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Research Article

D-optimal Design for Preparation and Optimization of Fast Dissolving Bosentan Nanosuspension

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Abstract

Purpose: Bosentan is a drug currently taken orally for the treatment of pulmonary arterial hypertension. However, the water solubility of bosentan is very low, resulting in low bioavailability. The aim of this study was preparation and optimization of bosentan nanosuspension to improve solubility and dissolution rate.

Methods: The different formulations designed by Design Expert® software. Nanosuspensions were prepared using precipitation method and the effects of stabilizer type and content and drug content on the particle size, polydispersity (PDI) and yield of nanosuspensions were investigated.

Results: Particle size, PDI and yield of the optimal nanosuspension formulation were 200.9 nm, 0.24 and 99.6%, respectively. Scanning electron microscopy (SEM) results showed spherical morphology for bosentan nanoparticles. Thermal analysis indicated that there was a partial crystalline structure and change in the pholymorphism of bosentan in the nanoparticles. In addition, reduction of particle size, significantly increased *in vitro* dissolution rate of the drug.

Conclusion: Optimization by design expert software was shown to be a successful method for optimization and prediction of responses by less than 10% error and formulation with 15.8 mg span 85 as an internal stabilizer and 45 mg drug content were introduced as the optimum formulation. The solubility of bosentan in the optimal formulation was 6.9 times higher than coarse bosentan and could be suggested as promising drug delivery systems for improving the dissolution rate and possibly the pharmacokinetic of bosentan.

Introduction

Pulmonary arterial hypertension (PAH) is a devastating and progressive disease that usually characterized by remodeling of the pulmonary vasculature.[1] The progressive vasculopathy increases the pulmonary arterial pressure and pulmonary vascular resistance, eventually culminating in limited patients exercise capacity, right ventricular failure and death.[2] Endothelin plays a major role in the pathogenesis of pulmonary arterial hypertension and induction of vasoconstriction.[3] Additionally, endothelin 1 is a smooth-muscle mitogen, and can potentially raise the vascular tone and pulmonary vascular hypertrophy.[4]

Bosentan is an orally active, selective and competitive non-peptide dual endothelin receptor (both ET_A and ET_B) antagonist and usually used in cure of PAH.[5] Bosentan is also being considered for treatment of other conditions such as Eisenmenger syndrome,[6] persistent pulmonary hypertension of the newborn,[7] digital ulcer prevention in patients with systemic sclerosis,[8] adolescent and adult patients who have undergone Fontan operation,[9] vascular remodeling and dysfunctional angiogenesis in diabetes[10] and possibly even depression.[11]

For treatment of PAH, bosetan is currently administered at the daily dose of 125-250 mg.[12] The maximum plasma peak is seen within 3-5 h after oral intake and the half-life of drug is 5.4 h.[13] Moreover, the bioavailability of bosentan after oral administration is approximately low (50%)[14] and variability is seen in bosentan absorption which may get back to its poor water solubility.[13] The water solubility of bosentan is low (1 mg/100 ml)[15] and classified in class II of BCS.[16] So, increasing the solubility of bosentan can improve its pharmacokinetics and dose reduction and variability in drug exposure among individuals can be solved.[17]

One of the strategies in solubility enhancement is particle size reduction.[18] Nanoparticle are colloidal drug delivery systems usually between 10-1000 nm in size. Due to the high surface to volume ratio, nanoparticles can provide better solubility, adsorption and improve therapeutic outcomes.[19] Pharmaceutical nanosuspensions can be designed to be taken orally and topically or through parenteral or inhalation routes.

The aim of this study is preparation, characterization and optimization of bosentan nanosuspention by using of experimental design to enhance the solubility and dissolution rate.

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Materials and Methods

Materials

Bosentan was provided by Pajouhesh Darou Arya, Iran. Sodium dodecyl sulfate (SDS) and HPβCD were purchased from Sigma Aldrich, USA. Tween 20, Tween 80, Span 85 and acetonitrile were from Merck. Acetone was from Applichem, Germany.

Preliminary study on nanosuspension formulation

Bosentan nanosuspensions were prepared by the antisolvent precipitation method in the presence of different types of surfactants in aqueous phase. First, the screening was done on the effect of some formulation parameters on the characterization of prepared nanoparticles as presented in Table 1. In summary, a combination of bosentan, 25 mg tween 80 (internal stabilizer) and 1 ml acetone were placed in a beaker and mixed (organic phase). In another beaker, the aqueous phase was prepared by dissolving of 2 mg/ml external stabilizer in distilled water. Subsequently, the organic phase was emulsified dropwise in aqueous phase. Emulsification was carried out using a homogenaizer (High Shear T25D, IKA, Germany) for 3 min in 18000 rpm. Then, the emulsion was treated with an ultrasonic probe (UP400S_Ultrasonic processor, Hielscher, Germany) at power input of 320W and a cycle of 0.7 per second for 3 min.

Next, acetone was eliminated by evaporation under reduced pressure using a rotary evaporator (Buchi, Switzerland). Nanoparticles were recovered by centrifugation (sigma, Germany) at 20000 rpm for 25 min at 25 °C.

Table 1. Screening formulation for evaluation effect of external stabilizer type, aqueous volume and drug content on the size and PDI of prepared nanosuspension formulation

Stabilizer type	Water amount	Drug content (mg)	Size (nm)	PDI
Tween80	25	25	2190	0.3
		15	Large	1.0
	40	25	3323	0.2
		15	2250	0.4
βCD	25	25	1426	0.6
		15	1561	0.9
	40	25	1216	0.6
		15	950	0.8
HPβCD	25	25	732	0.5
		15	440	0.8
	40	25	443	0.3
		15	400	0.4
SDS	25	25	2132	1.0
		15	601	0.9
	40	25	1652	0.3
		15	700	0.6

Experimental design

Experimental design is one of the most reliable methods for selection of the best formulation as optimized, when different factors and variables are involved. The information obtained from this mathematical method can yield the combination of variables that can make up the best formulation. In the current research, 17 formulations with 3 variables –two levels of qualitative variable and 3 levels of quantitative variable (low, medium and high) - were assessed using D-optimal design and Design Expert® V6 (DX6) software for design of experiments (DOE). Experimental factors and factor levels were determined in preliminary studies. The independent variables included the type and content of internal stabilizer as well as the drug concentration. The dependent outcomes were the size and yield of the formulations (Table 2). The relationships linking the main factors and their interactions to the responses were determined and presented as a general form in the following equation:

$$Y = \text{intercepts} + \sum \text{main effects} + \sum \text{interactions} \qquad \text{(Eq.1)}$$

Equation coefficients were calculated using coded values; hence the various terms were compared directly, regardless of magnitude. A positive parameter coefficient indicates that output increases at a higher level evaluation for a variable and vice versa. Values are given as mean±SD. Statistical significance of the results was determined using one-way analysis of variance (ANOVA), employing a confidence interval of 95%. The numerical output of ANOVA includes the F-value, stating magnitude of the impact of each factor and P-value as representative of the statistical significance with smaller figures signifying greater importance.

Table 2. Run parameters and responses for experimental design

Runs	Stabilizer type (C)	Stabilizer content (A)	Drug content (B)
R_1	Span 85	15	45
R_2	Tween 80	15	30
R_3	Span 85	15	45
R_4	Span 85	15	15
R_5	Span 85	30	45
R_6	Tween 80	30	30
R_7	Span 85	15	15
R_8	Tween 80	15	45
R_9	Tween 80	15	15
R_{10}	Span 85	22.5	30
R_{11}	Span 85	30	15
R_{12}	Tween 80	30	15
R_{13}	Tween 80	30	45
R_{14}	Tween 80	22.5	45
R_{15}	Span 85	30	45
R_{16}	Span 85	30	15
R_{17}	Tween 80	22.5	15

Physiochemical characterization of nanoparticles

Particle size analysis

The mean hydrodynamic size (z-average) of prepared nanoparticles was determined by photon correlation spectroscopy (Zetasizer®, Malvern Instruments, UK) at 25 °C. Particle sizes were analyzed immediately after preparation and without dilution. The experiments were performed in triplicates.

Yield

Bosentan nanosuspensions were centrifuged (Sigma, Germany) at 20000 rpm for 25 min. The concentration of drug in the supernatant was quantified using isocratic HPLC system (Waters 600E, USA) and C18 column (5 µm, 15 cm). The mobile phase consisted of potassium dihydrogen orthophosphate buffer (pH=4.7) and acetonitrile (45:55) at a flow rate of 1 ml/min with UV detection at 270 nm. All experiments were performed in triplicate. The yield was calculated by means of the equation below:

$$\text{Yield } (\%) = \frac{\text{Total drug} - \text{dissolved drug}}{\text{Total drug}} \times 100 \quad \text{(Eq. 2)}$$

Scanning electron microscopy (SEM)

The surface morphology of unprocessed bosentan and optimal nanoparticles (NPs) was evaluated using a scanning electron microscope (S-4160, Hitachi, Japan) at a voltage of 20 kV. Coarse bosentan and a few drops of the freshly prepared nanosuspensions were spread on stubs using double side carbon tape and then sputtered with gold using a sputter coater (BAL-TEC, Switzerland).

Differential scanning calorimetry (DSC)

A differential scanning calorimeter (Mettler Toledo, Switzerland) was employed to evaluate the thermal behavior of all materials used in the optimal nanoparticles. The equipment was calibrated using indium and zinc. Then, accurately weighed sample powders (8 mg) were heated in aluminum pans within the range of 20-330 °C at a scanning rate of 20 °C/min under nitrogen gas.

Dissolution study

The solubility of coarse bosentan and the freeze-dried optimized nanosuspension were studied in phosphate buffer (PBS) at a pH of 7.4 as dissolution medium. For this purpose, Amounts of powders equal to 20 mg of bosentan were dispersed in screw-capped glass vials, (100 ml) containing 50 ml of medium, by shaking at 50 rpm at 37 ± 0.5 °C in shaker incubator (LABOTEC, Germany). At predetermined time intervals (1, 5, 10 and 15 min) 1 ml of the dispersion were taken away and replaced with 1 ml of fresh PBS. The samples were filtered through 0.22 µ syringe filter and the amount of dissolved bosentan was determined using HPLC method that described previously. All tests were carried out in triplicate.

Results

Preliminary formulations

Bosentan nanosuspensions were successfully prepared using the antisolvent precipitation method. In the first step, the effect of various external stabilizer types, drug content and volume of aqueous phase were studied (Table 1) to achieve the most favorable particle size. As seen in Table 1, the smallest size of nanoparticles was seen when HPβCD was used as an external stabilizer. In addition, by increasing the drug content in formulation that HPβCD was as an external stabilizer, the size of particles increased. Furthermore, when a larger volume of aqueous phase applied in the formulation of nanoparticles, the size of formed particles was smaller. So, HPβCD was selected as an external stabilizer in next investigations.

In the next step, several formulations having Tween 20, Tween 80 and Span 85 as internal stabilizers were prepared. Particles that formed in presence of span 85 (220.3 nm) had smaller size as compare to tween 80 (550 nm) and tween 20 (1432 nm). Therefore, span 85 and tween 80 were selected for more investigation.

Experimental design

After determination of the type and levels of variables from the preliminary formulation studies, the Design Expert software was used to design of experiments.

Size measurements

The hydrodynamic size of prepared nanoparticles measured by nano zetasizer. The particle size of nanoparticles was in the range of 188.3-2816 nm and all formulations had a PDI of less than 0.7.

The quadratic model was the best fitted model on the data ($p< 0.001$). As presented in Table 3, the most effective parameter on the size of nanoparticle was stabilizer type with F-value 32.87 ($p<0.001$). The relation of parameters and size of particles presented in equation 3:

$$\text{Size} = +1104.19 + 64.92*A + 156.93*B + 508.78*C + 973.88*A2 - 1152.47*B2 - 92.87*A*B - 96.23*A*C + 124.38*B*C \quad \text{(Eq. 3)}$$

As shown in Figure 1, the produced particles in formulations with span 85 as an internal stabilizer had smaller size than formulations with tween 80. In addition, increase of drug content up to midpoint and stabilizer content in the lower and upper level increased the particle sizes.

PDI measurements

The PDI of nanoparticle formulations varied within ranges of 0.17-0.69. The reduced quadratic model is the best fitted model on PDI data with F value of 9.31 ($p<0.001$). As presented in Table 3, the only significant parameter affects the PDI was stabilizer content (F value = 4.83 and $p<0.05$). Increase in stabilizer content up to middle of studied range (22.5 mg), causes decrease in PDI. But more increase in stabilizer content results in the

higher polydispersity index (Figure 2). This fact is in agreement with positive value of stabilizer content. The relation of parameters and PDI of formulation presented in equation 4:

$$PDI=+0.48+0.063*A-0.042*B-0.017*C+0.18*A2-0.27*B2 \quad (Eq.4)$$

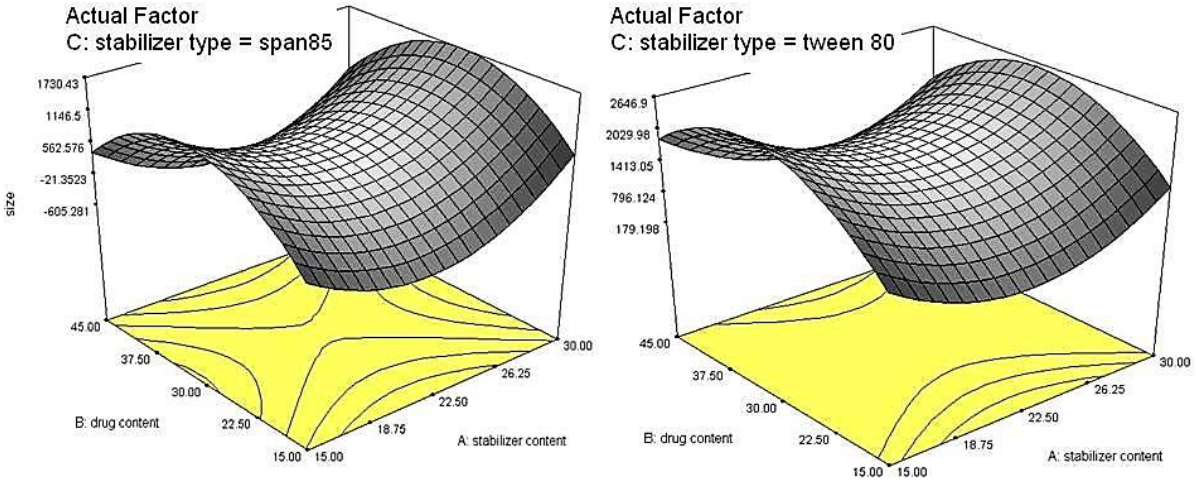

Figure 1. The effect of stabilizer content and drug content on the particle size

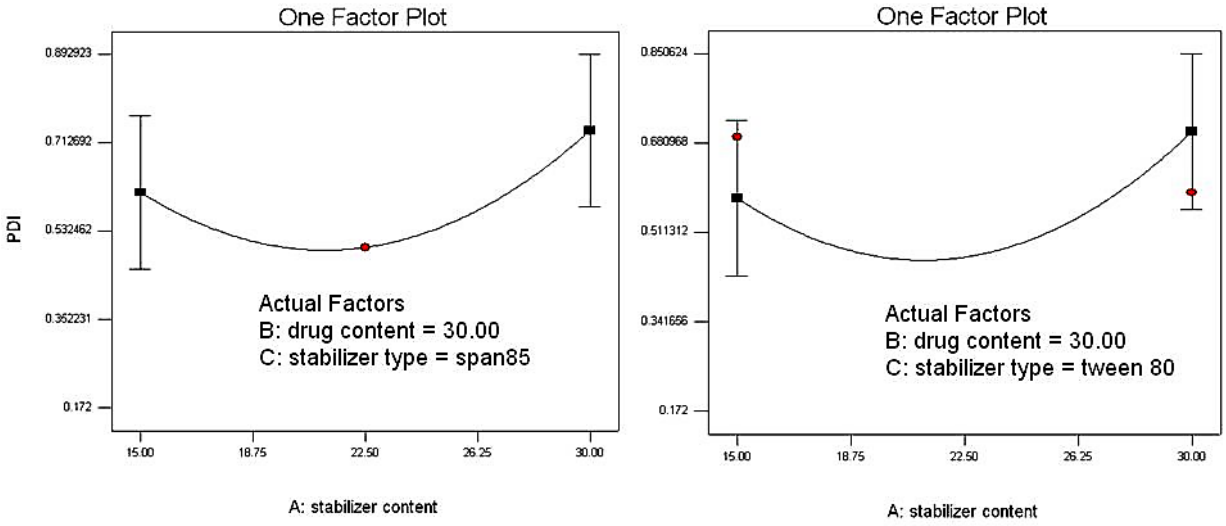

Figure 2. The effect of stabilizer content on the PDI

Table 3. The contribution and significance of different formulation parameters on different nanoparticle attributes

Parameters	F-value in size	F-value in yield	F-value in PDI
A	0.46	2.58	4.83*
B	2.68	24.11**	2.13
C	32.87**	1.31	0.42
A^2	17.50	-	6.16
B_2	24.51	-	15.19
AB	0.82	-	-
AC	1.01	-	-
BC	1.68	-	-

*: $P < 0.05$ and **: $P < 0.001$

Yield

The yield of fabricated nanoparticles was in the range of 80.79-96.97%. Furthermore, ANOVA analysis showed that the drug content is a factor significantly affects the yield (F value = 24.11, $p<0.001$). As shown in Figure 3, the increase in drug content leads to a relative increase in the yield of nanoparticles. The linear model that explains the relationship of parameters and yield of formulation presented in equation 5:

$$Yield = +92.28 +1.52*A+ 4.64* B- 0.98* C \quad (Eq.5)$$

Optimization

After confirming the polynomial equations relating the response and independent factors, the optimization

model was constructed by combining the size, PDI and yield of formulations. Optimization was performed by using a desirability function to obtain the levels of drug content, stabilizer type and content which maximized yield, while minimizing PDI and size of particles less than 500 nm. Simultaneously, the formulation with 15.8 mg span 85 as an internal stabilizer and drug content of 45 mg suggested as an optimized formulation. This formulation prepared and evaluated. Predicted and actual amounts of responses are compared and shown in Table 4. As seen in Table 4, the amount of responses for optimized formulation have lower than 10% difference with the predicted amount of D-optimal design.

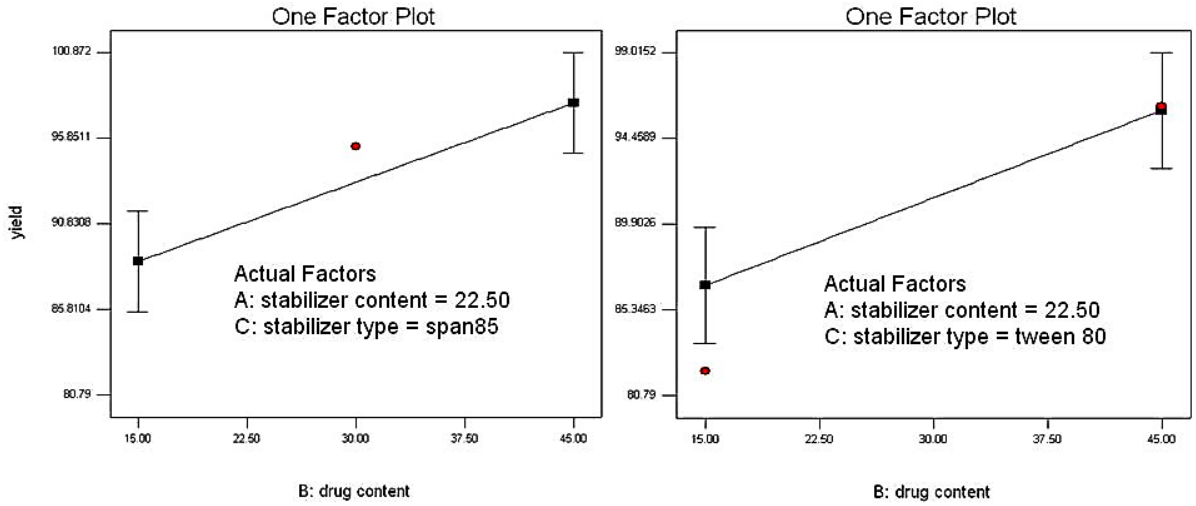

Figure 3. The effect of drug content on the yield of nanoparticles

Table 4. Comparison of actual and predicted responses for optimal formulation

	Size (nm)	PDI	Yield (%)
Predicted response	188.3	0.265	96.55
Actual response	200.7	0.285	99.54
Error (%)	6.6	7.7	3.1

Physiochemical properties of the nanoparticles

Morphology

The morphology of coarse bosentan and optimized nanoparticles were studied by SEM (Figure 4). Coarse bosentan particles were needle-shaped crystals or squama like with rough surfaces (Figure 4A); whereas, nanoparticles were relatively spherical in shape with some degree of agglomeration that could be related to the drying process (Figure 4B).

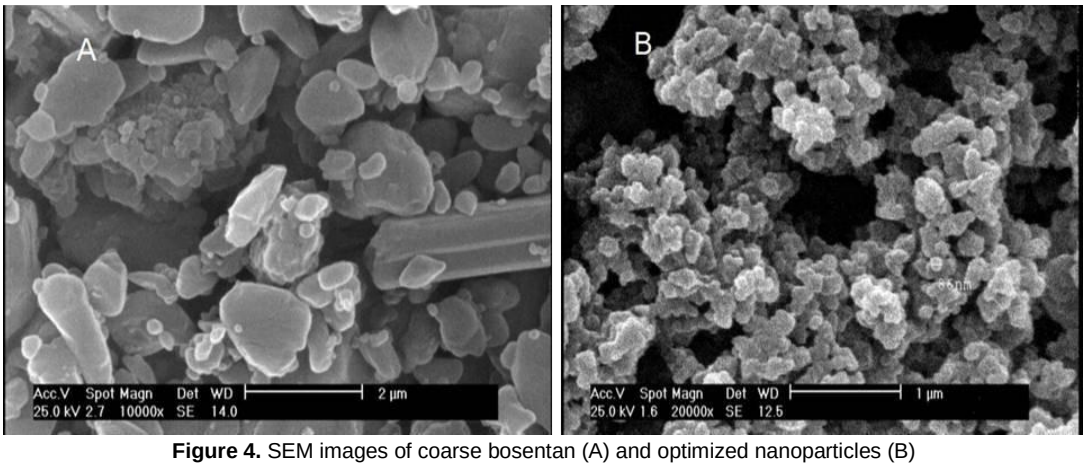

Figure 4. SEM images of coarse bosentan (A) and optimized nanoparticles (B)

Thermal analysis

Figure 5 shows the DSC thermograms of coarse bosentan, HPβCD and the lyophilized optimized formulation.

In Figure 5 was shown that coarse bosentan had 2 endothermic peaks in 130 °C and 80 °C. In the case of HPβCD, an extended endothermic feature is seen around 90 °C. In the optimized nanoparticles, the endothermic peak of bosentan in 80 °C and extended peak of HPβCD were disappeared and the endothermic peak of bosentan in 130 °C was seen by lower intensity.

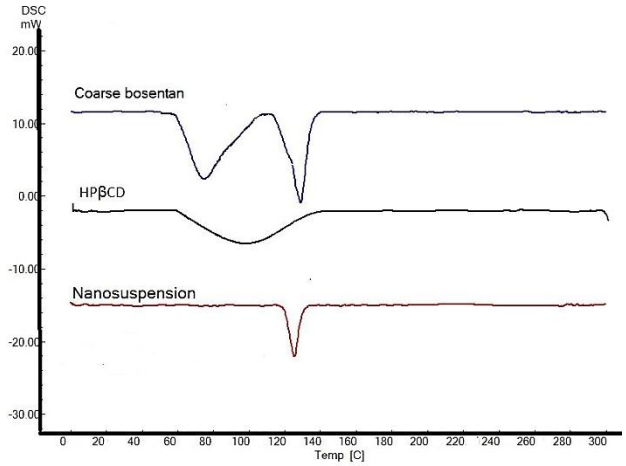

Figure 5. DSC thermograms of coarse bosentan, HPβCD and the lyophilized optimized nanosuspension

Dissolution study

The dissolution profiles of coarse bosentan and fabricated nanoparticles were studied and the results have been shown in Figure 6.

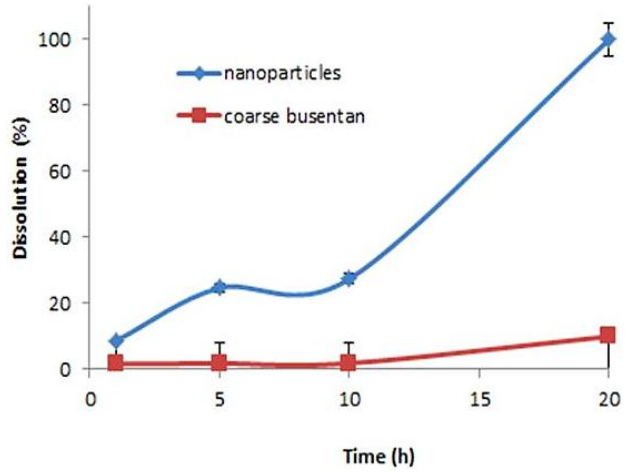

Figure 6. The dissolution profiles of coarse bosentan and fabricated nanoparticles

As seen in Figure 6, about 30% of drug content of nanoparticle formulation dissolved after 5 min and the drug content completely dissolved after 20 min. But, the maximum amount of dissolved coarse bosentan was 10% after 20 min.

The maximum solubility of optimized nanoparticles and coarse bosentan was measured in phosphate buffer (pH=7.4) after 24 h. The maximum solubility of lyophilized nanoparticles was 6.9 mg/100 ml, while coarse bosentan is was 1 mg/100ml. On the other words, the bosentan nanoparticles had around 7- fold higher solubility than unprocessed bosentan.

Discussion

Bosentan is a drug with low water solubility and belongs to class II of BCS.[20] Thus, increasing the solubility and dissolution rate can increase the absorption and efficacy of the drug.[21] Decreasing particle size and incorporating drugs in the structure of nanoparticles is a strategy widely used for increasing the solubility and dissolution rate of pharmaceuticals.[22]

In this study, nanosuspension formulations of bosentan were fabricated to improve its solubility by nanoprecipitation (antisolvent) method.

As indicated in the results section, formulations with Span 85 as an internal stabilizer had smaller size than Tween 80. This finding could be justified by focusing on the properties of these surfactants. Since Span85 has a higher tendency to organic phase compared to Tween 80 and drug was dissolved in organic phase, better coverage of drug particles in fabrication process was done by span 85 and result in smaller particles.

Increasing the stabilizer content initially lead to decreasing PDI; however, further increase results in increasing in PDI. The initial decrease in PDI can be attributed to favorable coverage of nanoparticles surface. However, further increase in stabilizer content can also lead to increase in viscosity of the solution and can lead to increase in nanoparticle aggregation and PDI.[23]

Increase in the drug content could directly increase the yield of formulation. One of the role of surfactants in formulations is solubility enhancing.[18] However, when the drug content increased and stabilizer amount was fixed, the ability of surfactant for dissolving is fixed and the amount of precipitated drug and as a result yiled was increased.

Subsequently, according to the predictions of experimental design software, the optimized formulation was suggested to be composed of 15.8 mg Span 85 and 45 mg of bosentan. The difference in experimental and predicted values was less than 10%, which refers to the ability of the software in prediction of parameters for optimized formulation.

In SEM images were shown that optimized nanoparticles have a spherical shape (compared to coarse bosentan which was crystal-like). This change in shape can influence the dissolution behavior of bosentan.

In DSC thermogram of coarse bosentan, 2 endothermic peaks were seen which are related to the polymorphism structures of bosentan.[24] In the case of bosentan nanoparticles, this endothermic peak of 80 °C was disappeared and it can be explain by changing this polymorph of bosentan to more stable of polymorph that be appear in 130 °C. On the other hand, the endothermic peak of nanoparticle had a lower intensity than coarse bosentan in this point. It can be a sign of partial crystallization of bosentan in nanoparticles which can be favorable in terms of drug dissolution.

As presented in Figure 6, the dissolution extent and rate of nanoparticles are higher than coarse bosentan. This phenomenon can be due to the increase in the effective surface area between drug and the solvent provided by size reduction in nanoparticles. Overall, nanoparticles increased the saturation solubility of bosentan to 6.9 mg/100ml which is 7 fold higher than the solubility of

unprocessed bosentan (1 mg/100 ml) which can be due to increase in dissolution pressure.[25]

Conclusion

The data confirm that antisolvent-precipitation method is a feasible method for preparation of bosentan nanosuspension. Besides, the experimental design software successfully could determine the optimal conditions to achieve the desired responses. This optimum condition could be proposed as a beginning to scale up and industrialization of bosentan nanoparticle formation to utilize in the form of tablets or processed in the presence of inhalable sugars to form a dry powder for inhalation purposes. As compared to unprocessed bosentan, the formed nanocrystalline structures can significantly enhance solubility properties and dissolution rate of the drug.

Ethical Issues

No ethical issues to be promulgated.

Conflict of Interest

The authors declare that there are no conflicts of interests.

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