International Journal for Holistic Research, Vol. 2, No. 2. Jan 2025

OPEN ACCESS

Print ISSN: 3009-6898
Online ISSN: 3009-6790

Design and Evaluation of Carvedilol Ethosomes using Box-Behnken Design

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Article history: Received: 20-03-2024 Revised: 24-03-2024 Accepted: 17-10-2024

Abstract: Carvedilol is a third-generation non-selective beta blocker commonly used in the long term management of hypertension in elderly people. Carvedilol is superior to older generation beta blockers in avoidance of fatigue due to low cerebral and cardiac blood flow because unlike other beta blockers, it works by decreasing peripheral resistance without any change in cardiac output. As the case with many beta blockers, carvedilol undergoes significant metabolism by liver enzymes, which leads to deficient oral bioavailability and the necessity of multiple dosing per day.

Carvedilol has been formulated as ethosomal vesicles using the cold method based on a four-factor three-level box-Behnken experimental design. The ethosomes were evaluated for vesicle size (Y1) and entrapment efficiency % (Y2). The effect of X1: phospholipid concentration, X2: Carvedilol concentration, X3: ethanol concentration, and X4: sonication time on Y1 and Y2 was evaluated and analysed using contour plots and second order polynomial equations.

The vesicle size ranged between 46.75 ± 8.0 nm and 259.3 ± 8.02nm and the entrapment efficiency % ranged between 86 % and 97 % for F7 and F23 respectively. Vesicle size (Y1) increased as the phospholipid concentration increased and sonication time decreased, yet the ethanol and Carvedilol concentrations gave concave curves with inflection points. There was an inverse relation between phospholipid concentration and EE%, but a positive effect (between Carvedilol concentration and EE% was observed.

Keywords: Box-Behnken Design, Carvedilol, Ethosomes, Transdermal system

1. Introduction

Cardiovascular diseases (CVD) are the most popular cause of mortality in industrialized countries, and hypertension is the most prevalent risk factor for cardiovascular diseases[1-3]. Patients suffering from hypertension require long-term treatment with a controlled-release dosage form. Most of blood pressure lowering drugs suffer from first-pass metabolism by liver enzymes, which leads to their poor bioavailability via the oral route. Due to extensive hepatic metabolism, antihypertensive drugs require multiple daily doses. Hence, such drugs are considered to be promising candidates for the development of transdermal formulations[4]. Carvedilol (CAR), is an anti-hypertensive drug, commonly used in the management of numerous cardiovascular diseases like cardiac arrhythmias, congestive heart failure, angina pectoris and hypertension [5]. Due to major hepatic metabolism and poor oral solubility, Carvedilol suffers from deficient oral bioavailablity, ranging from 25% to 35% [6, 7].Carvedilol may produce undesired cardiovascular adverse effects such as bradycardia and hypotension when taken orally[8]. CAR is a low molecular weight drug molecule (406.5) with high lipophilic profile and a favourable logarithmic partition coefficient (log p value 3.8). These physical properties make it ideal for percutaneous distribution[8]. Transdermal drug delivery (TDD) has a number of advantages over oral administration, including avoiding first-pass metabolism and gastrointestinal problems, lowering dosage frequency, and better control of plasma [1]levels[9]. Some pharmaceutical approaches such as liposomes, self-microemulsifying preparation, and solid lipid nanoparticles have been used to avoid the first pass effect of Carvedilol and to improve its oral bioavailability[10, 11]. Several routes including pulmonary, intranasal, and transdermal routes were also developed to bypass the extensive first-pass metabolism of Carvedilol[12-14].The innovation of transdermal deformable vesicular carriers, such as ethosomes, has piqued interest in transdermal medication administration in recent years. Ethosomes are phospholipid bilayer vesicles with rather high ethanol content (from 20 to 45 %)[15]. Ethosomes differ from liposomes in that they have a higher alcohol concentration. Drug percutaneous penetration is aided by ethosomes, and the phospholipid plays a role as well. Ethosomes have small size (nm to microns), a stable structure, and a high entrapment efficiency, allowing medications to be delayed. As a result, when compared to typical liposomes, ethosomes can transport drugs far deeper into the skin or directly into the blood circulation, thus improving drug transdermal permeation efficacy. The method by which ethosomes increase permeability is linked to their shape and high alcohol concentration. The increased mobility and fluidity

Cite this article: Maha M. Marzouk, et. al." Design and Evaluation of Carvedilol Ethosomes using Box-Behnken Design ", International Journal for Holistic Research, Vol. 2, No. 2. Jan 2025. DOI: 10.21608/ijhr.2024.278228.1006

of polar lipid molecules within the lipid membrane is due to the high quantity of alcohol. Pharmaceutical chemists were frequently faced with the task of determining the optimal mix of process and formulation variables that influenced product quality. The formulator can quickly comprehend the relationship between the variables and the experiment's outcome using Box–Behnken experimental design (BBD)[16].BBD provides various advantages, including requiring fewer experimental runs while using fewer resources, identifying interactions between variables, and constructing model equations for optimisation and prediction of a particular system's behaviour. The goal of this study was to create and analyse ethosomes containing Carvedilol for transdermal distribution in order to prevent substantial first-pass metabolism, which would result in increased bioavailability and fewer adverse effects.

2. Materials:

Carvedilol(CAR) was received as a gift sample from Global Napi for pharmaceutical industries (6th of October, Giza, Egypt). Phospholipon 90G (PL90G) was provided as a gift sample from Lipoid, GmbH, Ludwigshafen, Germany. Ethanol HPLC and Methanol HPLC were purchased from Fisher Scientific Co., UK. Potassium dihydrogen phosphate, disodium hydrogen phosphate were purchased from El Gomhouria Co., Kasr El Ainy, Cairo. All of the studies were conducted with double-distilled pyrogen-free water. Methylcellulose membrane, (molecular weight cut off =14,000) was procured from Sigma- Aldrich., USA.

3. Methods:

3. 1. Box-Behnken Experimental Design (BBD)

An experimental design software (Design Expert software, version 7 Minmeapolis, USA) was utilized to investigate the effects of independent formulation and processing variables (factors, X) on selected dependent variables (responses, Y). A three-level, four-factors BBD was generated to investigate the main effects, interaction effects and quadratic effects of factors X1: phospholipid concentration, X2: Carvedilol (mg); X3: Ethanol (%); X4: sonication time (min.) on the following responses: (Y1= vesicle size in nm) and (Y2=entrapment efficiency as %) and obtain the optimal levels of the four factors. The low, middle, and high levels of each variable were designated as -, 0, and + respectively. The following constrains were applied: Y1 – Minimize; Y2 – Maximize. The factors, levels and the measured vesicle size and entrapment efficiency are given in Table 1.

3.2. Preparation of CAR-loaded ethosomes

Carvedilol ethosomal formulations (CEFs) were prepared by classical cold method as previously mentioned elsewhere[17, 18]. Both phospholipid and Carvedilol were dissolved in ethanol in a well-sealed container. To summarise, distilled water was gently introduced drop by drop to the ethanolic solution, with steady stirring at 700 rpm by a magnetic stirrer (WiseStir, Korea). After complete water injection, stirring was continued for additional 5 minutes to guarantee homogeneity of the preparation. Throughout the process, the system was kept at 30°C[19]. To obtain large multilamellar vesicles (LMLV), the produced ethosomal dispersions were permitted to swell overnight at 4°C. LMLVs were probe sonicated (Model-Q125, Newtown, CT, USA) at 4 ºC in an ice bath at 40% output frequency to prepare smaller vesicles (at 40 W). The empty (blank) ethosomal dispersions were obtained as described above without adding any drug during the preparation process.

All the formulations were prepared according to BB experimental design as per the directions given in Table 2

Table 1: A four-factor, 3- levels Box-Behnken design along with the responses and the constraints

Independent variables (factors)	Levels, actual (coded)		
	Low (-1)	Medium (0)	High (+1)
X1: PL 90 G (g)	1	2	3
X2: drug (mg)	35	50	65
X3: ethanol (%v/v)	20	30	40
X4: sonication time (min.)	1	2	3
Dependent variables (responses)	**Constraints (Goals)**		
Y1: vesicle size (nm)	Minimize		
Y2: Entrapment Efficiency (EE %)	Maximize		

3.3. Characterization of CAR -loaded ethosomes:

3.3.1. Determination of vesicle size and polydispersity index:

Measurement of vesicle size (VS) and polydispersity index (PDI) was done by Dynamic light scattering using a Zetasizer Nano ZS90 (Malvern Instruments, UK). To avoid multi scattering, the dispersions were appropriately diluted with distilled water. From each dispersion, three separate samples were obtained[19].

3.3.2. Determination of CAR entrapment efficiency:

The entrapment efficiency (EE %) of ethosomes was measured by means of a cooling ultracentrifuge (Cooling Centrifuge, stratos centrifuge, Maximum 22,000rpm, Germany). First, accurate volume of each formulation (about 2 ml) was taken in 2- mL eppendorf tube which was centrifugated via a cooling centrifuge at 4° C at 20,000 rpm for 1 hour. The supernatant was separated, filtered with a 0.2 µm millipore membrane filter and diluted with 30% hydroethanolic solution. The unentrapped drug in the diluted supernatant was assayed spectrophotometrically at 241 nm[4, 20].

The % entrapment was calculated using the following formula[21]:

$$Percentage\ entrapment\ (EE\%) = \frac{(Total\ drug\ content - free\ drug\ content)}{Total\ drug\ content} * 100\ (1)$$

Table 2: Compositions of the 27 formulations of Carvedilol ethosomes in the Box Behnken design

Formulation Code	X1: PL 90 G (g)	X2: CAR (mg)	X3: Ethanol (% v/v)	X4: Sonication time (min.)
F1	2.0	50.0	40.0	1.0
F2	3.0	65.0	30.0	2.0
F3	1.0	50.0	30.0	1.0
F4	1.0	65.0	30.0	2.0
F5	2.0	35.0	30.0	3.0
F6	2.0	50.0	30.0	2.0
F7	3.0	50.0	30.0	1.0
F8	2.0	50.0	30.0	2.0
F9	1.0	35.0	30.0	2.0
F10	2.0	35.0	20.0	2.0
F11	1.0	50.0	40.0	2.0
F12	2.0	50.0	20.0	1.0
F13	2.0	50.0	20.0	3.0
F14	3.0	50.0	30.0	3.0
F15	2.0	65.0	30.0	3.0
F16	3.0	50.0	20.0	2.0
F17	2.0	50.0	30.0	2.0
F18	2.0	35.0	40.0	2.0
F19	1.0	50.0	20.0	2.0
F20	3.0	35.0	30.0	2.0
F21	2.0	65.0	30.0	1.0
F22	2.0	65.0	20.0	2.0
F23	1.0	50.0	30.0	3.0
F24	3.0	50.0	40.0	2.0
F25	2.0	65.0	40.0	2.0
F26	2.0	35.0	30.0	1.0
F27	2.0	50.0	40.0	3.0
F optimized	1.0	52.9	29.5	1.0

3.3.3. Dissolution *(in vitro release)* studies of CAR - loaded ethosomes

The in vitro release profiles of CAR from six ethosomal dispersions which had highest desirability values namely; F 1, F 3, F 10, F 12, F optimized , F 23 and CAR hydroethanolic solution were done using the dialysis bag method [19].

The dialysis bags (molecular weight cut off 14000, Sigma-Aldrich) were filled with drug-loaded vesicles containing (1 mg) Carvedilol and were sealed from both sides to prevent leaking. To preserve the pH of the skin, the sealed bags were suspended in 100 ml screw-capped glass jars filled with 1% (v/v) methanolic PBS pH5.5 [22].The experiment was carried out in a thermo-controlled shaking water bath (Memmert, 2000) at 100 rpm and 32 0.5° C to maintain temperature of skin.

At predetermined time intervals (1, 2, 3, 4, 5, 6, 8 and 24h), 2 mL samples were taken, replenished with same volume of fresh release medium[8]. After suitable dilution, the amount of drug in the withdrawn samples was assayed with a UV spectrophotometer (Shimadzu, 2401/PC, Japan) at 241 nm. The release profile of plain CAR suspension, suspended in distilled water, having 1 mg CAR, was done for comparison. The cumulative percentage of released drug was plotted against time. The kinetic parameters for the *in vitro* data were determined at the end of the experiment in order to estimate the best fit to a different kinetic model (zero, first order, or Higuchi model) in order to establish the release mechanism of Carvedilol from the selected ethosomes.

The Correlation Coefficient Parameter (R) was used to determine the right mode of release, with the highest correlation coefficient being the actual mode of release[22]. The experiment was run in triplicate. The formulation with the highest entrapment efficiency and highest release was selected for further studies.

3.3.4. Drug excipients interaction studies by Fourier Transform Infrared Spectroscopy:

Infrared spectra of pure Carvedilol, physical mixture of drug: phospholipon 90G and CAR-loaded ethosomes were scanned from 4000 to 400 cm^{-10} by FTIR (Perkin Elmer, USA). Dried potassium bromide disks were used in scanning of the FTIR spectra[22].

4. Results and Discussion:

4.1. Particle size and entrapment efficiency

The mean vesicle size of the current ethosomes was 110.39 ± 2.70 nm (n=27) and the mean poly dispersity index was 0.426 ± 0.177 (n=27). As clearly seen in Table 3 and Figure 1a,b, the particle sizes of the 27 formulations were in the nanosize range and varied between 46.75 ± 8.0 and 259.3 ± 8.02 nm. Previously published literature mentioned that this particle size range is favourable for transdermal delivery[23].

Generally, vesicles with a diameter of 300 nm or below are favourable for percutaneous delivery of their contents to the deeper skin layers[23]. Biodegradable lipid based nanovesicles like ethosomes have been reported for the encapsulation of large number of therapeutic molecules such as lamivudine, trihexyphenidyl and ligustrazine [17, 24, 25].

Determination of the encapsulation parameters, particularly the EE% for nanovesicle formulations is of extreme importance for evaluating the therapeutic effectiveness and delivery potentiality of the drug delivery system[26]. The entrapment efficiency of the ethosomes

was determined in an attempt to investigate the impact of ethosomal components, i.e., the amount of Carvedilol, phospholipid and ethanol on the entrapment capacity of the ethosomal vesicles.

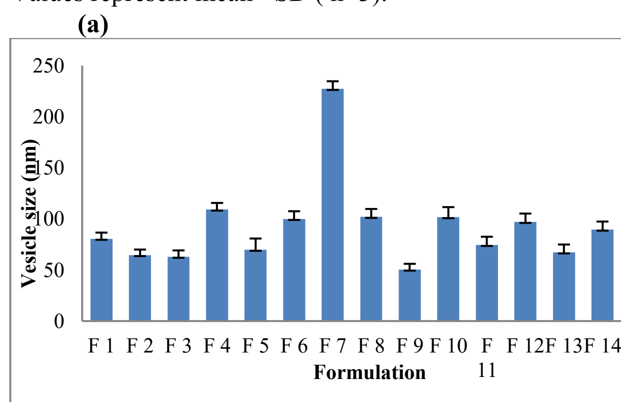
It is clearly illustrated in table 3 and Figure 2a & b, that all the formulations exhibited excellent entrapment efficiency which ranged between 86 % and 97 % for F 7 and F23 respectively. The mean entrapment efficiency was 92.17%.

Table 3: The observed responses of the 27 formulations

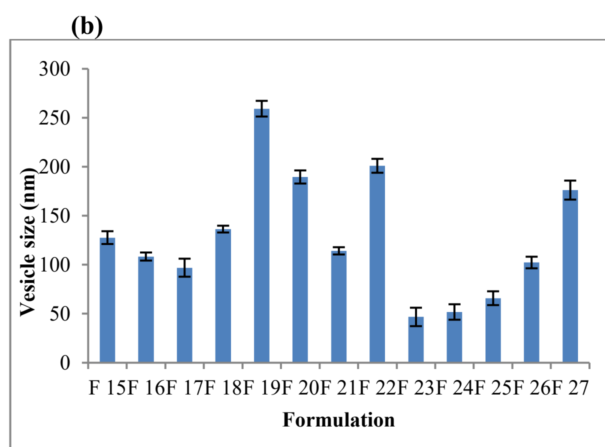
Formulation Code	VS (nm)*	EE%*	PDI*
F1	80.55 ± 6.01	96.0 ± 1.80	0.22 ± 0.01
F2	64.60 ± 5.40	90.0 ± 2.0	0.31 ± 0.06
F3	62.92 ± 6.24	96.0 ± 1.0	0.37 ± 0.06
F4	109.10 ± 6.52	96.60 ± 0.50	0.36 ± 0.09
F5	69.75 ± 11.01	88.30 ± 2.40	0.46 ± 0.02
F6	100 ± 7.50	91.90 ± 1.90	0.314 ± 0.04
F7	227.20 ± 7.47	86.0 ± 1.70	0.6 ± 0.08
F8	102 ± 7.71	91.50 ± 2.10	0.44 ± 0.08
F9	50.38 ± 5.65	89.60 ± 2.10	0.57 ± 0.05
F10	101.70 ± 9.81	95.30 ± 1.30	0.88 ± 0.04
F11	74.52 ± 8.01	90.10 ± 1.10	0.13 ± 0.03
F12	97.06 ± 8.15	94.10 ± 1.60	0.514 ± 0.06
F13	67.18 ± 7.76	92.10 ± 2.10	0.43 ± 0.06
F14	89.50 ± 7.89	90.70 ± 0.50	0.35 ± 0.05
F15	127.70 ± 6.53	94.10 ± 2.90	0.34 ± 0.05
F16	108.40 ± 4.12	86.90 ± 1.90	0.45 ± 0.03
F17	97.0 ± 9.20	91.0 ± 3.20	0.29 ± 0.08
F18	136.40 ± 3.47	89.80 ± 5.40	0.23 ± 0.05
F19	259.30 ± 8.02	96.90 ± 2.20	0.72 ± 0.05
F20	189.60 ± 6.70	88.50 ± 6.30	0.35 ± 0.07
F21	114.20 ± 3.70	92.20 ± 2.80	0.65 ± 0.04
F22	201.0 ± 7.10	93.40 ± 2.50	0.47 ± 0.02
F23	46.75± 8.0	97.10 ± 2.30	0.25 ± 0.09
F24	51.81 ± 7.89	91.20 ± 4.10	0.23 ± 0.05
F25	65.87 ± 7.06	92.0 ± 3.20	0.182 ± 0.07
F26	102.30 ± 5.93	90.70 ± 2.70	0.44 ± 0.03
F27	176.20 ± 9.70	96.50 ± 2.20	0.471 ± 0.03
F optimized	75.20 ± 5.54	88.10 ± 3.25	0.354 ± 0.125

*Values represent mean± SD (n=3).

(a)



(b)



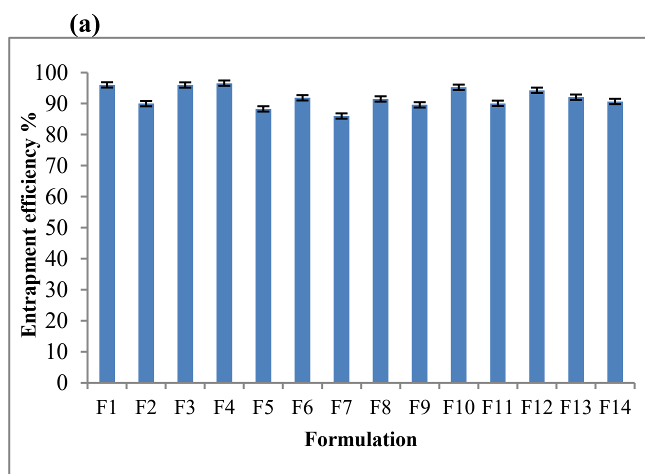
Values represent mean± SD (n=3)

Figure 1: Vesicle size of ethosomal formulations: a (F1-F14), b (F15- F27)

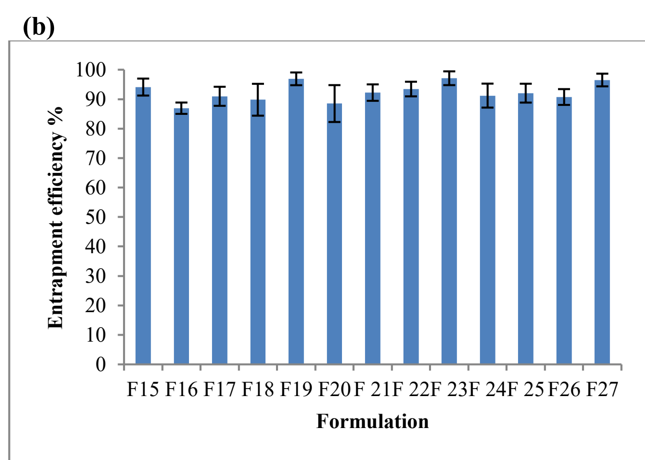
The entrapment efficiency decreased on increasing phospholipid concentration. However, the ethanol content affected the entrapment in an inverse way, where increase in entrapment was achieved upon increase in ethanol concentration. The increased amount of ethanol caused higher EE %. This is possibly due to better solubility of drug in higher % of ethanol present in the ethosomal vesicles. Similar findings were reported by Manish Chourasia *et al.* [27].The PDI values were below one, indicative of a monodispersed population

consisting of homogeneous vesicles.

(a)



(b)



Values represent mean ±SD (n=3)

Figure 2: entrapment efficiency % of various ethosomal formulations: a (F1-F14), b (F15-F27)

4.2. Fitting of data to the model

By fitting of the data for observed responses to various models, the best-fitted model for the two dependent variables (vesicle size and entrapment efficiency) was the quadratic model which was represented by a multiple linear regression equation[28]:

$Y = C_0 + C_1X_1 + C_2X_2 + C_3X_3 + C_4X_4 + C_{12}X_1X_2 + C_{13}X_1X_3 + C_{14}X_1X_4 + C_{23}X_2X_3 + C_{24}X_2X_4 + C_{34}X_2X_4 + C_{11}X_2 + C_{22}X_2^2 + C_{33}X_3^2 + C_{44}X_4^2$

Where:

Y = the measured response;

C_0= constant;

C_1, C_2, C_3 and C_4=linear Coefficients;

C_{12}, C_{13}, C_{14}, C_{23}, C_{24}, C_{34}= interaction Coefficients (between the four factors),

C_{11}, C_{22}, C_{33}, C_{44}=quadratic Coefficients

The regression coefficient (R^2) was employed for the selection of the best fit model. The regression coefficient was $R^2 = 0.65$ for particle size and $R^2 = 0.75$ for entrapment efficiency indicating a good fit to the model.

Vesicle Size:

The VS attained at different levels of X_1, X_2, X_3 and X_4 was fitted to multiple regression analysis to obtain a second-order polynomial equation:

$Y\,1 = -472.70 + 263.62\,X_1 + 9.67\,X_2 + 8.75\,X_3 - 62.01\,X_4 - 9.21X_1^2 - 3.06X_1X_2 + 0.86\,X_1X_3 - 39.55\,X_1X_4 + 0.09\,X_2^2 - 0.49\,X_2X_3 + 0.77\,X_2X_4 + 0.12\,X_3^2 + 3.14\,X_3X_4 + 0.68\,X_4^2$

Where the sign of the coefficient accounts for the collaborative (synergism) or antagonistic effect of the factor on the response, where a positive sign for a variable presents the synergistic effect and a negative sign predicts the antagonistic effect on response. Variables X_1, X_2, X_3 and (X_1X_3, X_2X_4 and X_3X_4) had a positive correlation on VS of CAR-loaded ethosomes. The variable X_4 showed an inverse effect on vesicle size. Upon increase in X_4 (sonication time) ,a decrease in the vesicle size would occur and vice versa[28]. As shown in the above equation, the coefficient of X_1 (phospholipid) was the highest among the positive coefficients of the four variables, indicating that X_1has the greatest substantial effect on vesicle size (b_1= 263.62). These findings comply with the results obtained by Hina Kauser *et al.* and Sarvesh Paliwal *et al.* [22, 28].The size of ethosomes decreased as the ethanol concentration increased up to a certain limit (30 % v/v) after which the size increased upon increase in ethanol. Mainly, the largest vesicles were obtained in F19 (259.3± 8.02 nm) having 20 % ethanol (low level), while the smallest vesicles were observed in formulation F23 (46.75± 8.0 nm) having 30 % ethanol (medium level). These findings are completely consistent with prior studies[28].This can be justified by the presence of high concentration of ethanol which provides a negative net charge on the surface of the ethosomal vesicular systems by modifying specific surface properties, thus causing the vesicles to shrink in size[31].

4.2.1. Statistical analysis of the effects of formulation factors on: Y_1; vesicle size:

Single factors - In Table 4 are listed the estimated effects of the formulation single factors as well as the two- factors interactions on the size of carvedilol ethosomes. Effects were characterized by the sign and magnitude of the obtained estimate, i.e. the larger the value, the more significant is the influence, whether in positive or in negative direction.

Single terms were used to describe the single factors (e.g. A…D for $X_1…X_4$, respectively), whereas double terms were used to describe the interactions between the factors (e.g. AB for X_1*X_2...AD for X_1*X_4). When p values were <0.05, the results were considered to be statistically significant.

In Table no. 4, the biggest positive effect was due to X_1 (phospholipid), followed by X_2 (carvedilol) and X_3 (ethanol). In other words, increase in their values will produce an increase in particle size, these observations come in parallel with studies reported by Hina kauser *et al.[28]*.

Table 4: Estimated effects of single factors and two-factors interactions on the vesicle size of Carvedilol ethosomes

Factor or Interaction	Estimate	P-value
A:X1: phospholipid (g)	263.615	0.172
B:X2: Carvedilol (mg)	9.670	0.598
C:X3: ethanol (% v/v)	8.750	0.849
D:X4: sonication time (min.)	-62.009	0.681
AB: X1*X2	-3.062	0.082
AC: X1*X3	0.857	0.729
AD: X1*X4	-39.550	0.128
BC: X2*X3	-0.488	0.010
BD: X2*X4	0.768	0.642
CD: X3*X4	3.138	0.218

ANOVA analysis revealed that the interaction BC (between factors X_2 and X_3) was the only statistically significant interaction ($p = 0.0105$) and had a negative effect on the vesicle size, however, the other effects were not statistically significant within the investigated range of variations.

The effect of X_4 (sonication time) has a large negative value (-62) indicating that an increase in sonication time will reduce the particle size. This was also reported by Gollavilli *et al.* [32].Yet, ANOVA analysis showed non significance of this effect ($p=0.681$)[29, 33-35]. Figure 3 illustrates graphically the effects of the single factors on vesicle size. It showed increase in size when phospholipid was increased from 1% to 3% and a decrease in size when sonication time was increased from 1 min. to 3 min. The effects of the factors X_2 (carvedilol) and X_3 (ethanol) exhibited inflection points within the investigated ranges where the size was decreased on increasing the drug and alcohol concentration until certain point after which the size increased on increasing both drug and alcohol concentration.

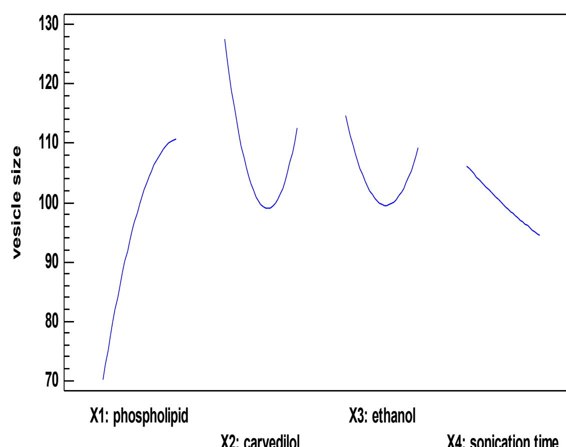


Figure 3: Effects of single factors on the vesicle size

This significant difference in the size of ethosomal formulations is because of the presence of different concentrations of ethanol. Ethanol most likely modifies the system's net charge and confers some extent of stearic stability, which may contribute to reduction in vesicle size.

Interactions – It can be concluded from Table 4 that the effects of the interactions showed either positive or negative values. A negative estimate indicates that the positive effect of one single factor will be reduced when the amount of another single factor is increased. The two-dimensional contour plot of the two-factors (X_1 and X_2) in Figure 4 illustrates this graphically. The plot clearly shows that the smallest particle size was achieved when both parameters were at their lowest values yet, the particle size increased at any other level of the two parameters.

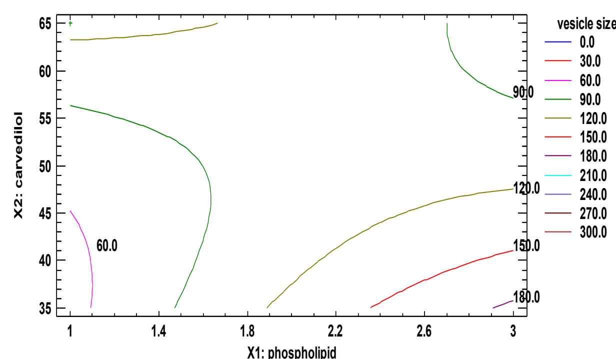


Figure 4: Contour plot showing the effect of X1 and X2 on vesicle size (nm)

4.2.2. Statistical analysis of the effects of formulation factors on: Y_2; entrapment efficiency %:

Single factors - In Table 5 are listed the estimated effects of the formulation single factors as well as the two- factors interactions on the entrapment efficiency of carvedilol nanovesicles.

As represented in Table 5, the estimates of X_1, X_3 and

X_4 have negative values whereas the estimate of X_2 has a positive sign, which means that the entrapment efficiency increased as the drug amount increased, but decreased at higher levels of the remaining factors. The effect of X_1 (phospholipid) was the only statistically significant ($p=0.001$) effect among the other three single factors.
The positive estimate of the effect of carvedilol (X_2) can be explained by the increase in amount of drug available for encapsulation, which increased the entrapment efficiency.
The negative estimate of the effect of phospholipid (X_1) on the entrapment efficiency was unusual since commonly increasing the phospholipid amount leads to rise in entrapment efficiency % of ethosomes due to the formation of multilamellar vesicular structures [36].

Table 5: Estimated effects of single factors and two-factors interactions on the entrapment efficiency of Carvedilol ethosomes

Factor or Interaction	*Estimate*	*P-value*
A:X1: phospholipid	-6.207	0.001
B:X2: Carvedilol	0.102	0.064
C:X3: ethanol	-1.843	0.706
D:X4: sonication time	-12.047	0.654
AB: X1*X2	-0.092	0.254
AC: X1*X3	0.277	0.033
AD: X1*X4	0.9	0.448
BC: X2*X3	0.007	0.392
BD: X2*X4	0.072	0.367
CD: X3*X4	1.268	0.591

It can be seen in Figure 5 which illustrated graphically the effects of $X_1X_2X_3$ and X_4 on entrapment efficiency that the maximum EE% was obtained with the lowest amount of phospholipid and no further improvement is possible within the selected range of the investigation. This can be explained by absence of cholesterol within the vesicles bilayers which caused the vesicles membrane to become less rigid and thus more permeable and leaky which caused the encapsulation of the drug to decrease. Such finding comes parallel with a study reported by Jia You Fang *et al.* where decrease in cholesterol content of estradiol proniosomes lead to significant decline in estradiol encapsulation [37]. Another possible explanation for the inverse relationship between lipid content and encapsulation efficiency is that the used lipid (phospholipon 90 G) contains double bonds that facilitate forming of a loose conjunction between the bent molecule and its adjacent molecule making the membrane more leaky [38]. Upon saturation of these double bonds, and the use of hydrogenated lecithin force the bilayer molecules to be in ideal ordered shape and therefore generating less leaky and more rigid vesicle membrane [45]. This hypothesis is substantiated by the proportional increase in entrapment efficiency of vinpocetine proniosomes upon use of hydrogenated lecithin which caused the vesicles to become less leaky and firmly retained vinpocetine within them [39]. It is also clear in Figure 5 that the entrapment decreased when the percentage of phospholipid (X_1) was increased but it increased when the percentage of drug (X_2) was increased. The effects of the percentage of ethanol (X_3) and sonication time (X_4) exhibited inflection points at which the effect changed from negative to positive trend upon increase in ethanol content (X_3) and sonication time (X_4).

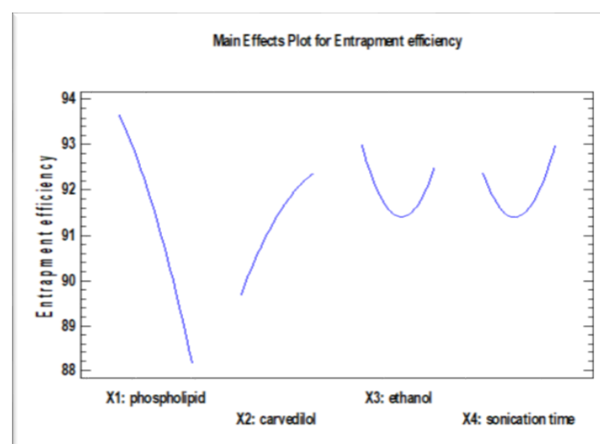


Figure 5: Effects of single factors on EE % of carvedilol ethosomes

AS for the EE %, a second-order polynomial equation (full model) was obtained by BBD:

$$Y2 = 135.34 - 6.207 X_1 + 0.102 X_2 - 1.84 X_3 - 12.05 X_4 - 0.52 X_1^2 - 0.09 X_1X_2 + 0.28 X_1X_3 + 0.9 X_1X_4 - 0.002 X_2^2 + 0.007 X_2X_3 + 0.072 X_2X_4 + 0.01 X_3^2 + 0.063 X_3X_4 + 1.27 X_4^2 \quad (1)$$

Interactions - It can be concluded from Table 5 that the effects of the interactions were either positive or negative but exhibited very low values. For example the interaction AB (between factors X_1 and X_2) had a negative estimate indicating that the positive effect of the factor X_2 was affected by the negative effect of the factor X1 as graphically displayed by the two-dimensional contour plot(Figure 6). As noticeable in the plot, the greatest EE% was obtained when X_1 was at the lowest level and X_2 was at the highest level. For any other level of the two factors, the entrapment efficiency decreased.

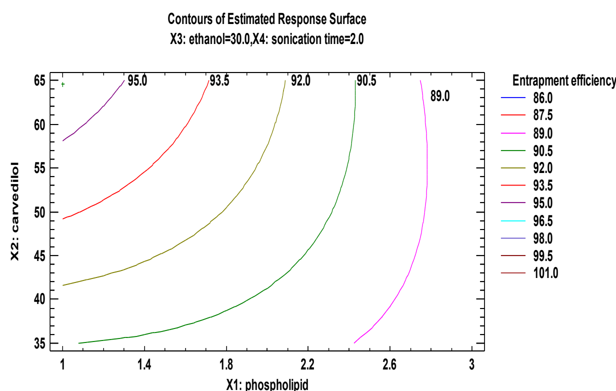


Figure 6: Contour plot showing the effect of X1 and X2 on entrapment efficiency %

Optimize desirability – The findings show that the responses were influenced by the factors in a variety of ways. To account for all of the components' effects, the desirability function was used with Design-Expert® software to optimise both responses at the same time (vesicle size and entrapment efficiency).
Furthermore, by maximising desirability the best formulation can be identified and recommended for further research. The selection criteria were based on the constraints indicated in Table 1, namely, VS minimization and EE% maximisation. The components of the optimized formula along with its observed response values are manifested in Table 2 and table 3 respectively.

4.3. In vitro release studies of CAR ethosomes

Depending on desirability values, the major six formulae (F1, F3, F23, F10, F12 and F optimized) with highest observed desirability were chosen to undergo further release studies. Table 6 illustrates the release profiles of the chosen ethosomal vesicles. The formulation F23 showed the highest release value after 24 h. The high release is beneficial for carvedilol delivery[39].
Fig. 7 shows the percentage of carvedilol released across the semipermeable membrane from ethosomes versus time. All the formulations showed a biphasic release pattern (figure 7). During the first 2 hours (initial phase), about 34 % of the drug was liberated and released.
This is called the burst effect where the unentrapped drug present on the ethosomal surface was released since drug release is a surface phenomenon. A comparatively controlled release of carvedilol from ethosomes was observed up to 24 h. This second phase elucidates the sustained release of carvedilol (entrapped) from the ethosomes. Similar results were reported by Wilson *et al.*[38].
It was found that vesicle size had an apparent influence on the drug release rate where a shorter average diffusion path for entrapped carvedilol was taken in the smaller nanovesicles which allowed quicker release of the entrapped drug in comparison with the bigger size vesicles having the same composition where F23 and F12 had the same composition as F3 and F 10 respectively, yet due to the smaller size of F 23 and F 12 in comparison to F 3 and F 10, F 23 and F 12 showed 70.25 % and 60 % release respectively. However, F 3 and F 10 showed less release as shown in table 6 and figure 7. This can be also explained on the basis that smaller vesicle size had larger surface area and thus led to higher burst release, while larger vesicles could sustain the drug release for more than 24 h.

Table 6: Cumulative drug concentration % from carvedilol loaded ethosomes and carvedilol hydroethanolic solution

Time (hr)	Cumulative µg % of carvedilol released in 1% methanolic phosphate buffer pH 5.5 ± SD (n=3)						
	CAR hydroethanolic solution	F optimized	F 1	F 3	F10	F12	F 23
0	0 ±0	0 ±0	0 ±0	0± 0	0 ± 0	0± 0	0± 0
1	20.25 ±1.26	31.63 ±2.60	24.50 ±2.60	30.20± 5.62	26.90± 5.26	24.60± 5.26	27.61± 2.62
2	30.20 ±2.88	34.95 ±8.20	30.20 ±5.62	34.28± 4.62	30.50± 7.26	32.65± 2.62	35.25± 4.62
3	40.6 ±4.61	35.61 ±3.64	35.92 ±4.21	40.20± 6.35	34.60± 6.26	36.58± 5.26	40.90± 5.20
4	45.90 ±2.90	37.20 ±4.56	40.51 ±6.9	44.52± 8.50	37.60± 4.90	39.48± 4.26	44.50± 4.56
5	52.67 ±9.94	39.11 ±7.52	47.55 ±6.942	50.40± 5.26	41.25± 6.25	39.77± 9.26	51.20± 6.32
6	59.60 ±7.94	40.50 ±4.26	50.10 ±7.52	55.90± 5.62	42.62± 9.26	41.86± 4.92	55.62± 9.26
8	65.24 ±12.39	45.23 ±9.25	52.60 ±4.92	58.90± 4.23	44.77± 8.25	42.47± 5.26	60.26± 7.25

24	80.50 ±9.32	62.20 ±12.02	61.33 ±10.23	66.45± 9.524	58.58± 11.02	60.0± 7.025	70.25± 4.26

On increasing ethanol conc. from 20 % (F10, F12) to 40% (F1) while keeping the concentration of phospholipid constant at 2 %, F1 showed higher release than F10 and F12 after 24 hours , where release was increased from 58.58 and 60.0 % to 61.33 % for F10, F12 and F1 respectively.

Increased fluidity of the vesicular bilayer membrane due to increase of alcohol concentration could explain the observed increase in CAR release with higher level of ethanolic content[27]. The concentration of the lipid was among the factors controlling the rate of drug release, as the lipid content increased, the burst effect and amount released after 24 h decreased as clearly seen in F 10 and F 12 which contained 2% lipid. They showed 30.5 % and 32.65 % burst release and 58.58 and 60 % drug release at 24 h, while F optimized which contained 1 % lipid showed 34.95 % burst release and 62.60 % ofdrug release at 24 h. this might be due to the lipophilicity of the drug that retarded the diffusion of the drug from the phospholipidic bilayer of the vesicles to the aqueous dissolution medium. This might also be due to increased thickness and integrity of the lipid vesicle, so the diffusion occurs more slowly and the release becomes more sustained [39].

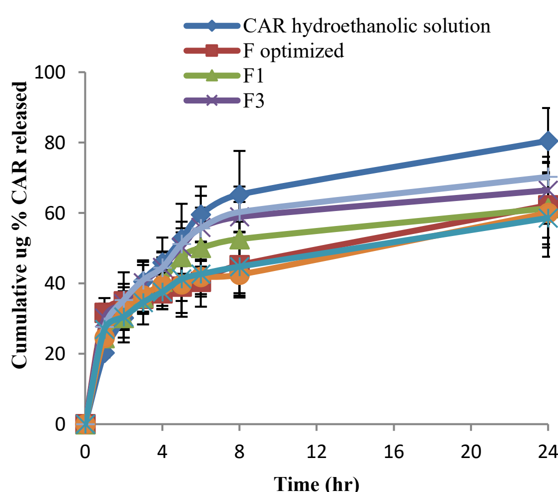


Figure 7: In vitro drug release profiles of (a) **Carvedilol hydroethanolic solution and six selected CAR ethosomes**

4.4. Fourier transform infrared spectroscopy

Figure (8a): showed remarkable characteristic peak for carvedilol at 3344 cm^{-1}corresponding to stretching of NH group of secondary amine, two peaks were visible at 2993.52 cm^{-1} and 2924.09 cm^{-1} assigned for (–CH-aliphatic stretching) group , sharp peaks appeared at 1500-1400 cm^{-1} assigned for C-C aromatic stretching, also another sharp bands appeared at 1253.73 cm^{-1} and 1099.433 cm^{-1} for C-N stretching and C-O stretching vibration respectively.

Figure (8b): showed shorting of the pure drug peak at 3344 cm^{-1} in the physical mixture. However, the rest of pure drug peaks were still present which indicated there was no major change in the chemical structure of the drug and the lipid in the mixture.

Figure (8c): Upon addition of phospholipid to our drug (carvedilol), remarkable change was achieved on the characteristic NH group of the drug that became more broad and the spike of the peak was absent suggesting that hydrogen bond between phospholipid groups and –NH group of our drug was formed. However, there are no changes in the characteristic peaks of phosphatidylcholine which means that the phospholipid is present in the outer shell of our formula. This behaviour indicates complete encapsulation of the drug in the ethosomes.

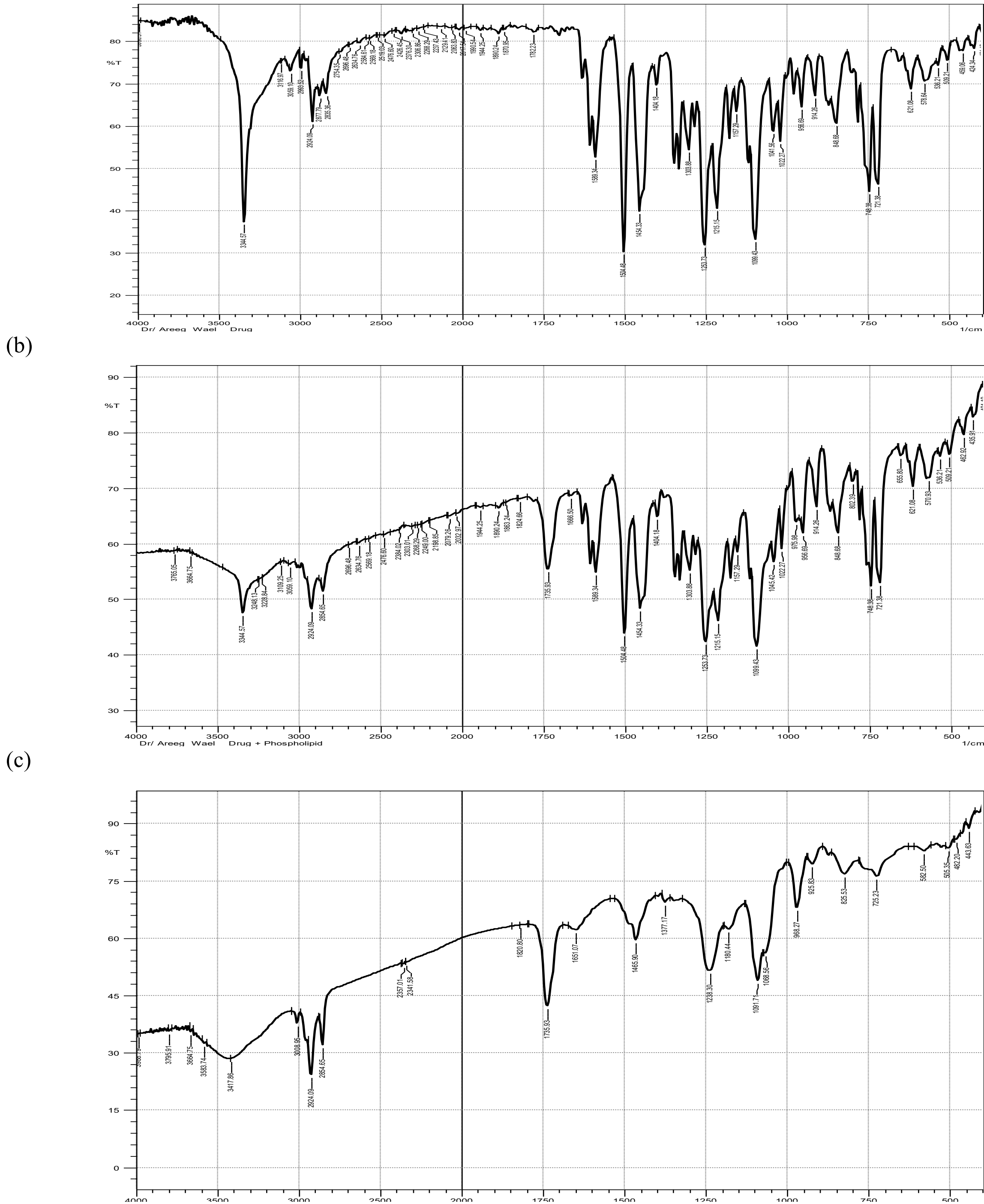


Figure 8: FTIR spectrum of a) pure carvedilol powder, b) phospholipid + CAR and c) CAR ethosome (F 23)

5. Conclusions:

Box-Behnken Design was employed to develop CAR ethosomes and study the effect of different amounts of components on the vesicle size and EE% of ethosomes to obtain an optimized formula with reasonable size and EE%. Our optimized formula showed a suitable size of 75.20 ± 5.54 nm and about 88 ± 3.25 % entrapment efficiency. Five different CAR ethosomal formulations along with the optimized formula suggested by Box Behnken Design were subjected to *in vitro release* studies. F3 and F23 showed the best release with 66.45± 9.524 % and 70.25 ±4.26 % drug release after 24 hours respectively. Based on these promising results, we are encouraged to approach further stability and pharmacokinetic studies on both F3 and F23 in the future.

Conflicts of interest:

The authors have no competing interests to declare that are relevant to the content of this article.

Funding:

Partial financial support was received from the National Research Center

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