Pharmaceutical Development and Technology

http://informahealthcare.com/phd
ISSN: 1083-7450 (print), 1097-9867 (electronic)

Pharm Dev Technol, Early Online: 1–11
 DOI: 10.3109/10837450.2013.795169

informa healthcare

ORIGINAL ARTICLE

Preparation, *in vitro* evaluation and statistical optimization of carvedilol-loaded solid lipid nanoparticles for lymphatic absorption via oral administration

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Abstract

Carvedilol-loaded solid lipid nanoparticles (SLNs) were prepared using solubility parameter (δ) to select the lipid, and hot homogenization to fabricate SLNs. The effect of concentration of Compritol 888 ATO (COMP) and Poloxamer 188 (P-188) on the particle size of blank SLNs was studied using the design of experiments. Further narrow concentration range of COMP and P-188 was selected and carvedilol-loaded SLNs were prepared to obtain an optimized formulation which was lyophilized (L-SLNs), transformed into enteric compression-coated tablet and evaluated for drug release, X-ray diffraction and cellular uptake mechanism. COMP was chosen as lipid due to its least value of $\Delta\delta$ with carvedilol. The optimized formulation (7.5% COMP, 5.0% P-188 and 1.11% carvedilol) had 161 nm particle size and 94.8% entrapment efficiency. The enteric-coated carvedilol-loaded SLNs tablet protected carvedilol from acidic environment and similar prolonged release profiles were obtained from L-SLNs, core tablet and enteric-coated tablet. Absence of crystalline carvedilol XRD peak indicated the presence of amorphous carvedilol in SLNs. Higher carvedilol uptake from SLNs compared to drug solution in the Caco-2 cell line exhibited a potential prolonged drug release. Moreover, upon cellular uptake, SLNs could then enter the lymphatic system which will avoid first pass metabolism and hence higher oral bioavailability.

Keywords

Caco-2 cells, carvedilol, cellular uptake mechanism, design of experiments, oral drug delivery, solid lipid nanoparticles

History

Received 22 January 2013
Revised 7 April 2013
Accepted 9 April 2013
Published online 22 May 2013

Introduction

The past few decades have seen significant advances in drug delivery technologies. Oral drug delivery is not only the largest and the oldest segment of the entire drug delivery market; it is also the fastest growing and the most preferred route for drug administration. Because a large number of recently developed chemical entities have poor aqueous solubility, many formulation approaches, such as salt formation, co-solvent, size reduction, complexation and lipid-based drug delivery systems have been evaluated to increase their bioavailability[1]. Lipophile delivery through oral route is extremely challenging because upon introduction into the aqueous biological environments, lipophilic molecules exhibit instability, precipitation, food interactions, non-specific targeting and toxic effects[2]. According to Biopharmaceutical Drug Disposition Classification System (BDDCS), based upon the prediction of drug disposition by interplay of transport, absorption and elimination, majority of the BCS Class II drugs not only have poor aqueous solubility but also have extensive first pass metabolism causing additional factor for poor bioavailability[3,4]. Therefore, the development of a delivery system, which not only regulates the release of the drug but also avoids the first pass metabolism, is needed to enhance the overall therapeutic outcomes.

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Utilization of solid lipid nanoparticles (SLNs) to regulate the release of drug has been evaluated in oral administration route[5]. It has been reported that oral bioavailability can be increased by encapsulating drug into SLNs where uptake of drug occurs through the lymphatic system[6,7]. SLNs generally are spherical in shape and are comprised of a solid lipid core stabilized by a surfactant interfacial region which combines the advantages of lipid emulsion systems and polymeric nanoparticle systems while overcoming the temporal and *in vivo* stability issues that are exhibited by other drug delivery approaches[8–10]. SLNs are biocompatible and biodegradable and can be produced easily without the use of organic solvents. In addition, due to solid nature of the lipid matrix, SLNs can be lyophilized and transformed into the conventional oral solid dosage forms such as tablets and capsules, thereby enhancing stability and patient compliance[8–10].

In order to evaluate the features of regulating the release of drug and avoiding the first pass metabolism through lymphatic uptake offered by SLNs[6,7], carvedilol was selected as the model drug. The properties of carvedilol, e.g. high first pass metabolism and low dose as well as its need for long-term treatment and repetitive dosing, make this drug an interesting candidate for oral administration where the release of the drug should be controlled and the first pass metabolism could be avoided to increase overall bioavailability by promoting lymphatic uptake of carvedilol-loaded SLNs. Carvedilol ($\log P = 4.2$) is practically insoluble in water, in gastric fluids (pH 1.1) and in intestinal fluids (pH 7.5). Although carvedilol is completely absorbed from the gastrointestinal tract after ingestion of the conventional tablets, the systemic

availability is about 25% due to its metabolism in liver. Therefore, carvedilol-loaded SLNs using solid safe lipid materials, low amount of surfactant and its feasibility of being transformed into tablets as a dosage form were evaluated in this investigation. Various lipids were screened and selected based upon the solubility parameter and then high-shear homogenization was employed to fabricate blank SLNs for evaluation of such formulation variables as concentrations of lipid and surfactant on the size of the nanoparticles. Based upon the results of particle size obtained from blank SLNs, narrow range of lipid and surfactant concentrations were further selected to produce targeted nanoparticles (less than 200 nm) and carvedilol-loaded SLNs were prepared using the design of experimental approach. Physicochemical properties of carvedilol-loaded SLNs, such as particle size, drug entrapment efficiency and *in vitro* drug release kinetic were evaluated and used to obtain an optimized formulation. The optimized formulation was then enteric compression coated and the powder XRD study was performed to evaluate the nature of the drug present in the prepared SLNs. *In vitro* cellular uptake studies of carvedilol from SLNs as well as from carvedilol solution and physical mixture of components of SLNs as controls were performed using Caco-2 cell line as an indirect indication of lymphatic absorption.

Materials and methods

Materials

Carvedilol was obtained as a free sample from Caraco Pharmaceuticals (Detroit, MI). Compritol 888 ATO (COMP) was obtained as a gift sample from Gattafosse (Paramus, NJ). Monobasic potassium phosphate, dibasic potassium phosphate, Poloxamer 188 (P-188), polyethylene glycol 400 (PEG) and HPLC grade methanol were purchased from VWR International (West Chester, PA). Starch 1500 and Eudragit L100-55 were kind gifts from Colorcon (Harleysville, PA) and Evonik Industries (Piscataway, NJ), respectively. Dialysis membrane (spectra/Por dialysis memberane MWCO: 6–8000) was obtained from Spectrum Labs (Rancho Dominguez, CA). The human umbilical vein endothelial cell (HUVEC) line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM/F12) with L-glutamine and 15 mM HEPES, fetal bovine serum, 1% streptomycin and trypsin were obtained from Sigma-Aldrich (St. Louis, MO). All chemicals were of analytical or technical grade and were used without further treatment.

Selection of lipid using solubility parameter

Group contribution method was used to calculate Hildebrand solubility parameter which requires energy of vaporization and the molar volume. The molar volume of the lipid was determined using group contribution method and energy of vaporization was calculated using Van Krevelen–Hoftyzer[11,12]. According to this method, the total solubility parameter (δ) is given by Equation (1). Van Krevelen–Hoftyzer method was employed to calculate the solubility parameter of carvedilol and various lipids and confirmed with the published literature values. Various lipids used in this investigation are outlined in Table 1.

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \quad (1)$$

where

δ = total solubility parameter,
δ_d = contribution from dispersion forces $\left(\delta_d = \frac{\sum F_{di}}{V}\right)$,
δ_p = contribution from polar force $\left(\delta_p = \frac{\sqrt{\sum F_{pi}^2}}{V}\right)$,
δ_h = contribution of hydrogen bonding $\left(\delta_h = \frac{\sqrt{\sum E_{hi}}}{V}\right)$,
F_{di} = molar attraction constant due to dispersion component,
F_{pi} = molar attraction constant due to polar component,
E_{hi} = hydrogen bonding energy,
V = molar volume.

Table 1. Solubility parameter of carvedilol and various lipids together with their differences.

Drug/lipid name	Solubility parameter $(MPa)^{1/2}$	Difference with carvedilol ($\Delta\delta$)
Carvedilol	25.38	
Myristic acid	17.64	7.73
Palmitic acid	17.54	7.83
Stearic acid	17.46	7.91
Behenic acid	17.34	8.03
Trilaurin	17.63	7.74
Trimyrisin	17.52	7.85
Tripalmitiin	17.44	7.93
Tristearin	17.36	8.01
Tribehenin	17.26	8.11
Mono-glyceryl behenate	20.15	5.22
Di-glyceryl behenate	18.08	7.29
Compritol ATO 888	21.42	3.96
Glyceryl monostearate (Imwitor 900 K)	20.37	5.00

Preparation of blank SLNs

Blank SLNs were prepared by the hot homogenization method. The lipid was melted at about 5–10 °C above its melting point. The aqueous surfactant solution was heated separately at the same temperature and added to the melted lipid. The mixture was homogenized at 25,000 rpm for 10 min using a high-shear homogenizer (Sentry Microprocessor, Kent City, MI). The resultant dispersion was diluted with water and allowed to cool at room temperature with constant stirring.

Design of experiments for blank SLNs

A series of blank SLN formulations using the design of experiment approach were prepared to evaluate the suitable range of lipid concentration and surfactant concentration that could produce the targeted particle size (less than 200 nm) of blank SLNs. This is the size of particles reported to be absorbed by means of lymphatic route following oral administration[6,7]. A fractional factorial design using Fusion Pro software (S-matrix Corporation, Eureka, CA) was applied and the concentrations of COMP and P-188 were set from 0.625% to 15.0% and from 0.25% to 5.0%, respectively. A total of 13 formulations were derived and shown in Table 2. Following the fabrication of blank SLNs, particle size was determined and used as response parameter for the correlation analysis with the COMP and P-188 concentration as independent parameters. In the obtained quadratic equation, only the coefficients that were statistically significant ($p < 0.05$) were retained. From the correlation obtained, concentration range values of COMP and P-188 were calculated in order to obtain the targeted particle size before loading the drug. Thereafter, a further narrow range could be selected for lipid (e.g. 5.0%–7.5%) and surfactant (e.g. 1.5%–5.0%), and concentration of an additional third formulation variable (i.e. drug concentration) for a full factorial design studies. This design of experiment approach offers many advantages, e.g. reduction in the total number of formulations containing three formulation variables, identification of interaction between formulation variables and detection of the

optimal response such as minimum particle size with maximum drug entrapment.

Preparation of carvedilol-loaded SLNs

Carvedilol-loaded SLNs were prepared using the same procedure that was adopted for the preparation of blank SLNs except that carvedilol was added to the lipid and allowed to dissolve in the lipid melt. After carvedilol dissolved in the lipid melt, the hot aqueous surfactant solution was added to it and the mixture was homogenized at 25 000 rpm for 10 min. The resulting dispersion was diluted with 10.0 mL of water (final preparation, 20.0 mL) and was allowed to cool at room temperature with constant stirring.

Table 2. Composition of blank SLN formulations obtained from the fractional factorial design and their respective particle size (data shown as mean ± standard deviation, $n = 3$).

Formulation code	Formulation variables		Particle size	
	COMP (% w/v)	P-188 (% w/v)	Mean (nm)	PI
P1	0.625	0.25	116 ± 4	0.162
P2	0.625	5.0	1224 ± 433	0.737
P3	0.625	5.0	1224 ± 433	0.737
P4	2.5	5.0	110 ± 15	0.167
P5	5.0	2.5	113 ± 10	0.228
P6	5.0	5.0	101 ± 14	0.222
P7	7.5	0.25	5918 ± 2917	0.286
P8	7.5	2.5	199 ± 7	0.200
P9	12.5	1.5	2442 ± 1118	0.249
P10	12.5	2.5	1034 ± 283	0.217
P11	15.0	0.25	9013 ± 4322	1.000
P12	15.0	5.0	331 ± 23	0.372
P13	15.0	5.0	331 ± 23	0.372

COMP: Compritol 888 ATO, P-188: Poloxamer 188, PI: polydispersity index

Design of experiments for carvedilol-loaded SLNs

A full factorial design using Fusion Pro software was employed to derive predictive model for carvedilol-loaded SLNs fabricated from COMP concentration, P-188 concentration and carvedilol concentration as formulation variables. A total of 27 formulations (Table 3) were derived. The concentrations of COMP and P-188 were selected based on the outcomes obtained from experiments performed using blank SLNs. Carvedilol concentrations were selected based on the preliminary experiments (data not shown). Lipid concentration was ranged from 5.0% to 7.5%, the concentration of P-188 was used from 1.5% to 5.0%, and carvedilol concentration was ranged from 0.25% to 1.5%. Particle size and entrapment efficiency were determined and used as the response parameters for correlation with the formulation variables. Based on the results obtained after the completion of experimental design, a mathematical model could be established and used for optimization, validation and prediction of carvedilol-loaded SLNs. This mathematical model involved a dependent variable Y and three independent variables A, B and C, and could be expressed as $Y = f(A, B, C)$ which was used for regression analysis. The three independent variables selected were lipid concentration, P-188 concentration and carvedilol concentration while the dependent variables included particle size and entrapment efficiency.

Regression analysis of design of experiment

The contribution of the three formulation variables was compared using analysis of variance (ANOVA) at $p = 0.5$ significance level.

Table 3. Composition of carvedilol-loaded SLNs formulations obtained from the full factorial design and their respective particle size and entrapment efficiency (data shown as mean ± standard deviation, $n = 3$).

Formulation code	Formulation variables			Particle size		*EE* (%)
	COMP (% w/v)	P-188 (% w/v)	Carvedilol (% w/v)	Mean (nm)	PI	
F1	5.0	1.5	0.25	146 ± 9	0.207	68.5 ± 0.9
F2	5.0	1.5	0.88	285 ± 24	0.195	90.3 ± 2.6
F3	5.0	1.5	1.5	1319 ± 46	0.412	93.5 ± 0.2
F4	5.0	3.3	0.25	101 ± 6	0.243	69.9 ± 3.2
F5	5.0	3.3	0.88	130 ± 8	0.183	89.5 ± 3.8
F6	5.0	3.3	1.5	2486 ± 83	0.601	94.6 ± 2.2
F7	5.0	5.0	0.25	97 ± 16	0.231	60.8 ± 2.9
F8	5.0	5.0	0.88	124 ± 3	0.128	86.4 ± 6.4
F9	5.0	5.0	1.5	414 ± 19	0.191	88.9 ± 2.0
F10	6.3	1.5	0.25	218 ± 27	0.241	71.5 ± 4.3
F11	6.3	1.5	0.88	279 ± 43	0.285	92.0 ± 3.8
F12	6.3	1.5	1.5	667 ± 12	0.289	93.6 ± 2.1
F13	6.3	3.3	0.25	124 ± 17	0.218	69.6 ± 2.9
F14	6.3	3.3	0.88	227 ± 66	0.232	86.8 ± 0.8
F15	6.3	3.3	1.5	406 ± 6	0.154	95.6 ± 2.3
F16	6.3	5.0	0.25	98 ± 7	0.233	67.3 ± 9.8
F17	6.3	5.0	0.88	110 ± 13	0.237	87.1 ± 4.2
F18	6.3	5.0	1.5	327 ± 42	0.154	93.3 ± 1.2
F19	7.5	1.5	0.25	289 ± 22	0.283	56.7 ± 6.9
F20	7.5	1.5	0.88	470 ± 10	0.209	87.3 ± 6.2
F21	7.5	1.5	1.5	1125 ± 290	0.324	95.0 ± 2.7
F22	7.5	3.3	0.25	189 ± 9	0.231	52.1 ± 4.1
F23	7.5	3.3	0.88	216 ± 39	0.22	88.0 ± 4.8
F24	7.5	3.3	1.5	263 ± 73	0.206	92.5 ± 3.1
F25	7.5	5.0	0.25	109 ± 8	0.236	62.7 ± 2.1
F26	7.5	5.0	0.88	153 ± 28	0.211	94.8 ± 2.3
F27	7.5	5.0	1.5	217 ± 144	0.209	93.7 ± 3.5

COMP: Compritol 888 ATO, P-188: Poloxamer 188, PI: polydispersity index, *EE*: entrapment efficiency.

A regression analysis was carried out to obtain a quadratic model in the form of:

$$Y = b_0 + b_1A + b_2B + b_3C + b_4(A * C) + b_5(C * B) + b_6(A * B) + b_7(curvature) \quad (2)$$

where

- Y is the response parameter associated with each factorial level combination,
- b_0 is an intercept (constant),
- b_i is the regression coefficient computed from obtained experimental values of Y,
- A, B and C stand for the formulation variables,
- $A*C$, $C*B$ and $A*B$ are for the interaction between the formulation variables,
- (curvature) is the quadratic term of the independent variables which was used to simulate the curvature of the designed sample space. A backward elimination procedure was used to fit the data to the quadratic model.

Characterization of carvedilol-loaded SLNs

Particle size

Photon correlation spectroscopy was employed to measure particle size distributions of carvedilol-loaded SLNs, which were diluted 10 times using distilled water before the determination. Delsa™ Nano Series Zeta Potential and Submicron Particle Size Analyzer (Brea, CA) were used at a scattering angle of 90° at room temperature.

Entrapment efficiency

Entrapment efficiency was determined using the dialysis bag method which separates un-entrapped drug from the carvedilol-loaded SLN dispersions[13]. Briefly, to determine un-entrapped drug, 1.0 mL of the 20.0 mL prepared formulation was placed in the dialysis bag (spectra/Por dialysis membrane MWCO: 6–8000) at 4 °C in a beaker containing 20.0 mL of pH 6.8 phosphate buffer with 30% PEG 400. At predetermined time intervals, samples (1.0 mL each) were withdrawn for 8 h and immediately replaced with the fresh medium. The collected samples were diluted with 1.0 mL of methanol and analyzed by the HPLC method described previously[14]. The following equation was used to calculate entrapment efficiency.

$$EE = \frac{D_t - D_m}{D_t} \times 100 \quad (3)$$

where

EE = entrapment efficiency,
D_t = total amount of drug initially added,
D_m = amount of drug in medium (un-entrapped).

The carvedilol-loaded SLN dispersion (without un-entrapped drug) obtained at the end of the above-mentioned procedure were also measured for entrapment efficiency and mass balance was studied.

Constrained optimization of carvedilol-loaded SLNs

To further identify an optimal formulation, a constrained optimization technique was used to generate the optimum setting for the carvedilol-loaded SLNs. Therefore, two main constraints were used to optimize the carvedilol-loaded SLNs: (i) minimization of the particle size below 200 nm to promote lymphatic uptake and (ii) greater than 90% entrapment efficiency. The optimized formulation was subjected to particle size analysis, entrapment efficiency determination and *in vitro* drug release study. The proposed higher oral bioavailability of carvedilol from SLNs is mainly due to desired particle size leading to higher cellular uptake for lymphatic delivery. To minimize the potential passive absorption of premature released carvedilol from SLNs, *in vitro* drug release study was performed to ensure the retarded release of carvedilol in the upper gastrointestinal tract before the cellular uptake of SLNs by Peyer's patches located in the distal ileum of the gastrointestinal tract. For the comparison of carvedilol release profile among formulations studied, *in vitro* drug release study was also performed in the environment of jejunum and ileum to minimize the release of carvedilol before the cellular uptake of SLNs. In addition, the release of carvedilol from SLNs, upon their uptake in the body, is a complex mechanism which was not well understood. Therefore, the parameters to be obtained from the carvedilol release profiles were not used for the constrained optimization of carvedilol-loaded SLNs.

Lyophilization and tableting of carvedilol-loaded SLNs

In order to obtain SLNs in a solid form, selected carvedilol-loaded SLNs dispersions were lyophilized using freeze dry/shell freeze system (Labconco, Kansas city, MO). Carvedilol-loaded SLN dispersions were diluted with 15% trehalose as a cryoprotectant at 1:1 ratio, frozen and lyophilized for 24 h to obtain formulation L-SLN[15]. Core tablets of optimized formulation were prepared on a Carver Press (model C, Fred S. Carver Inc., Menomonee Falls, WI) using a 7.0 mm concave punch and 0.5 ton compression pressure. Being directly compressible binder, diluent and disintegrant Starch 1500 was used at 1:1 ratio. The core tablets were compression coated with 30 mg of Eudragit L100-55 to resist release of carvedilol in the acidic environment. An 8 mm punch at a compression pressure of 1.5 ton was used to provide a thin coating on the lateral sides as well as on the upper and lower sides of the core tablet.

In vitro drug release study of carvedilol-loaded SLNs

The *in vitro* release profiles of carvedilol from selected formulations of carvedilol-loaded SLNs (Table 3) and the optimized formulation were determined using dialysis bag technique. In addition, the *in vitro* release profiles obtained from the dispersion of the optimized formulation were compared with lyophilized powder (formulation L-SLNs), compress core tablet and enteric coated tablet having the same formulation components as optimized formulation.

In order to confirm that the dialysis bags used (same as used previously for entrapment efficiency determination) acted as a filter only and allowed to separate the drug released from SLNs during the release medium sampling step, the distribution of carvedilol solution from the inside to the outside of the bags was also studied. The dialysis bags were soaked in distilled water for 12 h before use. The selected formulations of carvedilol-loaded SLNs (0.5 mL) were added to the dialysis bag and both ends of the bag were tied with clamps. In the case of solid dosage forms, 40 mg of accurately weighed lyophilized powders or each tablet containing 40 mg of lyophilized powders were added into the dialysis bag with 1.0 mL of dissolution medium. The bags were placed in a beaker containing 30.0 mL of dissolution medium, and the beakers were placed in a thermostatic shaker (VWR, Shell lab, Cornelius, OR) and then shaken horizontally 100 rpm at 37 °C. To simulate the acidic conditions of the stomach and the gastric emptying time, 0.1 N HCl (pH 1.2) was used as release medium and samples were withdrawn at 15 min intervals for 2 h. In addition, to simulate the environment of jejunum and ileum, phosphate buffer solution (pH 6.8) containing 1% v/v of Tween 80 was used. Aliquots (0.5 mL) were withdrawn at predetermined

time intervals and same volume was replaced with the fresh medium. The samples were analyzed by HPLC method to determine the drug content. All the studies were carried out in triplicate.

Powder X-ray diffraction studies

To study change in the degree of crystallinity of carvedilol when formulated as SLNs, pure materials and SLN formulations together with their physical mixture were characterized using Shimadzu X-Ray Diffractometer 6000 (Shimadzu, Columbia, MD). Samples were irradiated using a Cu target tube. A monochromator was used to select Kα 1 line. The scanning angle ranged from 10° to 50° of 2θ, steps were 0.02° of 2θ and the counting time was 1 s/step. The scanning rate was 2° per min. The current used was 30 mA and the voltage was 40 kV.

Cellular uptake of carvedilol-loaded SLNs

Due to the limitation of *in vitro* methods available to evaluate the lymphatic absorption of carvedilol-loaded SLNs, cellular uptake studies of carvedilol from SLNs as compared to from carvedilol solution and physical mixture of components of SLNs were performed using Caco-2 cell line, which has been reported to be an indirect indication of lymphatic absorption[16,17]. Human excised Caco-2 cell monolayer (HUVECs) was seeded in 48-well plates with complete fresh growth medium until cells were confluence. A stock dispersion of the optimized SLN formulation (FOPT) in DMEM (without serum and antibiotics) was prepared and various dilutions from this stock dispersion were used to study the effect of nanoparticles on uptake by the cells in the DMEM medium. To initiate the experiment, the medium in the wells was replaced with the various concentrations of nanoparticles and incubated for predetermined time. Since it was difficult to measure the number of nanoparticles, carvedilol concentration was used as an indication of nanoparticles uptake by the cells. This is based on the assumption that dilution of the stock dispersion resulted in various concentrations of nanoparticles as well as resulted in various concentrations of carvedilol accordingly. In addition, in order to differentiate the passive absorption of carvedilol from the active uptake of nanoparticles, several control experiments are needed. Therefore, drug solutions and components of optimized formulation as physical mixture (FOPT-PM) with same carvedilol concentrations of that from each respective concentration of nanoparticles in a DMEM media were prepared and evaluated. At the end of the incubation period, the nanoparticle dispersions or solutions containing media were removed from the wells and the cell monolayers were rinsed three times with PBS to remove un-uptaken cells, drug solutions and nanoparticles. For the cell lysate and solubilization, 0.1 mL of 1% triton-X 100 and 0.2 N NaOH in a 1:1 ratio was added to each well. To this, 0.4 mL of methanol was added, centrifuged and samples were analyzed using the previously described HPLC method for carvedilol concentration.

Results and discussion

Selection of lipid using solubility parameter

The value of solubility parameter for the carvedilol was compared with the solubility parameter of various lipid carriers (Table 1). The solubility parameter for carvedilol was found to be 25.38 $MPa^{1/2}$ while for a number of lipids evaluated in this investigation the values ranged between 17.26 $MPa^{1/2}$ (tribehenin) and 21.42 $MPa^{1/2}$ (Compritol ATO 888). It has been reported that miscibility between the drug and carrier increases when the difference in solubility parameters ($\Delta\delta$) between the drug and carrier[11,12] is less than 7 $MPa^{1/2}$. The solubility of the drug in the lipid and hence the entrapment and physical stability of the drug in the SLNs is likely to increase with decrease in $\Delta\delta$. Therefore, Compritol ATO 888 (COMP) having the least value of $\Delta\delta$ (3.96 $MPa^{1/2}$) with carvedilol was chosen as the suitable lipid for further studies.

Design of experiments for blank SLNs

The particle size obtained from 13 blank SLNs formulations (based on fractional factorial design) ranged between 101 ± 14 nm and 9013 ± 4322 nm (Table 2). At 0.625% level of COMP concentration, increasing P-188 concentration from 0.25% (formulation P1) to 5.0% (formulation P2) lead to increase in particle size from 116 ± 4 nm to 1224 ± 433 nm with polymodal distribution in the later case. When the concentration of COMP was increased to 5.0% level (formulations P5 and P6), the desired size range of SLNs (i.e. less than 200 nm) were obtained with both 2.5% and 5.0% concentration of P-188. Further increase in COMP concentration to 7.5%, 12.5% and 15.0% levels (formulations P7 through P13) lead to significant increase in particle size (except formulation P8 containing 7.5% COMP and 2.5% P-188) and did not produce SLNs of desired range irrespective of P-188 concentration used.

To better understand the comparative effect of COMP and P-188 on particle size of SLNs, a surface response curve was plotted (Figure 1). SLNs with larger size were observed when either COMP or P-188 concentrations were at the higher levels. In addition, with suitable concentration combination of COMP and P-188, SLNs having particle size less than 200 nm could be obtained. To further evaluate the quantitative effect of COMP and P-188 as well as their interactions on the particle size of SLNs, an analysis of quadratic model ($p < 0.0001$) was performed and an equation having r^2 value of 0.9989 was derived as below:

$$\begin{aligned}\text{Particle size} = {} & 13.8 + 8.37(\text{COMP}) - 4.52(\text{P-188}) \\ & + 14.05(\text{COMP})^2 + 4.62(\text{P-188})^2 \\ & - 18.38(\text{COMP} * \text{P-188})\end{aligned} \quad (4)$$

The positive coefficients of COMP ($p < 0.0001$) and negative coefficient of P-188 ($p < 0.0001$) indicate that increase in COMP concentration increased particle size whereas increasing P-188 concentration helped to decrease the particle size. Moreover, the curvature (higher order, COMP ($p = 0.0004$) and P-188 ($p = 0.0407$) terms) indicates that increasing P-188 concentration up to a certain level helped to decrease the particle size whereas beyond a certain level, multiple layers started to form followed by increase in particle size. Furthermore, increasing COMP concentration up to a certain level was able to form SLNs with desired size of less than 200 nm whereas beyond a certain concentration, larger particles were obtained. Thus, the combination of COMP and P-188 concentrations ($p < 0.0001$) appears to play a crucial role on the particles size of SLNs produced.

Design of experiments for carvedilol-loaded SLNs

The results obtained from the particle size analysis of blank SLNs helped to understand the effects of COMP and P-188 on the particle size of SLNs. To predict the particle size for selecting the suitable concentration ranges of COMP and P-188 before incorporating the drug, Equation (4) was applied and the results are tabulated in Table 4. It can be seen from Table 4 that below the 2.5% level of COMP, the predicted particle size would be less than 200 nm as long as the concentration of P-188 was less than 1.5%. However, at low concentration of COMP, the drug loading would be expected to be less. Further increase in COMP concentration (above 7.5%) predicted particle size was larger

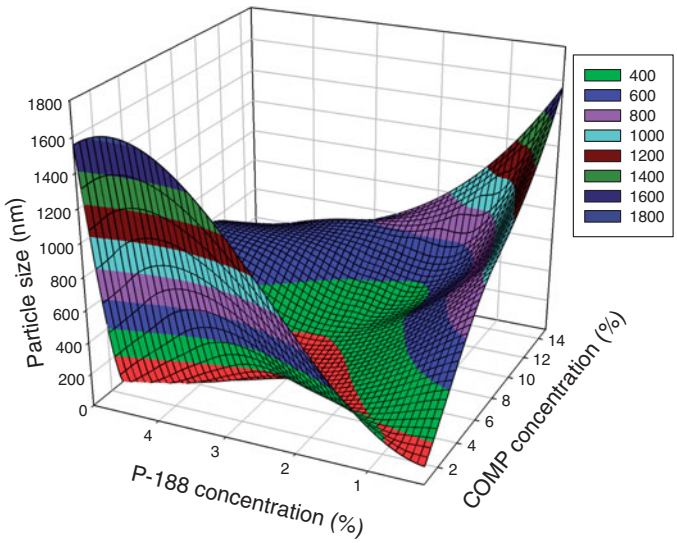

Figure 1. Response surface curve illustrating the effects of COMP and P-188 concentrations on particle size of blank SLNs.

Table 4. The selected concentration ranges of Compritol 888 ATO (COMP) and Poloxamer 188 (P-188) for the fabrication of carvedilol-loaded SLNs and their respective predicted particle size determined from the equation obtained in design of experiments for blank solid lipid nanoparticles.

COMP (% w/v)	P-188 (% w/v)	Predicted particle size (nm)
1.25	0.25	105
1.25	1.0	136
1.25	1.5	172
1.25	5.0	1207
2.5	0.25	118
2.5	1.0	126
2.5	1.5	145
2.5	5.0	295
5.0	0.25	214
5.0	1.0	169
5.0	1.5	155
5.0	5.0	154
5.0	7.5	1414
7.5	0.25	475
7.5	1.0	330
7.5	1.5	264
7.5	5.0	206
7.5	7.5	638
7.5	10.0	2155
12.5	0.25	2149
12.5	1.0	1497
12.5	1.5	1162
12.5	5.0	279
15.0	0.25	4062
15.0	1.0	2921
15.0	1.5	1162
15.0	5.0	322

than 200 nm in all the cases. Thus, the concentration range of COMP was selected from 5.0% to 7.5%. At this selected concentration range of COMP, the size of SLNs was predicted to be above 200 nm when concentration of P-188 was at low levels (i.e. 0.25% and 1.0%). Based on the approach of predicted particle size, selected concentration ranges for COMP and P-188 were 5.0% to 7.5% and 1.5% to 5.0%, respectively, in order to obtain the desired size of SLNs. However, after the loading of the drug onto the SLNs, the particle size of drug-loaded SLNs might increase further. Thus, the concentration range was selected such that, in the absence of drug, SLNs would be predicted to be less than

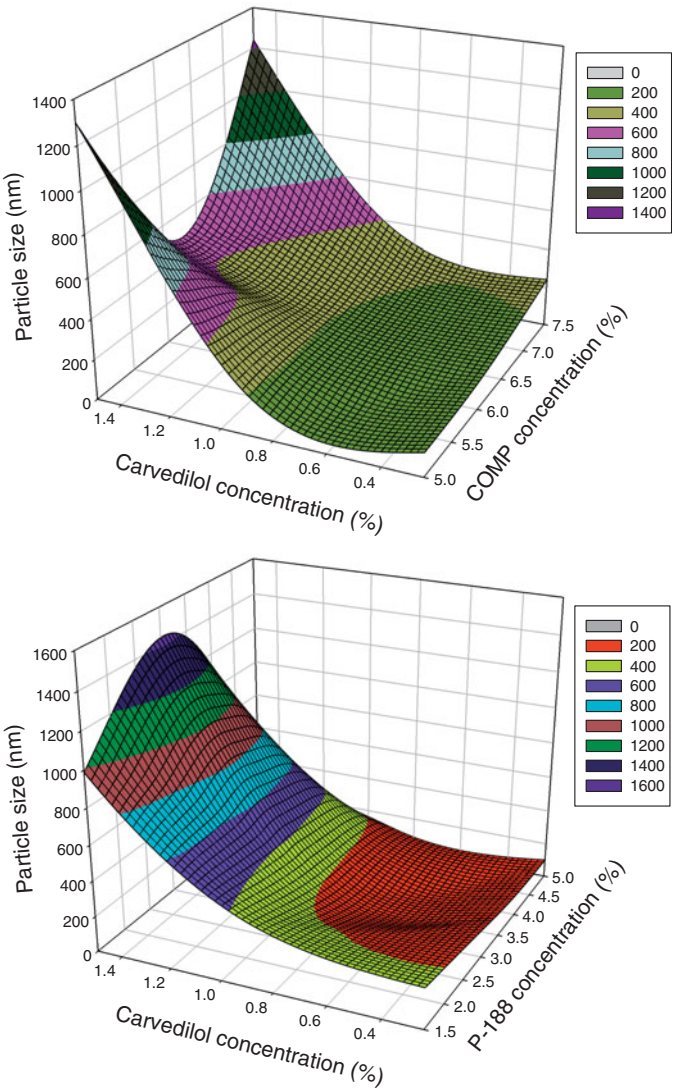

Figure 2. Response surface curve demonstrating the effects of COMP, P-188 and carvedilol concentrations on particle size of carvedilol-loaded SLNs.

200 nm. During the loading of drug onto the SLNs, drug concentration would be the only factor affecting the particle size and entrapment efficiency of drug.

Based on the application of Equation (4) and the predicted values of the particle size obtained, the selected concentration ranges of COMP and P-188 were able to minimize the number of formulations when an additional formulation variable (carvedilol concentration) was included for the fabrication of carvedilol-loaded SLNs. Therefore, a total of 27 formulations were derived and shown in Table 3 and subjected to the characterization of particle size and entrapment efficiency.

Characterization of carvedilol-loaded SLNs

Carvedilol-loaded SLNs were characterized by particle size, polydispersity index and entrapment efficiency.

Particle size

Particle size and polydispersity index (PI) of the 27 formulations obtained from the full factorial design are shown in Table 3. The magnitude of effect of the variables in the form of response surface curve is shown in Figure 2. The results indicate that the particle sizes of prepared formulations ranged from as low as 97 ± 16 nm (formulation F7) to as high as 2486 ± 83 nm

(formulation F6). It was observed that when COMP and P-188 concentrations were kept constant, increasing carvedilol concentration from 0.25% to 1.5% lead to significant increase in particle size.

Following the same approach of analyzing the particle size of blank SLNs, the quantitative effect of COMP, P-188, and carvedilol concentrations as well as their interactions on the particle size of carvedilol-loaded SLNs by means of a quadratic model ($p < 0.0001$) was evaluated and an equation having an r^2 value of 0.8985 was derived as below:

$$\begin{aligned}(\text{Particle size}) = {} & 5.32 - 0.48(\text{P-188}) \\ & + 0.64(\text{Carvedilol}) + 0.26(\text{Carvedilol})^2 \\ & - 0.26(\text{COMP} * \text{Carvedilol})\end{aligned} \quad (5)$$

The absence of the COMP term in Equation (5) confirms that the selected concentration range of COMP was appropriate. It also indicates that COMP did not affect directly the size of the carvedilol-loaded SLNs and the selected concentration range of COMP was able to produce desired size range of carvedilol-loaded SLNs. In addition, the negative coefficient value for P-188 variable ($p < 0.0001$) suggests that increase in surfactant concentration lead to decrease in particle size, which is also in agreement with the previous results obtained. For the carvedilol variable included in Equation (5), as the concentration of carvedilol increases, the particle size of carvedilol-loaded SLNs was observed to increase as the positive and highest coefficient value of carvedilol variable ($p < 0.0001$) obtained. This type of phenomenon has also been observed by several other researchers which could be because of increased entrapment of the carvedilol[18]. For instance, at 5% of COMP and 1.5% of P-188, increasing carvedilol concentration from 0.25% to 1.5% increased the particles size from 146 ± 9 nm to 1319 ± 46 nm (formulations F1, F2 and F3). The same trend was observed for the 6.3% and 7.5% COMP (formulations F10 to F27). At 0.25% of the carvedilol concentration, all the formulations were able to produce carvedilol-loaded SLNs below 200 nm except formulations F10 and F19 where P-188 concentration was insufficient to stabilize and fully cover the particles. In case of formulations F2, F11, F14, F20 and F23 where carvedilol concentration is 0.88%, the obtained carvedilol-loaded SLNs were larger than 200 nm due to insufficient surfactant concentration with respect to the lipid concentration. A further increase in carvedilol concentration to 1.5% level lead to carvedilol-loaded SLNs larger than 200 nm in all the cases. Apparently, higher carvedilol concentration resulted in greater entrapment which resulted in increased particle size.

Entrapment efficiency

The results of the entrapment efficiency of the 27 formulations obtained from the full factorial design are shown in Table 3. The magnitude of the effect of the variables was plotted using response surface curve and is shown in Figure 3. The entrapment efficiency of carvedilol-loaded SLNs ranged between $52.1 \pm 4.1\%$ (formulation F22) and $95.6 \pm 2.3\%$ (formulation F15). Keeping P-188 and carvedilol concentrations constant, increase in COMP concentration led to increase in entrapment efficiency due to lipophilic nature of the COMP. For example, at 5% P-188 level, increase in COMP concentration from 5% to 7.5% lead to increase in entrapment efficiency from $86.4 \pm 6.4\%$ (formulation F8) to $94.8 \pm 2.3\%$ (formulation F26). Entrapment efficiency also increased when carvedilol concentration was increased from 0.25% to 1.5%. On the other hand, entrapment efficiency decreased with increase in P-188 concentration due to increased

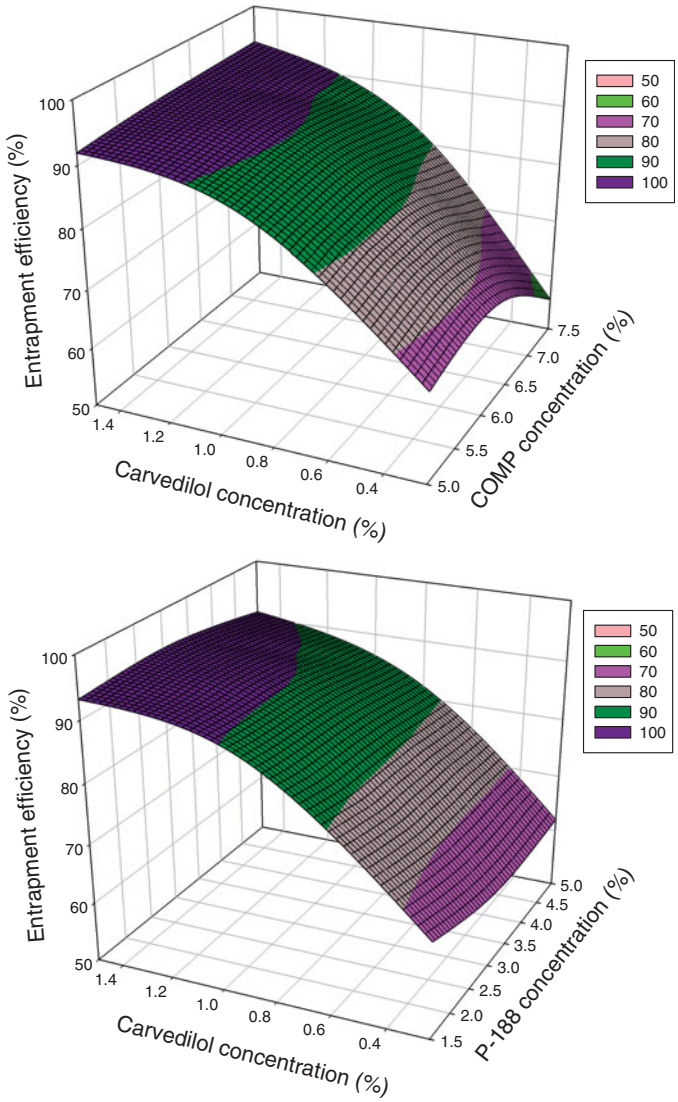

Figure 3. Response surface curve demonstrating the effects of COMP, P-188 and carvedilol concentrations on entrapment efficiency of carvedilol-loaded SLNs.

solubility of carvedilol in hot aqueous surfactant (i.e. P-188) solution, thus resulting in the loss of entrapment during fabrication of SLNs stage.

Following the same approach of analyzing the particle size of carvedilol-loaded SLNs, the quantitative effect of COMP, P-188, and carvedilol concentrations as well as their interactions on the entrapment efficiency of carvedilol-loaded SLNs using a quadratic model ($p < 0.0001$) was evaluated and the following equation having an r^2 value of 0.9557 was derived:

$$\begin{aligned}(\text{Entrapment efficiency}) = {} & 7796.1 + 2321.17(\text{Carvedilol}) \\ & - 1485.06(\text{Carvedilol})^2 \\ & + 352.67(\text{COMP} * \text{P-188}) \\ & + 350.83(\text{COMP} * \text{Carvedilol})\end{aligned} \quad (6)$$

Based on the results from Equation (6), at the selected concentration ranges of COMP, P-188, and carvedilol, carvedilol ($p < 0.0001$) as well as the interaction of COMP with P-188 ($p = 0.014$) and carvedilol ($p = 0.0151$), respectively, were the formulation variables affecting the entrapment efficiency of carvedilol-loaded SLNs. The positive coefficient value of carvedilol variable confirmed the assumption that incorporation of more drug leads to more entrapment because of lipophilic

nature of carrier (i.e. COMP) which can accommodate more drug. It was expected that increase in lipid concentration will lead to increase in entrapment due to decreased tendency of the drug migration into the aqueous phase. At higher lipid concentration, it will provide more space to accommodate the drug[19]. However, the off trend was observed in the case of 1.5% of P-188 where increasing lipid concentration did not increase entrapment efficiency. This may have been due to insufficient surfactant concentration which may not have been able to form a stable nanoparticulate system causing poor entrapment.

Constrained optimization of carvedilol-loaded SLNs

From the selected concentration ranges of COMP, P-188 and carvedilol, many of the 27 formulations generated from the full factorial design showed particle size of carvedilol-loaded SLNs below 200 nm. However, to further identify an optimal formulation, a constrained optimization technique was applied to generate the optimum setting for the carvedilol-loaded SLNs. From the constrained optimization, an optimized formulation (FOPT) with the composition of 7.5%, 5.0% and 1.11% of COMP, P-188 and carvedilol, respectively, was obtained. Based on the optimization approach, the theoretically predicted values for particle size and entrapment efficiency of optimized formulation were expected to be 151 nm and 94.6%, respectively. The experimentally obtained values of response parameters for this optimized formulation were 161 nm and 94.8% of entrapment efficiency which are in close agreement with the predicted values, suggesting the feasibility of this optimization approach.

In vitro release study of carvedilol-loaded SLNs

The *in vitro* release profiles of the selected carvedilol-loaded SLN formulations (Table 3) as well as the optimized formulation and carvedilol solution in phosphate buffer solution (pH 6.8) are shown in Figure 4. Carvedilol solution showed rapid and complete distribution from inside to outside of the dialysis bag within 1 h confirming that the dialysis bag functioned as a filter and was not a barrier of drug distribution. Entrapping carvedilol into a lipid by SLNs formulations were able to retard the drug release. Furthermore, the differences in release profiles of carvedilol from various SLNs formulations were due to differences in concentrations of COMP, P-188 and carvedilol as formulation variables used to fabricate the carvedilol-loaded SLNs and thereby their outcomes of particle size and entrapment resulted from the differences of formulation variables. It was found that increasing carvedilol concentration from 0.25% (formulations F1, F7, F19 and F25) to 1.5% (formulations F3, F9, F21 and F27) lead to significant decrease in drug release rate. At low carvedilol concentration, about 60% to 100% drug was released at the end of 24 h whereas less than 40% of drug release was obtained when carvedilol concentration was increased from 0.25% to 1.5%. The decreased drug release could be attributed to the increased particle size and high entrapment attributed from higher carvedilol concentration used. However, keeping drug concentration and COMP concentration constant, increasing surfactant concentration led to increase in drug release. At 0.25% carvedilol concentration, when 1.5% of P-188 concentration (formulations F1 and F19) was used, about 70% drug was released at the end of 24 h whereas drug release increased to 100% when P-188 concentration was increased to 5.0% (formulations F7 and F25). Similar findings were obtained when carvedilol concentration was at 1.5% level (formulations F3 and F21 at 1.5% P-188 level as well as formulations F9 and F27 at 5.0% P-188 level). As expected, the optimized formulation was able to retard drug release and prolong the release over a period of 24 h. This may have been due to decreased particle size followed by increased surface area and decreased diffusion path length. In addition, entrapment was observed to decrease with increase in P-188 concentration and thus free drug could have played a role in increased drug release. On the other hand, drug release was observed to decrease when carvedilol and P-188 concentrations were kept constant and COMP concentration was increased from 5% to 7.5%.

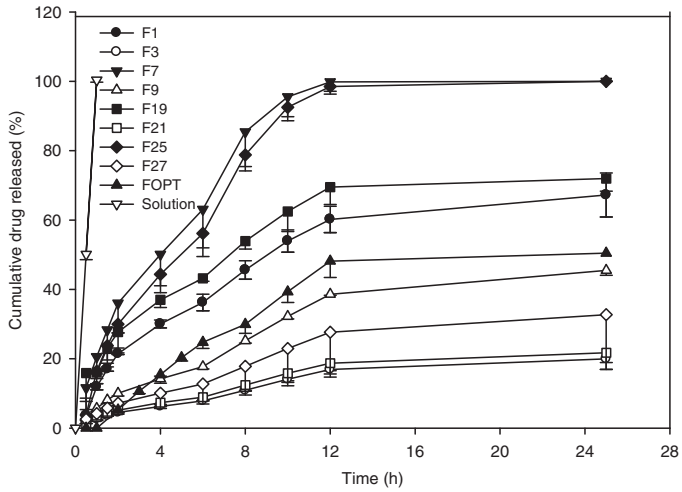

Figure 4. The *in vitro* release profiles of the selected formulations of carvedilol-loaded SLNs together with the optimized formulation (FOPT) as well as carvedilol solution as a control in phosphate buffer solution (pH 6.8) by dialysis membrane diffusion technique (data shown as mean ± standard deviation, $n = 3$).

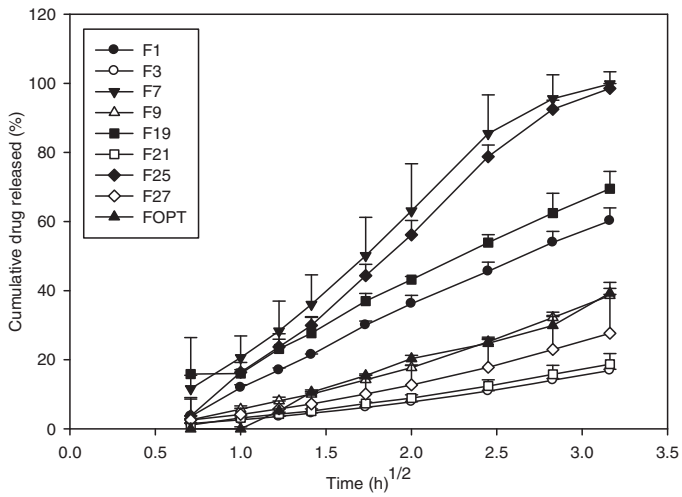

Figure 5. Comparative relationships of cumulative amount of carvedilol released as a function of the square root of time from the selected formulations of carvedilol-loaded SLNs together with the optimized formulation (FOPT) in phosphate buffer solution (pH 6.8) by dialysis membrane diffusion technique (data shown as mean ± standard deviation, $n = 3$).

In order to better understand the release mechanism of drug from SLNs formulations, the release profiles displayed in Figure 4 was plotted as cumulative drug released versus square root of time and shown in Figure 5. A linear relationship with regression value of 0.98 or greater was obtained in all selected carvedilol-loaded SLNs studied, thereby confirming the diffusion model for release mechanism from matrix type of delivery systems. In the present investigation, hot homogenization method was used to fabricate carvedilol-loaded SLNs suggesting the possible model could be combination of drug-enriched surface and solid solution where drug is molecularly dispersed in the lipid matrix[10].

Complete drug release from SLN formulations was obtained in an acidic medium (0.1 N HCl, pH 1.2) within 2 h of study due to the weakly basic nature of the drug and smaller particle size. This finding indicates a need to protect SLNs from the stomach if improved bioavailability has to be achieved by active absorption from the Peyer's patches located particularly in the distal ileum of the intestinal tract. Therefore, the *in vitro* release profiles obtained from the optimized formulation as a liquid dispersion, as well as from other solid dosage forms such as lyophilized powder (L-SLNs), compressed core tablet, and enteric coated tablet having the same formulation components as optimized formulation were compared (Figure 6). All dosage forms were studied in PBS (pH 6.8) as release medium for 12 h except that the enteric coated tablet was kept in 0.1 N HCl (pH 1.2) for 2 h and then placing it in PBS (pH 6.8) for 10 h. Interestingly, similar release pattern was observed with lyophilized SLNs and un-lyophilized SLNs (i.e. optimized formulation). However, higher drug release rate was observed from lyophilized L-SLNs as compared to un-lyophilized SLNs. The possible reason could be attributed to the effect of cryoprotectant (trehalose) and possible structural change that might have occurred during lyophilization process. In addition, similar drug release profiles were obtained from L-SLNs, compressed core tablet and enteric coated tablet confirming the ability of prepared tablets to exhibit the same release kinetics as L-SLNs. Furthermore, prolonged controlled release profiles of carvedilol over the period of 12 h from these solid dosage forms were obtained. Therefore, it can be concluded that the carvedilol-loaded SLNs based enteric-coated tablet was able to protect the carvedilol from the acidic environment of the stomach and drug release was retarded before the uptake of carvedilol-loaded SLNs from small intestine region.

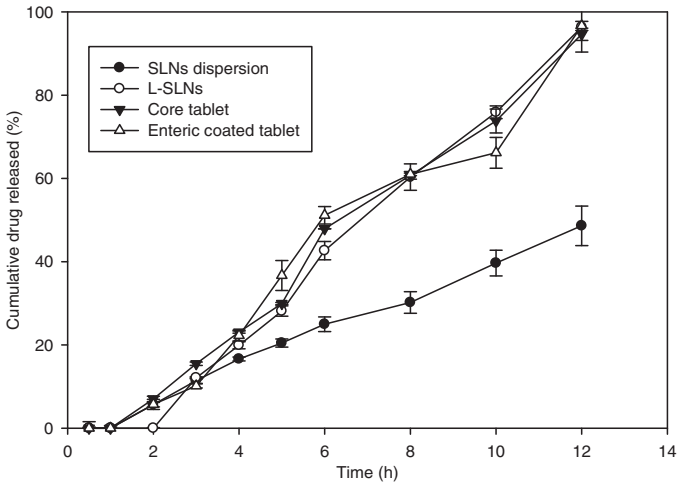

Figure 6. Comparison of the *in vitro* release profiles obtained from the optimized formulation as a liquid dispersion form as well as from other type of solid dosage forms such as lyophilized powder (L-SLNs), compress core tablet, and enteric-coated tablet having the same formulation components as optimized formulation (data shown as mean ± standard deviation, $n = 3$).

Powder X-ray diffraction

X-ray diffraction patterns of carvedilol, COMP, P-188, physical mixture of FOPT and lyophilized SLNs of FOPT are shown in Figure 7. Carvedilol showed characteristic crystalline peaks from 15° to 30°, with the peak at 24° being the most prominent with intensity of 6 000 cps. COMP showed crystalline peak at 21° with intensity of 12 400 cps whereas PLX 188 (which is semi-crystalline in nature) showed peaks at 19° and 23.5° of 2θ. In the case of FOPT-based physical mixture, similar peaks were observed but with lesser intensity. When carvedilol was incorporated into SLNs, absence of crystalline peak for carvedilol and decrease in the peak of COPM and P-188 indicates conversion of crystalline carvedilol to amorphous form in the SLNs.

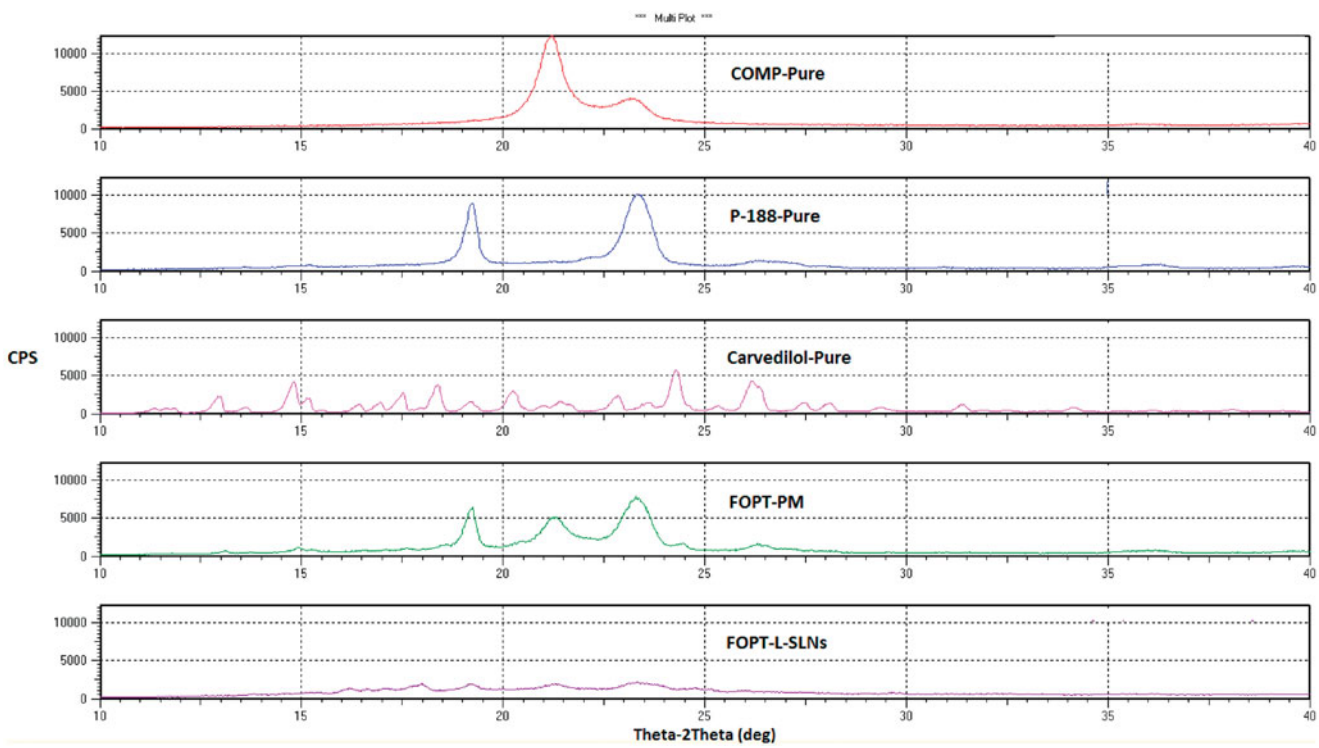

Figure 7. X-ray diffraction pattern of pure, COMP, P-188, carvedilol and optimized formulation (FOPT)-based physical mixture as well as its lyophilized SLNs.

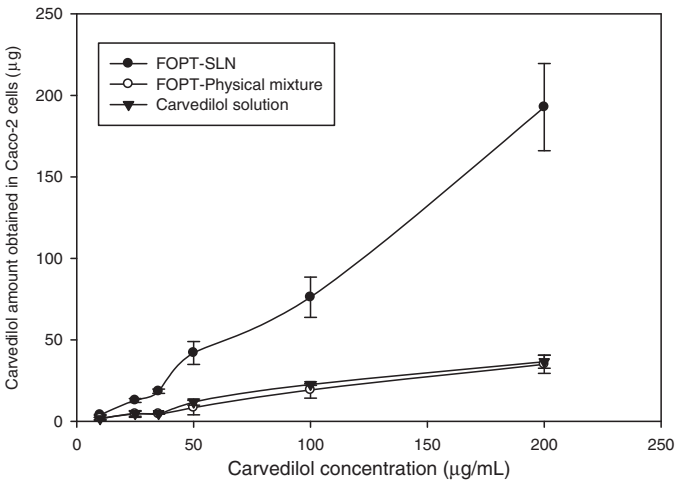

Figure 8. The cellular uptake mechanism of nanoparticles from the optimized formulation (FOPT) of SLNs together with their physical mixtures and carvedilol solutions as control groups by *in vitro* model using Caco-2 cell line (data shown as mean ± standard deviation, $n = 3$).

Cellular uptake of carvedilol-loaded SLNs

Higher amount of carvedilol uptake were found in all cases of FOPTs as compared to the drug solutions and physical mixtures of FOPT, irrespective of carvedilol concentrations studied (Figure 8). The uptake by Caco-2 was found to be dependent on the concentration of carvedilol and hence the concentration of nanoparticles in the medium. It was observed that when the concentration of carvedilol was increased from 10 µg/mL to 200 µg/mL, the amount of drug uptake increased from carvedilol solutions and physical mixture of FOPTs. This finding indicates a characteristic of passive transport of drug molecules from carvedilol solutions and physical mixture of FOPTs. The similar higher cellular uptake of drug from drug-loaded SLNs as compared to the drug solution has been reported[20,21]. The higher amount of carvedilol from FOPTs as compared to the carvedilol solution and physical mixture of FOPTs suggested the crucial role of SLNs as a carrier for preferential cellular uptake through Caco-2 cells.

Caco-2 cells have been widely developed, used and accepted tool for the investigation of transport across the small intestinal epithelium of many substances[22]. The direct relationship of Caco-2 cell line with the lymphatic absorption has not been evaluated to the best of our knowledge. However, Caco-2 cell line has been used to determine the effect of lipid-based excipients as formulation variable for the lymphatic delivery of therapeutic agents. Amongst the lipidic excipients evaluated, the long-chain fatty acids forms lipoprotein assembly by associating with intestinal lipoproteins in the enterocytes and thereby facilitates the lymphatic transport of therapeutic agents[16,17,23–25]. Thus, Caco-2 cell line could have a potential application as screening tool for lipid-based formulations targeted to lymphatic absorption. The long-chain fatty acid (COMP) was used in the present investigation for the preparation of FOPTs. Therefore, FOPTs, after the cellular uptake, association of COMP with the intestinal lipoprotein might have potential to promote the lymphatic absorption of carvedilol.

Conclusion

Solubility parameter was found to be a useful tool for the selection of lipid to fabricate SLNs. Design of experiments with blank SLNs helped to understand the interaction of formulation variables and minimized the number of experiments with the drug. Carvedilol-loaded SLNs with minimal particle size and high entrapment was successfully developed using statistical optimization. The *in vitro* drug release study ensured the retarded release of carvedilol in the gastrointestinal tract before the cellular uptake of SLNs. Followed by cellular uptake from small intestinal region, carvedilol-loaded SLNs could have potential to gain access to the lymphatic system which will avoid the first pass metabolism and hence higher bioavailability.

Acknowledgements

The authors acknowledge St. John's University for providing financial assistance and research facilities to carry out this research.

Declaration of interest

The authors declare no conflict of interest (monetary or otherwise) in conducting this research. The authors alone are responsible for the content and writing of the paper.

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