

Methotrexate Amorphous Solid Dispersions: Development, Physicochemical Characterization, and Solubility Limitations

Dispersões sólidas amorfas de metotrexato: desenvolvimento, caracterização físico-química e limitações na solubilidade.

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ABSTRACT

Methotrexate (MTX) is a widely used drug for autoimmune diseases and cancers, but suffers from low oral bioavailability due to its poor solubility and permeability. This study aimed to enhance MTX solubility by developing amorphous solid dispersions using Poloxamer 407, PVP K-30, and PEG 6000 through solvent evaporation. The dispersions were characterized using DSC, PXRD, and FTIR to confirm amorphization, and their solubility was tested in a pH 6.8 phosphate buffer compared to free MTX. The results showed that amorphization was successfully achieved, as confirmed by PXRD analyses and supported by DSC and FTIR findings. However, the amorphous solid dispersions did not improve MTX solubility (3.28 mg/ml and 3.72 mg/ml) over the free drug, consistent with previous studies indicating minimal solubility gains for MTX in both amorphous (4.06 mg/ml) and crystalline forms (4.57 mg/ml). These findings suggest that amorphization alone may be insufficient to enhance MTX solubility. The study concludes that MTX amorphous solid dispersions are technically feasible but do not offer significant solubility benefits. Alternative strategies, such as nanoencapsulation, may be more promising for improving MTX bioavailability. These results underscore the need for innovative approaches in MTX delivery systems to overcome its inherent limitations.

Keywords: Drug; Bioavailability; Poloxamer 407; Autoimmune diseases; Cancers.

RESUMO

O metotrexato (MTX) é um medicamento amplamente utilizado para doenças autoimunes e cânceres, mas sofre de baixa biodisponibilidade oral devido à sua baixa solubilidade e permeabilidade. Este estudo teve como objetivo aumentar a solubilidade do MTX desenvolvendo dispersões sólidas amorfas usando Poloxamer 407, PVP K-30 e PEG 6000 por meio da evaporação do solvente. As dispersões foram caracterizadas usando DSC, PXRD e FTIR para confirmar a amorfização, e sua solubilidade foi testada em um tampão fosfato de pH 6,8 em comparação com MTX livre. Os resultados mostraram que a amorfização foi alcançada com sucesso, conforme confirmado pelas análises de PXRD e apoiado pelos achados de DSC e FTIR. No entanto, as dispersões sólidas amorfas não melhoraram a solubilidade do MTX (3,28 mg/ml e 3,72 mg/ml) em relação ao fármaco livre, consistente com estudos anteriores que indicavam ganhos mínimos de solubilidade para o MTX tanto em formas amorfas (4,06 mg/ml) quanto cristalinas (4,57 mg/ml). Esses achados sugerem que a amorfização sozinha pode ser insuficiente para aumentar a solubilidade do MTX. O estudo conclui que dispersões sólidas amorfas de MTX são tecnicamente viáveis, mas

não oferecem benefícios significativos de solubilidade. Estratégias alternativas, como a nanoencapsulação, podem ser mais promissoras para melhorar a biodisponibilidade do MTX. Esses resultados ressaltam a necessidade de abordagens inovadoras nos sistemas de entrega de MTX para superar suas limitações.

Palavras-chave: Fármaco; Biodisponibilidade; Poloxamer 407; Doenças autoimunes; Cânceres.

INTRODUCTION

Methotrexate (MTX) is widely utilized as a disease-modifying drug in the treatment of rheumatoid arthritis, psoriasis, inflammatory conditions such as Crohn's disease, and various cancers, including those of the head and neck, breast, lung, lymphoma, colorectal cancer, and leukemia. MTX functions as a folic acid antagonist, primarily inhibiting cell proliferation. It achieves this by inhibiting dihydrofolate reductase, an enzyme crucial for the metabolic processes involving folic acid derivatives. However, MTX and its metabolites are associated with several serious side effects, including gastrointestinal and hepatic toxicity, bone marrow suppression, and stomatitis, among others (Agafonov, Ivanov, Terekhova, 2021; Hwang et al., 2024).

Although MTX is a hydrophilic molecule ($\log P = 1.85$), it is practically insoluble in water and mostly ionized at physiological pH, on account of two deprotonated carboxylic groups, which prevent it from crossing the cell membranes (Alvarez-Figueroa; Delgado-Charro; Blanco-Méndez, 2021). The drug MTX belongs to class IV in the Biopharmaceutical Classification System (BCS) because it has low solubility and permeability, which makes it have low bioavailability (about 18% for an oral dose $> 40 \text{ mg.m}^{-2}$). Due to this physicochemical profile, obtaining adequate oral formulations for MTX still represents a challenge (Giri et al., 2022). Solid dispersions emerge as a promising drug delivery for class IV drug solubility improvement (Bhairam et al., 2022; Kumari et al., 2023; Nair et al., 2020; Panizzon et al., 2019; Nart et al., 2015).

Sekiguchi and Obi first reported in 1961 that a eutectic mixture of sulfathiazole and urea could enhance drug absorption compared with the conventional crystalline drug, demonstrating that modification of the drug's physical state could improve its pharmaceutical performance. Although the term solid dispersion (SD) was not used in their original study, this work is widely regarded as a landmark in the development of SD technology (Sekiguchi, Obi, 1961). In 1971, Chiou and Riegelman subsequently formalized the concept of SD systems, defining them as dispersions of one or more active ingredients in an inert carrier in the solid state and describing their preparation by fusion, solvent, or melting-solvent methods (Chiou, Riegelman, 1971).

As the understanding of these systems evolved, their classification was increasingly related to the physical state and molecular organization of both the drug and the carrier. Janssens and Van den Mooter (2009) described SD as a broad class of solid-state systems that may include eutectic mixtures, solid solutions, and amorphous systems, depending on the physical state of the components and the degree of miscibility between the drug and carrier. In a solid solution, the drug is molecularly dispersed within a solid carrier, whereas amorphous systems may exhibit molecularly dispersed drug or drug-rich amorphous domains. These distinctions highlight that solid dispersions are not defined solely by their preparation method, but also by the physical state and spatial organization of their components (Janssens, Van den Mooter, 2009).

More recently, the development of polymer-based amorphous solid dispersions has broadened the

application of this technology beyond the original eutectic systems. In these formulations, poorly water-soluble drugs are incorporated into polymeric matrices in an amorphous or molecularly dispersed state, with the aim of reducing crystallinity, increasing apparent solubility, enhancing dissolution, and ultimately improving oral bioavailability. The contemporary concept therefore encompasses a range of drug-polymer solid-state arrangements, including molecular dispersions and amorphous drug-rich phases, with the resulting performance being strongly influenced by drug-polymer interactions, miscibility, physical stability, and the tendency of the drug to recrystallize (Baghel, Cathcart, O'Reilly, 2016). Thus, while the original concept arose from the formation of eutectic mixtures, solid dispersion technology has evolved into a broader formulation strategy for modifying the solid state of poorly water-soluble drugs and improving their biopharmaceutical performance.

Three studies can be found in the literature exploring the SD platform for MTX bioavailability improvement to date. These studies explore the wider definition of SD, considering a system composed of hydrophilic carriers that dissolve the poorly soluble drug and release it into the dissolving medium as tiny particles (ranging from micro- to nanoscale), thereby increasing surface area and enhancing solubility. Two studies produced microcrystalline dispersion, in which Differential Scanning Calorimetry (DSC), Powder X-Ray Diffraction (PXRD), and Fourier Transform Infrared Spectroscopy (FTIR) results showed the crystalline state of dispersed MTX, as well as the absence of chemical interactions between MTX and the hydrophilic matrix, resulting in a two-phase system different from a solid solution or amorphous matrix (Agafonov, Ivanov, Terekhova, 2021; Giri et al., 2022). These studies showed the microcrystalline SD structure, with MTX not molecularly dissolved or in an amorphous state. Solid-state changes can modulate

physicochemical and biopharmaceutical properties (Zhou et al., 2024; Jangid et al., 2020). Therefore, we found an opportunity to explore SD as a drug delivery system for MTX by changing the MTX crystalline form as a different strategy from previous studies on MTX SDs.

The Cambridge Structural Database (CSD) reports the existence of three different structural forms of MTX, as follows: two tetrahydrates, both tetragonal forms showing space group $P 4_2 2_1$, which differ on cell unit dimensions: $10.343(2)\text{\AA}$ b $10.343(2)\text{\AA}$ c $45.521(6)\text{\AA}$, α 90° β 90° γ 90° (deposition number 1143754) or a $10.372(1)\text{\AA}$ b $10.372(1)\text{\AA}$ c $45.546(7)\text{\AA}$, α 90° β 90° γ 90° (deposition number 1143755) and one in salt form (deposition number: 1151162; space group: $P 1$ (1); unit cell dimensions: a $8.4435(6)\text{\AA}$ b $10.6655(7)\text{\AA}$ c $19.199(1)\text{\AA}$, α $92.251(5)^\circ$ β $98.869(5)^\circ$ γ $92.881(5)^\circ$) (CCDC, [202-]), which contains strontium as a contra-ion. According to Chan and Gonda (1990), besides the hydrate forms, MTX exists in an amorphous state, which was obtained by dissolving and drying the drug in methanol. The amorphous MTX has been proven to be 4-fold more soluble than the crystalline forms. This finding aligns well with the concept of a solid solution within the definitions of SD and applies to the solvent evaporation method. MTX can be molecularly dissolved in methanol, with hydrophilic polymers also soluble in the solvent, and this solution can be converted into a solid solution having amorphous MTX. This approach differed from the previous studies of MTX-based SD because it proposed a solid-state variation, which was not done by the prior art.

Therefore, this work aimed to develop an SD of MTX using the solvent evaporation technique with methanol, targeting a solid solution-type dispersion containing MTX in an amorphous state. It could bring an innovative step for the SD platform to improve MTX solubility and bioavailability.

METHODS

Materials

Methanol was obtained from Dinâmica Química Contemporânea Ltda. (Indaiatuba, Brazil), as well as from Anidrol Produtos para Laboratórios Ltda. (Diadema, Brazil). Poloxamer 188 (P188), poloxamer 407 (P407), diethylene glycol monoethyl ether (DGME), 30-vinylpyrrolidone (PVP K-30), and poly(ethylene glycol) 6000 (PEG 6000) were procured from Sigma-Aldrich (St. Louis, USA), Vetec (Rio de Janeiro, Brazil), and Inlab (São Paulo, Brazil), respectively.

Solid dispersion obtention

The samples for the preparation of MTX-loaded SDs, as detailed in Table 1, were packaged in 1 mL vials and protected from light. Initially, the samples were stirred for two minutes using a vortex mixer, followed by mixing in a Cole-Parmer TR-200 tube orbital shaker (Antyllia Scientific Ltda., Stone, United Kingdom). After 24 hours of stirring, the samples were vacuum-dried at 40 °C for 60 minutes using a Centrivap concentrator (Kansas City, USA) and subsequently stored in a desiccator. For characterization and solubility evaluation, additional samples were prepared using the same procedure, but the drying step was performed using a rotary evaporator at 47 °C and 80 rpm. The dried samples were then stored in a desiccator until further analysis.

Table 1: Experimental design for SDs obtention containing 0.7 mg.mL⁻¹ methotrexate (MTX).

Sample	Weight of excipients for 1 mL of MTX solution in methanol				
	P188 (g) ^{a)}	DGME (g) ^{b)}	P407 (g) ^{c)}	PVP K-30 (g) ^{d)}	PEG 6000 (g) ^{e)}
A	-	-	-	-	-
B	-	0.10	-	-	-
C	0.10	0	-	-	-
D	0.10	0.10	-	-	-
E	0.05	-	-	-	-
F	0.05	0.10	-	-	-
G	-	0.05	-	-	-
H	0.10	0.05	-	-	-
I	0.05	0.05	-	-	-
J	-	-	-	-	-
K	-	-	-	0.010	-
L	-	-	0.010	-	-
M	-	-	0.010	0.010	-
N	-	-	0.025	-	-
O	-	-	0.025	0.010	-
P	-	-	-	0.025	-
Q	-	-	0.010	0.025	-
R	-	-	0.025	0.025	-
S	-	-	-	-	0.010
T	-	-	-	-	0.025
U	-	-	0.01	-	0.01
V	-	-	-	0.01	0.01

Note: a) P188 = Poloxamer 188, b) DGME = diethylene glycol monoethyl ether, c) P407 = poloxamer 407, d) PVP K-30 = 30-vinylpyrrolidone, and e) PEG 6000 = poly(ethylene glycol) 6000.

The polymer type and concentration were the variable parameters in the experimental design with three levels of variation. The criteria for selecting the prototypes included the homogeneity of the SDs, the absence of phase separation, and their solid appearance.

PXRD, FTIR, and DSC

Physical mixtures having the same composition as the SDs samples U and V were prepared by mixing the components using a mortar and pestle. Sample A was used as a comparative control group of having MTX without the polymers, but submitted to the solvent evaporation method. Also, the MTX without any treatment was used as a comparative control group.

The samples U, V, PM U, PM V, A, and original MTX were analyzed and characterized by powder X-ray diffraction (PXRD), Fourier Transform Infrared Spectroscopy (FTIR), and Differential Scanning Calorimetry (DSC).

PXRD range selection was over 3° to $35^\circ 2\theta$. The analysis employed $K\alpha$ Cu radiation ($\lambda = 1.5406 \text{ \AA}$) at settings of 40 kV and 30 mA, with an optical step of 0.02° and a scanning rate of $1^\circ 2\theta \cdot \text{min}^{-1}$ (Pedreiro, 2012). All measurements were conducted on an Ultima IV diffractometer (Rigaku, Tokyo, Japan).

For FTIR analyses, samples were prepared as KBr tablets using an Affinity (Shimadzu, Tokyo, Japan) spectrophotometer. The spectra were recorded from 600 to 4000 cm^{-1} with a resolution of 4.0 cm^{-1} and 34 scans.

A NETZSCH Syrius 3500 calorimeter (Kanagawa, Japan) was used for the DSC study. The analysis was conducted in a closed aluminum crucible, heating from 25°C to 250°C at a $10^\circ\text{C} \cdot \text{min}^{-1}$ rate, under a nitrogen atmosphere at a flow rate of $50 \text{ mL} \cdot \text{min}^{-1}$ (Pedreiro, 2012).

Validation of an analytical method for MTX quantification by UV-spectrophotometry

The method was developed and validated following Q2(R2) guidelines from the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH,

2022). To find the maximum absorption wavelength, a $10 \text{ }\mu\text{g} \cdot \text{mL}^{-1}$ MTX methanolic solution spectrum was recorded from 200 to 400 nm. To discard any possible interference, UV spectra were obtained from methanolic solutions ($10 \text{ }\mu\text{g} \cdot \text{mL}^{-1}$) of the solid dispersions (SDs) constituents: P407, PVP K-30, PEG 6000. These spectra were compared with the MTX spectrum for the Selectivity conference.

A calibration curve was constructed from a methanolic solution of MTX at a concentration of $100 \text{ }\mu\text{g} \cdot \text{mL}^{-1}$ and subsequently diluted, in triplicate, with the same solvent to achieve concentrations of 1, 2.5, 5, 7.5, and $10 \text{ }\mu\text{g} \cdot \text{mL}^{-1}$. The absorbances of these solutions were measured at the selected wavelength and plotted against their concentrations to create the standard curve. The appropriateness of the linear regression model was determined by assessing variance homogeneity through the Cochran test. Additionally, the suitability of the adjustment was evaluated using the linear regression coefficient (r), the distribution of residuals (using the Shapiro-Wilk test and plotting residuals against concentration), the significance of the angular coefficient (F), and through simultaneous plotting of calculated and experimental absorbances versus concentration.

The accuracy was evaluated by the analyte addition method. MTX solution was added to SDs' suspensions at three concentration levels: $1 \text{ }\mu\text{g} \cdot \text{mL}^{-1}$, $5 \text{ }\mu\text{g} \cdot \text{mL}^{-1}$, and $8.5 \text{ }\mu\text{g} \cdot \text{mL}^{-1}$. The recovery (R) was calculated by the following equation 1 (AOAC, 2016):

$$R (\%) = \frac{C_f - C_u}{C_a} \times 100 \quad (1)$$

C_f is the MTX concentration in the analyte-added sample, C_u is the MTX concentration in the sample without analyte addition, and C_a is the concentration of the analyte added to the sample. Recovery values between 97% and 103% were accepted.

Intraday precision was assessed using nine independently prepared methanolic solutions of MTX at concentrations of 1, 5, and 10 $\mu\text{g.mL}^{-1}$, each tested in triplicate. The precision was calculated as relative standard deviation (RSD) (2):

$$RSD(\%) = \frac{SD}{\bar{x}} \times 100 \quad (2)$$

SD is the standard deviation, and $\bar{x}$ is the concentration mean.

For the evaluation of interday precision, new solutions were prepared and measured on a different day, following the same procedure used for the intraday precision assay. The relative standard deviation (RSD) was calculated from the eighteen solutions.

The detection limit (DL) and the quantification limit (QL) were calculated from three standard curves following equations 3 and 4, where SD is the intercept standard deviation, and CI is the curve inclination.

$$DL = 3.3 \times \frac{SD}{CI} \quad (3) \quad DQ = 10 \times \frac{SD}{CI} \quad (4)$$

Solubility assay

For the solubility assay, U, V, PM U, PM V, A, and original MTX, each with an excess mass equivalent to 14 mg of MTX, were prepared in small glass flasks. Samples were added to 2 mL of pH 6.8 phosphate buffer solution. The samples were maintained at $37 \pm 1^\circ\text{C}$ while stirring at 150 rpm in an orbital shaker (WEG S.A., Jaraguá do Sul, Brazil), equipped with a temperature controller (Novus Produtos Eletrônicos Ltda., Canoas,

Brazil). Samples were collected at 60 minutes, filtered using a hydrophilic PTFE filter (45 μm), and quantified using a previously validated UV spectrophotometric method, as detailed in the Supplementary Material. Results were expressed as mean $\pm$ standard deviation. Parametric data were analyzed using ANOVA followed by the Tukey test, while non-parametric data were compared using the Kruskal-Wallis test, utilizing R software.

RESULTS

Solid dispersion obtention

The samples containing DGME and/or P188 (labeled B to I in Table 1) resulted in liquid dispersions instead of solids. All samples composed of P407 and/or PVP K-30 (labeled K to R in Table 1) appeared gel-like after drying. The samples M, N, R, P, and Q exhibited phase separation, while K and L were homogeneous. Among the samples containing PEG 6000, S and T showed solid and liquid aspects, respectively. The samples U and V, in which PEG 6000 was blended with P407 or PVP K-30, were solid. The polymers P407 and PVP K-30 did not exhibit synergistic effects, but they did yield solid samples when utilized individually (samples L and K), similar to PEG 6000 (sample S). Conversely, the samples that incorporated P407 or PVP K-30 in association with PEG 6000, specifically samples U and V, respectively, visually demonstrated the formation of SD, which were selected for the following characterization.

PXRD

Figure 1A presents the PXRD results for SD U, with the corresponding physical mixture and raw materials. The crystalline polymers, represented by PEG 6000 and P407, exhibited Bragg peaks at $19.28^\circ 2\theta$ (PEG 6000), $19.14^\circ 2\theta$ (P407), and $23.30^\circ 2\theta$ (both), which is consistent with previously reported findings (El-Badry et al, 2013; Patil, Gaikwad, 2011). The MTX diffractogram reveals several medium- to high-intensity Bragg

peaks, with the most prominent peak appearing at $9.4^\circ 2\theta$, confirming its crystallinity. An enlarged view of these diffractograms (Figure 1B) reveals that the Bragg peak at $9.4^\circ 2\theta$ is specific to MTX since it is absent in the polymers PEG 6000 and P407. The presence of this Bragg peak exclusively in the physical mixture (U) and its absence in the SD U sample confirms the amorphization of MTX in the latter.

Figure 1C illustrates that SD V, PM V, and PEG 6000 exhibited identical diffraction patterns, characterized by two prominent Bragg peaks at 19.12 - $19.28^\circ 2\theta$ and 23.18 - $23.82^\circ 2\theta$. The diffractogram of PVP K-30 was characteristic of an amorphous sample. Notably, an enlarged view of the diffractograms (Figure 1D) could not confirm the absence of the Bragg peak at $9.4^\circ 2\theta$ in SD V due to interference from PVP K-30 in this region. As a result, it remains inconclusive whether MTX amorphization occurs in SD V.

The methanol treatment induced amorphization of the drug, as evidenced by the absence of crystalline signals in the diffractogram of this sample. Thus, it was concluded that methanol contributes to the amorphization of MTX, both independently and in the presence of the polymer.

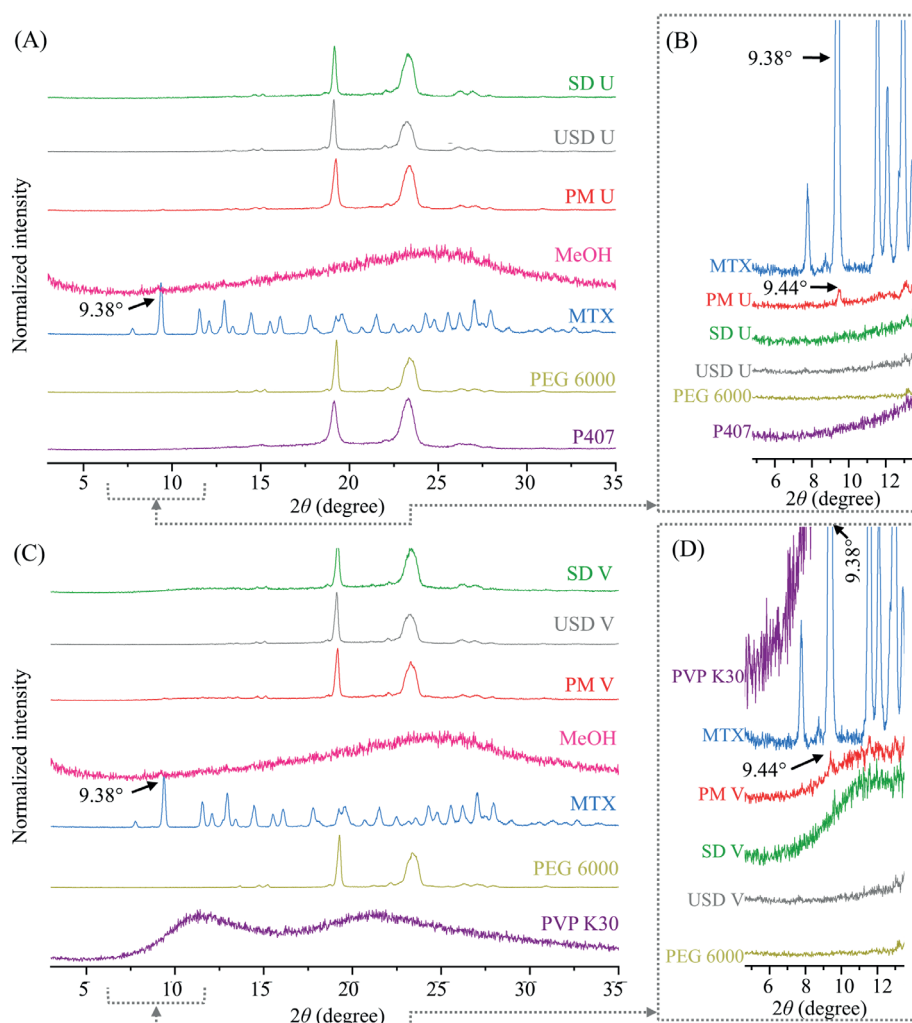


Figure 1: PXRD results.

Legend: Powder X-ray diffractograms of SDs U (A) and V (C) compared to the respective physical mixture, unloaded solid dispersion, and raw materials. A and C were expanded from 6° to 12° 2θ (B and D, respectively) to show one peak from MTX found in the physical mixtures and absent in SD U. SD = solid dispersion, USD = unloaded solid dispersion, MTX = methotrexate, MeOH = SD A, P407 = poloxamer 407, PEG 6000 = polyethylene glycol 6000, and PVP-K30 = polyvinylpyrrolidone K-30.

FTIR

The FTIR results are shown in Figure 2. The hydroxyl stretching appeared as a medium-intensity broad band with peaks at 3439 cm^{-1} (PEG 6000), 3452 cm^{-1} (PM U), and 3429 cm^{-1} (U). The SD V spectra displayed slight shifts compared to the raw materials and physical mixture: the carbonyl stretching occurs at 1661 cm^{-1} in SD V and at 1659 cm^{-1} in both PVP K30 and PM V; the hydroxyl stretching shows its maximum absorption at 3450 cm^{-1} in SD V, 3444 cm^{-1} in PM V, and 3455 cm^{-1} in PVP K30. An absorption band at 1732 cm^{-1} was observed in the SD U spectrum; however, it was absent in both the unloaded solid dispersion and the physical mixture U, indicating an intermolecular interaction in SD U that was absent in A and the PM U. These FTIR findings effectively distinguish SD U from the corresponding physical mixture and the raw materials.

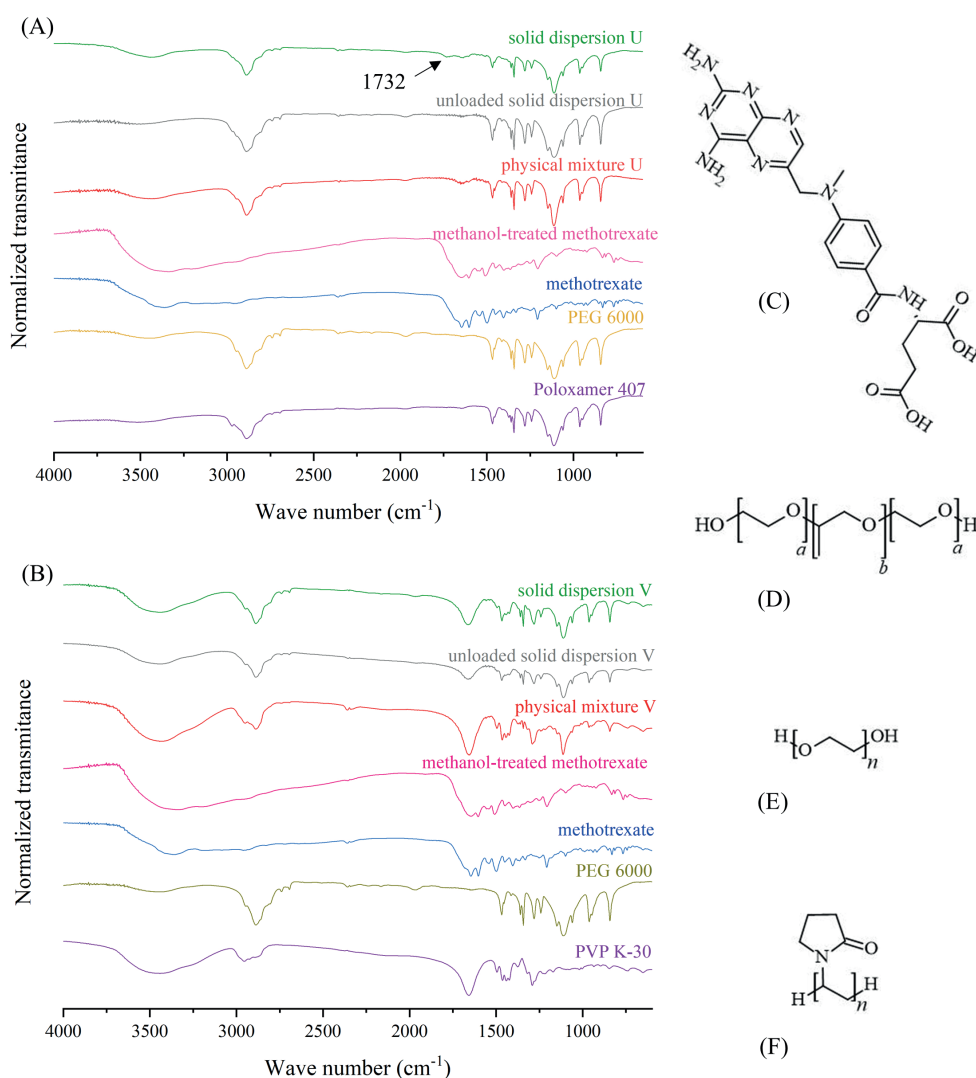


Figure 2: FTIR results.

Legend: Infrared spectra of SDs U (A) and V (B) compared to their respective physical mixtures, the unloaded solid dispersion, and the raw materials. The chemical structures of these raw materials include methotrexate (C), Poloxamer 407 (D), polyethylene glycol 6000 (PEG 6000) (E), and polyvinylpyrrolidone K-30 (PVP K-30) (F). Notably, the absorption band observed in solid dispersion U at 1732 nm highlights a significant difference between this sample and the others.

DSC

The DSC curves are presented in Figure 3. The SD U curve exhibited an endothermic event at 60.8 °C (Tonset), which falls between the melting points of the polymers, Tonset = 57.6 °C and Tonset = 61.8 °C for P407 and PEG 6000, respectively. In contrast, the PM U sample showed two distinct melting points, highlighting the difference between this sample and SD U, confirming an intermolecular interaction between MTX and the polymers in SD U. The melting point of MTX (Tonset = 117.1 °C) was not visible in the SD U or PM U curves, making it difficult to conclude MTX amorphization. Although the methanol-treated MTX curve lacks a melting point and exhibits a profile consistent with an amorphous state, these results suggest a possible change in the drug crystallinity due to the solid dispersion obtained. However, additional experiments are required to confirm this hypothesis. The DSC curve of SD V displayed an endothermic event at 55.0 °C (Tonset), which is lower than the melting points of its individual excipients and isolated components. This suggests the presence of a eutectic mixture, a type of solid dispersion, as described by Mooter (Mooter, 2012).

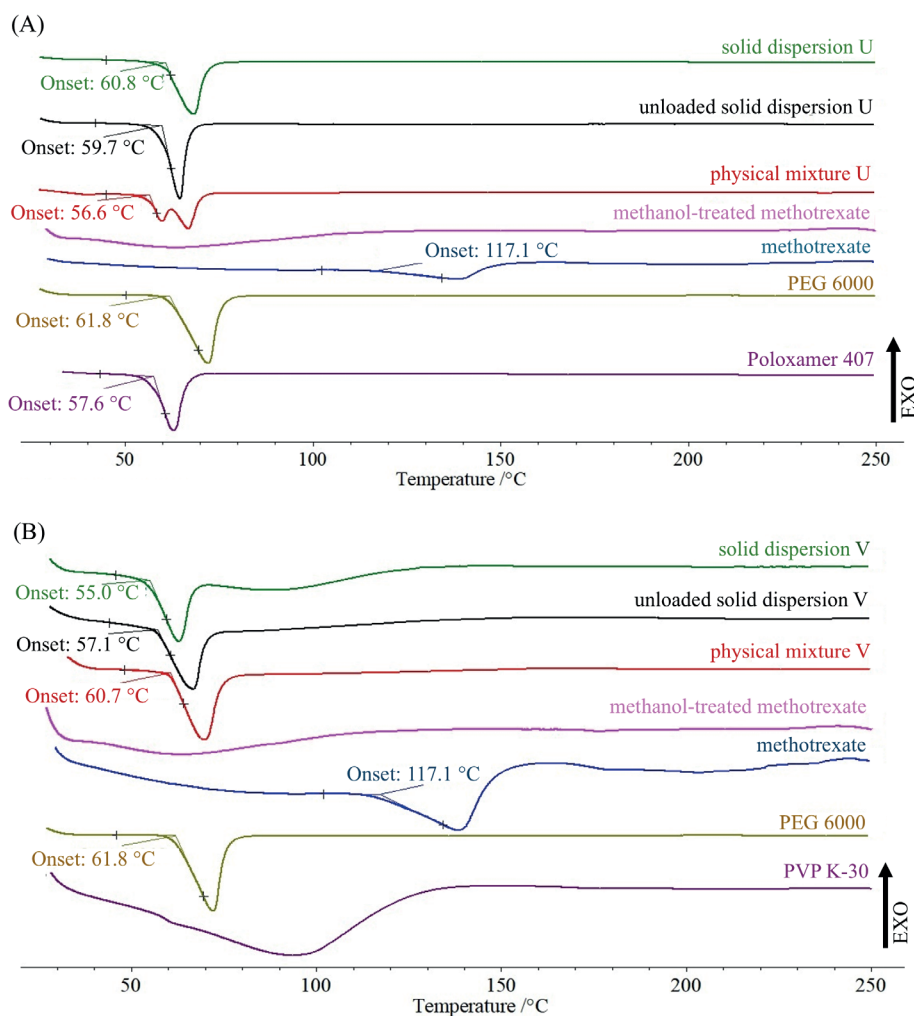


Figure 3: DSC curves.

Legend: Differential scanning calorimetry (DSC) curves of the solid dispersions U (A) and V (B) compared to the respective physical mixture, unloaded solid dispersion, and raw materials. PEG 6000 = polyethylene glycol 6000 and PVP-K30 = polyvinylpyrrolidone K-30

Results of the validation of an analytical method for MTX quantification by UV-spectrophotometry

The selectivity was confirmed by obtaining the MTX, P407, PEG 6000, and PVP K-30 spectra in methanolic solution at 10 µg.mL⁻¹, from 200 to 400 nm. The maximum absorption of MTX occurred at 298 nm. Since the excipients demonstrated insignificant absorbance (0.011, 0.001, and 0.002 for PVP K-30, P407, and PEG 6000, respectively) compared with the drug (0.446), such a wavelength was selected for quantifying MTX.

Visual linearity was observed within the concentration range of 1 to 10 µg.mL⁻¹. The data distribution exhibited homoscedasticity, tested by the Cochran test, as the calculated value ($C_{\text{calculated}} 0.445813$) was less than the tabulated value ($C_{\text{tabulated}} 0.6868$). Consequently, linear regression analysis was conducted using the ordinary least squares method, yielding the equation: $y = 0.0446886x - 0.0201138$, with an r of 0.9992, indicating a strong correlation between the variables. The significance of the slope (angular coefficient) was confirmed with a calculated F -value of 1.008×10^4 and a p -value of 2.2×10^{-16} . The normality of the residuals was assessed using the Shapiro-Wilk test, yielding $W = 0.93336$ and $p = 0.3061$, and the residuals showed random distribution concerning concentration. The calculated absorbance values closely matched the experimental data, further supporting the reliability of the mathematical model used.

The recovery values for the concentrations 1, 5, and 10 µg.mL⁻¹ were 101.44%, 97.41%, and 97.84%, respectively. Thus, the method demonstrated accuracy according to AOAC (AOAC, 2016).

The precision aims to evaluate how close the values obtained by the analytical method are to each other. Intraday precision was measured by the relative standard deviation (RSD) recorded for samples prepared at 1, 5 and 10 µg.mL⁻¹. The results obtained

were 1.009 µg.mL⁻¹ ± 0.022 µg.mL⁻¹, RSD = 2.210; 5.059 µg.mL⁻¹ ± 0.044 µg.mL⁻¹, RSD = 0.880; and 10.080 µg.mL⁻¹ ± 0.093 µg.mL⁻¹, RSD = 0.920. Precision was confirmed at all concentration levels following (AOAC, 2016).

Interday precision results were 1.501 µg.mL⁻¹ ± 0.060 µg.mL⁻¹, RSD=4.050%; 4.969 µg.mL⁻¹ ± 0.129 µg.mL⁻¹, RSD=2.590; and 10.139 µg.mL⁻¹ ± 0.249 µg.mL⁻¹, RSD= 2.460. Interday precision was demonstrated at concentrations of 5 µg.mL⁻¹ and 10 µg.mL⁻¹; however, it was not observed at the lowest concentration, where the RSD reached 4.050%. This exceeds the acceptable limit of 2.70% as set by AOAC (AOAC, 2016). Nevertheless, the lack of interday precision at 1 µg.mL⁻¹ does not invalidate the analytical method, as this issue can be addressed through daily standard deviation measurements. Furthermore, all other validation parameters were successfully met.

The DL refers to the lowest concentration of an analyte that can be detected in a sample using the established methodology, although it cannot be quantified at this level. In contrast, the QL is the lowest concentration at which the analyte can be accurately and precisely measured (MS, 2023). In the method proposed, the DL and QL were determined to be 0.12 µg.mL⁻¹, and 0.39 µg.mL⁻¹, respectively.

Solubility assay

The pH 6.8 phosphate buffer was chosen as the vehicle in the solubility test, since it was the solution where MTX was found to have the highest solubility value of 2 mg/mL in previous work (Bernardes, 2021). Other pH values were not considered due to the very low solubility of the drug. Saturation solubility was found at 60 min. Figure 4 presents the solubility results. The solubility of MTX (4.57 ± 0.09 mg/mL) was not significantly different from that of MeOH-treated MTX (4.06 ± 0.05 mg/mL), $p = 0.11$, indicating that amorphization did not enhance MTX solubility, contrary to the findings reported by Chan and Gonda

(1990). These authors found a 4-fold increase in solubility of the amorphized MTX tested in distilled water media during 48 h.

The physical mixtures, PM U (2.82 ± 0.24 mg/mL) and PM V (2.92 ± 0.39 mg/mL) ($p = 0.99$), resulted in similar solubility values, both of which were lower than that of pure MTX, suggesting that the polymers in these mixtures retained MTX release. Likewise, SD U (3.72 ± 0.17 mg/mL) and SD V (3.28 ± 0.19 mg/mL) demonstrated statistical equivalence in solubility ($p = 0.21$), with values also lower than pure MTX. Although the solubility from SD U was lower than MTX ($p = 0.00$), it was equivalent to Me-OH-treated MTX sample A ($p = 0.43$), and higher than PM U ($p = 0.00$). SD V did not change the solubility of PM V ($p = 0.37$). These findings collectively deny the initial hypothesis, as MTX amorphization within the solid dispersion matrix did not improve the drug's solubility.

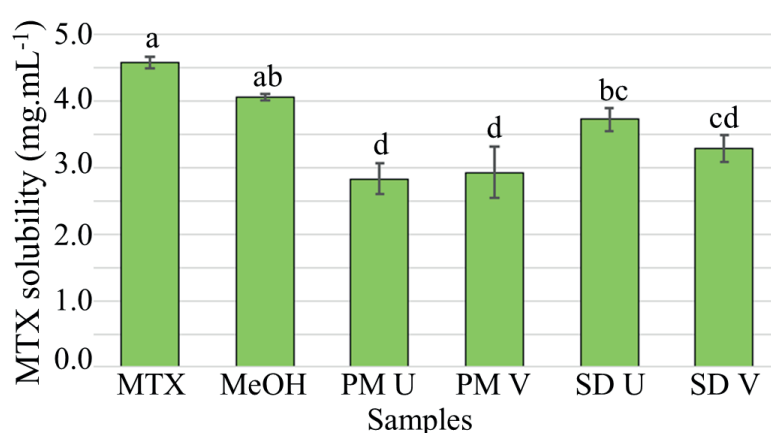


Figure 4: Solubility results.

Legend: Methotrexate (MTX) solubility in pH 6.8 phosphate buffer at 37 °C, after 60 minutes under stirring, from different samples: the original drug (MTX), sample A methanol-treated MTX (MeOH), solid dispersion U (SD U), solid dispersion V (SD V), and the respective physical mixtures U (PM U) and V (PM V). Equal letters indicate statistical equality.

DISCUSSION

This study aimed to develop MTX SDs by exploring alternative production methods that have not been previously investigated. Existing MTX-based SDs in the literature typically yield a matrix mixture of the drug and polymers. While SDs can contain the drug in crystalline form, they can also consist of a single-phase system where the drug is converted to an amorphous form in a polymer matrix, known as a solid solution (Bhujbal et al., 2021; Iyer et al., 2021; Qian et al., 2021; Saffoon et al., 2011). Previous studies on MTX-containing SDs have focused on the solubilizing effect of polymers without modifying the drug's solid state (Agafonov, Ivanov, Terekhova, 2021; Alali et al., 2023; Giri et al., 2022).

In this study, an SD was successfully prepared, with MTX adopting an amorphous state in the SD U sample. Notably, Chan and Gonda (1990) reported that the amorphous form of MTX is approximately four times more soluble than its hydrated crystalline forms. The amorphous state of MTX is typically achieved by dissolving the drug in methanol, followed by solvent evaporation, a common method used for SDs manufacturing. The solvent evaporation technique can potentially induce MTX amorphization while incorporating hydrophilic polymers, aiming to create a one-phase solid amorphous solution. Such a configuration was explored in this work to enhance MTX solubility through an additive or synergistic effect.

In this work, the PXRD results confirmed amorphization of MTX in SD U, and we demonstrated that our methodology induced amorphization in the absence or in the presence of polymers for this sample. Additionally, the observed changes in the thermal profile and FTIR spectra of the SD U, compared to those of the physical mixture and raw materials, suggest the formation of a novel system. This outcome contrasts with previous findings by Giri et al. (2022) and Agafonov, Ivanov, and Terekhova (2021), which maintained MTX in a crystalline state.

To test our hypothesis, we conducted a solubility assay. Unfortunately, the MTX solid dispersions with PEG 6000, P407, or PVP K-30 showed lower solubility in pH 6.8 phosphate buffer than the free drug. This reduction in solubility may be attributed to the interaction with polymers, which likely slowed MTX dissolution. Similar results were found by Agafonov, Ivanov, and Terekhova (2021), who also used P407 as a hydrophilic and solubilizer polymer. SD was prepared by the fusion method: the MTX and P407 were heated to 70°C, then cooled to room temperature to solidify. PXRD patterns indicate relatively good crystallinity of MTX in the SD. DSC curves showed a similarity of T_{max} for the physical mixture and solid dispersion and pointed out an absence of chemical interactions between MTX and P407. FTIR spectra of the physical mixture and solid dispersion are identical and exhibit the typical peaks of both MTX and the polymer. The influence of P407 on MTX solubility in phosphate buffer pH 6.8 was insignificant. This result had an impact on the dissolution profile, in which the apparent permeability coefficients were slightly decreased for the solid dispersions (Agafonov, Ivanov, Terekhova, 2021). Giri et al. (2022) produced SD by a solvent evaporation method, dispersing the MTX in an aqueous medium as an alternative to avoid organic solvents. A non-dissolved MTX suspension was preferred to prevent the drug's instabilities. The suspension was spray-dried, resulting

in the SDs. Hydrophilic polymers tested were sodium carboxymethyl cellulose (Na-CMC) and sodium lauryl sulfate (SLS). DSC, PXRD, and FTIR curves showed the drug was still in a crystalline structure and had not undergone amorphization. Water solubility was 2.2 mg/ml, a considerable increase compared to the free drug, which is practically insoluble in water. However, the solubility in biorelevant media was not tested.

We hypothesized that the amorphization of MTX would affect the solubility in the absence of polymers, which did not happen. Associating our results with the Agafonov, Ivanov, and Terekhova (2021) findings, we can conclude that if the MTX in the solid dispersion is in crystalline or amorphous form, it does not affect the solubility.

Previous studies have shown that free MTX exhibits a solubility of approximately 2.2 mg/mL in bio-relevant buffer at pH 7.4 (Alvarez-Figueroa, Delgado-Charro, Blanco-Méndez, 2021). In our study, the solubility of free MTX in phosphate buffer at pH 6.8 was 4.9 mg/mL. This demonstrates that a two-fold increase in MTX solubility can be achieved simply by adjusting the pH, which raises questions about the necessity of complex manufacturing processes, such as spray drying, to produce solid dispersions.

The technique employed in this study, which involves the solvent evaporation method, should be evaluated regarding the potential presence of residual solvent. According to ICH guideline Q3C(R9), methanol is classified as a Class 2 solvent, which includes solvents with low toxic potential and acceptable exposure levels that are considered safe for use in pharmaceutical products (ICH, 2021). The permissible limit of methanol in a pharmaceutical product must not exceed 3000 parts per million (or 0.3%). Therefore, complementary thermogravimetric analysis studies should be conducted to verify compliance with these limits.

Therefore, our findings associated with the prior

art can lead to the conclusion that solid dispersion cannot be the most appropriate drug delivery platform for improving MTX solubility. Neither the drug's amorphization nor traditional solid dispersion containing MTX in crystalline particles gave an inventive step in MTX solubility improvement. Not only crystallinity conformation, but the influence of different polymers, manufacturing processes, and polymers' interaction did not have a considerable synergic effect on the solubility of MTX.

This discussion highlights the need to critically assess the true applicability of solid dispersion as a platform for enhancing MTX solubility. While MTX solubility remains a substantial challenge, alternative approaches such as nanoencapsulation could offer promising solutions. By modifying the coating and particle size of nanocarriers, both active and passive transport through biological barriers may be enhanced (Austria et al., 2025; Liu et al., 2024; Yusuf et al., 2023), potentially overcoming physicochemical limitations that hinder MTX's biological efficacy.

This study provides valuable insights for pharmaceutical scientists exploring the fundamental principles of MTX-based solid dispersions. This work offers a critical comparison with the existing method by evaluating an innovative amorphous MTX solid dispersion structure composed of polymers, including P407, PVP-K30, and PEG 6000, giving a critical opinion on the application of solid dispersion for MTX.

These findings contribute to a deeper understanding of the applicability and limitations of the solid dispersion drug delivery platform for improving MTX solubility.

CONCLUSION

In this study, amorphous MTX was combined with the polymers P407, PVP K-30, and PEG 6000 to develop solid dispersions through the solvent evaporation method. DSC, FTIR, and PXRD analyses confirmed MTX

amorphization in the SD U sample. However, further characterization is necessary for the SD V sample to confirm its amorphization. While the results indicate that producing amorphous MTX solid dispersions is feasible, the data did not support the hypothesis of enhanced MTX solubility. A critical comparison with existing studies reinforces our findings, suggesting that solid dispersion may be a limited drug delivery platform for improving MTX solubility. These findings highlight the need for additional research to fully elucidate the potential and limitations of solid dispersion technology for MTX and to consider alternative approaches that may provide more effective solutions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DECLARATION OF ARTIFICIAL INTELLIGENCE USE

The authors declare that Grammarly (Superhuman Platform Inc., San Francisco, CA, USA) was used as an artificial intelligence-assisted language editing tool for English language review and proofreading during the manuscript preparation stage. ChatGPT (OpenAI, San Francisco, CA, USA) was also used to assist in reviewing and organizing the bibliographic reference lists. Following the use of these tools, all content was validated by the authors. The authors assume full responsibility for the accuracy, integrity, and final version of the manuscript submitted to BJHP.

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