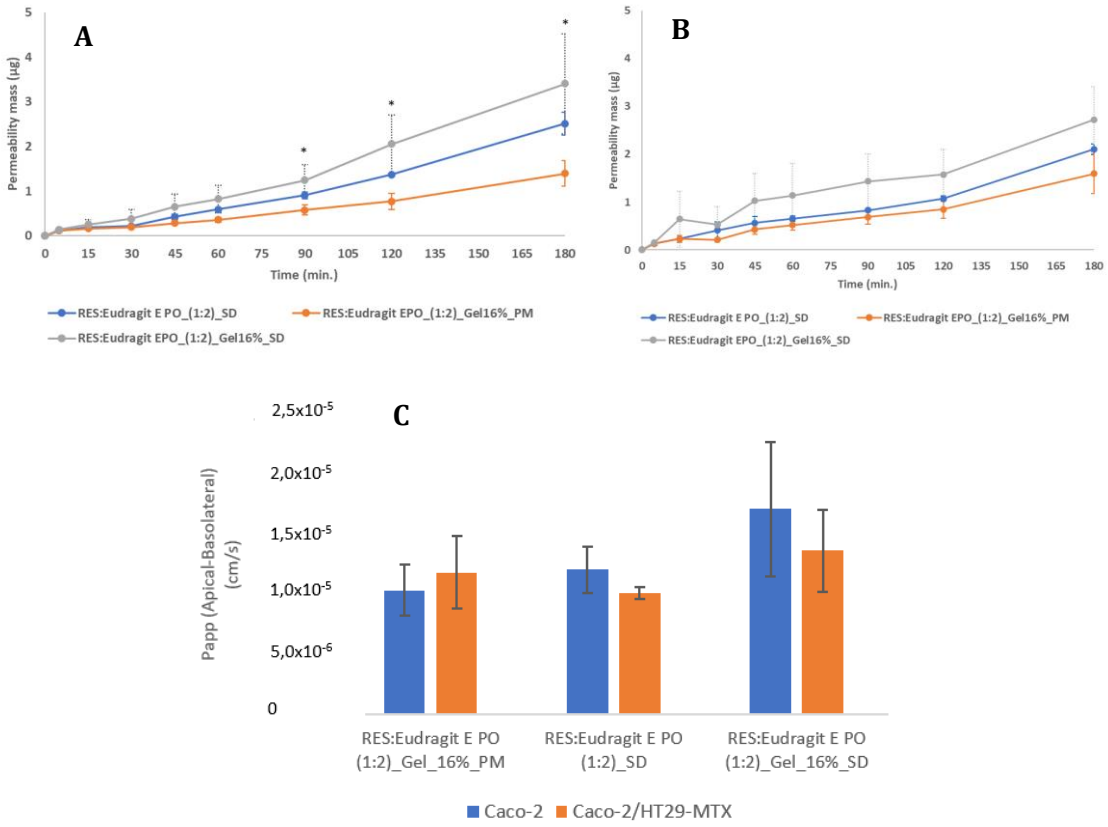


Figure 73: RES permeability in Caco-2 cell based intestinal *in vitro* model – A, and in Caco-2/HT29-MTX cell based intestinal *in vitro* model – B. Apparent permeability (P_{app}) of RES third-generation SD, PM, and second-generation SD – C. RES theoretical concentration administered in each sample: 50 μg/mL. (mean±SD, *n*=3), $^{*}p < 0.05$ comparing third-generation SD with PM.

TEER values were constant across the study for both models indicating an intact cellular monolayer membrane with restrict paracellular absorption through the tight junctions, and the validity of the permeability results obtained.

In both cell models, third-generation SD presented higher permeability, compared to its PM and second-generation SD. Despite the presence of mucus secreting cells (HT29-MTX), similar results were obtained for both models, suggesting no interference of mucus in the performance of third-generation SD and consequently enhanced permeability of RES (267). In Caco-2, at 90 minutes and following timepoints the difference between third-generation SD and its PM was statistically significant (Figure 73 – A). At 180 minutes the average RES permeated was 2.4 and 1.7-fold higher in Caco-2 and Caco-2/HT29-MTX respectively compared to its PM (Figure 73 – A and B).

The Biopharmaceutical Classification System (BCS), as defined by Amidon et al. (20), inserts RES in the second class of drugs characterized by low water solubility and high intestinal membrane permeability. The apparent permeability values obtained in both cell models for all tested samples confirm that classification, indicating high permeability and high intestinal absorption ($P_{app} > 10x10^{-6}$ cm/s). The third-generation SD presented the highest values in both models (Figure 73 – C).

The improved RES permeability observed in the third-generation SD, compared to its PM can be explained by the physical and chemical interaction of RES with Eudragit E PO and Gelucire 44/14 in the SD. Gelucire 44/14 primary mechanism to increase the bioavailability of orally administered low soluble drugs like RES is through improved dissolution rates in the gastrointestinal tract. Furthermore, there is increasing evidence that Gelucire 44/14 can inhibit presystemic drug metabolism and can reduce P-gp mediated efflux, which contributes to an improved RES bioavailability (257-259). These two additional mechanisms can also have contributed to the higher RES permeability from the third-generation SD compared to the second-generation SD tested.

The mechanism by which excipients like Gelucire 44/14 inhibit P-gp activity is currently unknown, however theories include altering cell membrane integrity, blocking binding sites competitively, non-competitively or allosterically, interfering with ATP hydrolysis and creating a futile cycle of ATP hydrolysis (257-259).

2. *In vivo* pharmacokinetic assessment

2.1. Objective

To assess RES pharmacokinetics after oral administration an *in vivo* study was conducted in Wistar male rats.

The following formulations were tested:

- **RES:Eudragit E PO (1:2) SD – Second-generation SD**
- **RES:Eudragit E PO (1:2)_Gelucire 44/14_16%_PM**
- **RES:Eudragit E PO (1:2)_Gelucire 44/14_16%_SD – Third-generation SD**

2.2. Introduction

Literature on the bioavailability of RES suggests that this compound is rapidly absorbed, metabolized, and excreted in humans and animals, indicating a poor bioavailability of RES (269). RES has high oral absorption but has rapid and extensive metabolism without adverse effects in both rodents and humans, resulting in very low quantities of unchanged RES in the systemic circulation. In humans, about 70% of orally administered RES (25 mg) is rapidly absorbed (< 30 min.) and metabolized with a peak plasma level of ≈ 2 µM of RES metabolites and a half-life of 9-10 h. There is a significant interpersonal variability in drug absorption and metabolic processes. The extent to which the human colon can absorb and metabolize RES depends on the hepatic function and the metabolic activity of the intestinal microflora (183, 270-274).

RES undergoes extensive phase I (oxidation, reduction, and hydrolysis) and phase II (glucuronic acid, sulphate and methyl conjugations) biochemical changes immediately after ingestion, being metabolized into both glucuronic acid and sulphate conjugations of the phenolic groups in liver and intestinal epithelial cells. Hydrogenation of the aliphatic double bound also occurs (198, 275).

RES has poor water solubility and therefore needs to be bound to plasma proteins to ensure its body distribution and bioavailability. RES can bind to serum proteins like lipoproteins, hemoglobin, and albumin which facilitate its carrier mediated cellular uptake and then it can passively diffuse through the plasma membrane (276, 277).

All the metabolites of RES are eliminated from the organism and excretion is almost equally distributed between urine and feces. An almost complete elimination of RES and metabolites from tissues is observed 72 h after a single dose (274, 278).

2.3. Pharmacokinetic study

The *in vivo* pharmacokinetic studies were performed using, male Wistar rats, weighing approximately 250 grams, purchased from Charles River Laboratories (France). The animal study protocol complied with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and the Portuguese law on animal welfare (*Decreto-Lei 113/2013*). Three groups of rats (one per formulation) of 5 animals each were tested.

Rats were randomly separated into 5 animals per cage and placed on wood litter, with free access to pellet chow diet (2014 Envigo) and tap water. The animal cages were maintained in a 12-hour light/dark cycle (07:00 to 19:00 hours) in a controlled ambient temperature of 22 ± 2ºC and relative humidity of 50 ± 20%. The day before food was removed.

On the day of administration, rats were weighed, and each formulation was orally administered via gastric gavage at a dose of 200 mg/kg of RES via dispersion state in 6 mL of hydroxypropyl methylcellulose 0.5% (w/v) in water before dosing.

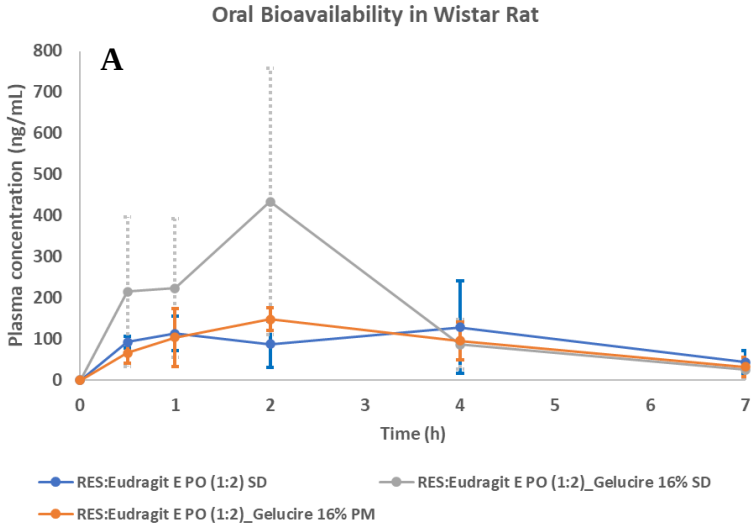
Approximately 150 µL of blood samples were collected by lateral tail vein at pre-determined timepoints (pre-dose, 0.5, 1, 2, 4 h), with exception of the last timepoint (7 h), in which samples were collected by cardiac puncture, after an overdose of pentobarbital.

Plasma was isolated through centrifugation at 1500 rpm for 15 minutes (4ºC) and samples were stored at -80ºC. Samples were assayed for RES by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The analytical method was validated according to the ICH guidelines.

The C_{max}, T_{max} and $AUC_{0\text{-}7h}$ were calculated for each group using GraphPad Prism, (GraphPad software Inc., CA, USA).

2.4. Results and discussion

The third-generation SD was able to unquestionably enhance RES bioavailability, while its PM and second-generation SD presented a very similar pharmacokinetic profile (Figure 74 – A). In the third-generation SD formulation, higher RES concentration in plasma compared to PM and second-generation SD was observed immediately after oral administration, with a 2.3 and 3.2-fold increase respectively after 30 minutes. After 60 minutes, the RES permeated with third-generation SD was 2.0 and 2.2-fold higher compared to second-generation SD and PM respectively. After 4 hours RES plasma concentration in the third-generation SD decreased to values similar to those in the other 2 formulations and continued to decrease to lower values after 7 hours.



B

Formulation	AUC_{0-7h} (ng*h/mL)	C_{max} (ng/mL)	T_{max} (h)
RES:Eudragit E PO (1:2) SD	650.1±218.1	128.7±112.8	4
RES:Eudragit E PO (1:2)_Gelucire 44/14_16% PM	620.4±102.7	148.1±28.04	2
RES:Eudragit E PO (1:2)_Gelucire 44/14_16% SD	1181±396.3	433.8±323.76	2

Figure 74: Plasma concentration-time profiles of RES third-generation SD, PM, and second-generation SD after oral administration of samples containing 200 mg/kg of RES – A. Pharmacokinetic parameters for RES third-generation SD, PM, and second-generation SD in Wistar rats – B. (mean±SD, *n*=5).

The third-generation SD clearly presented the highest values for AUC_{0-7h} with a 1.9 and 1.8-fold increase compared to its PM and second-generation SD. The third-generation SD was also 2.9 and 3.4-fold higher in C_{max}, than PM and second-generation SD respectively (Figure 74 – B).

As previously discussed with regards *in vitro* intestinal permeability assessment, the higher RES bioavailability obtained with the third-generation SD over the other 2 formulations, can be explained not only by the dissolution improvement, but also by the potential interference of Gelucire 44/14, with RES metabolism, and inhibition of P-gp mediated efflux. The presence of excipients like Gelucire 44/14 in the SD allows for greater bioavailability of orally administered RES (257-259). Increased RES permeability and bioavailability by surfactants (Gelucire 44/14 and poloxamer 407) in SDs due to reduction of efflux transport and metabolism, had already been demonstrated by Vasconcelos et al. (279). In this study, a RES:Soluplus (1:2)_poloxamer 407_15% SD was administered to rats at a RES dose of 100 mg/kg, obtaining an AUC_{0-7h} of 279±54 ng_*h/mL, and a C_{max} of 134±78 $ng_/mL$. The third-generation SD developed in the current work, allowed an increase of more than 2 and 1.5-fold respectively for both parameters (corrected values considering different doses administered in the two studies), compared with the formulation developed by those authors.

The main role of excipients in formulations is to create technological conditions that allow a robust and suitable manufacturing process for the pharmaceutical form and deliver the desired drug quality and performance. Lipid excipients like Gelucire 44/14 create excellent conditions for improved permeability into the enterocytes. Gelucire 44/14 has interesting properties in improving the solubility of low soluble drugs such as RES, due to its unique composition of surfactants (mono and diesters of polyethylene glycol, cosurfactants (monoglycerides), and oily phase (di and triglycerides) (280).

After oral administration, RES is absorbed at a relatively high rate through the small intestine (191). The small and non-polar character of RES may allow for its absorption across the membranes by passive diffusion, yet there are evidences that RES is mostly transported across the intestinal epithelium cell via ATP-dependent binding cassette (ABC) transporters (192).

After drug penetration into the cytosol, it is subjected to efflux by several transporters. Efflux transporters like P-glycoprotein (P-gp or MDR1 – the most significant), encoded by the MDR1 gene; multidrug resistance-associated proteins (MRP1 and 2); lung

resistance protein (LRP); and breast cancer resistance protein (BCRP = ABCG2) are efflux transporters present in the gastrointestinal tract (281-283).

Degradation by metabolizing enzymes of the cytochrome P450 (CYP), uridine diphosphate-glucuronosyltransferase (UGT) and sulfotransferase (SULT) families also contributes to poor oral administered drug bioavailability (267).

P-gp is a 170-kDa transmembrane protein member of the ATP binding cassette (ABC) transporter family, which utilizes energy released by ATP hydrolysis. It is localized at the apical secretory surface of various tissues (e.g., liver, kidney, gastrointestinal tract, blood-brain barrier) where it mediates the active transmembrane transport of a variety of lipophilic substrates, which tend to be large, aromatic, and amphiphilic. P-gp can extrude/exclude a wide range of structurally diverse xenobiotics and limits the oral absorption of several drugs such as RES by transporting them back from the intestinal cells into the gut lumen (257-259, 281).

Various models have been proposed to try to clarify and explain the efflux pump mechanism by P-gp. The pore model by which the compound is transported out of the cell though a protein channel. The flippase model where the compound is incorporated in the inner face of the plasma membrane and then is transported to the extracellular face through the action of P-gp. The hydrophobic vacuum cleaner model which combines both the pore and the flippase model (284, 285).

There is increasing evidence that some lipid excipients like Gelucire 44/14 are capable of inhibiting P-gp mediated drug efflux back to the intestine (16, 258, 279). Several mechanisms have been proposed. Surfactants have been shown to modulate P-gp activity by changing the fluidity of the lipid membrane environment of P-gp leading to a reduction of ATPase activity (286, 287). Other study showed that pluronic block copolymers sensitize multidrug resistance cell lines by decreasing the affinity of P-gp for ATP, decreasing the ATPase activity in combination with depletion of intracellular ATP (288). Lipid excipients like Gelucire 44/14 actively affect P-gp efflux mechanism not only by altering the membrane fluidity or ATPase activity but also by downregulation of P-gp expression.

The enhancement in RES oral bioavailability observed in rat model with the third-generation SD was explained by the physicochemical interaction demonstrated between drug and hydrophilic polymer (RES and Eudragit E PO respectively), and consequently, drug amorphization, particle size reduction, improved wettability and higher porosity that led to improved solubility and dissolution. As a result, a greater amount of RES was

solubilized and prone to being absorbed by the intestinal wall. *In vitro* studies demonstrated that the increase in RES solubility determines a partial saturation of its metabolism with a consequent improvement in its bioavailability (221).

Gelucire 44/14, presystemic drug metabolism inhibition and interference in P-gp efflux mechanism also contributed to the higher C_{max} and AUC in plasma observed with the third-generation SD.

Protecting RES from rapid metabolization in the gastrointestinal tract and liver is also a general mechanism which can increase bioavailability. Given that CYP, UGT, and SULT are the key enzymes which conjugate RES, any intervention that decreases their rate of reaction to it should increase concentration of the parent compound (210).

Strategies to increase and extend in time RES permeability by SD formulation using the same excipients, would encompass the use of a higher quantity of Gelucire 44/14 in the SD and consequently higher inhibition of efflux mechanisms due to more available surfactant and increased RES metabolism interference. Alternatively other surfactants with known efflux pumps inhibition such as Tweens® or Pluronics®, polysaccharides, polyethylene glycols and derivates, amphiphilic block copolymers, dendrimers and thiolated polymers, should be investigated to assess their performance (289).

The higher *in vivo* RES concentration allowed by the third-generation SD makes it easier to obtain some of the physiological benefits demonstrated with RES.

2.5. Conclusion

Enhanced oral bioavailability was obtained not only by solubility and dissolution improvement, but also by the interference of Gelucire 44/14 with RES metabolism, and inhibition of P-gp mediated efflux. The presence of excipients like Gelucire 44/14 in the SD allows for greater bioavailability of orally administered RES, making it easier to obtain some of the physiological benefits demonstrated by this molecule.

VIII. Conclusions

Resveratrol (RES) is a natural product that possesses a very high antioxidant potential, exhibits antitumor activity, and is considered a potential candidate for prevention and treatment of several types of cancer. Other therapeutic indications reported include anti-inflammatory, cardioprotective, vasorelaxant, phytoestrogenic and neuroprotective activities. Nonetheless, Resveratrol as well as other class II compounds from the Biopharmaceutics Classification System (BCS) present a major challenge for the pharmaceutical industry in order to develop strategies to improve solubility in water, and consequently bioavailability. Those formulation strategies are crucial to overcome the solubility problems and allow its therapeutic use.

With that in mind, the general purpose of this project was to develop a Resveratrol drug delivery system with improved solubility and consequently enhanced oral bioavailability, by the solvent evaporation method (Lyophilization/Freeze-drying).

Several hydrophilic polymers were initially screened and characterized with the aim to disperse Resveratrol at a molecular level and consequently induce its amorphization and solubility improvement. Resveratrol being practically insoluble in water cannot be adequately freeze-dried with water-based formulations.

A mixture of *tert*-butanol (TBA) + acetate buffer pH 4.5 solvent system was used to solubilize Resveratrol and the hydrophilic polymers tested. *Tert*-butanol (TBA) a class 3 solvent with low toxicity, high vapour pressure, low melting point and accepted by FDA, was selected due to be an excellent freeze-drying medium. These organic co-solvent systems present many interesting advantages: high freezing temperatures, very short sublimation time, low sublimation enthalpies, high equilibrium vapor/solid pressure values, and low toxicity.

Eudragit E PO was the hydrophilic polymer selected due to higher Resveratrol solubility increase achieved by the solid dispersions in physiological relevant pH buffers. Eudragit E PO is a nonhygroscopic hydrophilic cationic polymer consisting of methyl methacrylate, N-N-dimethylaminoethyl methacrylate and butyl methacrylate monomers (1:2:1), possesses tertiary amines that ionize at the acidic pH to make the polymer highly soluble in fluids when pH is below 5.

Several surfactants were assessed to complete the third-generation solid dispersion formulation qualitative composition. Gelucire 44/14 was the surfactant selected due to higher Resveratrol solubility improvement obtained compared with other surfactants tested, and additionally considering its effect on presystemic drug metabolism inhibition and interference in P-gp mediated efflux, that contribute to an improved Resveratrol

bioavailability. Gelucire 44/14 is also a non-ionic surfactant having a less irritant effect than cationic or anionic surfactants. Gelucire 44/14 is obtained by an alcoholysis reaction between coconut oil and polyethylene glycol-32 (PEG-32) under controlled conditions. It consists of glycerides and PEG esters of fatty acids of varying chain length.

Hydrophilic polymer and surfactant content in the third-generation solid dispersion were optimized to allow Resveratrol amorphization and relevant solubility improvement. The final mass of formulation was also considered for the excipients quantitative composition in order to be suitable for later development of a solid oral dosage form with favourable patient compliance. Resveratrol:Eudragit E PO (1:2)_Gelucire 44/14 16% in TBA/Acetate buffer pH 4.5 (75:25) was the final formulation selected. The drug:hydrophilic polymer ratio in the formulation was 1:2 (w/w). Gelucire 44/14 was present at 16% (w/w) of Resveratrol.

Solid dispersions were produced by the bulk lyophilization method. This method is performed in trays allowing the production of higher quantities of solid dispersion per batch to be used in the production of oral solid dosage forms. To optimize the lyophilization cycle by the bulk method, several critical factors were investigated at freezing, primary and secondary drying phases based on a DoE approach. A tray filling height of 1 cm was established. As a result of freezing optimization, a cooling rate of 1ºC/min. was selected because it is a good compromise, avoiding some possible phase separation during freezing with slow cooling, and allowing the formation of relatively large ice crystals, which facilitates sublimation. A target supercooling temperature of -40ºC and a supercooling time of 2 hours were selected. A target shelf temperature of 40ºC and a chamber pressure of 75 mTorr were adequate to allow a shorter primary drying period without compromising product integrity.

Usually, secondary drying is performed at a much higher temperature than the used for primary drying so that desorption of solvents may occur at a practical rate, nevertheless in our case primary drying was already performed at 40ºC. Drying for 18 hours at 40ºC was considered adequate in terms of product stability and residual moisture level obtained.

The formulation was prepared by an optimized (shortened) and robust bulk lyophilization process, with a duration of approximately 23 hours. The formulation developed was dried at a high target product temperature (40ºC), without product collapse. This allowed primary and secondary drying to be carried out at the same temperature, with clear advantages in terms of reducing manufacturing process time and saving energy costs.

Solid dispersions prepared by the optimized bulk lyophilization method were characterized for solid state, solubility, dissolution rate and stability.

The level of Resveratrol amorphization in the solid dispersion was assessed by differential scanning calorimetry (DSC), X-ray powder diffraction (XRPD), Fourier-transform infrared spectroscopy (FTIR), polarized light microscopy (PLM), and scanning electron microscopy (SEM).

DSC results revealed that pure Resveratrol melts around 265.5ºC. In solid dispersion a small endothermic event was observed around the melting temperature of Resveratrol, which did not increase after 1 month at 40ºC/75% RH. The pronounced reduction in the endothermic event compared to Resveratrol alone, and the fact that it did not increase during stress study, can indicate amorphization and improved solubility for Resveratrol in the solid dispersion, which was confirmed in solubility assessment.

By XRPD characterization it was observed that in the third-generation solid dispersion, Resveratrol was converted from the crystalline to the amorphous state, despite the presence of a small peak at around 31-32º, that do not correspond to any peak in the Resveratrol alone neither in PM XRPD patterns. Its origin can be explained by some sodium remnants from solvent in the formulation that was not completely removed.

The formation of various molecular interactions between Resveratrol and Eudragit E PO in the solid dispersion was also confirmed by FTIR. The solid dispersion spectra revealed changes in the Resveratrol molecule and consequently the formation of a new structure with the hydrophilic polymer.

By PLM faded particles without birefringence were observed with the solid dispersion sample, which is a signal of Resveratrol amorphization.

With SEM imaging more porous particles with rough contours were observed in solid dispersion compared to physical mixture. The drug disappeared in the solid dispersion, suggesting conversion to the amorphous state dispersed in the polymer.

The results obtained present clear evidence that an amorphous solid dispersion prepared by the bulk lyophilization method was obtained. Resveratrol in the solid dispersion/lyophilized formulation appeared amorphous and chemically interacted with Eudragit E PO.

Particle size and shape analysed by automated morphological imaging demonstrated that third-generation solid dispersion prepared by the lyophilization method presented

more slightly spherical particles with more than 3-fold decrease in mean particle size, D_{50}, and D_{90}, compared to Resveratrol.

It was also noticed an increase of around 20% in porosity from Resveratrol to third-generation solid dispersion particles.

Solubility assessment studies in buffers 1.2, 4.5 and 6.8, confirmed that third-generation solid dispersion, presented more than 7-fold increase in Resveratrol solubility compared to pure Resveratrol and higher solubility than second-generation solid dispersion in all tested buffers. At pH 6.8, solid dispersion presented a 10-fold increase in Resveratrol solubility compared to physical mixture. Eudragit E PO being a cationic polymer precipitates above pH 5.5, explaining the lower solubility of Resveratrol obtained in the physical mixture at pH 6.8. This is also evidence for chemical interactions between Resveratrol, Eudragit E PO and Gelucire 44/14 in the solid dispersion compared to the physical mixture.

Resveratrol solubility after 1 month at 40ºC/75% RH packaged or unpackaged remained relatively constant in all buffer conditions. These results indicate that the third-generation solid dispersion appears to be stable. The maintenance of the amorphous physical state of the particles was also confirmed by DSC.

At pH 1.2 as observed for solubility, third-generation solid dispersion and its physical mixture exhibited a similar dissolution rate with more than 2-fold increase compared to pure Resveratrol after 30 minutes. At pH 6.8, an increase in Resveratrol dissolution for the third-generation solid dispersion is clear, compared to physical mixture and pure Resveratrol, with an increase of almost 5-fold compared to pure Resveratrol after 30 minutes.

The developed formulation exhibited improved solubility in the range of relevant physiological pH values, enhanced dissolution in acidic pH (gastric environment) and was stable in amorphous form for 1 month packaged or unpackaged under accelerated ICH conditions (40ºC±75% RH).

Resveratrol conversion from the crystalline to the amorphous state, particle size reduction, increased porosity, weakening of aggregation and agglomeration, solubilizing effect of the polymer and wettability improvement are some mechanisms that explain the dissolution and solubility improvement observed with the developed Resveratrol:Eudragit E PO (1:2)_Gelucire 44/14 16% solid dispersion compared to physical mixture and pure Resveratrol.

Solid dispersion produced was formulated into an immediate release tablet containing 100 mg of Resveratrol with an individual target weight of 750 mg. Cellactose® (75% of lactose and 25% cellulose), as diluent/binder, crospovidone as disintegrant, and steraric acid as lubricant were the excipients selected to produce tablets by dry granulation. Solid dispersion tablets showed higher average hardness and higher dissolution rate, with a 4.6 and 8.7-fold increase in dissolved Resveratrol at pH 6.8 after 15 minutes compared with Resveratrol and physical mixture tablets respectively, despite their longer disintegration time. Furthermore, the improvement in dissolution of Resveratrol powder at pH 1.2 (stomach pH) from the solid dispersion compared to Resveratrol alone allows in solid dispersion a greater amount of Resveratrol to be solubilized and available for absorption in the intestine.

To confirm whether the developed third-generation solid dispersion allowed to obtain an enhanced Resveratrol oral bioavailability compared to its physical mixture and second-generation solid dispersion, an *in vitro* study in cell-based models for intestinal permeability, and an *in vivo* pharmacokinetic study in Wistar rat model were conducted.

The third-generation solid dispersion presented higher permeability in Caco-2 and Caco-2/HT29-MTX models, compared to its physical mixture and second-generation solid dispersion. After 180 minutes the average Resveratrol permeated was 2.4 and 1.7-fold higher in Caco-2 and Caco-2/HT29-MTX respectively compared to physical mixture.

The improved Resveratrol permeability observed in the third-generation solid dispersion, compared to its physical mixture was explained by the physical and chemical interaction of Resveratrol with Eudragit E PO and Gelucire 44/14 observed in the first. Furthermore, based on the literature and results obtained, despite Gelucire 44/14 primary function as a surfactant and dissolution enhancer, this lipid excipient has inhibited Resveratrol metabolism and reduced P-gp mediated efflux (257-259). These two additional mechanisms have contributed to the higher Resveratrol permeability observed in third-generation solid dispersion compared to second-generation solid dispersion.

Pharmacokinetic studies in Wistar rats corroborated the results obtained with the *in vitro* permeability assessment. The third-generation solid dispersion improved the bioavailability of Resveratrol, presenting a higher concentration in plasma compared to physical mixture and second-generation solid dispersion, with a 2.3 and 3.2-fold increase respectively after 30 minutes. Higher values for $AUC_{0\text{-}7h}$ were also obtained, with a 1.9 and 1.8-fold increase compared to physical mixture and second-generation solid

dispersion. A 2.9 and 3.4-fold higher in C_{max}, compared to physical mixture and second-generation solid dispersion respectively was observed.

The observed enhancement in Resveratrol oral bioavailability in Wistar rat model with the third-generation solid dispersion was explained by the physicochemical interaction between Resveratrol and Eudragit E PO, and consequently drug amorphization, particle size reduction improved wettability and higher porosity that led to an improved solubility and dissolution. As a result, a greater amount of Resveratrol was solubilized and prone to being absorbed by the intestinal wall. Gelucire 44/14, presystemic drug metabolism inhibition and interference in P-gp efflux mechanism also contributed to a higher C_{max} and AUC in plasma observed with the third-generation solid dispersion.

The presence of excipients like Gelucire 44/14 in the solid dispersion allows for greater bioavailability of orally administered Resveratrol, making it easier to obtain some of the physiological benefits demonstrated by this molecule.

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