Synthesis and Characterization of Ezetimibe Pharmaceutical Cocrystal: A Reaction Crystallization Method to Improve Physicochemical Properties and Hypolipemic Activity Evaluation

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Ezetimibe has significant lipid-lowering activity but presents low aqueous solubility. Co-crystallization techniques can be used to overcome this limitation. However, it is not yet clear whether the solubility of the cocrystal can be maintained after oral administration. In the present study, an ezetim-imidazole cocrystal was synthesized by the reaction crystallization method, a promising strategy in cocrystal engineering. After conducting characterization tests, we determined the solubility and thermodynamic stability of the cocrystal. Also, the solubility advantage for both water and biorelevant fasted state simulated intestinal fluid (FaSSIF) was calculated. Dissolution was determined under supersaturated conditions, and the hypolipidemic activity of the cocrystal was assessed in vivo. The cocrystal exhibited high solubility and thermodynamic stability in FaSSIF. The solubility advantage was calculated as 22.4 in water and 11.8 in FaSSIF. The dissolution of the cocrystal resulted in supersaturation levels ($\sigma_{\text{max}}$) of 1.3 in water and 3.3 in FaSSIF and the area under the curve (AUC) increased from 478.49 (ezetimibe) to 2963.62 µg min mL$^{-1}$ (cocrystal) in FaSSIF. The atherogenic index of the animals that received the cocrystal was similar to that of the control group. These results demonstrate that the inherent advantages of the co-crystallization process were maintained, enabling us to elucidate the behavior of the cocrystal.

Keywords: ezetimibe, cocrystal, solubility, hypolipemic activity, supersaturation

Introduction

Improving the elementary properties of drugs guides and directs the pharmaceutical industry in promoting technological advances.¹ Furthermore, 40% of the approved drugs and nearly 90% of new chemical entities comprise low aqueous solubility molecules.² Drug solubility is a significant parameter in pharmaceutical systems since dissolution limits absorption. Therefore, drugs with poor solubility in aqueous media will have a reduced dissolution rate, implying low bioavailability.³ Thus, several strategies can be used to increase the solubility of drugs while keeping the pharmacological activity unaltered.⁴⁻⁶ From this perspective, cocrystallization enhances the physicochemical properties of the drugs, such as stability, solubility, compressibility, and bioavailability.⁷ Pharmaceutical cocrystals comprise two or more solid organic components in a stoichiometric ratio. At least one of these is an active pharmaceutical ingredient (API), and the other is a pharmaceutically acceptable molecule, a co-former that interacts via non-covalent bonds.⁸ Several techniques can prepare cocrystals.⁹⁻¹⁴

The API used in this work was ezetimibe (EZE) ((1-(4-fluorophenyl)-3(R)-3-(4-fluorophenyl)-3(S)-hydroxypropyl)-4(S)-(4-hydroxyphenyl)-2-azetidione) (Figure 1), which is the first of a new class of anti hyperlipemic drugs that selectively inhibits intestinal absorption of

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Figure 1. Ezetimibe chemical structure.
dietary and biliary cholesterol. There are two crystalline forms of the drug: anhydrous and monohydrate.

EZE is well tolerated and safe for treating primary hypercholesterolemia. Concomitant ingestion of the drug with food has no effect on its absorption. It is a class II molecule according to the Biopharmaceutical Classification System (BCS) as it has high intestinal permeability (log P = 4.56), but it is practically insoluble in aqueous media. The solubility of the anhydrous and hydrate forms is approximately 0.012 and 0.008 mg mL⁻¹, respectively.

To mitigate the limitations mentioned above, some studies have previously attempted to enhance the solubility of EZE. Additionally, co-crystallization techniques can be utilized. Most investigations reported in the literature on ezetimibe cocrystals focus on synthesizing new crystalline solids through screening studies to select co-formers and/or comparing different methods for co-crystallization.

In the present study, we utilized a cocrystal that has already been described in the literature, however, it was obtained through the reaction crystallization method (RCM), a promising strategy in cocrystal engineering. RCM is advantageous over other techniques for synthesizing cocrystals since the methodology is simple, practical, scalable, and does not require high equipment costs.

Furthermore, although the pharmaceutical cocrystals of ezetimibe have been elucidated, there is a need for studies regarding whether the solubility of the cocrystal will be maintained after oral administration. Therefore, it is necessary to examine the solubility and thermodynamic stability of the cocrystal, determine its dissolution behavior under supersaturated conditions, and evaluate its lipid-lowering efficacy in an in vivo study. In addition to characterization tests via X-ray powder diffraction (XRPD), Fourier transformed infrared (FTIR) spectrophotometer, differential scanning calorimetry (DSC), and scanning electron microscopy (SEM), in vitro studies were conducted to determine the thermodynamic solubility, solution-state stability, and dissolution behavior of the cocrystal in non-sink supersaturation conditions. The studies were performed in biorelevant medium that simulates the composition of intestinal fluids in the fasted (FaSSIF) state, as well as in water. The therapeutic effect of the cocrystal was evaluated by measuring its lipid-lowering activity in an in vivo study.

Experimental

Chemistry

Methods and materials

EZE, with a purity of 99.50%, was acquired from SM Pharmaceuticals in Bangalore, India, while imidazole (IMID), with a purity of 99.60%, was obtained from Êxodo Científica (São Paulo, Brazil). Ultrapure water from Merck Millipore’s Simplicity System (Darmstadt, Germany) was utilized in all experiments. All of the reagents and chemicals used were of analytical grade.

Cocrystal synthesis

The cocrystal used has already been described in the literature, however, instead of using the mechanochemistry method with a mixture ethyl acetate:heptane, we obtained the cocrystal through the RCM, without using organic solvent. Initially, 1.16 g of IMID (properties: white powder with the formula C₃H₅N₂, a molecular weight of 68.0 g mol⁻¹, and pKₐ of 14.9) was weighed on an analytical balance (Shimadzu, AUX220, Japan). The sample was solubilized in 10 mL of aqueous sodium lauryl sulfate 2% (m/v) and maintained on a stirring plate (Fisatom, 754A, São Paulo, Brazil) at room temperature. Subsequently, 7 g of EZE were slowly added to the solution. EZE is a white crystalline powder with a molecular formula of C₁₇H₁₆F₂N₂O₄, 409.4 g mol⁻¹ molecular weight, and a pKₐ 9.4. The solution was stirred magnetically for forty-eight hours at 25 °C. The objective was to maintain the solution in a supersaturated state with respect to the cocrystal formation while ensuring it remained either saturated or unsaturated with respect to the individual components. This way, the cocrystals that precipitate from the solution will be pure.

The resulting suspension was vacuum-filtered, and the solid phase was collected on quantitative filter paper. The item was subsequently dried in an oven at 40 °C for three hours. The cocrystal was finally stored in a desiccator. Cocrystal stoichiometry was quantified by high-performance liquid chromatography (HPLC) methodology.

Chromatographic conditions

HPLC quantifies both the drug and co-former. The HPLC method for EZE involved using a Luna® C18 150 × 4.6 mm column, with a mobile phase consisting of 0.03 mol L⁻¹ acetonitrile (65 v/v): phosphate buffer (35 v/v), pH of 3.5. The flow rate was set at 0.6 mL min⁻¹, a detection wavelength of 247 nm, an injection volume of 20 µL, a temperature of 45 °C, and a total run time of 6.5 min. In this study, IMID was quantified using a Luna® SCX column with dimensions of 150 × 4.6 mm. The mobile phase consisted of 10 mmol L⁻¹ methanol (35 v/v): phosphate buffer, pH 7.1 (65 v/v), the flow rate was set at 0.8 mL min⁻¹, and detection was performed at a wavelength of 220 nm. An injection volume of 20 µL was used, and the temperature was maintained at 40 °C to ensure accuracy.
X-ray powder diffraction (XRPD)

X-ray powder diffraction (XRPD) patterns were obtained using a Bruker D8 Phaser powder diffractometer (Waltham, USA) with Cu Kα radiation (λ = 1.5418 Å) operating at 30 kV and 30 mA. X-ray diffraction data were collected in the range of 5 to 35° (2θ), with a step time of 1 s and an increment of 0.05°. The XRPD data were processed using Origin 8.0 software.²⁹

Fourier-transformed infrared spectroscopy (FTIR)

The FTIR spectrum was obtained using an FTIR spectrophotometer (PerkinElmer Frontier IR/NIR, Waltham, USA), in the scan range of 4000-600 cm⁻¹. An average of more than thirty-two scans was taken with a spectral resolution of 4 cm⁻¹. Additionally, a background spectrum (white) was obtained. The spectral information was obtained through diffuse reflection, which involved measuring the incidence and reflection of light on the powdered drugs. The powder was applied in sufficient quantity to cover the support disc for the sample. The FTIR data was processed using Origin 8.0 software.²⁹

Thermal characterization

Differential scanning calorimetry (DSC) curves were obtained using an automatic DSC/TGA thermal analytical system (PerkinElmer, STA 6000, Waltham, USA). Using an alumina crucible, 5 mg of the sample were weighed and heated from 25 to 600 °C at a heating rate of 10 °C min⁻¹ in a dynamic atmosphere of N₂ at a flow rate of 100 mL min⁻¹. DSC data were analyzed using Origin 8.0 software.²⁹

Scanning electron microscopy (SEM)

The surface morphology was investigated using photomicrographs obtained from a scanning electron microscope (Hitachi, TM 3000, Ibaraki, Japan) with a filament voltage of 5 kV. The samples were mounted onto aluminum supports and fixed in place using double-sided carbon tape. Photomicrographs were captured at magnifications of 100×, 200×, and 2000×.

Measurement of the eutectic point, eutectic constant (Kₑₑ), thermodynamic solubility, and the cocrystal’s solubility advantage

The eutectic point was measured to determine the thermodynamic solubility and solution-state stability of the cocrystal in water and FaSSIF biorelevant medium. It consisted of sodium taurocholate (3 mmol L⁻¹), soy lecithin (0.75 mmol L⁻¹), sodium chloride (105.9 mmol L⁻¹), sodium hydroxide (8.7 mmol L⁻¹), and monobasic sodium phosphate (28.4 mmol L⁻¹), with a pH of 6.5.³⁰

The eutectic point was reached by suspending 700 mg of the cocrystal and 100 mg of EZE in 10 mL of water, 1500 mg of the cocrystal, and 200 mg of EZE in 10 mL of FaSSIF. Prepared suspensions were kept at a constant temperature of 25 ± 0.5 °C with continuous stirring for 72 h using a shaker incubator (New Lab, NL 343-01, São Paulo, Brazil). The existence of drug and cocrystal solid forms in equilibrium was checked at 24, 48 and 72 h and confirmed by XRPD. The eutectic concentration of the drug and co-former was determined using the HPLC method, and the pH of the final solution was measured before analysis. The experiments were conducted in triplicate. The equilibrium solubility of the cocrystal (Sₑₑ,cocrystal) was calculated by utilizing equation 1 for 1:1 cocrystal, which involves measuring the total eutectic concentrations of the drug ([drug]ₑₑ,T) and co-former ([co-former]ₑₑ,T):³¹

\[
S_{\text{cocrystal}}^{1:1} = \sqrt{[\text{drug}]_{\text{ex},T} [\text{co-former}]_{\text{ex},T}} \quad (1)
\]

The eutectic constant (Kₑₑ) was calculated to predict the solubility and stability of the cocrystal. Drug and co-former concentration values at the eutectic point were determined after 24 h and used in the Kₑₑ equation (equation 2):³¹

\[
K_{\text{ex}} = [\text{co-former}]_{\text{ex},T} /[\text{drug}]_{\text{ex},T} \quad (2)
\]

The cocrystal’s solubility advantage (SA) indicates its potential to convert/precipitate to the constituent drug when in solution, such as during dissolution or pharmaceutical processes.³² The SA was also calculated using equation 3:

\[
SA = S_{\text{cocrystal}} / S_{\text{drug}} \quad (3)
\]

Dissolution under non-sink conditions

For the powder dissolution experiments under non-sink (supersaturating) conditions, 20 mg of EZE or the equivalent amount of the cocrystal were added to 50 mL of water or FaSSIF. The system was agitated in a shaker incubator at 110 rpm and at room temperature of 25 ± 0.5 °C (using water) and at 37 ± 0.5 °C (using FaSSIF), to consider the human-bodily temperature. A 5 mL sample was collected at intervals of 2, 5, 7, 10, 15, 30, 60, 90, 120, and 150 min after the beginning of the study. Each aliquot was centrifuged, filtered through a 0.45 μm polyamide membrane, and then analyzed using the HPLC method. The experiment was conducted in triplicate. The pH was
determined at the end of the study. The dissolution behavior of the cocrystal was analyzed in terms of the: maximum dissolution concentration ($C_{\text{max}}$), the time to achieve the $C_{\text{max}}$ ($T_{\text{max}}$), maximum supersaturation ($\sigma_{\text{max}}$), and the area under the curve (AUC) for the cocrystal.

$$\sigma_{\text{max}} = \frac{C_{\text{max}}}{S_{\text{drug}}}$$  \hspace{1cm} (4)

**In vivo hypolipemic study**

Twenty-four male Wistar rats weighing 250 ± 20 g and aged two months were used in the study. The experiment adhered to the International Guidelines for the Care and Use of Laboratory Animals. The Ethics Committee approved the experimental protocol for the use of animals (CEUA: 37/2021) at the Universidade Estadual do Centro-Oeste do Paraná-UNICENTRO, Brazil.

The animals were randomly divided into four groups (n = 6): a control group, a group treated with a high-fat (HD), a group treated with pure drug EZE, and a group treated with cocrystals (COC). The animals were housed in propylene cages and maintained in a controlled environment with 12-h light/dark cycles and a room temperature of 23 ± 1°C. The experiment lasted for a total of twelve weeks. All rats, except for the control group, were induced with hyperlipidemia until the eighth week. The diet consisted of a mixture of standard chow (100 g), fat (18 g), and cholesterol (2 g) for the experimental group. The control group received only a standard diet. All animals were kept with free access to water.

Treatment was subsequently initiated and conducted for a period of four weeks. The groups treated with pure EZE and COC received a dose equivalent to 1.13 mg kg$^{-1}$ of EZE, which was calculated based on the human dose of 10 mg for an adult with an average weight of 70 kg. The dose was suspended in 0.4 mL of a sodium carboxymethylcellulose vehicle (0.75% m/v). Each suspension was individually prepared and immediately administered via gavage. The control group and HD group received only the volume equivalent to the vehicle.

**Biochemical assessment**

Blood samples were obtained via the cardiac puncture procedure after seven, fourteen, and twenty-eight days of treatment. The animals were previously anesthetized with ketamine hydrochloride (80 mg kg$^{-1}$) associated with xylazine hydrochloride (15 mg kg$^{-1}$). After the final collection, the animals were euthanized by administering a lethal dose of pentobarbital (180 mg kg$^{-1}$) applied intraperitoneally.

The blood samples were collected in microtubes containing separator gel and then centrifuged at 3500 rpm for thirty minutes to obtain the serum. Serum concentrations of total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), liver enzymes: glutamic-oxaloacetic transaminase (TGO/AST) and glutamic-pyruvic transaminase (TGP/ALT). Ultrasensitive C-reactive protein (us-CRP) were determined using in vitro kits developed and standardized by Labtest Diagnóstica S.A. (Minas Gerais, Brazil) and analyzed on biochemistry equipment (Sinnowa, SX-160, São Paulo, Brazil). The concentrations of low-density lipoprotein (LDL) were calculated using the Friedewald equation. The data were represented as mg dL$^{-1}$ and International units (IU L$^{-1}$) for liver enzymes.

The atherogenic index value (AI), which measures the extent of atherosclerotic lesions based on serum lipids, was determined by calculating the ratio of TC to HDL.$^{36}$

**Data analysis**

The data were represented as mean ± standard deviation for statistical in of the animal assay. The hypolipemic effect of the pure drug and cocrystals was analyzed using Friedman’s analysis of variance (ANOVA) test, followed by Durbin-Conover post hoc analysis. The groups were compared at each time point using the Kruskal-Wallis test, followed by the Durbin-Conover post hoc test. Results were considered significant when $p < 0.05$, using JAMOVI software version 1.8.4.$^{37}$ The Kruskal-Wallis test was used to analyze the data and determine the AI, with Dunn’s post hoc test conducted at a significance level of 95%.

**Results and Discussion**

**Cocrystal synthesis and characterization**

Pharmaceutical cocrystal of EZE and IMID were successfully obtained by RCM. The characterization techniques made it possible to confirm structural alterations of the crystalline solid obtained from the isolated elements. XRPD is a fingerprint characterization method for cocrystals. The crystalline state of pure EZE, IMID, and cocrystal (COC) are shown in Figure 2.

The XRPD pattern of the cocrystal displayed three distinct diffraction peaks ($\theta = 13.6 \pm 0.2, 18.8 \pm 0.2$, and $21.4 \pm 0.2^\circ$) that cannot be attributed to its precursors. This indicates the formation of a novel crystalline structure. The literature describes the characterization of the pharmaceutical cocrystal of ezetimibe and imidazole using XRPD.$^{24}$ Although the results are consistent, the signal intensity (count per second, CPS) varies due to four parameters: the potency
(watts) used in the measurements, the type of detector, the interaction time (in seconds), and the opening of the slit (0.1, 0.2, or 1 mm). The interaction between the X-ray radiation and the sample produces peaks with CPS relative to the combination of four parameters, resulting in a signal-to-noise ratio. Therefore, the CPS is relative.

Changes in the interaction between the drug and co-former functional groups can be detected using FTIR spectroscopy. The characteristic peaks and their shifts of EZE, IMID, the physical mixture of EZE and IMID (PM), and COC are illustrated in Figure 3.

A comparison of spectra reveals that several band shifts occurred between the starting components and the cocrystal. The FTIR spectrum of EZE exhibits strong peaks at 3260.0 cm\(^{-1}\) (corresponding to O−H stretching vibration), 1717.0 cm\(^{-1}\) (indicating C=O β-lactam stretching vibration), and 1506 cm\(^{-1}\) (representing benzene ring C=C stretching vibration). IMID exhibits absorption bands at 1060.0 cm\(^{-1}\) (CH-in plane deformation), 3131.0 cm\(^{-1}\) (N−H bond stretching), and 1550.0 cm\(^{-1}\) (ring breathing).

In the cocrystal, there was a shift of the O−H band of the EZE to 3255 cm\(^{-1}\), as well as a shift in the N−H band of IMID N−H group to 3123 cm\(^{-1}\). These changes suggested the possibility hydrogen-bonding interactions between the hydroxyl group of EZE and the amine group of IMID.

The imidazole co-former is composed of a five-membered aromatic structure that contains two nitrogen atoms. This molecule is an amphoteric and highly polar heterocycle.\(^{38}\) According to Zhang et al.,\(^{39}\) this co-former exists in two tautomeric forms, which allows the hydrogen atom to be positioned on either of the nitrogen atoms. The nitrogen in the electron-rich heterocycle can participate in both accepting and donating protons, leading to the formation of several weak interactions. Thus, the structure of imidazole may promote the enhanced solubility of elements attached to it by rapidly forming hydrogen-bonding interactions.

Thermal characterization can provide information related to the melting, phase transition, and decomposition of drugs, and drugs for the determination of their physicochemical state. Figure 4 shows the thermal behavior of the samples as measured by DSC.

The DSC curves for EZE, IMID, and the cocrystal showed a single melting peak each, at temperatures of 163.1, 90.2, and 75.9 °C, respectively. In cocrystal melting, the endotherm was a lower peak than either that of EZE or its co-former. The result was similar to that reported by Wen et al.\(^{23}\) The shift in the melting point of the cocrystal may be attributed to a change in the crystal arrangement of EZE and IMID in the cocrystal form, leading to a distinct crystal lattice.\(^{40}\)
Due to changes in the crystalline phase of cocrystals, it is expected that they will exhibit differences in particle size and morphology in most cases. Figure 5 displays photomicrographs of EZE, IMID, a physical mixture of EZE and IMID (PM), and the cocrystal (COC). Small, fine, spherical EZE particles can be observed in Figure 5a. This characteristic is common in microparticles containing hydrophobic drugs, and it has been reported in previous studies. The IMID (shown in Figure 5b) exhibited elongated particle shapes and a rough surface. In contrast, the morphology of the cocrystal’s surface (Figure 5c) indicates the presence of irregularly shaped particles with a smooth texture. Finally, Figure 5d shows the aggregation of fine spherical EZE particles on the surface of the elongated IMID particles.

Measurement of the eutectic point, eutectic constant ($K_{eu}$), thermodynamic solubility, and the cocrystal’s solubility advantage

After reaching equilibrium in water and FaSSIF, characteristic peaks of the API and cocrystal were confirmed through XRPD, as presented in the Supplementary Information section (Figures S1 and S2). These peaks indicate the presence of solid forms in the suspension and were used for analysis. The variation in API and co-former concentrations over time was less than 5%, even during suspension equilibrium. That being said, it is suggested that the eutectic point ($P_{eu}$) has been reached. The eutectic concentrations of the active pharmaceutical ingredient (API) and co-former, as well as the $S^{1:1 \text{cocrystal}}$, $K_{eu}$, SA, and pH values, are presented in Table 1.

In water, the cocrystal equilibrium solubility ($S^{1:1 \text{cocrystal}}$) was approximately 10 times greater than that of the pure drug. However, the $K_{eu}$ value, which predicts the relationship between cocrystal solubility and thermodynamic stability, was 500. For cocrystals with a stoichiometric ratio of 1:1, $K_{eu}$ values greater than 1 indicate thermodynamic instability but better solubility compared to the API. On the other hand, $K_{eu}$ values less than 1 indicate thermodynamic stability but lower solubility. The higher the $K_{eu}$ value, the lower the thermodynamic stability of the cocrystal.
The SA was approximately 22.4 and represents the driving force for drug precipitation during cocrystal dissolution. High values indicate inherent risks for cocrystal instability and potential conversion to the drug, which is a more favorable condition for occurrence. To better predict in vivo behavior, biorelevant methods that simulate the composition of intestinal fluids are widely used as an alternative to simply using buffer solutions. The FaSSIF consists of solubilizing agents, such as bile salts and lecithin, as well as micellar surfactants. These components work together to facilitate the wetting and solubilization of lipophilic compounds by forming micelles. The process is known as micellar solubilization. As the cocrystal dissolves, the hydrophobic drug enters the core of the micelles, improving dissolution performance. Furthermore, the surfactant, by decreasing the surface tension and the free energy of the solution, provides greater wettability of the drug, thus increasing dissolution.

In FaSSIF, the equilibrium solubility of the cocrystal was approximately six times greater than that of the pure drug. While the $K_{eu}$ and SA values were approximately 140.0 and 12.0, respectively. The solubility of the cocrystal was higher than that of the pure drug. However, the increase in solubility was smaller when compared to the study conducted in water.

According to Lipert et al., solubilizing agents contribute to increase the solubility of both the cocrystal and its constituent drug. However, in the case of cocrystals formed by a hydrophobic drug and a hydrophilic co-former, the interaction between the micellar surfactants and the co-former is less pronounced, resulting in a greater solubilization of the drug. Moreover, the reduction in the $K_{eu}$ value indicates a modification in the correlation between EZE and IMID concentrations at the eutectic point when solubilizing agents are present, resulting in an elevation in drug concentration at the expense of the co-former. A decrease in the SA value predicts a lower risk of phase transformation and a reduction in drug crystallization kinetics.

Another factor that may have contributed to the increased solubility of the cocrystal in FaSSIF is related to the acidic pH of the medium. Since the co-former has a basic characteristic in an acid medium, it is protonated, generating a positive ion that can interact with the negative sites of the drug. The slight increase in pH of FaSSIF from 6.5 to 7.1, is due to the properties of the imidazole. Being an organic base with a propensity to protonate in slightly acidic media, it leads to the neutralization of the medium, justifying the observed increase.

The results obtained indicate that, under conditions similar to the physiological environment, the performance of the cocrystal improved. This suggests that the therapeutic effect of the cocrystal could be enhanced, as the bioavailability of the drug is limited by its solubility.

### Dissolution under non-sink conditions

Establishing the dissolution pattern of a cocrystal is critical for predicting in vivo absorption behavior, particularly for BCS class II drugs, where absorption is restricted by the rate of dissolution. Non-sink conditions are typically used to sustain a state of supersaturation in a solution. The dissolution of the drug and cocrystal in water (as shown in Figure 6a) and FaSSIF (as shown in Figure 6b) are presented. Additionally, the kinetic parameters of dissolution are summarized in Table 2.

The equilibrium solubility of EZE in water and FaSSIF was 3.7 and 12.0 µg mL$^{-1}$, respectively, as shown by the dashed line in the graph. The dose used in the study was (400 µg mL$^{-1}$): 20 mg.

The dissolution of the cocrystal in water (Figure 6a) was significantly higher than that of the pure drug, exhibiting a common dissolution behavior known as the “spring-parachute” effect, as reported by Guzman et al.

In the case of the cocrystal, the maximum dissolution concentration ($C_{max}$) of EZE was 5.0 µg mL$^{-1}$, and was achieved at 5 min ($T_{max}$), followed by a gradual decrease in concentration to an equilibrium value within 150 min.

In the dissolution of the cocrystal in FaSSIF (as shown in Figure 6b), the drug was released at a higher rate, with the $C_{max}$ of EZE reaching 40.0 µg mL$^{-1}$ and being maintained for 10 min ($T_{max}$). The results indicate that the “parachute” effect offered a comfortable period window considered sufficient for the cocrystal to be absorbed into the systemic circulation before it releases the active constituent. Maintaining a higher level of supersaturation for a longer period after the cocrystal dissolves in the gastrointestinal tract is expected to enhance the dissolution advantage provided by the cocrystals.

### Table 1. Values of the eutectic concentrations, $S^{11\text{e cosyntal}}$, $K_{eu}$, SA, and pH were measured in both water and FaSSIF after reaching equilibrium

<table>
<thead>
<tr>
<th></th>
<th>$[\text{EZE}]_{eu}$ (mmol L$^{-1}$)</th>
<th>$[\text{IMID}]_{eu}$ (mmol L$^{-1}$)</th>
<th>$S^{11\text{e cosyntal}}$ (mmol L$^{-1}$)</th>
<th>$K_{eu}$</th>
<th>SA</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.02 ± 0.14</td>
<td>5.0 ± 0.57</td>
<td>0.2 ± 0.42</td>
<td>500.0</td>
<td>22.4</td>
<td>7.1</td>
</tr>
<tr>
<td>FaSSIF</td>
<td>0.06 ± 0.48</td>
<td>4.2 ± 0.46</td>
<td>0.4 ± 0.56</td>
<td>140.0</td>
<td>11.8</td>
<td>7.1</td>
</tr>
</tbody>
</table>

$S^{11\text{e cosyntal}}$: cocrystal equilibrium solubility; EZE: ezetimibe; IMID: imidazole; $P_{eu}$: eutectic point; $K_{eu}$: eutectic constant; SA: solubility advantage.
The data in Table 2 indicate that the $\sigma_{\text{max}}$ in FaSSIF was superior to the study in water, 3.3, and 1.3, respectively, favoring the dissolution of the cocrystal and maintaining drug supersaturation. Recent investigations have indicated that bile salts, which act as endogenous surfactants, can effectively inhibit crystallization, delay precipitation, and potentially prolong the supersaturation state of drugs in the body.\textsuperscript{51,52}

AUC represents the exposure of the drug, and in the case of cocrystals, it is directly proportional to dissolution and inversely proportional to precipitation.\textsuperscript{53} Moreover, the kinetics of dissolution, supersaturation, and precipitation is driven by high SA values. This condition can be confirmed when it is observed that the lowest SA value (11.8), as expressed in Table 1, also occurred in FaSSIF. The higher AUC value (2963.62 ± 9.1 µg min mL$^{-1}$) in FaSSIF suggests that the kinetics of cocrystal phase transition were hindered, leading to sustained drug supersaturation. These findings suggest that the cocrystal could enhance drug exposure \textit{in vivo}, leading to an increase in bioavailability and subsequent pharmacological activity of ezetimibe.

\textbf{In vivo hypolipemic study}

The induction of hyperlipidemia created a significant increase ($p < 0.05$) in the serum concentration of 33.33% of TC, 17.75% of TG, 55.68% of LDL, and a 12.00% reduction of HDL. In addition, TGP/ALT and TGO/AST levels increased by 39.75 and 17.14%, respectively, and us-CRP was elevated by 41.17% compared to the control group. The values indicate that the high-fat diet was adequate to induce an increase in serum lipid levels. The lipid profile result was similar to other studies that employed a hyperlipemic diet in an \textit{in vivo} model.\textsuperscript{54} A previous study\textsuperscript{55} indicate that high concentrations of TC and lipids contribute to hepatic steatosis and inflammation as factors predisposing to liver diseases. The plasma concentration of TC, TG, LDL, HDL, TGO/AST, TGP/ALT, and us-CRP after seven, fourteen, and twenty-eight days of treatment are shown in Figure 7.

The animals that received pure EZE showed a significant reduction ($p < 0.05$) in TC levels compared to the moments from seven to twenty-eight days of treatment. Furthermore, the results differed significantly ($p < 0.05$) from the control group. While the group treated with cocrystal showed a significant reduction ($p < 0.05$) of the values at the exact moment, however, there was a significant difference ($p < 0.05$) compared to the HD group. The data obtained indicate that in both groups, TC levels were reduced, an expected result since the principal effect of the drug is to use lipid-lowering activity. Although, the group treated with cocrystals showed levels similar to that of healthy animals. Similar results were achieved in other studies\textsuperscript{56} investigating decreased serum TC levels in induced hyperlipidemia in rats through different drug delivery strategies.

\begin{table}[h!]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Media & Solid form & $C_{\text{max}}$ / (µg mL$^{-1}$) & $T_{\text{max}}$ / min & $\sigma_{\text{max}}$ & AUC / (µg min mL$^{-1}$) & pH media \\
\hline
Water & cocrystal & 5.0 ± 0.14 & 5 & 1.3 ± 0.08 & 411.12 ± 7.5 & 7.0 \\
 & drug & 0.53 ± 0.01 & 7 & & 78.38 ± 1.4 & \\
 & & & & & & \\
FaSSIF & cocrystal & 40.0 ± 0.59 & 10 & 3.3 ± 0.07 & 2963.62 ± 9.1 & 7.1 \\
 & drug & 3.50 0 ± 0.02 & 30 & & 478.49 ± 6.9 & \\
\hline
\end{tabular}
\caption{Cocrystal and drug dissolution kinetic parameters in FaSSIF and water}
\end{table}

The data in Table 2 indicate that the $\sigma_{\text{max}}$ in FaSSIF was superior to the study in water, 3.3, and 1.3, respectively, favoring the dissolution of the cocrystal and maintaining drug supersaturation. Recent investigations have indicated that bile salts, which act as endogenous surfactants, can effectively inhibit crystallization, delay precipitation, and potentially prolong the supersaturation state of drugs in the body.\textsuperscript{51,52}

AUC represents the exposure of the drug, and in the case of cocrystals, it is directly proportional to dissolution and inversely proportional to precipitation.\textsuperscript{53} Moreover, the kinetics of dissolution, supersaturation, and precipitation is driven by high SA values. This condition can be confirmed when it is observed that the lowest SA value (11.8), as expressed in Table 1, also occurred in FaSSIF. The higher AUC value (2963.62 ± 9.1 µg min mL$^{-1}$) in FaSSIF suggests that the kinetics of cocrystal phase transition were hindered, leading to sustained drug supersaturation. These findings suggest that the cocrystal could enhance drug exposure \textit{in vivo}, leading to an increase in bioavailability and subsequent pharmacological activity of ezetimibe.

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{Figure6.png}
\caption{Powder dissolution under non-sink conditions of EZE and COC in water (a) and FaSSIF (b). The dashed line indicates the equilibrium solubility of the drug.}
\end{figure}
Figure 7. Serum levels of (a) TC, (b) TG, (c) LDL, (d) HDL, (e) TGO, (f) TGP, and (g) us-CRP after seven, fourteen, and twenty-eight days of treatment. Mean ± standard deviation (n = 6) of the following groups: control, high-fat diet (HD), pure drug (pure EZE), and cocystal (COC). *Significant difference ($p < 0.05$) compared the moments (7, 14, and 28 days). (a) Significant difference ($p < 0.05$) compared to the group control. (b) Significant difference ($p < 0.05$) compared to the group’s high-fat diet. (c) Significant difference ($p < 0.05$) compared to the group pure EZE.
The TG levels of the group treated with pure EZE and cocrystal showed a significant reduction ($p<0.05$) compared to the moments from seven to twenty-eight days of treatment, besides a significant decrease ($p<0.05$) in the HD group. In addition, these values presented no significant differences ($p<0.05$) with the control group. The LDL concentration in animals treated with pure EZE was significantly reduced ($p<0.05$) compared to the time points from seven to twenty-eight days of treatment, and significant difference ($p<0.05$) from the control group at the end of treatment. Animals treated with cocrystal showed a significant reduction ($p<0.05$) after fourteen and twenty-eight days compared to the HD group. According to Phan et al., EZE increases the expression of LDL receptors by inhibiting cholesterol absorption, contributing to greater uptake of endogenous cholesterol. The larger and faster absorption of the drug may explain what happens when in the form of cocrystals.

The animals treated with pure EZE showed a significant increase ($p<0.05$) in HDL levels at seven and fourteen to twenty-eight days of treatment. The group treated with cocrystal showed a significant difference ($p<0.05$) at time points seven to twenty-eight days of treatment and a significant increase ($p<0.05$) compared to the HD group.

The better therapeutic effect of cocrystal, when compared to the pure EZE, may have been caused by increased drug solubility, providing greater permeation and reduced variability in bioavailability, resulting in a decrease in TC, TG, and LDL levels and an increase in HDL. Similar studies investigating the advantages of cocrystallization in the therapeutic efficacy of hypolipemic drugs obtained satisfactory results in decreasing serum lipid concentration in a hyperlipidemia model.

The TGP/ALT levels of the group that received pure EZE presented a significant reduction ($p<0.05$) compared to the moments from seven to twenty-eight days of treatment and in relation the control group, with a decrease of 22.35%. The group treated with cocrystal also presented a significant reduction in levels ($p<0.05$) during the same period but with a decrease of 31.76%. Furthermore, the HD group’s values differed significantly ($p<0.05$).

The TGO_AST levels of the group treated with pure EZE showed a significant reduction ($p<0.05$) from fourteen to twenty-eight days of treatment, with a decrease of 11.23%. On the other hand, the group that received cocrystal presented a significant reduction ($p<0.05$) from seven to twenty-eight days of treatment, with a decrease of 22.59%. Besides, after twenty-eight days of treatment, the levels were significantly different ($p<0.05$) from the HD group and treated with pure EZE.

Elevated serum activity of these enzymes has been linked to significant damage to the integrity of the liver. However, less liver injury can explain decreased values of transaminase levels in the group treated with cocrystals due to a decrease in fat accumulation in hepatocytes for the animals of this group.

Besides being a biomarker in inflammatory processes, us-CRP can change the phenotype of endothelial cells and act as a mediator of vascular disease. Evidence directs the association between lipid disorders and inflammatory processes, making inflammation an essential regulator in the development of cardiovascular diseases. According to Goff et al., low cardiovascular risk values are considered concentrations lower than 0.1 mg dL$^{-1}$; average risk between 0.1 and 0.3 mg dL$^{-1}$; and high-risk values greater than 0.3 mg dL$^{-1}$. This study observed that the us-CRP values of animals treated with pure EZE and cocrystal did not present a significant reduction ($p<0.05$) in the moments or differences between the groups, a fact that may be related to the total period of treatment adopted. Studies by Mutty et al. indicate a significant reduction in us-CRP levels in rabbits after administration of a diet enriched with fats within eight weeks of treatment. According to Jain et al., the levels of us-CRP in diabetic rats after seven weeks of treatment presented significant differences in values compared to animals that did not receive the intervention.

The remarkable and sensitive AI parameter is a good indicator of possible cardiac risks. Its determination allows for predicting the relationship between serum lipid levels and the occurrence of cardiovascular diseases. AI of the HD groups, treated with pure EZE and cocrystal, calculated after twenty-eight days of treatment, can be seen in Table 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.60 ± 0.09</td>
</tr>
<tr>
<td>HD</td>
<td>4.56 ± 0.14*</td>
</tr>
<tr>
<td>Pure EZE</td>
<td>3.94 ± 0.1*</td>
</tr>
<tr>
<td>Cocrystal</td>
<td>3.62 ± 0.18*</td>
</tr>
</tbody>
</table>

Data expressed as mean ± standard deviation (n = 6) where *means the statistical difference between the control group ($p<0.05$) and HD group ($p<0.05$). AI: atherogenic index; HD: high-fat diet; EZE: ezetimibe.

The AI of the group treated with pure EZE showed a significant difference ($p<0.05$) value compared to the control group; however, the group treated with cocrystal showed a significant difference ($p<0.05$) value compared to the HD group. The increased solubility and dissolution of the drug when acting as a cocrystal may have contributed to its better and faster absorption, allowing the serum levels.
of TC and HDL, necessary for the determination of AI, to be similar to those of animals who received a standard diet.

Conclusions

In this investigation, a pharmaceutical cocrystal of ezetimibe and imidazole was successfully characterized using the reaction crystallization method. The solubility and thermodynamic stability of the cocrystal were found to be higher in a biorelevant medium compared to water. This was supported by a decrease in SA, indicating slower phase transition kinetics of the cocrystal. Dissolution under supersaturation conditions of the cocrystal in FaSSIF resulted in a higher level of supersaturation and an increase in the area under the curve. This suggests that the cocrystal was able to sustain supersaturation more effectively in a more efficient manner. The solubilizing agents in FaSSIF may have contributed to the increased stability of the cocrystal, resulting in better dissolution and a state of supersaturation that enhances the performance of the drug.

The in vivo hypolipemic activity of the cocrystal showed a significant improvement ($p < 0.05$) in the levels of TC, TG, LDL, and HDL, particularly between days seven and twenty-eight of treatment in the group that received a high-fat diet (HD). Although, the us-CRP concentration of the other groups, possibly due to the total treatment time difference ($p < 0.05$) compared to the HD group. From this perspective, the developed cocrystal shows promise and further studies are necessary to better understand its properties. This will contribute to its potential manufacture on an industrial scale.

Supplementary Information

Supplementary data corresponding to the XRPD for measurement of the eutectic point are available free of charge at http://jbcs.sbq.org.br as PDF file.

Author Contributions

Débora F. V. Ronik was responsible for the investigation, writing original, assays work, biological and assays work; Andressa P. Hosni for the biological assays work; Rafaela C. Brancalione for the biological assays work; Isabela F. B. Bicaia was responsible was assays work; Larissa S. Bernardi for the writing review, editing; Paulo R. de Oliveira was responsible for the conceptualization, writing review and editing, and project administration. All authors have read and agreed to the published version of the manuscript.

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