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DICLOFENAC SODIUM LOADED NIOSOMAL GEL FOR EFFECTIVE TRANSDERMAL DELIVERY USING THREE SQUARE FULL FACTORIAL DESIGN

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ABSTRACT
The aim of the present study was to develop niosomal carriers for transdermal delivery of diclofenac for relieving pain and exploring possible mechanism of better skin penetration and permeation of niosomal vesicles. Niosomes were prepared by ethanol injection method. Optimized formulation was obtained using the three squared factorial design study. Characterization was done for the tested formulations followed by particle size, percentage of drug content, percentage of entrapment efficiency, zeta potential and in *vitro* drug release and the optimized niosomes was subjected to develop niosomal gel using Carbopol 980 NF and characterized for viscosity studies, *ex vivo* drug release studies, pharmacokinetic study and stability studies etc. The niosomes obtained were large spherical unilamellar vesicles with an average particle size of 886-268nm; polydispersity index of 0.122-0.371; zeta potential of -39 to -49mV; entrapment efficiency of 30.90% to 74.25%. Based on optimum particle size, highest % EE and maximum % drug release F8 niosomes were selected to prepare diclofenac sodium loaded niosomal gel and plain gel using carbopol 980 NF. The niosomes and niosomal gel provided controlled plasma concentration in rats. From the observations made in pharmacokinetic studies the biological half life of the diclofenac niosomal gel has been increased along with reduced elimination rate. It has been proved that niosomal gels are promising tools in the treatment by transdermal route.

KEYWORDS: Factorial design, Niosomal gel, Transdermal drug delivery, Span 60, Cholesterol.

INTRODUCTION
The two prerequisites for an ideal drug delivery include, Firstly it needs to deliver the drug according to the requirement of the body. Further, it should channel the active entity to the area of action. Conventional dosage forms which include prolonged dosage forms are unable to meet any one of these. Currently, no available drug delivery system comports ideally, but genuine efforts have been made to accomplish them via distinct novel approaches in drug delivery.[1,2] In recent years, vesicles have become an alternative in drug delivery. Encapsulation of a drug in vesicular structures can be considered to extend the survival of drug in systemic circulation. Moreover, reduces the toxicity if selective uptake can be accomplished.[3] Vesicular drug delivery minimizes the cost of therapy by ameliorating bioavailability of medication particularly in case of poorly soluble drugs. These systems retard drug elimination of rapidly metabolizable drugs and acts as controlled release systems.[4]

Niosomes are microscopic lamellar structures formed by combination of non-ionic surfactant and cholesterol followed by hydration in aqueous media.[5] The vesicles are formed by inclusion of cholesterol and small amount of anionic surfactant such as dicetyl phosphate.[6] Earlier, we have attempted to avoid the disadvantages of loading diclofenac into liposomes for transdermal delivery in our study preparation, characterization and pharmacokinetic evaluation of diclofenac sodium liposomes.[7] From the results, we have concluded that the liposomes shows limited solubility which leads to low encapsulation efficiency. And the liposomes have limited penetration due to their rigid nature. Moreover, niosomes were documented to obtain better stability than liposomes.[8] Hence we considered the niosomes are alternative dosage forms for transdermal delivery. Generally niosomes are prepared with non-ionic surfactants like tweens, spans (different grades like span 20, span 40, span 60 and span 80). Among them span 60 was chosen because span 60 exists physically in solid form and has utmost penetration when compared to other span grades.[9] Carbopol 980 NF was used as base for niosomal dispersion because it is hydrophilic, biocompatible and has good bio adhesive properties.[10] It was not evaluated earlier for its applicability in the niosomal gel

formulations for transdermal delivery.[11] Preparation of niosomal diclofenac sodium gel was investigated by.[12]

Diclofenac sodium is an antiinflammatory drug which is used for dealing with pain usually at an oral maximum dose of 225mg daily. The recurrent side effects in the course therapy with diclofenac sodium are mostly gastrointestinal disturbances that are generally mild and reversible but in some cases are peptic ulcer and gastrointestinal bleeding.[13] An ethanol injection method was used for the preparation of niosomes using three squared factorial design, satisfactory and reproducible results were obtained. It was reported early that an amount of span 60 and cholesterol were found to be critical in the preparation and stabilization of niosomes.[12] Hence, span 60 and cholesterol were selected as independent variables. Vesicle size and entrapment efficiency were selected as dependent variables. The present study was aimed at development of novel niosomal carriers for transdermal delivery of diclofenac and to explore possible mechanism for better skin penetration and permeation.

MATERIALS AND METHODS

Diclofenac sodium was procured from MSN laboratories, Hyderabad (India). Span-60, stearic acid, carbopol 980 NF, cholesterol and Hcl were procured from Sigma Aldrich Chemical Company (India).

Estimation of Diclofenac sodium in present investigation

In the present work UV visible spectrophotometric method was used for estimation diclofenac sodium. The UV absorbance was measured at 276 nm. The calibration curve constructed in the range of 2 to 20 µg/ml concentration of diclofenac sodium using pH 6.8 phosphate buffers.[14]

Optimization of process parameters

Experimental design

To study the effect of variables on characterisation performance of niosomes, different batches were prepared using three squared factorial design. Amount of surfactant (X_1) and cholesterol (X_2) were selected as two independent variables which were varied at three levels low level (-1), medium level (0), high level (+1). Amount of stearic acid as charge inducer (20 mg) and drug diclofenac (20mg) were kept constant. Vesicle sizes (Y_1), entrapment efficiency (Y_2) were selected as dependent variables. Design-Expert® DX 8 software was used for the generation and evaluation of statistical experimental design (table 1).

Table 1: 3^2 factorial design of diclofenac sodium loaded niosomes.

Batch Code	Amount of span 60	Amount of cholesterol	Drug (mg)	Stearic acid(mg)	Dispersion phase
	(mg)	(mg)	Constant	Constant	(6.8 pH phosphate buffer)
-	X_1	X_2	-	-	-
F1	30	25	20	20	10
F2	30	50	20	20	10
F3	30	75	20	20	10
F4	60	25	20	20	10
F5	60	50	20	20	10
F6	60	75	20	20	10
F7	90	25	20	20	10
F8	90	50	20	20	10
F9	90	75	20	20	10

Preparation of niosomes

Accurately weighed amounts of span 60, cholesterol, stearic acid and drug were taken in a beaker and dissolved in 1ml ethanol. The surfactant mixture in ethanol was injected slowly through 14-gauge needle in to a beaker containing 10ml of pH 6.8 phosphate buffer maintained at a temperature of 60ºC under stirring at 500rpm (remi magnetic stirrer) using a teflon-coated bead. The system was subjected to evaporation for 15 minutes to remove ethanol. The aqueous phase immediately turned milky because of niosome formation. Buffer was added to adjust the volume of final niosomal suspension to 10ml. Furthermore it was refrigerated for 2 hours for effective vesicle sealing.[15]

Preparation of niosomal gel

Three different niosomal gel formulations were prepared 0.5%, 1% and 1.5% w/v carbopol 980 NF gel bases. Then the niosomal suspension was centrifuged to separate the un-entrapped drug. The obtained pellet was re-suspended in the pH 6.8 phosphate buffer. Initially required amount of carbopol 980 NF was added to water and kept overnight for complete hydration of polymer chains. Triethanolamine was used to neutralize the pH of the gel to 6-7 and induce gelling. Niosomal dispersion was incorporated in the hydrated carbopol 980 NF to obtain a final concentration of 0.14% w/w of diclofenac.

Preparation of plain gel

Diclofenac sodium was incorporated in 0.5%, 1%, 1.5% w/v carbopol 980 NF gel bases by trituration and stirred by using a glass rod to get 0.2% w/w of smooth homogenous diclofenac plain gel.

Optimization of formulation using three square factorial design

The data obtained from factorial design study was subjected to multiple regression analysis using 'Design-Expert® DX 8 software' and fitted in the following equation.

$Y= \beta_0+ \beta_1X_1+ \beta_2X_2+ \beta_{12}X_1X_2 +\beta_{11}X_1X_1+ \beta_{22}X_2X_2$

Where Y is the dependent variable, β_0 is the arithmetic mean response of the nine runs; β_1 and β_2 are the estimated coefficients for the independent factors X_1 and X_2 respectively. β_{11}, β_{22} and β_{12} are the estimated coefficients for the interaction terms X_1X_1, X_2X_2 and X_1X_2 respectively. The main effects (X_1 and X_2) represent the average result of changing one factor at a time from its low to high value. The interaction terms (X_1X_2) show how the response changes when two factors are simultaneously changed. The polynomial terms (X_1X_1, X_2X_2) are included to investigate non linearity. The effect of the variables was interpreted considering the magnitude of coefficient and the mathematical sign it carries (positive or negative). The adequacy of fitted model was checked by analysis of variance (ANOVA). To study the main and interaction effects of the independent variables, response surface plots were constructed using 'Design-Expert® DX 10 software.

Characterization of the tested formulations

Fourier Transform Infrared (FTIR) Spectroscopy

Appropriate amounts of diclofenac sodium, Span 60, cholesterol, stearic acid and lyophilized sample of optimized niosomal dispersion were mixed separately with KBr. The IR spectra of the resultant mixtures were recorded on a Bruker FTIR spectrophotometer equipped with Opus software.[6]

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) curves of the pristine diclofenac and drug loaded gel were recorded using TA instrument. The analysis was performed by heating the samples at the rate of 10ºC/ min under inert atmosphere.

Entrapment Efficiency Determination

The un-entrapped drug was separated from niosomes using centrifugation method. Niosomal dispersion was centrifuged at 16000 rpm for 90 min at a controlled temperature of 4°C (Remi cooling centrifuge). Supernatant containing unentrapped drug was withdrawn and measured UV spectrophotometrically at 276 nm against phosphate buffer saline (John and Lonnie, 1998) (pH 6.8). All the determinations were made in triplicate. The amount of drug entrapped in niosomes was determined as follows

$EE (\%) = [(C_d - C)/C_d]*100$

Where C_d is the concentration of total drug and C is the concentration of un-entrapped drug

Percent Drug Content

Niosomal suspension equivalent to 2 mg was taken and lysed with methanol. Drug content was determined by subsequent dilutions with the buffer and measuring the absorbance at a λ_{max} of 276 nm.

Vesicle Size Measurement

The mean particle size of the niosomal dispersion was determined by photon correlation spectroscopy using Zetasizer 300 HSA (Malvern Instruments). Analysis was carried out at 30±2°C temperature keeping the angle of detection at 90°. The mean vesicle size was expressed in terms of *d* (0.9) nm.[15]

Zeta potential (ζ) determination

The zeta potential of the niosomes was measured with the laser doppler electrophoretic mobility measurements using Zetasizer 300 HSA (Malvern Instruments) at a temperature of 30±2°C.[15]

Visualization by Scanning Electron Microscopy

A drop of vesicle dispersion was applied on a carbon film-covered copper grid. Excess dispersion was blotted from the grid with filter paper to form a thin-film specimen. The sample was then stained with 2 % uranyl acetate, air dried and examined under transmission electron microscope (Hitachi, H-7500) at a magnification of 60000 X.[16]

Viscosity studies

Various concentrations (0.5%, 1% and 1.5%w/v) of niosomal gel formulations were subjected to rheological studies. The rheological studies were performed by using Brookfield cone and plate rheometer model LV-DV III. The viscosity properties of the tested formulations were studied at 25±0.1ºC for 0.5, 1 and 1.5% w/v concentrations of tested products. Required quantity of gel samples were placed on the viscometer plate and analysed for its viscosity (η), shear stress (τ) and shear rate at various speeds and at 100 rpm.

***In-vitro* Drug Release Study**

In vitro permeation studies were performed using vertical Franz diffusion cells with an effective diffusional area of 4.52 cm^2. 2 ml volume of different formulations was placed in the donor compartment. 25 ml of phosphate buffer pH 6.8 was used as receptor medium to ensure sink condition. The receptor compartment was maintained at 37°C and stirred by a magnetic bar at 100 rpm. The donor compartment was separated from the receptor compartment by cellulose dialyzing membrane (Membra–Cel MD 34-14, cut-off 14kD) which was soaked in the receptor medium overnight. At predetermined time intervals (0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 h), 1 ml aliquots were withdrawn from the sampling port and were replaced with an equal volume of fresh buffer to maintain constant volume. The samples were analyzed spectrophotometrically at 276 nm in reference with the constructed calibration curve.[17]

Drug Release Kinetics

In order to understand the kinetics and mechanism of drug release, the result of *in vitro* drug release study of optimized batch of niosomes was fitted with various kinetic equations like zero order (cumulative % released vs. time), first order (log % drug remaining vs. time), Higuchi's model (cumulative % drug released vs. square root of time), Peppas (log % drug released vs. log time) and erosion ((1-Q) $^{1/3}$ vs. time). K and r values were calculated for the linear curve obtained by regression analysis of the above plots.

***Ex Vivo* Drug Release Studies by Franz Diffusion Cell**

Ex vivo permeation studies were conducted using rat skin by Franz diffusion cell. The albino (Wistar strain) rats used for this experiment were sacrificed. The abdominal hair of the sacrificed rat was neatly shaved using a hand razor. The rat abdominal skin was surgically removed and subcutaneous fat was carefully cleaned. The skin membrane was soaked in the phosphate buffer prior to the experiment. The excised rat skin was mounted on the receptor compartment with the stratum corneum side facing upwards into the donor compartment. The formulations; niosomal suspension, niosomal gel and diclofenac sodium plain gel were studied, and each study was replicated three times. The receptor compartment was maintained at 32±0.5°C and stirred by a magnetic stirrer at 100 rpm. One mL of aliquots was withdrawn at different time intervals (1, 2, 3, 4, 6, 8, 10, 12, and 24 h) from the sampling port and was replaced with an equal volume of fresh buffer to maintain constant volume. The drug concentrations in the *ex vivo* studies were estimated by UV spectrophotometrically at a λmax of 276 nm.

Transdermal Flux

Transdermal flux (J) for the three formulations across the rat skin was calculated from the slope of the cumulative drug permeated per unit area verses time plot. The permeability coefficient (Kp) was calculated from the transdermal flux and the applied concentration in the donor compartment (C_{donor}) as per the following equation,

$Kp = J / C_{donor}$

Pharmacokinetic Study

Based on the literature survey, the design and procedure followed for the pharmacodynamic studies on rat model were developed. In the current study, wistar male rats, weighing 200±250 gm were used. The animals were divided into three groups, each group containing four animals. Group I was treated with niosomal dispersion, group II was treated with diclofenac-niosomal gel and group III was treated with plain gel. The dose equivalent to 2 mg was taken from all the tested products. The selected products were applied on the dorsal site of the abdominal skin of the rats. Samples of 300 µL aliquots of blood were collected from the rats' retro-orbital sinus into micro centrifuge tubes containing dipotassium ethylene diaminetetraacetic acid at 0, 0.5, 1, 1.5, 2, 4, 6, 8, 10 and 12 h post dose. . The 100 µL of blank plasma was taken into Eppendorf micro centrifuge tube and 20µL of Aceclofenac internal standard was added to it. 100µL of thawed test plasma sample was taken and 200µL of methanol was added to the tube as a precipitating agent and the solution was vortexed for five min and then centrifuged for 15min at 5000rpm. The supernatant solution was separated and filtered through 0.45µm pore size filter and 20µL of the solution was injected directly into the HPLC loop injector. The peak area and peak ratios of the different samples were recorded. These values were taken for analysis by PK Solver software trial.[18,19,20,21]

Stability Study

The formulations were packed in high density polyethylene (HDPE) screw capped bottles and kept in humidity chambers maintained at 5±3°C and 25± 2°C/60 ± 5% RH. Samples were withdrawn at predetermined time intervals at initial, 3 months and 6 months. The optimized niosomes were analyzed for characteristics such as percent drug content, particle size, zeta potential, %EE and niosomal gel samples were analyzed for characteristics such as percent drug content, particle size, zeta potential, %EE and viscosity.[22,23,24]

RESULTS AND DISCUSSION

Estimation of diclofenac sodium in the present investigation

The method obeyed beer's law in the concentration range of 2-20µg/mL for pH 6.8 phosphate buffers. The 'r^2' value was found to be more than 0.999 for pH 6.8 phosphate buffers, which indicated a positive correlation between the concentration of diclofenac sodium and corresponding absorbance values. The S.D values were found to be low, which indicated that the methods used were reproducible. The method was found to be suitable in the present investigation for estimation of diclofenac sodium "Fig. 1".

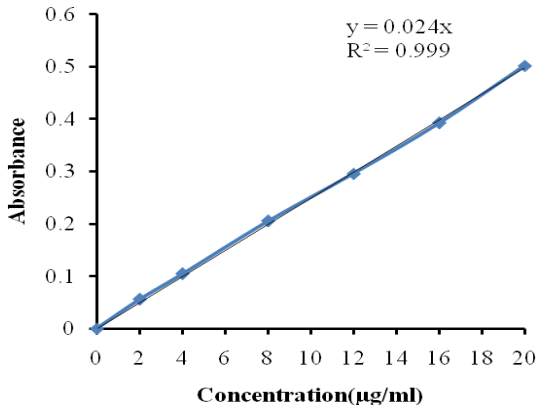


Fig. 1: Calibration curve for the estimation of diclofenac sodium in pH 6.8 buffer.

Characterization of test formulations

FTIR studies

FTIR studies were performed to detect the possible interactions between drug and the excipients. The FTIR

spectra of pure drug diclofenac sodium span 60, cholesterol and optimized formulation F8 were shown in "Fig. 2". The major absorption peaks for the pure drug and the excipients were well in support with the theoretical prediction with respect to the functional groups. Presence of excipients did not produce any major shift in the principal peaks of diclofenac and also presence of one ingredient did not produce shift in peaks of other ingredients. FTIR spectral analysis proved compatibility of drug and excipients used in the study.

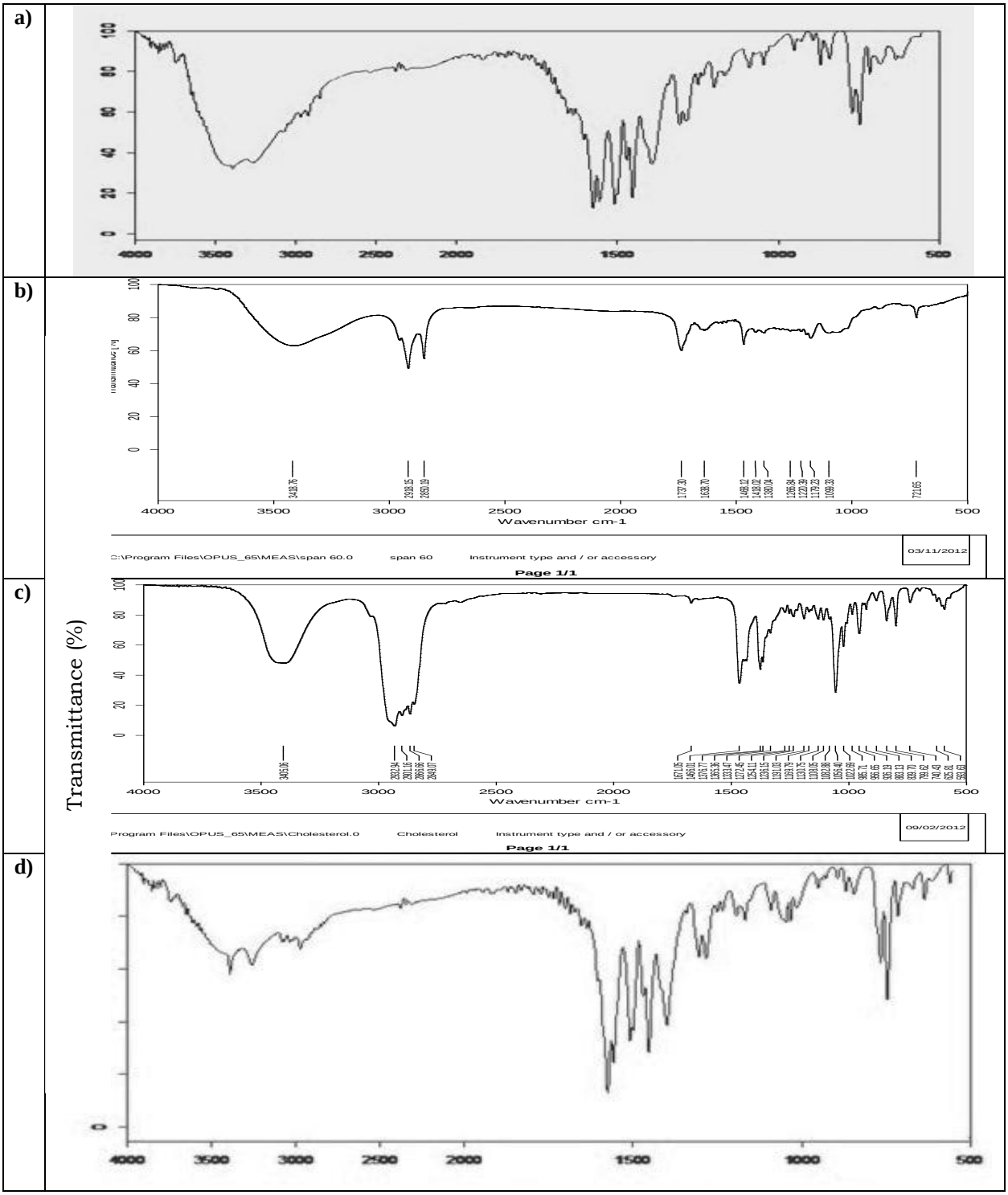


Fig. 2: FTIR spectra of (a) Pure drug (b) span 60 (c) cholesterol (d) Diclofenac niosomal suspension

Differential Scanning Calorimetry (DSC)

The results of DSC were shown in "Fig. 3". The DSC thermogram of diclofenac sodium shows 282°C which indicated that the pure drug was found its crystalline state whereas from the thermogram of liposomal gel shows 102°C. This suggests diclofenac was not in crystalline state but the drug was intercalated into niosomal bilayers and carbopol 980 NF. This melting

point depression might be due to small particle size (nanometer range), their high specific surface area and the presence of surfactant and cholesterol.

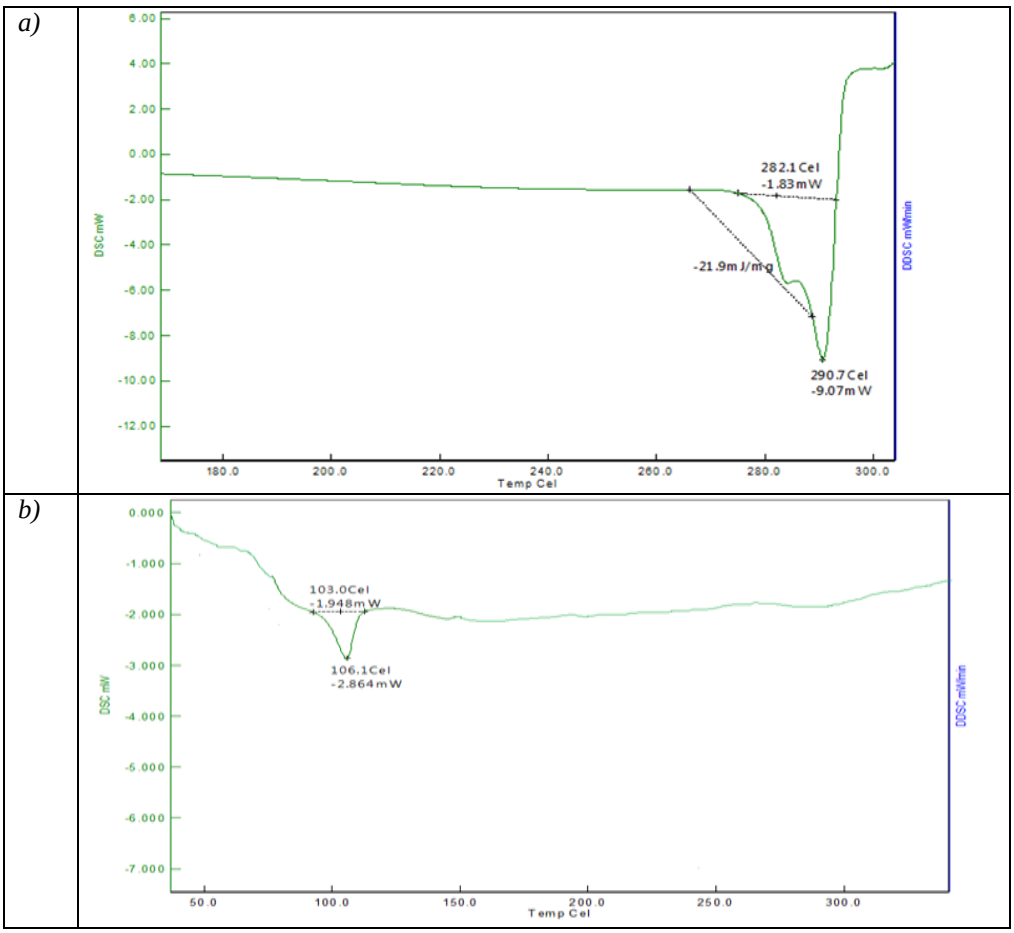


Fig. 3: DSC Thermograms of a) pure diclofenac sodium b) diclofenac sodium niosomal gel.

Visualization by scanning electron microscopy

The niosomes obtained were spherical large unilamellar vesicles as shown in "Fig. 4".

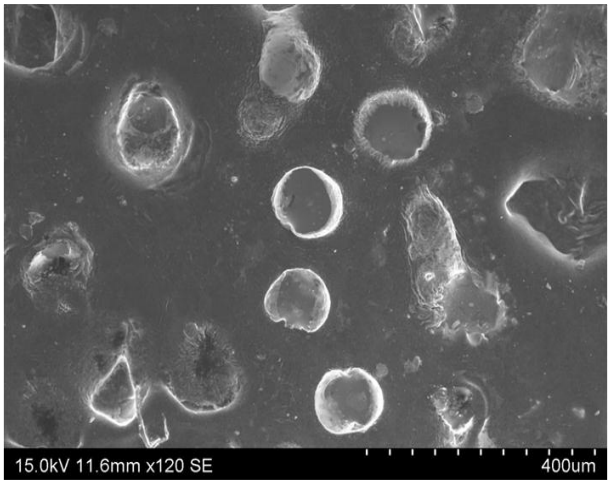


Fig. 4: Scanning electron micrograph of niosomal vesicle.

Particle size

The mean vesicle size was in the range 886–268 nm, and it was strongly affected by the selected variables. The polydispersity index (PdI) was in the range of 0.122-0.371 which indicated a narrow vesicle size distribution "Fig. 5".

Zeta potential

Zeta potential values of prepared niosomal dispersions ranged from -39 to -49 mV. Niosomes were reported to have greater stability when compared to other colloidal dosage forms "Fig. 6".

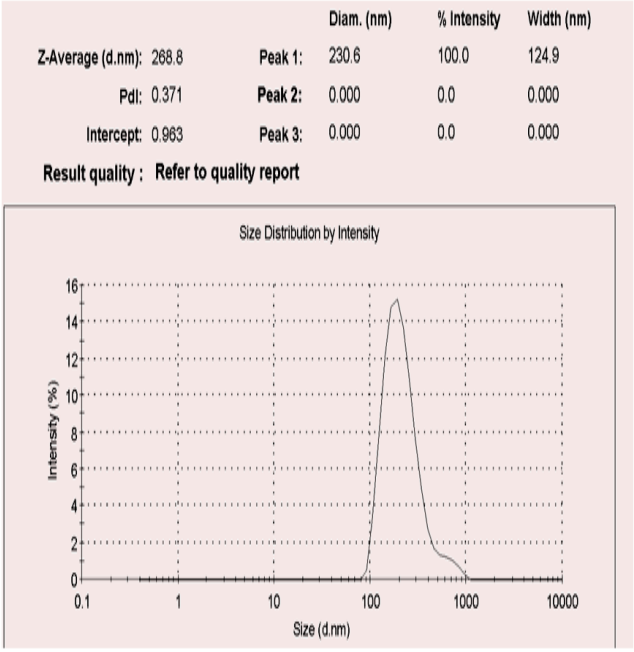


Fig. 5: Particle size of F8 formulation.

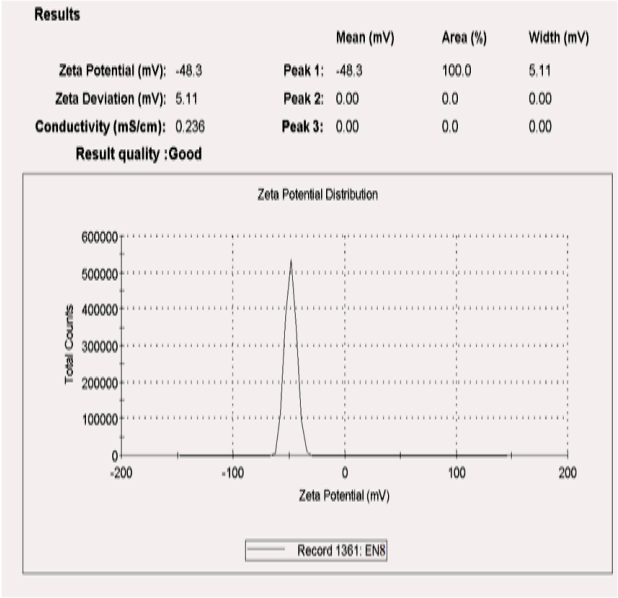


Fig. 6: Zeta potential of F8 formulation.

Percent entrapment efficiency

The percent entrapment efficiency of niosomes was determined after separating entrapped and unentrapped drug by ultra centrifugation. It varied from 30.90 % to 74.25 % for all the formulations. The highest entrapment efficiency of 74.25 % was observed for F8 formulation.

Percent drug content

The percentage drug content of the niosomal formulations varied between 99.25 to 101.55 %. Hence, all the formulations were within the standard limits. This indicates uniform distribution of drug in each niosomal formulation.

Viscosity studies

Viscosity decreased up to the end of the lag phase; after the lag phase, a steady state was observed with a constant shear rate. The average viscosity of a 1% w/v plain gel and niosomal gel were found to be the lowest (1441.15 and 1219.05 Cps) among the three gels. Thus, 1% w/v gel concentration was optimized for both gels. All the viscosity graphs show a pseudo-plastic flow in the graph drawn between viscosity vs. shear rate. The results of the viscosity studies were as shown in the (table 2).

Table 2: Viscosity, shear stress and shear rate values of tested gel formulations.

	Minimum	Maximum	Average
1 % w/v plain gel			
Viscosity(Cps)	1345.3	1537.9	1441.15
Shear stress(dynes sq/ cm)	8.34	10.97	
Shear rate(1/sec)	11.98	120.19	
1 % w/v niosomal gel			
Viscosity (Cps)	1159.8	1278.3	1219.05
Shear stress(dynes sq/ cm)	6.15	8.32	
Shear rate(1/sec)	10.22	115.3	

Each value represents (n=3)

Percent Drug content and content uniformity of gels

The percent drug content for niosomal gel and plain gel was found to be 98.75 % and 98.39 % respectively and the content uniformity obtained was 98.60±1.04 % and 99.41±0.96 % (mean±s.d., n=3) for niosomal gel and plain gel respectively.

Table 3: Physicochemical characterization of diclofenac niosomes.

Batch code	Drug content ± s.d.[a] (%)	Particle size (nm)	PdI	Zeta potential (mV)	Entrapment efficiency ± s.d.[a] (%)
F1	98.4±0.50	886	0.316	-39.8	30.90±1.81
F2	101.55±0.97	628	0.250	-42.5	50.70±2.75
F3	99.55±0.10	756	0.354	-38.8	34.00±3.01
F4	98.25±0.32	531	0.252	-38.7	45.00±2.65
F5	98.3±0.85	460	0.122	-44.6	59.63±1.88
F6	100.15±0.67	495.7	0.223	-42.2	51.52±2.52
F7	99.2±0.70	298.7	0.318	-43.1	63.75±3.06
F8	98.75±0.85	268.8	0.371	-48.3	74.25±2.32
F9	99.7±1.00	280	0.320	-49.4	56.74±0.86

a=each value represents mean±s.d. (n=3)

***In-Vitro* release studies**

The study revealed that the release of the drug from the formulation depends on the relative amounts of surfactant and cholesterol present. Among all the formulations F8 showed a maximum drug release of 98.89% after 24hrs. The percentage release of the drug increased with increased concentration of surfactant and at a certain level of surfactant, the percentage release decreased at higher levels of cholesterol. The release profile of diclofenac sodium was shown in "Fig. 7" and (table 4). As the formulation F8 showed optimum particle size, highest entrapment efficiency and maximum drug release, it was optimized and used for further studies.

Release kinetics

The data obtained from the invitro release of optimized formulation was fitted to various kinetic equations like zero order, first order, higuchi, peppas and erosion to determine the mechanism of drug release. The release kinetics was as shown in (table 5). The drug release followed zero order kinetics as indicated by its higher 'r' value (0.97) than first order (0.93). The mechanism of drug release was by diffusion as observed from the higher 'r' value (0.99) of Higuchi plot than erosion (0.97) and the type of diffusion was non-Fickian as indicated by 'n' value of Peppas plot (0.58).

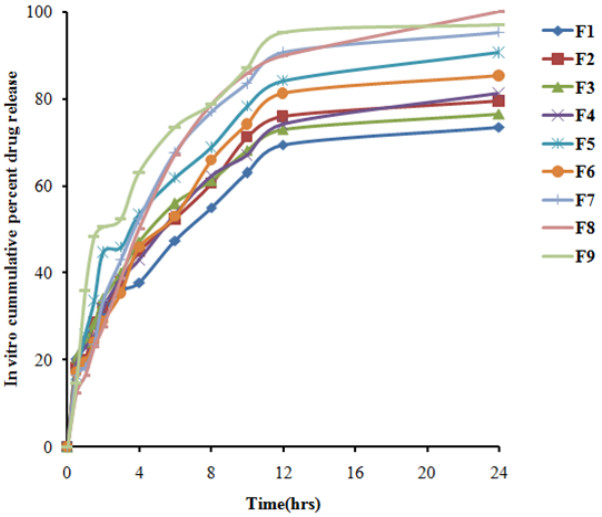


Fig. 7: *In vitro* drug release profile of formulations.

Table 4: Cumulative % drug released from diclofenac niosomes

Time (hrs)	In vitro cumulative percent drug release (mean±s.d. (n=3))								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
0.5	13.2±0.89	16.3±1.65	17.4±2.11	12.78±1.52	13.3±2.57	14.6±1.58	12.5±0.98	10.5±1.83	8.31±2.64
1	15.1±1.05	18.0±1.54	19.3±1.67	15.69±2.31	15.8±1.62	16.1±1.42	15.4±2.36	13.1±0.79	11.49±1.35
1.5	18.4±1.65	20.2±2.31	21.8±2.64	20.21±1.85	18.4±1.54	18.9±1.60	18.4±2.15	18.4±1.48	14.21±0.96
2	21.6±0.92	22.7±2.28	25.1±0.98	24.91±1.63	21.9±2.16	21.3±0.48	21.9±1.95	23.4±2.46	17.48±2.14
3	24.8±0.74	25.9±0.91	28.8±0.51	30.48±0.99	27.6±0.59	25.8±2.42	26.3±0.67	28.6±±1.24	25.81±2.01
4	27.8±1.58	29.1±0.89	32.6±2.64	34.87±1.21	32.5±1.69	29.3±2.36	31.3±0.94	34.7±0.96	32.33±1.54
6	32.8±1.93	34.8±1.91	36.9±1.87	40.76±1.64	36.9±0.82	34.2±0.92	40.5±1.35	41.6±0.84	40.10±1.63
8	39.7±0.98	39.4±2.06	41.1±1.24	46.70±2.81	42.8±2.57	39.6±1.65	46.5±2.31	48.4±1.64	47.32±2.51
10	45.7±2.01	44.7±2.51	45.8±0.84	51.34±2.35	47.4±2.30	45.7±1.25	52.6±1.54	54.2±1.87	55.21±0.98
12	51.3±1.24	50.1±1.08	49.1±1.54	58.89±1.12	55.3±1.24	55.9±1.39	58.5±2.11	60.3±2.04	62.99±0.85
24	79.7±1.38	76.4±1.58	73.5±0.99	90.55±1.34	85.3±1.18	81.1±0.98	95.3±1.89	98.9±1.38	97.25±2.32

Table 5: Release kinetics of F8 formulation

Batch Code	Zero order		First order		Higuchi	Erosion	Peppas	
	K_o	R	K_1	r	r	R	r	N
F8	3.84	**0.97**	0.16	0.93	**0.99**	0.97	0.99	0.58

Effect of variables on particle size and % entrapment efficiency and *in vitro* drug release

The purpose of 3^2 factorial experimental designs was to conduct a comprehensive study of the effect of Span 60 (X_1) and cholesterol (X_2) and their interactions using a suitable statistical tool (Design-Expert® DX 10 software) by applying one way ANOVA at 0.05 levels. Model parameters obtained from analysis of variance (ANOVA) for the response and model coefficients estimated by the regression are used to describe the effect of independent variables on the responses. The three-dimensional response surface graphs and corresponding two-dimensional contour plots were generated by the Design-Expert® DX 10 software. The three-dimensional response surface graph is very useful in learning about the main and interaction effects of the independent variables, whereas two-dimensional contour plot gives a representation of values of the response. The data of the particle size, percent entrapment efficiency and *in vitro* drug release was fitted to quadratic polynomial model. From the ANOVA results of the model relating vesicle size, entrapment efficiency and in vitro drug release as response, it was observed that model terms X_1 (amount of surfactant) for vesicle size and X_1, X_2X_2 (cholesterol-cholesterol interaction) for entrapment efficiency were found to be significant ($p<0.05$). The polynomial quadratic model was suggested for three dependent factors particles size, percent encapsulation efficiency and in vitro drug release. R-square value was found to be 0.9676, 0.9729 and 0.9013 for particle size, percent entrapment efficiency and *in vitro* drug release respectively. R-square value which was near to 1 indicated good model for the selected variables (table 6). From the equations, it was observed that relative amounts of span 60 and cholesterol were found to play an important role in determining vesicle size, entrapment efficiency and *in vitro* drug release (table 7 to 9). The vesicle size was found to be decreased as concentration of Span 60 increased (from the negative coefficient of X_1). Cholesterol also decreases the vesicle size (from negative coefficient of X_2) but the effect was not found to be statistically significant. The percent entrapment efficiency was found to be increased with increase in the amount of Span 60 and cholesterol (from positive coefficients of X_1 and X_2). From the data, it was observed that very high cholesterol content had a lowering effect on drug entrapment. This is explained by the fact that cholesterol beyond certain level disrupts the regular bilayer structure leading to loss of entrapped drug. This effect of cholesterol was also not found to be statistically significant. The coefficient of X_2X_2, that is, cholesterol–cholesterol interaction effect pointed out a curvilinear relationship with the entrapment efficiency. The response surface plot and contour plots for particle size, %entrapment efficiency and *in vitro* drug release were shown in "Fig. 8.1-8.3".

Table 6: Niosomal summary of results of regression analysis for responses Y_1, Y_2 and Y_3.

Model	SS	DF	MS	R^2	Adj R^2	Pred R^2	Sd	P	Remarks
Particle size(Y_1)									
Polynomial equation= +451.10-237.08*A-3067B+27.83*AB+15.11*AA+80.06*BB									
Quadratic	22335.06	2	11167.53	0.9406	0.6829	0.9676	45.32	0.0450	Significant
%Entrapment efficiency(Y_2)									
Polynomial equation= +60.40+13.19*A+0.43*B-2.53*AB+0.48*AA-13.73*BB									
Quadratic	553.32	2	276.66	0.9503	0.7523	0.9729	2.75	0.004	Significant
***In vitro* drug release(Y_3)**									
Polynomial equation= +97.01+0.76*A-0.025*B+11.14*AB+4.69*AA-17.07BB									
Quadratic	779.93	2	389.97	0.8191	0.1653	0.9013	4.83	0.0035	Significant

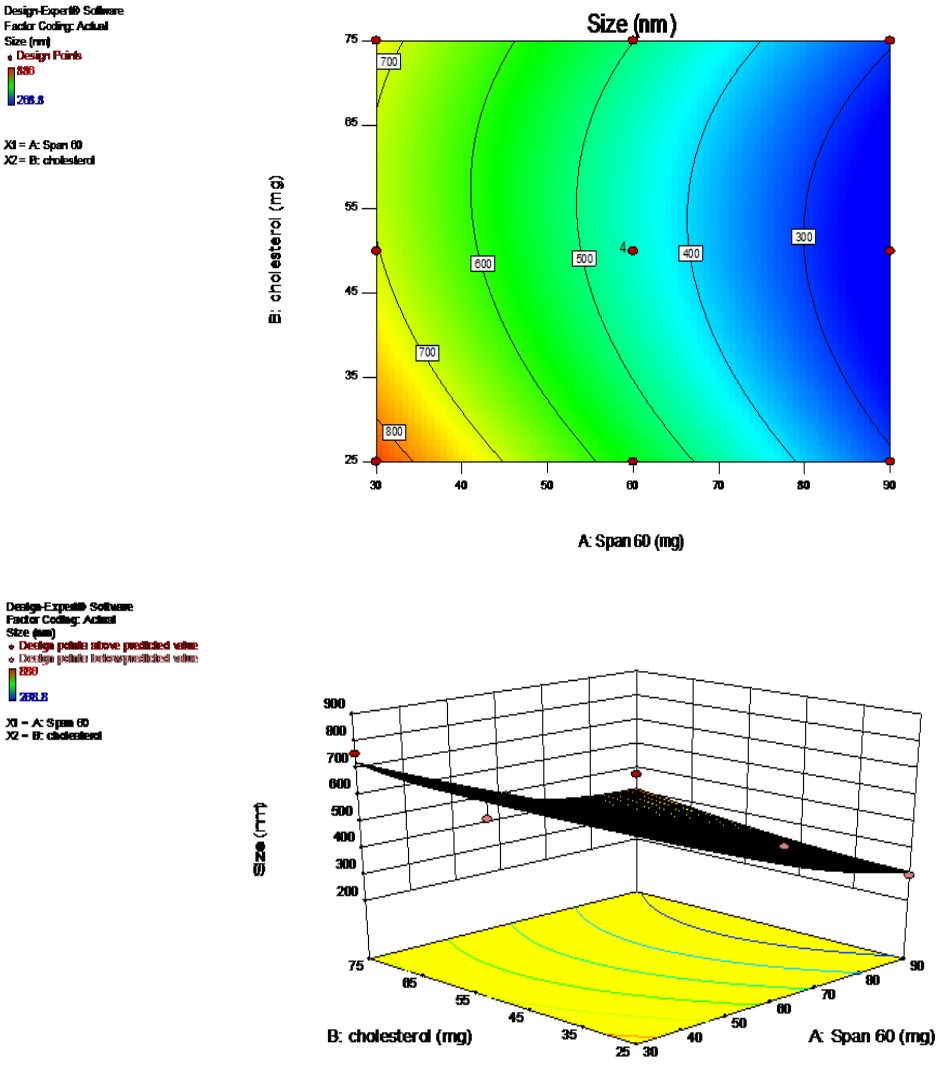


Fig. 8.1: Surface and counter plots for first dependent factor (Size) (Y_1)

Table 7: ANOVA for particle size distribution (Y_1).

Source	SS	DF	MS	F-value		p-value
Model	3.683E+005	5	73665.14	35.86	0.0002	Significant
A-Span 60	3.373E+005	1	3.373E+005	164.19	< 0.0001	
B-cholesterol	5642.67	1	5642.67	2.75	0.1485	
AB	3096.92	1	3096.92	1.51	0.2655	
A^2	609.03	1	609.03	0.30	0.6057	
B^2	17093.34	1	17093.34	8.32	0.0279	
Residual	12324.12	6	2054.02			
Lack of Fit	12324.12	3	4108.04			
Pure Error	0.000	3	0.000			
Cor Total	3.806E+005	11				

The Model F-value of 35.86 implies the model is significant

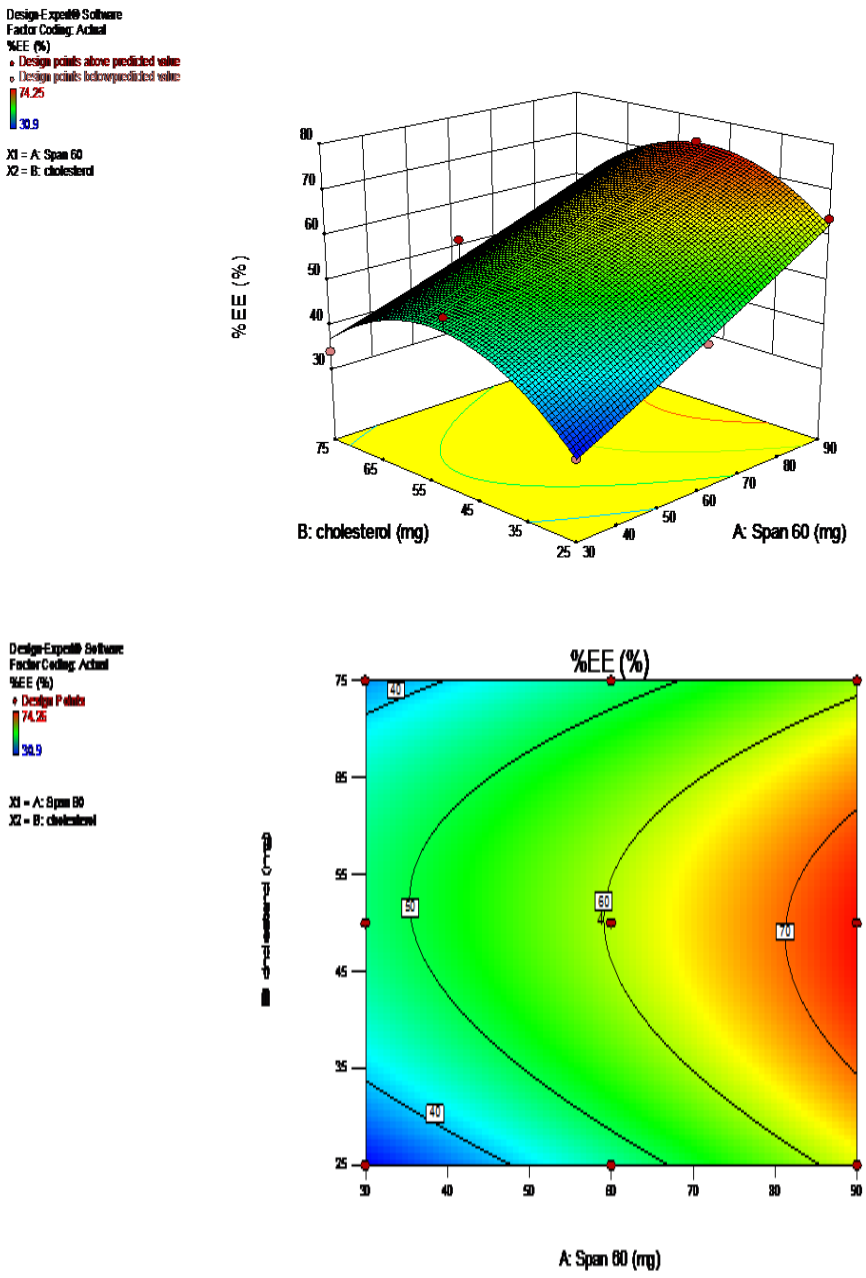


Fig. 8.2: Surface and counter plots for second dependent factor (%EE) (Y_2).

Table 8: ANOVA for Percent encapsulation efficiency (Y_2).

Source	SS	DF	MS	F-value		P-value
Model	1623.87	5	324.77	43.08	0.0001	Significant
A-Span 60	1043.86	1	1043.86	138.48	< 0.0001	
B-cholesterol	1.14	1	1.14	0.15	0.7113	
AB	25.55	1	25.55	3.39	0.1152	
A^2	0.62	1	0.62	0.082	0.7843	
B^2	502.98	1	502.98	66.72	0.0002	
Residual	45.23	6	7.54			
Lack of Fit	45.23	3	15.08			
Pure Error	0.000	3	0.000			
Cor Total	1669.10	11				

The Model F-value of 43.08 implies the model is significant.

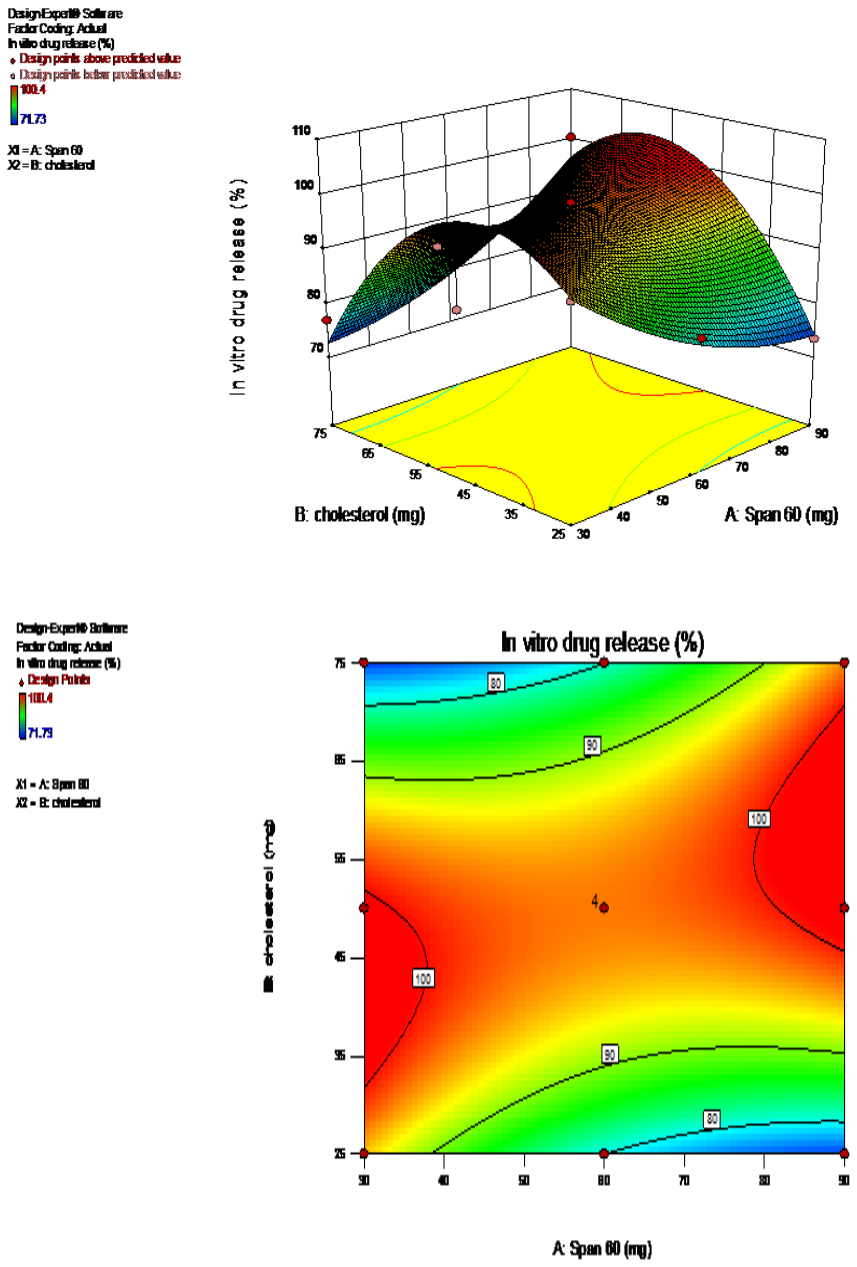


Fig. 8.3: Surface and counter plots for third dependent factor (*in vitro* drug release) (Y_3)

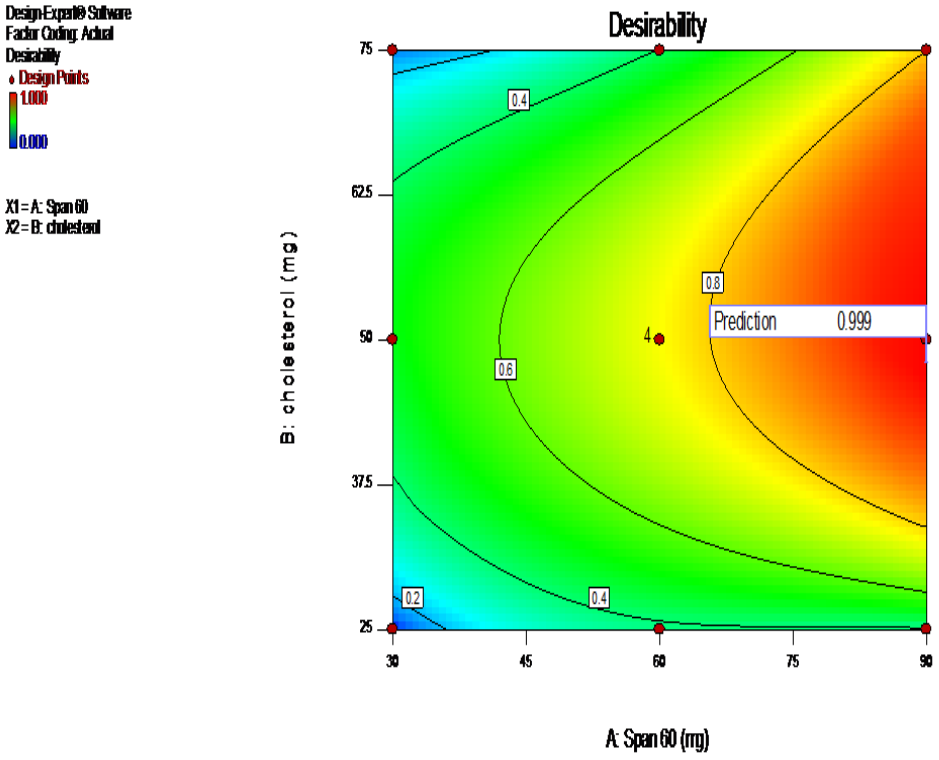
Table 9: ANOVA for *in vitro* drug release (Y_3).

Source	SS	DF	MS	F-value		p-value
Model	1280.05	5	256.01	10.96	0.005	Significant
A-Span 60	3.50	1	3.50	0.15	0.7122	
B-cholesterol	3.750E-003	1	3.750E-003	1.605E-004	0.9903	
AB	496.62	1	496.62	21.26	0.0037	
A^2	58.56	1	58.56	2.51	0.1644	
B^2	776.91	1	776.91	33.26	0.0012	
Residual	140.17	6	23.36			
Lack of Fit	140.17	3	46.72			
Pure Error	0.000	3	0.000			
Cor Total	1420.23	11				

The Model F-value of 10.96 implies the model is significant.

Optimization of vesicular dispersions

The next objective was to optimize the three responses with different targets. The optimized formulation was obtained by applying constraints (goals) on dependent (responses) and independent variables (factors). Optimized formulation was selected based on the criteria of minimum particle size (nm), maximum % EE and *in vitro* drug release. The optimization was carried out by both numerical optimization and graphical optimization techniques. The desirability and overlay plots obtained in the analysis by 3^2 factorial designs were given in "Fig.9" for a desirability value of 0.999. It was observed that the values indicated by the design are essentially the same as the values used in the original design adopted for preparation and characterization of F8 formulation. Hence, it was decided to proceed with further work by taking F8, niosomes of diclofenac sodium (lipid: cholesterol 90:50), as the optimized formulation. It was concluded from this analysis that the predicted liposomal formulation composition was the same as that of the F8 batch; however the experiment was repeated to reconfirm the result. The percent relative error values for all the response parameters were below 5%. The experimental results were in agreement with the predicted values, which confirmed the predictability and the validity of the model shown in (table 9). So, use of optimization through adoption of a 3^2 factorial design was successfully carried out to find the compositions of the vesicles that can have the best values of the chosen dependent variables, such as, size, % encapsulation efficiency and *in vitro* drug permeation. The formulations identified by this technique as those with the best properties, were the same as F8 diclofenac sodium loaded niosomes. Hence these were considered as optimized and were taken for incorporation into gels and for proceeding for further work.



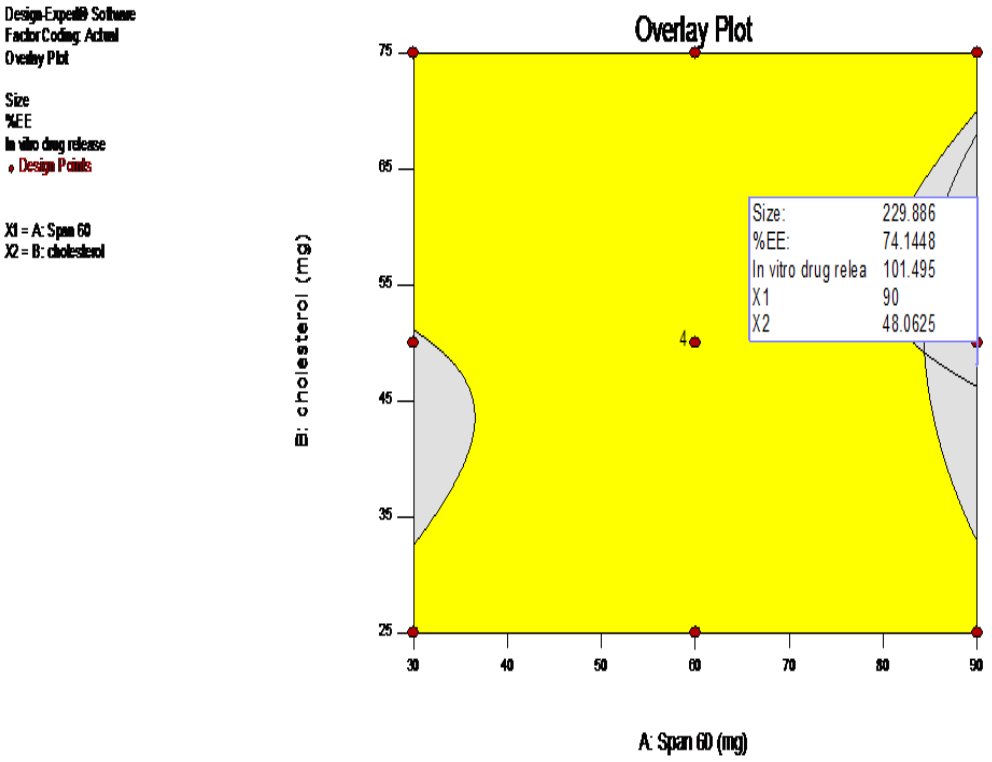


Fig. 9: Desirability and overlay plots.

Table 10: Cross validation of observed and predicted responses of niosomes

Response	Observed	Predicted	% Relative error
	Niosomes		
Y_1	229 nm	230.12	1.56
Y_2	74.02%	75.42	4.21
Y_3	101.4%	100.1	0.89

Pharmacokinetic study

The prepared niosomal dispersion, niosomal gel and plain gel were selected for pharmacokinetic study. All the PK and PD studies were reviewed and approved by the Institutional Animal Ethics Committee, A.U. College of Pharmaceutical Sciences, Andhra University (Reg. No. 516/01/A/CPCSEA). The pharmacokinetic parameters of all tested products are compared and the results are shown in "Fig. 10" and (table 11).

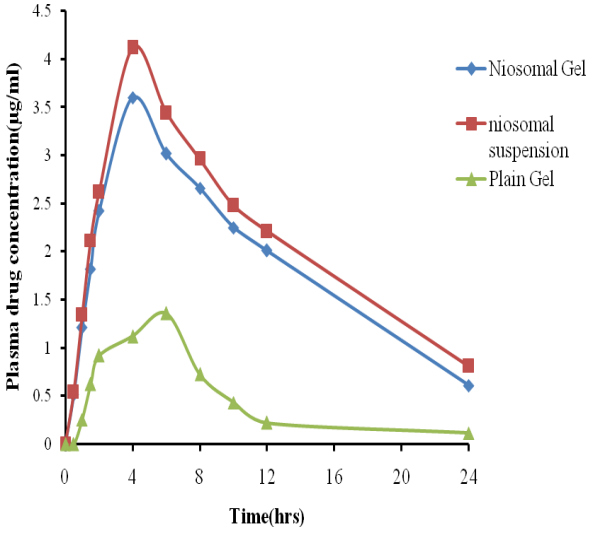


Fig. 10: Comparative plasma drug concentrations of tested products.

Comparative *ex-vivo* permeation studies

Comparative *ex vivo* permeation studies were conducted for optimized batch of niosomal suspension, niosomal

gel prepared using optimized batch and for plain gel. The results are given in the (table 12). The permeation of drug from niosomal gel is more compared to plain gel as encapsulation of diclofenac in niosomes produced an enhancement of permeation compared to plain gel.

Transdermal flux

Transdermal flux (J) for the three formulations across the rat skin was calculated from the slope of the cumulative drug permeated per unit area verses time plot. The permeability coefficient (Kp) was calculated from the transdermal flux and the applied concentration in the donor compartment (C_{donor}). Transdermal flux and Kp of niosomal suspension were found to be higher compared to niosomal gel and plain gel. The results were shown in the (table 13). Higher cumulative amount of drug permeated and steady state transdermal flux from the niosomal gel formulation can be explained by solubilisation, penetration and permeation enhancement effect of amphiphiles of the niosomes bilayers.

Table 11: Pharmacokinetic parameters of all tested products in rats.

Parameters	Niosomal suspension	Niosomal gel	Plain Gel	P-value	Remarks
T_{max} (Hrs)	4±0.01	4±0.00	6±0.00	<0.001	S
C_{max} (µg/ml)	4.12±0.13	3.6±0.00	1.36±0.10	<0.001	S
K_{el} (hr^{-1})	0.0807±0.01	0.097±0.02	0.328±0.09	<0.001	S
$t_{½}$ (Hrs)	8.58±0.4	7.13±0.10	2.10±0.10	<0.001	S
AUC_{0-24hr} (µg.hrs/mL)	51.47±0.5	46.44±0.93	7.82±1.8	<0.001	S
$AUC_{0-\infty}$ (µg.hrs/mL)	61.50±0.6	52.72±2.02	8.18±2.6	<0.001	S
$AUMC_{0-\infty}$ (µg.hrs/mL)	847.22±12.5	639.63±10.9	41.46±19.9	<0.001	S
MRT (Hrs)	13.77±0.2	12.0±0.47	5.06±0.70	<0.001	S
V_d (µg/ml)	0.402	0.390	0.743	<0.001	S
Clearance (Cl_t) (µg/ml/hr)	0.0325	0.0379	0.244	<0.001	S

S is significant for all the tested formulations

Table 12: Ex vivo permeation studies of niosomal suspension, niosomal gel and plain gel

Time (hrs)	Cumulative % drug permeated		
	Niosomal suspension	Niosomal gel	Plain gel
0.5	8.24±0.96	5.03±1.24	2.86±1.72
1	10.31±1.13	8.68±1.51	4.33±0.96
1.5	14.57±1.58	11.38±0.62	7.85±0.54
2	18.54±1.62	14.65±1.37	10.56±1.86
3	22.38±2.01	17.61±1.75	14.84±1.28
4	26.65±0.67	21.89±2.47	17.34±1.71
6	31.54±2.14	27.68±0.86	22.46±2.67
8	39.33±1.85	31.97±1.36	27.37±1.11
10	44.69±1.34	38.46±2.24	31.59±1.54
12	50.64±0.81	41.25±1.41	35.89±1.68
24	87.02±2.54	75.48±1.57	56.88±2.11

Table 13: Ex vivo permeation parameters

Formulation	Cumulative amount permeated ($\mu g/cm^2$)	Transdermal flux ($\mu g/cm^2h^{-1}$)	Permeability coefficient (cm/hr)
Niosomal suspension	770.07	30.10	7.52×10^{-3}
Niosomal gel	667.97	26.29	6.5×10^{-3}
Plain gel	503.41	20.84	5.2×10^{-3}

Stability study

The results of the stability study indicated that the percent entrapment efficiency of the niosomal dispersion decreased from 74% to 68% at 2-8°C whereas at 25±2/60%±5%RH°C, the percentage encapsulation efficiency decreased from 74% to 63% after 3 months. The drug leakage from the niosomes was very less at 2-8°C. This may be attributed to the phase transition of the surfactant at 25±2/60%±5%RH°C causing vesicle leakage during storage. Hence it was concluded that the optimum storage condition for the niosomes was found to be between 2-8 °C.

CONCLUSION

A 3^2 full factorial design has been employed to develop diclofenac loaded niosomes by using ethanol injection method. The statistical optimization reduced the number of experiments to be carried for obtaining formulations

with desired properties. The derived polynomial equations, response and contour plots helped in predicting the values of selected independent variables for preparation of optimum niosomal systems with desired properties. Niosomes showed an optimum particle size, % EE, and higher in *vitro* drug release. Among all the niosomal formulations, F8 batch showed desired optimum results such as lowest particle size, highest % EE and higher *in vitro* drug release of 98.6% and 99.4% at 24 h. Based on (using desirability and overlay plots) optimum particle size, highest % EE and maximum % drug release F8 niosomes were selected to prepare diclofenac sodium loaded niosomal gel and plain gel using carbopol 980 NF. Carbopol 980 NF provides the versatile properties of flexibility, hydrophilicity and biocompatibility. The 1% w/v of niosomal and plain gels were optimized on the basis of desired pseudo plastic flow. *In vitro* and *ex vivo* permeation of niosomal gels shows controlled permeation rate of drug up to 24h as compared to plain gel. The drug and excipients interaction was characterized by FTIR and DSC confirms the compatibility of drug and excipients used in formulation. The *in vivo* pharmacokinetic study of tested products by rats indicated that diclofenac sodium loaded niosomes and their niosomal gel showed superior results compared to plain gel. The niosomes and their niosomal gel provided controlled plasma concentration in rats. The results obtained in the present study were compared to the results obtained from our previous study diclofenac sodium loaded liposomes for transdermal drug delivery. The liposomes were prepared by using the combination of HSPC and cholesterol whereas in niosomes Span-60 and cholesterol were used. The prepared liposomal and niosomal dispersions were evaluated for various physicochemical characteristics like particle size, zeta potential, encapsulation efficiency and *in vitro* drug release. When compared to liposomes, niosomes were found to have higher encapsulation efficiency and increased permeability and these properties make niosomes superior to liposomes. Further, the stability of niosomes was higher when compared to liposomes. This can be attributed to the higher phase transition temperature of Span-60 than HSPC. Additionally, the surfactants used for the preparation of niosomes provide assistance for significant penetration of compounds by adsorption at interfaces, by altering the biological membranes and by modification of the function of the stratum corneum. Transdermal controlled drug delivery gels can lead to decrease in number of applications and thus to better patient compliance. The observations made in the pharmacokinetic study were reduced elimination and increased biological half life for diclofenac from the niosomal gels. So the prepared niosomal gels can be considered as promising tools in the treatment of anti-inflammatory by transdermal route.

CONFLICT OF INTEREST

None declared

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