Response Surface Methodology for Optimization of Process Variables of Atorvastatin Suspension Preparation by Microprecipitation Method Using Desirability Function

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Abstract

Background: Atorvastatin (AT), as a synthetic lipid-lowering agent, is a highly crystalline substance having poor solubility and low bioavailability. The objective of the present research was to improve the microprecipitation method of AT suspension preparation.

Methods: Microprecipitation parameters were improved using Box-Behnken experimental design method. The suspension was formulated with Brij 35 (stabilizer agent) using methanol as solvent and water as non-solvent, respectively. DSC, XRD, FTIR studies were performed for characterization of the microcrystals. With the aim of evaluating the effect of independent variables, the amounts of organic solvent (X1), emulsifier concentration (X2), stirring rate (X3), and volume of aqueous solvent (X4) on dependent variables, drug content (DC), particle size (PS), drug released after 5 minutes (Q5), Gibbs free energy change (ΔG°), crystal yield (CY) and saturated solubility (SS), a full factorial was used.

Results: The results of DSC, XRD, and FTIR showed that there was not any interaction between AT and Brij 35. This research demonstrated a reduction in crystallinity in agglomerates. The microcrystals showed that micromeritics characteristics were significantly improved compared to pure AT. The content of drug and yield crystal was in the limit of 42.58-110.24% and 58.33-98.18% in all formulations, respectively. It was shown that the prepared microcrystals had a higher rate of release compared to the untreated AT powder (P < 0.05). Size reduction of AT is needed for improving the solubility. Solubility and drug release rates of AT was enhanced with the microprecipitation method.

Conclusion: The results showed that microcrystals significantly increased AT dissolution rate.

Introduction

In order to overcome the problems arising from the limited solubility and dissolution rate, intrinsic modification of chemical or physical properties of drug molecules and extrinsic modification of drug formulations can be used. Many procedures have been used for improving drugs dissolution rate and bioavailability, including salt complexes, prodrug formation, changes in the solid state, and particle size reduction.

Precipitation technologies can be divided into 4 categories as follows: precipitation by addition of liquid solvent-antisolvent, precipitation with supercritical fluid, precipitation by eliminating the solvent and precipitation. Among them, precipitation by pouring liquid solvent-antisolvent is the simplest and most commonly used method. The drug compound solved in a solvent is mixed with a miscible antisolvent under stirring or sonication. The supersaturated drug in the antisolvent quickly creates a large number of nuclei, and then the nuclei grow and form the microparticles. Agitation and ultrasound are a feasible mixing method to accelerate molecular diffusion and mass transfer, which controls the nucleation and crystallization processes.

Stabilizers should be added as insolvent or anti-solvent to prevent the molecular association and crystal growth. Stabilizer shows an extremely important function in particle size decrement and stability of the suspension. Stabilizers can spontaneously adsorb on and cover the newly formed particle surface to (a) reduce the free energy of system and interfacial tension between particles; (b) form a dense hydrophilic layer around hydrophobic...
particles, provide steric hindrance and steep repulsions between the particles (steric stabilization); (c) charge the particle surface if the stabilizer has ionizable groups, which increase the repulsive force (electrostatic stabilization); (d) combine the steric and electrostatic stabilization.\(^9\)

The rate of dissolution of a drug is considered very important as it is the rate-limiting stage for the absorption of drugs after eating by mouth. This item is for several hydrophobic; particularly BCS category II drugs with very low solubility in water. Fundamentally, when the solubility volume of the highest dose is more than 250 ml, the solubility is considered low.\(^8\) Atorvastatin (AT) calcium, the calcium salt of AT, is a synthetic lipid-lowering agent. The primary uses of AT are for the treatment of dyslipidemia and prevention of cardiovascular disease. Half-life of AT is short and very good intestinal permeability. Although, the drug has low oral bioavailability (12%) owing to low solubility in water (0.1 mg/mL), hepatic first-pass metabolism and crystalline character.\(^10\)

Generally, the Noyes-Whitney and Prandtl equations are used in micro-sizing poorly water-soluble compounds. These equations explaining how the reduction decrease of size the particle causes an enhanced in the surface area and a reduce in the diffusion layer thickness and thus supply an increased dissolution rate.\(^11\) The increase in the solubility with particles of micronized is represented by the Ostwald–Freundlich, and Kelvin equations, which the size of particles and particle curvature was related with solubility.\(^12\) Therefore, in particles having a submicron size, the solubility of saturation relates not only on the drug components, the dissolution medium, and temperature but as well on the size of particle.\(^13\) Changing particle size can affect the concentration around the particles at time \(t (Ct)\). Based on the Eq. 1, and enhanced in the area of surface \((A)\) of a drug could lead to a very fast dissolution process, particularly under sink situations \((C<<Cs)\).\(^14\)

\[
\frac{dC}{dt} = DAK(C_S - C_x)/Vh \quad \text{Eq. (1)}
\]

In dispersed solid-liquid systems, decrease of the size of the particle leads to an enhancement of the rate of dissolution of a sparingly soluble substance by reducing the thickness of the diffusion layer \((h)\) around each particle.\(^15\) When \(h\) reduce, both the \(Cs\) and the gradient of concentration \((Cs-Ct)/h)\) will enhance and as a result, the rate of dissolution will enhance.\(^16\)

The aim of this research was to perform a statistical analyze on the formulation of AT suspension by precipitation method. In this regard, to provide polynomial equations and construct surface and contour plots; a 4-factor with 3-level Box-Behnken design using Design-Expert 10.0.7. was applied by desirability function.

Materials and Methods

AT calcium was obtained from Darou Pakhsh Company, Iran. Hydroxypropyl Methyl Cellulose (HPMC, K 4M), Brij 35, Tween 80, dichloromethane, acetone, isopropyl alcohol, methanol, sodium hydroxide and potassium phosphate, monobasic used in this study were from Merck company (Darmstadt, Germany).

Experimental design

The experimental design applied in the present research was a modified Box-Behnken design for 4 variables in 3 levels by Design-Expert.\(^17\) This design was regarded as a suitable method for researching quadratic response surfaces and constructing second-order polynomial models. In the study, 4 independent formulation variables were examined, including dispersing medium organic solvent volume \((X1)\), stabilizer \((X2)\), the rate of stirring \((X3)\) and non-solvent volume \((X4)\). The dependent variables investigated were the size of particle \((PS, Y_1)\), content of drug \((DC, Y_2)\), drug released after 15 minute \((Q_{15}, Y_3)\), crystal yield \((CY, Y_4)\), Gibbs free energy change \((\Delta G^*, Y_5)\), and saturated solubility \((SS, Y_6)\). The design had 25 experimental points, which included 3 replications. Data were evaluated to match the polynomial equation to \(Y\).\(^17\)

The response surface methodology (RSM) is a technique that permits to identify factors included in a procedure and evaluates their relative importance. Moreover, any interaction between the factors may be recognized. Construction of an RSM design includes the choice of factors and the selection of responses.

In statistics, stepwise regression is a manner of matching regression models in which the selection of prediction factors is performed by an automatic procedure. In each stage, a variable is attended for adding to or subtraction from the set of descriptive variables located several predetermine scale. The stepwise regression includes of repeatedly increasing and eliminating predictors, in the predictive model with the aim to the subset of variables in the data set leading to the best carrying out model that is a model that reduces prediction error. Moreover, any interaction between the variables may be recognized.\(^17\)

Solubility study

To determine the saturation solubility, the drug was added to a solvent to make a saturated solution (suspension). AT powder was added to 10 ml of different solvents like ethanol, propylene glycol, acetone, methanol, dichloromethane and water, and the samples were stirred in an incubator shaker at 25°C for 48 hours. Then, the content of each sample was removed by a 0.45 µm nylon filter. The filtrates were diluted with the same solvent and determined spectrophotometrically using UV spectrophotometer (UV- 160, Shimadzu) at a maximum wavelength of the drug in each solvent.\(^18\)

Preparation of microcrystals

After the solubility test, methanol was selected as a good solvent for AT because of its high solubility and volatility (Table 1). 500 mg of AT is dissolved in 5 to 25 ml of methanol (as solvent) and it was quickly poured into 50 to 150 ml of water (as non-solvent) consisted of
Preparation of Atorvastatin Suspension by Microprecipitation Method

Characterization of AT particles

Drug content assay

Twenty milligrams of each formulation of microcrystal was weighed and poured to a 10 ml flask, then dissolved in 10 ml methanol, and the mixture was stirred. Then, after proper dilution, the amount of AT was measured by UV spectrophotometer at 246 nm.¹⁹

The crystal yield of particles

The percentage of crystal yield is the ratio of the amount of micromized powder (A) from each of the formulations to the total amount of dispersed solids in the dispersion phase (B) according to the following equation:²⁰

\[
\text{Crystal Yield} = \left( \frac{A}{B} \right) \times 100
\]

Determination of solubility of micromized particles

The saturation solubility of microsized AT formulations and untreated AT powder were assayed, by adding an extra amount of each microcrystal to 10 ml of distilled water into the flask for a period of 48 hours at a constant temperature of 37°C. The amount of each flask was filtered through 0.45 µm nylon filter and the filtrate was next diluted with purified water and analyzed spectrophotometrically using UV Spectrophotometer.²¹

Table 1. The results of the solubility of AT in different solvents.

| Solvent        | Solubility (mg/ml±SD) |
|----------------|------------------------|
| Dichloromethane| 1.18±0.004             |
| Isopropyl Alcohol| 1.69±0.009             |
| Methanol       | 0.91±0.003             |
| Ethanol        | 0.43±0.005             |
| Water          | 0.26±0.007             |

Table 2. The variables and values used for the design of experiments of prepared AT formulations.

| Formulation code | Variable levels in the coded form | PS (µm) | DC (%) | Q₅ (%) | CY (%) | ΔGₛ (J/mol) | Sₛ (mg/ml) |
|------------------|----------------------------------|---------|--------|--------|--------|-------------|------------|
| F1               | X₁ 25, X₂ 0.1, X₃ 14000, X₄ 100| 3.026   | 72.15  | 107    | 69.09  | -15155.3    | 12.87      |
| F2               | X₁ 25, X₂ 0.2, X₃ 20000, X₄ 100| 22.28   | 105.13 | 102.99 | 74.54  | -16494.8    | 21.64      |
| F3               | X₁ 15, X₂ 0.1, X₃ 26000, X₄ 50 | 15.37   | 104.62 | 62.09  | 73.33  | -15714.9    | 15.99      |
| F4               | X₁ 25, X₂ 0.1, X₃ 26000, X₄ 100| 33.56   | 84.57  | 81.13  | 61.66  | -17130.3    | 27.69      |
| F5               | X₁ 15, X₂ 0.1, X₃ 14000, X₄ 100| 14.17   | 110.24 | 92.04  | 78.18  | -14835.9    | 11.37      |
| F6               | X₁ 15, X₂ 0.1, X₃ 20000, X₄ 100| 9.89    | 88.48  | 111.42 | 98.18  | -15816      | 16.63      |
| F7               | X₁ 15, X₂ 0.2, X₃ 26000, X₄ 100| 22.10   | 107.32 | 64.73  | 76.36  | -16748.8    | 23.88      |
| F8               | X₁ 5, X₂ 0.05, X₃ 20000, X₄ 100| 42.46   | 110.23 | 77.48  | 78.46  | -16416.2    | 20.99      |
| F9               | X₁ 25, X₂ 0.1, X₃ 20000, X₄ 100| 11.90   | 106.24 | 63.12  | 74.54  | -18194.4    | 41.84      |
| F10              | X₁ 5, X₂ 0.1, X₃ 20000, X₄ 150 | 15.52   | 63.17  | 101.76 | 76.36  | -15719.7    | 16.02      |
| F11              | X₁ 5, X₂ 0.1, X₃ 26000, X₄ 100 | 13.79   | 103.03 | 100.92 | 87.27  | -15560.4    | 15.06      |
| F12              | X₁ 5, X₂ 0.2, X₃ 20000, X₄ 100 | 14.90   | 54.32  | 99.84  | 83.63  | -15314.5    | 13.69      |
| F13              | X₁ 5, X₂ 0.1, X₃ 20000, X₄ 50  | 8.28    | 42.54  | 96.34  | 86.54  | -15080.1    | 12.50      |
| F14              | X₁ 15, X₂ 0.1, X₃ 26000, X₄ 150| 18.96   | 86.90  | 96.49  | 63.38  | -16819      | 24.54      |
| F15              | X₁ 15, X₂ 0.2, X₃ 14000, X₄ 100| 23.06   | 107.02 | 98.63  | 83.80  | -15408.8    | 14.20      |
| F16              | X₁ 15, X₂ 0.2, X₃ 20000, X₄ 50  | 20.79   | 102.62 | 95.21  | 58.33  | -14785.5    | 11.15      |
| F17              | X₁ 15, X₂ 0.2, X₃ 26000, X₄ 150| 6.082   | 107.73 | 97.84  | 74.54  | -14502.3    | 9.99       |
| F18              | X₁ 15, X₂ 0.1, X₃ 14000, X₄ 150| 16.72   | 79.56  | 78.92  | 63.36  | -14994.1    | 12.09      |
| F19              | X₁ 25, X₂ 0.1, X₃ 20000, X₄ 100| 6.082   | 87.63  | 100.97 | 78.18  | -14748.2    | 10.99      |
| F20              | X₁ 15, X₂ 0.05, X₃ 14000, X₄ 100| 0.55    | 62.71  | 100.92 | 63.63  | -15907.4    | 17.23      |
| F21              | X₁ 25, X₂ 0.05, X₃ 20000, X₄ 100| 1.12    | 90.37  | 101.14 | 68.57  | -16746.6    | 23.86      |
| F22              | X₁ 15, X₂ 0.05, X₃ 20000, X₄ 150| 0.60    | 105.08 | 61.90  | 65     | -17162.7    | 28.04      |
| F23              | X₁ 15, X₂ 0.05, X₃ 26000, X₄ 100| 0.59    | 110.02 | 101.41 | 72.38  | -14623.3    | 10.47      |
| F24              | X₁ 5, X₂ 0.1, X₃ 14000, X₄ 100 | 0.58    | 78.65  | 98.39  | 65     | -14992      | 12.08      |
| F25              | X₁ 15, X₂ 0.05, X₃ 20000, X₄ 50 | 0.56    | 106.43 | 74.96  | 81.81  | -15210.8    | 13.15      |

*Volume of organic solvent; *Emulsifier concentration; *RPM of homogenizer; *Dispersing medium (volume of aqueous solvent); *Particle size; *Drug content; *Amount of drug release after 5 minutes; *Crystal yield; *Gibbs free energy change; *Saturated solubility

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Calculation of Gibbs free energy of particles
Gibbs free energy (J mol⁻¹) was calculated as the solubility of microcrystals to the solubility of pure AT powder based on the below equation:²²

\[ G^o_r = -2.303RT \log S_s / S_r \]  

Eq. (3)

Where \( G^o_r \) is the solubility of the microcrystal in water and \( S_s \) is the solubility of pure AT powder in water, \( R = 8.31 \text{ J k}^{-1}\text{mol}^{-1} \) and \( T = 310.15^\circ\text{C} \).

Optical photography of microcrystals
Using a digital camera (12.1 MP), images were taken from the samples with appropriate magnification.

Determination of microcrystals particle size
A laser particle size analyzer was used to measure the sizes of particles. The suspensions were dried via lyophilization and the obtained micronized particles (after doing an ultrasound) were used to measure.

Micromeritics study
The ratio of the mass of the particle given volume, with respect to the porosity of the particles, is called the apparent (bulk) density which is calculated according to Eq. 4:²³

\[ \text{Apparent density (mg/cm}^3\text{)} = \frac{\text{Mass of particles (mg)}}{\text{Apparent volume of the particles (cm}^3\text{)}} \]  

Eq. (4)

Apparent and tapped densities were as well as measured using a 10 ml graded cylinder. The microcrystals were placed to the cylinder and tapped mechanically for 200 times. Next, the tapped volume was recorded, and as a result, the tapped and apparent densities were determined. Compressibility index (Ci) or Carr’s index value of particles was estimated based on the below equation:²⁴

\[ \text{Carr's index} \% = \left( \frac{\text{tapped density} - \text{bulk density}}{\text{tapped density}} \right) \times 100 / \text{tapped density} \]  

Eq. (5)

Hausner’s ratio of microcrystals was measured by dividing the tapped density to the bulk density using the following equation:²⁵

\[ \text{Hausner’s ratio} = \frac{\text{Tapped density}}{\text{Bulk densit}} \]  

Eq. (6)

The angle of repose for different microcrystals was measured in accordance with stabilized funnel standing method.²⁶ A conical pile was made when microcrystals were added onto a horizontal surface. The angle of repose was calculated according to Eq. 7, the internal angle between the surface of the pile and the horizontal surface was determined as the angle of repose.

\[ \theta = \tan^{-1}h / r \]  

Eq. (7)

Where \( \theta \) is the repose angle, \( r \) is the radius, and \( h \) is the height of the pile.

X-ray powder diffractometry (X-RPD)
XRD was carried out at room temperature using an instrument (Siemens D5000, Munich, Germany), using nickel Cuka radiation (a voltage of 40 KV and a current of 20 mA). Samples were scanned 2°/min over a limit of 20-70° and with 0.02° Θ.

Fourier transforms infrared spectroscopy (FT-IR)
Fourier Transform Infrared Spectroscopy (FTIR) analysis of the pure AT, Brij 35, microcrystals, and the physical mixture was performed by an automatized apparatus (Bruker, Tensor 27, USA). The range of scanning was 400 to 4000 cm⁻¹ and the resolution was 1 cm⁻¹.

Differential scanning calorimetry (DSC)
A differential scanning calorimeter (DSC 60, Shimadzu, Japan) was applied to monitor the thermal incident during heating. The samples (2 mg) were placed into the aluminum pan and heated from 30 to 300°C at a rate of heating of 10°C per min.

In vitro release studies
The in vitro release investigations of AT loaded microcrystals were done at 37°C in 900 mL of phosphate buffer, pH=6.8, consisted of 0.2% (w/v) Tween 80.²⁷ 100 mg of each batch of microcrystals were filled into capsule No.0 and each sample was placed to 900 mL of medium of dissolution in the flask. The media of dissolution was shaken at 75 RPM based on the USP paddle manner. Five mL of formulations were removed at regular time intervals and then was replaced with a similar volume of fresh medium. Next appropriate dilution, the content of drug each sample was measured using a UV spectrophotometer assay, respectively. It should be noted that each test was repeated 3 times.

Results and Discussion
Prerequisite tools needed for Expert-Design included statistical analysis by ANOVA, diagnostic analysis and response surface analysis for the expectation to observe how 4 factors affect responses and their interactions. Full and reduced mathematical models were derived for each response. The important factors in the equations were chosen with a stepwise (forward and backward) deletion for the computes of regression analysis. According to the probability function, insignificant terms (significance level = 5%, \( P > 0.05 \)) were excluded to derive reduced models. Reduced equations derived for each independent variable are shown in Table 3. Correlation coefficient determined the amount of variation about the mean. Adequate precision assessed the limit of predicted values at design points to the mean prediction error and determined signal to noise ratio. An acceptable ratio was greater than 4.

One of the ways of micronization is dissolving the drug (water-insoluble) in the solvent (methanol) and adding non-solvent (water) with a stabilizer to the drug solution.
by mixing at high rates. The insoluble drug dissolves in an organic solvent, and the stabilizing agent prevents the accumulation of particles, and then, under homogenizer, the non-solvent is rapidly poured into the drug solution, and then the prepared suspension is dried by lyophilization (Figure 1).

**Saturated solubility**

Solubility is measured by the equilibrium of intermolecular forces between the solute and solvent, which produces the solution that is accompanied by entropy change. Untreated AT applied in the research was determined to have low solubility. Microcrystals of AT were prepared by microprecipitation method. Pouring the organic phase to the aqueous phase lead to precipitation of AT particles. Production of microcrystals refers to the formation of an additional surface area and interfaces. Solubility results showed that the crystals that were prepared using Brij 35 has shown the highest solubility of the drug in water (9.99-41.84 mg/ml) as compared with the untreated drug (0.036 mg/ml) (Table 2).

An increment in the amount of emulsifier and non-solvent decreased the saturated solubility, whereas an increase in the concentration of solvent and RPM of homogenizer created an increase in saturated solubility since the coefficient X1 and X3 bears a positive sign. The increment in methanol and the rate of stirring outcomes in enhanced formulations solved amounts (Table 2 and 3).

Influence of individual terms in the above equations can be depicted by their corresponding coefficients. The negative sign of all the coefficients of factors as X2 and X4 (individually) represented their inverse influence on all the responses, while, the interaction between factors (as X2X3) showed high coefficient (+4.11) compared to other items. The amount of the solvent and rate of stirring of formulations were the effective factors in the saturated solubility ($R^2=92.82$). Stabilizers that were applied in the preparation of microcrystals stabilized these particles and prevented their growth. Microprecipitation manner for preparation of microcrystals was obtained to be efficient (Figure 2A).

The model F-value of 3.31 suggests the model is significant.

Table 3. Equations of the response surface regression model.

| Term           | Equation of regression coefficients                                                                 |
|----------------|-------------------------------------------------------------------------------------------------------|
| PS versus X1, X2 | PS = 12.92-1.46X1 +5.28X2+12.18X1X2                                                                 |
| DC versus X1, X2 | DC = 98.36+ 7.85X1-0.058X2+17.67X1X2-15.19X1X1                                                      |
| Q5 versus X2, X3, X4 | Q5= 98.49+10.05X2-5.76X3+4.51X4-8.60X2X3+11.88X3X4-4.29X3X4-11.92X4X4-19.79X2X3X3                 |
| CY versus X1, X2, X3, X4 | CY = 98.18-4.22X1+1.76X2+1.33X3-2.22X4+8.26X2X4-9.58X1X1-12.42X2X2-14.72X3X3-12.72X4X4          |
| $\Delta G^{tr}$ versus X1, X3, X4 | $\Delta G_{tr}^=$=15783.28-448.85X1-441.93X3-10.37X4+10.21X1X4                                   |
| Ss versus X1, X2, X3, X4 | Ss = 17.52+4.05X1-1.60X2+3.15X3-0.36X4-8.59X1X4+4.11X2X3                                   |

Figure 1. Optical microscopic photograph of crystals of AT.
There was only a 2.24% chance that an this large F-value might happen owing to noise. Values of “Prob> F” less than 0.05 showed model terms were significant. “Adeq Precision” determined the signal to noise ratio. This ratio of 7.989> 4 indicated an adequate signal.

**Drug content**

The content of drug was obtained to be good and uniform among the various samples of the prepared crystals (higher than 100%) (Table 2). The drug content (DC) in the samples had a direct relationship with the volumes of solvent and indirect relationship with the emulsifier concentration (the sign of the coefficients of the independent variables is positive and negative, respectively). That meant, the DC of the outcoming crystals was dependent on an independent variable with a regression coefficient of 76.52 and had a direct correlation with the interaction between factors X1 and X2 (Table 3 and 4).

The model F-value of 2.96 indicated the model was significant. There was only a 4.52% chance that an F-value this large might happen owing to noise. Values of “Prob> F” less than 0.05, exhibited model terms were significant. “Adeq Precision” assessed the signal to noise ratio. This ratio of 6.704>4 indicated an adequate signal (Table 4). The drug content was higher than 100% in all formulations (F2, F3, F5, F7, F8, F9, F11, F13, F16, F17, F22, F23, and F25).
Crystal yield

Obtained data from the crystal yield of the selected samples were displayed in Table 2. The amount of the drug in the formulations was determined to be in the range of 58.33-98.18%. According to the coefficients written in Table 3 and 4, the crystal yield in the formulation was negatively correlated to the amount of solvent and non-solvent medium and positively correlated to the emulsifier concentration and rate of the homogenizer. All of the variables were the most effective factors in the crystal yield ($R^2 = 87.95$). The production of crystal particles in the F6 formulation was the highest among the other formulations (98.18%) (Figure 2B).

The model F-value of 2.73 indicated the model was significant. There was only a 4.15% chance that an F-value this large might happen owing to noise. Values of “Prob>F” less than 0.05 showed model terms were significant. “Adeq Precision” determines the signal to noise ratio. This ratio of 8.127>4 indicated an adequate signal (Table 4).

The most common method for reducing the size of hydrophobic particles is micronization. In this way, small particles are produced by the formation of irregularities in large crystals. Chanmeil et al. reported an improvement in the solubility and bioavailability of insoluble and poorly soluble drugs by micronization with a jet mill and fluid energy mill. The in situ micronization method is the appropriate method for producing microsized drugs since they have a more uniform particle size distribution and lower cohesive in comparison with the grinding drugs. Rasenak and Müller observed that in situ micronization with the aid of a stabilizing polymer (such as Bridge 35) covers the hydrophobic surfaces of the drug and prevents the crystalline growth of the drug. The “Top-down” and “bottom-up” methods are the existing methods to prepare microcrystals. In the “bottom-up” methods, the molecules are solved and precipitated by adding the solvent to a non-solvent, such as spray-drying, supercritical fluid (SCF) method, spray-freezing into the liquid process, evaporative precipitation into an aqueous solution (EPAS) and solvent change process. A useful way to prepare microsized particles of drug is the anti-solvent method. As a rapid and direct process, this technique can be done easily. Rasenack et al successfully prepared micronized drug particles such as beclomethasone-17,21-dipropionate and betamethasone-17-valerate with the pouring of HPMC by a solvent change process. But they failed in the preparation of prednisolone.

Gibbs free energy

The Gibbs free energy of the formulations was decreased with increasing the amount of solvent, rate of stirring and non-solvent medium of the independent variables (Table 3). The mounts of solvent (X1), RPM of homogenizer(X2), and non-solvent medium (X4) individually and interaction between factors X1X4 were the most effective factor in the Gibbs free energy ($R^2 = 85.91$) (Table 4).

The model F-value of 3.37 indicated the model is significant. There was only a 2.92% chance that an F-value this large might happen owing to noise. Values of “Prob>F” less than 0.05 showed model terms were significant. “Adeq Precision” determined the signal to noise ratio. This ratio of 8.071>4 indicated an adequate signal (Table 4).

As the Gibbs free energy change, depending on the creation of the additional interface, was negative, the microcrystals made were thermodynamically unstable and would tend to decrease their total energy by agglomeration. Gibbs free energy change was negative for all formulations utilized at different concentrations displaying the spontaneous nature of drug solubilization.

Particle size

The disorder in the crystalline network can cause physical and chemical instability. Micronized powders exhibited high-energy particles, weak flowing properties, and large particle distribution. Reducing the size of particle in the form of adsorption of stabilizers on the particle surface inhibits the crystalline growth of micronized particles and changes the morphology of crystals. The moisture content of the powder can be increased by adsorption of hydrophilic agents (as a stabilizer). Therefore, particle precipitation in the presence of stabilizing agents can have a positive effect on dissolution rate.

\[
\frac{dm}{dt} = \frac{DA(C_s - C)}{h}
\]

In accordance with Eq. 8, the solubility of the drug increases by decreasing the particle size. Because, when the particle size decreases, the surface area of particles enhances, leading to greater dissolution rate. Moreover, according to the Ostwald-Freundlich equation the solubility of drug particles is related to the decrease of the size of particle. In this equation, $\ln S_s/S_u$ is the molar solubility ratio of the formulations in water to the solubility of untreated drug in water, $\gamma$ is the surface tension, $M$ is the amount of soluble mass, $R = 8.31 \text{ JK}^{-1}\text{ mol}^{-1}$ is the gas constant, $T$ is absolute temperature, $\rho$ is the density of the particles and $r$ is the radius of the particles.

\[
\ln \frac{S_u}{S_s} = \frac{2\gamma M}{RT \rho r}
\]

According to the Eq. 9, decreasing particle size results in the increase of solubility of microcrystals. In the
microprecipitation method, displacement of two solvents (good solvent and non-solvent) is performed immediately in the presence of stabilizer agents. In F1, F17, F19, F20, F21, F22, F23, F24, and F25 formulations, disorders, and decreases of particle size in the crystals prepared from the drug and the hydrophilic stabilizing agent were observed. Stabilizer (as Brij 35) coats the surfaces of the hydrophobic drug (At) and prevents the crystalline growth of the particles.

The particle size (PS) of formulations has an indirect relationship with the amounts of solvent (the sign of the coefficients of the independent variables is negative) and direct relationship with the emulsifier concentration (the sign of the coefficients of the independent variables is positive). Stabilizer had a significant effect on the particle size of crystals prepared with high coefficient (+5.28) compared to other items (Figure 2C). In other words, the PS of crystals is dependent on an independent variable with a regression coefficient of 89.44 (Table 3 and 4). The F-value of 3.56 showed the model was significant. There was only a 3.17% chance that an F-value this large might happen due to owing to noise. Values of "Prob>F" less than 0.05 exhibited model terms were significant. "Adeq Precision" determined the signal to noise ratio. This ratio of 9.235 > 4 indicated an adequate signal (Table 4). The mean particle size of the pure drug (volume diameter) is 6.96 ± 0.49 μm. The particle size of formulations (0.56-33.56 μm) had changed in comparison with the pure drug.

Flowability characteristics of formulations

The effect of variables on the flowability characteristics of microcrystals and physicochemical characteristics of samples are displayed in Table 5. Bulk density was found to be between 0.01±0.00 and 0.44 ±0.03 g/cm³ and tapped density between 0.01±0.01 and 0.58±0.02 g/cm³ for all samples. From density data, Carr’s index was estimated and was showed to be between 20.90±0.00 and 100 ±0.78 %. The angle of repose was indicated to be in the limit of 5.02±0.11 to 43.96±2.31.

A Hausner ratio of less than 1.20 is representative of good flowability of the material, whilst the value of 1.5 or higher shows poor flow of the material and exceptionally few formulations cross the delimits of Hausner ratio.

The Hausner ratio and Carr index is applied to assess the flow characteristics of powders. The Hausner ratio and Carr’s index values for formulations suggest the good flow properties for formulation were prepared (Table 5). In the F2 formulation (with 15 ml acetone, 0.05% (%w/w) Brij 35, 100 ml water and 20,000 RPM rate of homogenizer) and F12 formulation (with 15 ml acetone, 0.1 (%w/w) brij 35, 150 ml water, and 20,000 RPM rate of homogenizer) showed the best results for flowability.

The F2 and F12 formulations showed Carr’s index of 21.78% and 22.85%, Hausner’s ratio of 1.22 and 1.29 and F1, F17 and F19 formulations in comparison with the pure drug were insignificant (P>0.05) and in F20, F21, F22, F23, F24, and F25, was significant (P<0.05).

| Formulation code | Bulk density (g/cm³± SD) | Tapped density (g/cm³ ± SD) | Carr’s index (%±SD) | Hausner ratio (°θ ± SD) | Angle of repose (°± SD) |
|------------------|---------------------------|-----------------------------|---------------------|-------------------------|------------------------|
| F1               | 0.01±0.00                 | 0.014±0.01                 | 37.85±0.01          | 1.60±0.00               | 8.53±0.20              |
| F2               | 0.073±0.02                | 0.089±0.02                 | 21.78±0.02          | 1.22±0.00               | 27.75±0.51             |
| F3               | 0.009±0.01                | 0.017±0.01                 | 47.05±0.01          | 1.88±0.00               | 15.1±0.50              |
| F4               | 0.37±0.02                 | 0.74±0.02                  | 100.00±0.78         | 2.00±0.00               | 33.66±0.33             |
| F5               | 0.041±0.00                | 0.064±0.00                 | 35.93±0.25          | 1.56±0.00               | 36.36±0.12             |
| F6               | 0.029±0.01                | 0.04±0.01                  | 27.50±0.00          | 1.37±0.00               | 15.10±0.45             |
| F7               | 0.115±0.12                | 0.158±0.11                 | 36.86±0.11          | 1.37±0.01               | 17.52±0.32             |
| F8               | 0.011±0.00                | 0.019±0.00                 | 42.10±0.01          | 1.72±0.01               | 8.92±0.21              |
| F9               | 0.045±0.00                | 0.077±0.00                 | 41.55±0.02          | 1.71±0.02               | 33.68±0.10             |
| F10              | 0.07±0.00                 | 0.11±0.00                  | 35.00±0.15          | 1.50±0.00               | 13.59±0.35             |
| F11              | 0.08±0.02                 | 0.12±0.05                  | 50.00±0.12          | 1.50±0.00               | 30.96±3.20             |
| F12              | 0.054±0.00                | 0.07±0.00                  | 22.85±0.03          | 1.29±0.00               | 31.75±0.12             |
| F13              | 0.028±0.00                | 0.052±0.00                 | 46.15±0.02          | 1.85±0.01               | 9.59±0.21              |
| F14              | 0.013±0.01                | 0.028±0.01                 | 53.57±0.01          | 2.15±0.00               | 5.02±0.11              |
| F15              | 0.076±0.01                | 0.110±0.01                 | 45.11±0.03          | 1.45±0.00               | 32.12±0.12             |
| F16              | 0.10±0.02                 | 0.14±0.00                  | 40.00±0.03          | 1.40±0.00               | 37.56±0.23             |
| F17              | 0.315±0.03                | 0.41±0.00                  | 30.03±0.05          | 1.30±0.00               | 14.89±0.12             |
| F18              | 0.043±0.02                | 0.065±0.02                 | 52.11±0.10          | 1.52±0.02               | 23.46±1.52             |
| F19              | 0.148±0.01                | 0.215±0.01                 | 45.07±0.11          | 1.45±0.01               | 18.92±0.87             |
| F20              | 0.438±0.03                | 0.583±0.02                 | 33.32±0.55          | 1.33±0.01               | 6.51±0.00              |
| F21              | 0.015±0.01                | 0.022±0.00                 | 50.68±2.31          | 1.50±0.00               | 24.44±0.00             |
| F22              | 0.023±0.00                | 0.047±0.01                 | 51.06±0.51          | 2.04±0.01               | 11.85±0.23             |
| F23              | 0.037±0.00                | 0.052±0.00                 | 28.65±0.12          | 1.40±0.01               | 43.96±2.31             |
| F24              | 0.042±0.00                | 0.063±0.02                 | 33.33±2.35          | 1.50±0.00               | 8.70±0.23              |
| F25              | 0.025±0.00                | 0.037±0.01                 | 32.43±0.01          | 1.45±0.01               | 12.95±0.33             |
| Untreated AT powder | 0.025±0.00                | 0.037±0.01                 | 20.9±0.00          | 0.93±0.00               | 36.86±0.00             |
Preparation of Atorvastatin Suspension by Microprecipitation Method

The results of formulations showed that the repose angle was smaller than 20°, the Carr's index was smaller than 25%, and Hausner's ratio is smaller than 1.25 that showed good flowability for selected particles. In F2 and F12 formulations, organic solvent (methanol, 15 ml), homogenizer with 20,000 RPM, amount of stabilizer (Brij 35, 0.05-0.1 %w/w) and anti-solvent (water, 150 ml) were used. The repose angle improved the flowability of the micronized particles of AT compared with untreated AT powder due to changes in the crystalline network arrangement. The size of the micronized particles was smaller than the pure powder of the drug (6.96±0.40 µm) because the surface of the hydrophobic particles was coated with a stabilizing agent such as Brij 35, the resulting micronized particles were produced with more contact surfaces, less cohesion between particles and more free-flowing compared with pure AT powder.

**DSC**

The AT pure indicated a relatively sharp endometrial peak of 156.48°C, showing the crystalline nature of the drug. The Brij 35 displayed a melting endothermic peak at 41.89°C (Figure 3). In the F2 and F9 formulations, the very wide melting peak of the drug was observed at 135.95 and 142.28°C, respectively. The results showed that the melting peak in micronized formulations was shifted to lower temperatures with low intensity. In other words, the crystalline state of the drug in micronized formulations was stabilized.

**XRD**

X-Ray diffraction studies were used to determine the presence and absence of the drug and polymer by considering some indicative peak heights in the diffraction of the formulations with the pure drug. AT is a crystalline drug. The pure drug had sharp peaks in the regions of 8°, 9.3° and 20° (Figure 4). These were not observed in F2 and F9 formulations and the intensity of peak decreased, which indicated a reduction in crystallinity of the drug.

**FTIR**

The IR spectrum of pure AT (Figure 5) exhibited property peaks at 2955.15 cm⁻¹ (C–H– stretching), 1313.56 cm⁻¹ (C–N– stretching), 3059.15 cm⁻¹ (C–O–H– stretching alcoholic group), 1564.97 cm⁻¹ (C=O– stretching amidic group), 3403.27 cm⁻¹ (N–H – stretching), 1656.97 cm⁻¹ (C=C– stretching), 751.62 cm⁻¹, 696.95 cm⁻¹ (C–F– stretching), 1104.39 cm⁻¹ (O–H bending). It might be the feasibility of intermolecular hydrogen bonding between molecules.

Brij 35 spectra (Figure 5) showed a wide peak at 3414.46 cm⁻¹ indicating bound water, peak -C=CH₃ stretch at 2882.40 cm⁻¹, carbonyl bands, 1965 cm⁻¹, and the strong Brij 35 absorption peak of a C–O stretch at 1101.33 cm⁻¹. The spectra of pure AT was similar to the spectrum resulted in the addition of stabilizer (with a slight shift). This indicated that there was no interaction between drug and stabilizer.

**Release**

The dissolution profiles of the formulations and the untreated drug are shown in Figure 6. All of the formulations prepared with surfactant (Brij 35) illustrated the faster rate of dissolution, with almost more than 90-100% of the drug being released within 5 minutes considered to 31% for the pure drug (without surfactant). This effect can be illustrated by an enhanced specific surface area which was hydrophilized owing to the adsorbed hydrophilic polymers. Surfactants applied in the process have been shown to reduce the aggregation tendencies of particles compared to pure drug. According to Table 6, in all of the formulations, the burst release was observed in the initial release, which was due to the adsorption of the stabilizing agent (Brij 35) on the surface of particles. Three phenomena could contribute to an increase in the diffusion of the dispersed drug in the hydrophilic phase: increasing the drug dissolution owing to the decrease of
size of particle and wetting of the particles increases the water permeability in the drug. In formulations, fast release (in 5 minutes) could be result from the increased water permeability at hydrophilic surfaces of micronized particles of AT. The dissolution rate and diffusion of the drug from the level of the stabilizing polymer (precipitation the drug) determine the drug release pattern.

Also, the high release rate in micronized particles may be associated with increasing hydrophilic Brij channels on the surface of the crystals or increasing the concentration gradient in the formulations and dissolution medium. The rate of drug release within one hour for F7, F8, F9, F22, and F25 formulations was 71.12%, 80.9%, 74.94%, 65.77%, and 77.75%, respectively, and for other formulations was approximately 100%.

Figure 4. XRD patterns of a) Brij 35, b) At, c) F2, and d) F9 formulations.

Figure 5. FTIR spectra of a) At, b) F2, and c) F9 and d) Brij 35.
According to Table 6 and Figure 6, the released drug in the fifth minute of the micronized AT formulations was compared with the pure (untreated) powder (P<0.05). The release rate of hydrophilic micronized particles is more than that of the hydrophobic particles of AT (P<0.05). On the other hand, the dissolution efficiency of the micronized formulation (≈100%) was higher than pure powder (47.44%). All of the prepared formulations had no similarity with pure powder (f1>15). Q5 of all formulations was obtained to be in the range of 62.09-111.42% (Table 2). Q5 in the samples has an only inverse relation with the rate of stirring and has a direct relationship with emulsifier concentration and non-solvent medium and interaction between factors X2X3, X3X4, X3X3, X4X4, and X2X3X3 (Table 3 and 4). Where an increase in X2 (emulsifier) and X4 (non-solvent) increased the Q5, but the interaction of X2X4, leads to a decrease of Q5 (except factor X3X3) (Figure 2D). The F-value of 3.39 suggested the model was significant. There was only a 1.81% chance that an F-value this large might be found owing to noise. Values of “Prob>F” less than 0.0500 show model terms were significant. “Adeq Precision” determined the signal to noise ratio. This ratio of 7.086 > 4 indicated an adequate signal (Table 4).

Conclusion
Increasing the dissolution rate of micronized particles caused a reduction in the size of particle size, change in crystalline behavior, the producing of hydrophilic levels, and increased the wettability of the drug during micronization. Based on the Gibbs free energy changes for micronized AT formulations, the negative values (smaller amounts than Gibbss free energy of pure AT powder) indicated a higher solubility of the prepared formulations. The results of FTIR, XRD, and DSC exhibited no chemical interactions between the drug and the stabilizing agent. The peak of the AT was reduced in the micronized formulation, indicating decreased crystallinity of the drug. In general, it can be concluded that the in situ microprecipitation technique by solvent change was an effective tool for microcrystals formation by improving the pharmacological properties of the drug for increasing the dissolution rate of a low-solubility drug such as AT. In this study, Box-Behnken design was applied for pharmaceutical development of AT powder, containing hydrophilic surfactants as Brij 35, reported to be solubility enhancers and crystallization inhibitors for the development of AT microcrystals. In the optimized AT microcrystal, they enhanced the dissolution rate and inhibit crystallization of AT. The derived polynomial equation and plots help in predicting the values of chose independent variables for preparation of optimum AT microcrystal samples with suitable characteristics (P>0.05).

Figure 6. Cumulative percentage release of At from formulations prepared and pure At powder.
Table 6. The values of release parameters of different prepared formulations.

| Formulation code | Rel50a (%)±SD | Rel10b (%)±SD | Rel50c (%)±SD | DE (%) | MDTd min | ηe |
|------------------|---------------|---------------|---------------|--------|---------|----|
| F1               | 0.49±0.107    | 8.41±10.722   | 107.25±8.58   | 102.77 | 2.51    | 15<|
| F2               | 102.99±3.84   | 2.57±10.33    | 103.41±6.91   | 99     | 2.56    | 15<|
| F3               | 15.31±2.09    | 13.37±6.23    | 12.19±4.66    | 61.86  | 8.59    | 15<|
| F4               | 81.13±0.53    | 96.99±1.44    | 103.80±1.26   | 94.19  | 5.55    | 15<|
| F5               | 92.04±13.79   | 99.72±12.94   | 102.05±9.31   | 94.15  | 4.64    | 15<|
| F6               | 111.42±11.39  | 110.89±17.00  | 110.97±16.23  | 106.18 | 2.59    | 15<|
| F7               | 64.73±2.48    | 68.50±7.18    | 71.12±6.93    | 66.49  | 3.91    | 15<|
| F8               | 77.48±5.59    | 80.78±0.19    | 80.90±2.16    | 76.94  | 2.94    | 15<|
| F9               | 63.12±8.62    | 72.23±3.37    | 74.94±3.24    | 69.69  | 9.82    | 15<|
| F10              | 101.76±3.93   | 102.31±2.85   | 103.58±3.60   | 98.51  | 2.94    | 15<|
| F11              | 100.92±6.37   | 103.21±7.01   | 104.58±5.70   | 99.15  | 3.11    | 15<|
| F12              | 99.84±5.78    | 101.57±3.51   | 102.23±2.63   | 97.52  | 2.77    | 15<|
| F13              | 96.34±0.68    | 100.98±2.73   | 103.29±4.61   | 96.76  | 3.79    | 15<|
| F14              | 96.49±4.66    | 99.40±4.87    | 100.62±6.84   | 95.55  | 3.02    | 15<|
| F15              | 98.63±1.60    | 98.52±2.18    | 100.07±1.38   | 94.73  | 3.20    | 15<|
| F16              | 95.21±3.50    | 97.72±2.78    | 99.41±1.76    | 93.72  | 3.43    | 15<|
| F17              | 97.84±0.69    | 98.92±4.70    | 102.23±5.76   | 96.30  | 3.48    | 15<|
| F18              | 78.92±6.06    | 92.76±12.15   | 97.92±2.31    | 88.86  | 5.55    | 15<|
| F19              | 100.97±2.53   | 101.49±5.88   | 102.36±4.11   | 97.75  | 2.70    | 15<|
| F20              | 100.92±5.90   | 101.46±2.55   | 101.55±1.60   | 98.56  | 2.94    | 15<|
| F21              | 101.14±2.58   | 101.92±0.55   | 101.05±5.58   | 97.35  | 2.20    | 15<|
| F22              | 61.90±10.72   | 62.44±11.38   | 65.37±10.87   | 91.74  | 3.88    | 15<|
| F23              | 101.41±7.43   | 102.48±7.80   | 103.08±9.44   | 97.81  | 3.06    | 15<|
| F24              | 98.39±1.13    | 99.98±2.01    | 101.09±1.69   | 95.5   | 3.32    | 15<|
| F25              | 74.96±2.70    | 75.76±1.97    | 75.77±4.38    | 72.42  | 2.65    | 15<|
| Untreated AT powder | 30.00±4.73 | 47.66±0.91 | 56.72±1.07 | 47.44 | 9.52 | 0 |

a amount of drug release after 5 min; b amount of drug release after 30 min; c amount of drug release after 60 min; d dissolution efficiency; e Mean dissolution time; f Differential factor (0<f<15).

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Conflict of Interests
The authors declare no conflict of interests.

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