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Preparation and Optimization of Idoxuridine Liposomes

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The technique of three variables at three levels (3^3) factorial design was used to derive simple reduced second order polynomial equation for constructing contour plots to obtain predetermined percent drug entrapment within liposomes of idoxuridine prepared by reverse phase evaporation method. Three independent variables selected were volume of organic phase (x_1), volume of aqueous phase (x_2), and drug/phosphatidylcholine/cholesterol in molar ratio (x_3). Based on factorial design, twenty-seven batches of idoxuridine liposomes were prepared. Prepared liposomal batches were evaluated for size, lamellarity, and percent drug entrapment. The percent drug entrapment (dependent variable) and the transformed values of independent variables were subjected to multiple regression to establish a second order polynomial equation (full model). To simplify the equation, F-statistic was applied to reduce polynomial equation (reduced model) by neglecting insignificant ($p>0.05$) terms. The coefficient value for independent variable; drug/phosphatidylcholine/cholesterol in molar ratio (x_3) was found to be maximum ($b_3=17.96$) and hence the variable x_3 was considered to be a major contributing variable for percent drug entrapment within liposomes prepared by reverse phase evaporation method. The reduced polynomial equation was used to plot three two-dimensional contour plots at a fixed levels of –1, 0 and 1 of major contributing variable (x_3) to obtain various combinations of values of two other independent variables (x_1 and x_2) at predetermined percent drug entrapment. The conformity of the established equation was checked by preparing three batches three times taking values of the independent variables from the contour plots for prefixed value of percent drug entrapment. Prefixed percent drug entrapment values were taken for designing the experiment and results obtained experimentally were compared using student 't' test and difference between experimentally obtained and theoretically calculated values of percent drug entrapment was found to be statistically insignificant ($p>0.05$). Hence, finding of this study establishes the role of the derived equation and plotted contour plots in predicting the values of independent variables for preparation and optimization of idoxuridine liposomes by reverse phase evaporation method having predetermined percent drug entrapment.

5-Iodo-2'-deoxyuridine (idoxuridine, IDU) has been found effective with the best benefit/risk ratio on *Herpes simplex virus* (HSV) infections[1,2]. Although effective topically, idoxuridine is too toxic for systemic use. It poorly penetrates the stratum corneum when administered in a hydrophilic vehicle like polyethylene glycol (PEG) while lipophilic vehicles such as dimethyl sulfoxide (DMSO) strongly enhances its penetration, through both human and guinea pig skin[3,4]. IDU is marketed as a 10% solution in DMSO, which has some drawbacks, such as the need to apply the solution with a brush; the poor adhesion to the skin surface and, therefore, the loss of active ingredient with the risk to stain or grease the clothes and unpleasant smell of the solvent. Therefore, liposomal topical delivery of IDU is expected to improve the therapeutic efficiency by enhancing penetration and distribution of the drug in skin layers by encapsulation of drug in lipid vesicles as shown by various

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drugs[5-12] by maintaining a sufficient level of drug in skin layers due to formation of drug reservoir and low systemic absorption. Thus, preparation of IDU liposomes was an attempt to develop liposomes of IDU with size range from 2.0 to 5.0 μm for topical drug delivery[13].

Many methods have been used to prepare liposomes[14]. IDU is slightly soluble in water and insoluble in most of the organic solvents, it is thus reasonable to expect that trapping the drug within aqueous compartment of liposomes (unilamellar) or within large aqueous core area of liposomes (oligolamellar) will be more efficient than trapping this drug in the small interstitial water layers in multilamellar liposomes. Hence, liposomes of IDU were prepared by reverse phase evaporation (REV) method to obtain maximum percent drug entrapment (PDE). Many independent formulation variables like volume of aqueous to organic phase and drug/phosphatidylcholine (PC)/cholesterol (CHOL) in molar ratio affect the preparation of liposomes using this method. The ratio of aqueous to organic phase is most important variable in REV method for proper emulsification and formation of fine aqueous droplet surrounded by phospholipid, i.e. liposomes, with uniform size, shape, and high PDE.

For economic reasons, it is necessary to optimize these variables for obtaining IDU liposomes with high PDE using minimum PC and optimum CHOL. It is difficult to assess the effect of the variables individually or in combination. Hence, the aim of this investigation was to derive a mathematical model suitable for predicting the quantitative values of selected independent variables to prepare IDU liposomes having predetermined PDE by REV method.

MATERIALS AND METHODS

IDU and PC (type-E 80) were obtained as gift samples from Allergan Pharmaceutical Ltd., Inc. Ireland, and Lipoid Gmbh, Germany, respectively. CHOL was purchased from S.D. Fine Chemicals, Mumbai, α-Tocopherol was purchased from Himedia, Mumbai and Dialysis sacks [Mol wt. cut of 12,000] was purchased from Sigma Chemical Co. St Louis, MO. All other chemicals and solvents were of analytical reagent grade.

Preparation of liposomes:

Three independent formulation variables were taken at their three levels; low, medium and high, which were represented by the transformed values of –1, 0, and 1 respectively. Values of these selected variables at different levels are shown in Table 1. Twenty-seven batches of liposomes of IDU were prepared by REV method[15] according to experimental design shown in Table 2. PC, CHOL, and α-tocopherol (1% of PC by weight) were dissolved in diethyl ether (organic phase) in a glass boiling tube (Quick fit neck B-24). Drug solution (5.4 μmol/ml) in purified water was injected rapidly into lipid solution through 23-gauge hypodermic needle using 5 ml syringe. The tube was closed with glass stopper and sonicated for 5 min. in a bath sonicator (Model V33, frequency-22 KHz, 120 W, Vibronics Pvt. Ltd, Mumbai). The tube was attached to a rotary evaporator to dry the contents at 37° under vacuum (250 mm of Hg) until a gel was formed. Remove the tube releasing the vacuum from the evaporator and subjected to vigorous mechanical agitation for 5 min. on vortex mixer for collapsing gel to fluid. The tube once again was fitted to rotary flash evaporator for the removal of organic solvent. A cycle of 10 min. drying and 5 min. vortexing was again repeated twice. Final liposomal suspension was subjected to complete removal of last traces of organic solvent in a rotary flash evaporator for 15 min under vacuum (600 mm of Hg). Each batch was prepared three times and stored in refrigerator. The prepared liposome batches were evaluated for PDE, size, and lamellarity.

TABLE 1: A 3^3 FACTORIAL DESIGN FOR IDU LIPOSOMES

Variables	Level		
	Low	Medium	High
x_1	4.0	6.0	8.0
x_2	1.0	1.5	2.0
x_3	1:1:1	1:2:1	1:4:1
Transformed values	-1	0	1

x_1-volume of organic phase (ml), x_2-volume of aqueous phase (ml), x_3-drug/PC/CHOL (molar ratio)

Estimation of entrapped drug and other components:

IDU within liposomes was estimated after removing unentrapped IDU by the method of dialysis[16]. The dialysis was carried out by taking liposomal suspension in dialysis sack (donor compartment) dipped in a beaker containing 200 ml of purified water (receiver compartment). The beaker was kept on a magnetic stirrer adjusting magnetic needle's rotation to 100-120 rpm and run for 4 h. After 4 h, the solution of receptor compartment was estimated for unentrapped IDU at 287 nm using Hitachi U-2000 Spectrophotometer. The PDE in the liposomes was calculated from

TABLE 2: EXPERIMENTAL DESIGN, PDE AND GEOMETRIC MEAN DIAMETERS OF IDU LIPOSOMES.

Batch No.	x_1	x_2	x_3	PDE[§] (±SEM)	Free IDU (±SEM)	dg*	σg#
1	-1	-1	-1	30.8 (1.16)	68.1 (0.55)	3.2	1.4
2	0	-1	-1	48.6 (1.23)	50.1 (0.59)	3.2	1.6
3	1	-1	-1	31.6 (0.84)	65.4 (072)	3.0	2.2
4	-1	0	-1	19.2 (0.59)	76.8 (1.08)	2.9	2.2
5	0	0	-1	42.6 (0.92)	52.4 (1.31)	3.2	1.8
6	1	0	-1	40.2 (0.47)	54.6 (1.04)	3.0	1.5
7	-1	1	-1	11.6 (0.78)	85.4 (1.16)	2.7	2.2
8	0	1	-1	18.7 (0.78)	76.8 (0.59)	2.8	1.8
9	1	1	-1	26.5 (1.25)	70.6 (0.80)	2.9	1.6
10	-1	-1	0	68.4 (1.29)	28.8 (1.49)	4.2	1.5
11	0	-1	0	58.6 (1.12)	38.4 (0.67)	3.6	1.2
12	1	-1	0	59.6 (0.80)	36.4 (0.90)	3.5	1.8
13	-1	0	0	21.5 (0.72)	72.6 (1.25)	3.0	2.2
14	0	0	0	74.4 (0.35)	22.6 (1.14)	3.5	2.3
15	1	0	0	68.9 (0.69)	28.2 (0.49)	3.6	1.8
16	-1	1	0	20.8 (0.73)	75.6 (0.57)	4.2	2.2
17	0	1	0	25.6 (0.43)	70.6 (1.20)	4.0	2.0
18	1	1	0	72.7 (0.20)	22.6 (1.72)	3.8	1.5
19	-1	-1	1	80.6 (0.96)	16.8 (0.76)	4.8	1.2
20	0	-1	1	83.5 (0.69)	12.6 (1.25)	4.6	1.5
21	1	-1	1	78.8 (0.96)	16.8 (1.55)	3.7	1.6
22	-1	0	1	28.0 (0.96)	26.9 (063)	2.9	1.8
23	0	0	1	82.5 (0.84)	32.8 (0.35)	4.9	1.2
24	1	0	1	79.5 (1.12)	17.6 (0.92)	4.8	1.2
25	-1	1	1	34.5 (0.96)	61.8 (1.33)	4.5	1.2
26	0	1	1	42.8 (0.96)	52.4 (1.55)	4.7	1.5
27	-1	1	1	82.9 (0.84)	14.6 (1.10)	4.9	1.6

[§](n=3), *geometric mean diameter, #geometric standard deviation, x_1-volume of organic phase, x_2 - volume of aqueous phase and x_3 drug/PC/CHOL (molar ratio).

the difference between the initial drug added and the drug detected in the receptor compartment of the dialysis unit. PC and CHOL were quantified by the ion-pair complexing[17] and Zlatkis[18] methods, respectively. The data of recovered PC and CHOL are not shown. The mean PDE of all the batches is recorded in the Table 2.

Determination of geometric mean diameter of liposomes by photomicrography:

Samples of IDU liposomes prepared were observed under Olympus (BX 40F4, Tokyo, Japan) microscope with a polarizing attachment to study their size and lamellarity after suitable dilution[19]. Linear diameter of 200 liposomes was measured for every sample. Mean geometric diameter (d_g) and geometric standard deviation (σ_g) were calculated by plotting logarithms of size of liposomes against the cumulative % frequency undersize on a probability scale. Results are shown in Table 2. Fig.1 shows a photomicrograph of IDU liposomes which were used further for making liposomal gels.

Data processing:

A technique of 3^3 factorial design[20] selecting three prime independent formulation variables at their three different levels, affecting the eminence of liposomes was used to design the experimental batches for the preparation of IDU liposomes by REV method. These variables affecting the PDE in REV method were volume of organic phase (x_1), volume of aqueous phase (x_2) and Drug/PC/CHOL in molar ratio (x_3). Twenty-seven batches of different combinations were prepared by taking values of selected variables; x_1, x_2 and x_3 at different levels as shown in Table 2. The prepared batches were evaluated for PDE; a dependent variable, size and lamellarity. The results are recorded in Table 2. Mathematical modeling of the preparation of IDU liposomes by REV method was carried out by using second order polynomial equation[21]described as, $Y=b_0+b_1x_1+b_2x_2+b_3x_3+b_{11}x_1^2+b_{22}x_2^2+b_3^3x_{32}+b_{12}x_1x_2+b_{13}x_1x_3+b_{23}x_2x_3+b_{123}x_1x_2x_3$- (1), where, Y is the dependent variable (PDE) while b_i, b_{ij} and b_{ijk} represent the regression coefficients for second order polynomial and x_i represents the levels of the independent formulation variables i.e., volume of organic phase (x_1), volume of aqueous phase (x_2), and drug/ PC/CHOL in molar ratio (x_3). A full model (Eqn. 2) is established after putting the values of regression coefficients in Eqn.1. Neglecting insignificant ($p>0.05$) terms from the full model establishes a reduced model (Eqn. 3), which facilitates the optimization technique by plotting contour plots keeping one major contributing independent formulation variable constant and varying other two independent formulation variables, to establish the relationship between independent and dependent formulation variables.

Multiple regression:

Transformed values of independent variables; volume of organic phase (x_1), volume of aqueous phase (x_2) and Drug/PC/CHOL in molar ratio (x_3) and their products as in Eqn.1 along with the PDE values (dependent variable) were subjected to multiple regression to determine the coefficients (b_o, b_i, b_{ij}, b_{ijk}) and the p-values of each term of the equation. A second order polynomial equation was derived by substituting the values of b_o, b_i, b_{ij}, and b_{ijk} in Eqn.1. This equation represents a full model (Eqn.2). The terms of full model having p values insignificant ($p>0.05$) have negligible contribution in obtaining dependent variable and thus neglected. Neglecting noncontributing terms of Eqn.2, a reduced polynomial equation obtained is Eqn.3. Results of ANOVA of full model and reduced model was carried out and then F-statistic[22] was applied to check whether the insignificant terms can be omitted or not from the full model (Table 3). The coefficient value of variable, drug/PC/CHOL

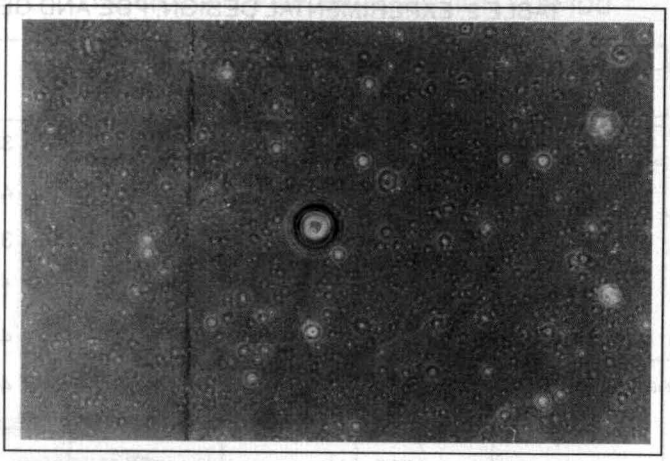

Fig. 1: Photomicrograph of IDU liposomes

IDU liposomes were prepared by REV method. Magnification 1000 X, Geometric mean diameter- 3.5 μm (2.3)].

TABLE 3: RESULTS OF ANOVA OF FULL AND REDUCED MODELS

	df	SS	MS	F Value
Regression (A)	10	12983.8	1298.38	8.25
(B)	4	12249.9	3062.5	19.5
Residuals (A)	16	2726.9 (E2)	45.4	
(B)	22	3460.7 (E1)	157.3	

(A): full model, (B): reduced model, ND=number of beta parameters being tested=6 (those having $p>0.05$), F calculated=$[(SSE_1-SSE_2)/ND/(SSE_2/df\ Residual)=122.3/45.4=2.69$

in molar ratio (x_3) in reduced model (Eqn.3) was found to be highest (b_3 = 17.96) and expected to be major contributing in the preparation of IDU liposomes by REV method. Hence, it was fixed at –1, 0, and 1 level varying other two independent variables for establishing contour plots.

Construction of contour plots:

Two-dimensional contour plots were established using reduced polynomial equation (Eqn.3). Values of x_1 and x_2 were computed at prefixed values of PDE and three contour plots were established between x_1 and x_2 at fixed level of –1, 0, and 1 of x_3 as shown in figures 2a, 2b, and 2c.

Checkpoint:

A check point experiment was performed to confirm the utility of established contour plots and reduced polynomial equation (Eqn.3) in the preparation of IDU liposomes by REV method. Values of independent variables (x_1 and x_2) were taken from three check points each on contour plots plotted at fixed level of –1, 0, and 1 of x_3 and the values of PDE (dependent variable) was calculated by substituting the values in the reduced polynomial equation. Liposomes of IDU were prepared experimentally by taking the amounts of the independent variables (x_1 and x_2) on the same checkpoints. Each batch was prepared three times and mean PDE values were determined (Table 4). Difference of theoretically computed values of PDE and mean values of experimentally obtained PDE was compared by using student 't' test method.

RESULTS AND DISCUSSION

Twenty-seven batches of IDU liposomes were prepared by REV method by using 3^3 factorial design (Table 1) varying three independent variables; volume of organic phase (x_1), volume of aqueous phase (x_2) and Drug/PC/CHOL in molar ratio (x_3). The PDE (dependent variable) of prepared batches was determined and the results are recorded in Table 2. Prepared liposomes were also observed under 1000X magnification using Olympus (BX 40 F4, Japan) microscope to examine their size and lamellarity. The liposomes were found to be oligolamellar with large aqueous core. The mean geometric diameter of oligolamellar type vesicles varies from 2.7 to 4.9 μm with geometric standard deviation ($ó_g$) from 1.2 to 2.3 as shown in Table 2. Maximum drug entrapment achieved in liposomes prepared by REV method was 83.5% at 1 level of x_3 (1:4:1), 0 level of x_1 (6 ml) and –1 level of x_2 (1 ml) but a substantial high drug entrapment (74.4%) was achieved even at 0 level of x_3 (1:2:1), 0 level of x_1 (6 ml) and 0 level of x_2 (1.5 ml).

The PDE (dependant variable) obtained at various levels of three independent variables (x_1, x_2 and x_3) were subjected to multiple regression. A second order polynomial Eqn.2 (full model) was obtained. $PDE=56.94+12.25x_1-11.09x_2+17.69x_3-5.20x_1^2-1.79x_2^2-4.07x_3^2+10.02x_1x_2-2.77x_2x_3+5.51x_1x_3+2.39x_1x_2x_3$—(2), The main effects of x_1, x_2 and x_3 represent the average result of changing one variable at a time from its low to high value. The interactions (x_1 x_2, x_1 x_3, x_2 x_3 and x_1 x_2 x_3) show how the PDE changes when two or more variables were simultaneously changed. The PDE values for the 27 batches showed a wide variation starting from a minimum of 20.8% to maximum of 83.5% (Table 2). This is reflected by the wide range of coefficients of the terms of Eqn.2 representing the effect of individual and combined variables. Small values of coefficients of terms x_1^2, x_2^2, x_3^2, x_2 x_3, x_1 x_3, and $x_1x_2x_3$ (having $p>0.05$) in Eqn.2 are regarded as least contributing in the preparation of IDU liposomes by REV method. Hence, these terms are neglected from full model (Eqn.2) considering insignificant[20] and a reduced polynomial equation (Eqn. 3) obtained following multiple regression of PDE and significant terms ($p<0.05$) of Eqn. 2 as, $PDE=49.39+12.51x_1+1.36x_2+17.96x_3+10.415x_1x_2$—(3). F-statistic[21] of the results of ANOVA of full model and reduced model confirmed omission of insignificant terms of Eqn. 2. Since the calculated F-value (2.69) is less than the tabled F-value (2.74) (α=0.05, v_1=6 and v_2=16), it was concluded that the neglected terms do not significantly contributing in predicting of PDE. When the coefficient values of three independent key variables (x_1, x_2, and x_3) in Eqn. 3 were compared, the value for variable x_3 (b_3=17.96) was found to be maximum and hence the variable x_3 was considered to be a major contributing variable for PDE of IDU liposomes by REV method.

The reduced model (Eqn. 3) was used to plot three two-dimensional contour plots (figs. 2a, 2b, and 2c) at fixed levels of –1, 0, and 1 of major contributing variable (x_3) respectively and computing the values of x_1 and x_2 between –1 to 1 at prefixed value of PDE.

Fig. 2a shows the possible contour plots drawn at –1 level of x_3 for prefixed values of 10%, 20%, 30% and 40%. All contour plots are found to be curvilinear signifying non-linear relation ship between x_1 and x_2 variables. It was elucidated from the contour that at –1 level of x_3, maximum PDE (40%) could be obtained with x_1 range at –1 level (4.0 ml) to 0.869 level (7.74 ml) and x_2 at –0.968 level (1.18 ml) to 0.998 level (1.99 ml). Thus, the range of aqueous to organic phase ratio at –1 level of x_3 was found to be in the range of 1:3.4 to 1:3.9.

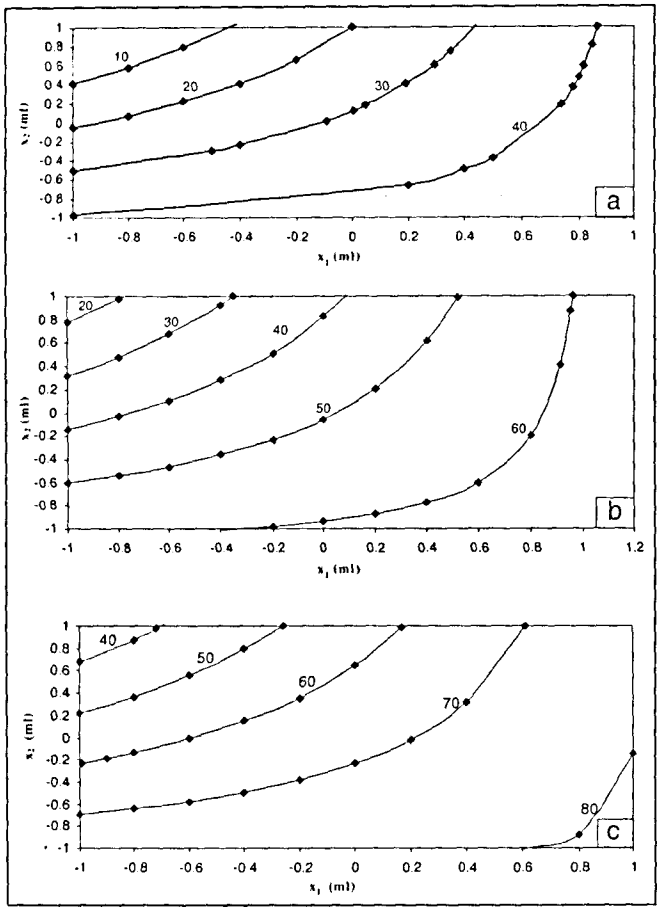

Fig. 2: Contour plots for predetermined PDE

(a) at -1 level of variable x_3, (b) at 0 level of variable x_3 and (c) at 1 level of variable x_3. x_1 – volume of organic phase (ml), x_2 – volume of aqueous phase (ml) and x_3 – drug/PC/CHOL (molar ratio).

Fig. 2b shows the possible contour plots at 0 level of x_3 for prefixed PDE values of 20%, 30%, 40%, 50% and 60%. All the contour plots were found to be curvilinear in nature suggesting that there is no linear relation between the two independent variables (x_1 and x_2). The maximum PDE (60%) at this level of x_3 can be computed with x_1 range at –0.4 (6.8 µl to 0.958 level (7.92 ml) and x_2 range at –1 level (1.0 ml) to 0.999 level (2.0 ml). Thus, the range of aqueous to organic phase ratio for high PDE at this level of x_3, ranges from 1:3.9 to 1:6.8.

Similarly, fig. 2c shows the possible contour plots plotted at 1 level of x_3 for prefixed PDE values of 40%, 50%, 60%, 70% and 80%. All the contour plots were found to be curvilinear thereby suggesting no direct linear relationship between x_1 and x_2 variables. The maximum PDE (80%) can be computed with x_1 range at 0.6 level (7.2 ml) to 1 level (8.0 ml) and x_2 range at –1 level (1.0 ml) to –0.146 level (1.3.7 ml). This computation attributed that for such high PDE at high level of x_3, range of aqueous to organic phase ratio for better emulsification was 1: 5.8 to 1: 7.2. Similarly, for 70% PDE, aqueous to organic phase ratio was computed with x_1 range at –1 level (4.0 ml) to 0.61 level (7.22 µl) and x_2 range at –0.696 level (1.248 ml) to 0.996 level (2.0 ml), suggesting aqueous to organic phase ratio range from 1: 3.2 to 1:3.6. This clearly indicates that aqueous to organic phase ratio range increases to double but the PDE decreases from 80% to 70% i.e. a fall of only 10% PDE. This computation clearly suggested that REV method does not depend on the concentration of lipid but its main crucial step is to obtain a optimum ratio of aqueous to organic phase for better emulsification at all levels of x_3 (drug/PC/CHOL in molar ratio).

Findings of this investigation confirm the earlier results[14], which claimed 1:3 as the ratio of aqueous to organic phase when diethyl ether was taken as organic phase. Conversely, it is evident from the results that plotted contours can be used in predicting the range of ratio of aqueous phase to organic phase in a precise manner.

Three check points were selected each on three plotted contours at fixed levels of –1, 0, and 1 of x_3 (Table 4). The computed PDE values from contours at –1, 0, and 1 level were found to be 40%, 60%, and 70% respectively. Liposomes at these three checkpoints were prepared experimentally by REV method using the amounts of x_1 and x_2 at the selected checkpoints. The experiment was repeated three times and the experimentally obtained mean PDE values were 38.6(±0.837)%, 61.5(±0.286)%, and 68.7(±0.388)% corresponding to its theoretically computed values. When experimentally obtained and theoretically computed PDE values were compared using student 't' test, the difference was found insignificant ($p>0.05$). This confirms the role of a derived reduced polynomial equation and contour plots in the preparation of IDU liposomes of predetermined PDE by REV method.

Findings of these studies conclusively demonstrate the use of 3^3 factorial design, derived reduced polynomial equation and two-dimensional contour plots. Finally, contour plots significantly reduce the cost of optimization of formulation and the efforts of a formulator by using certain ranges of aqueous to organic phase ratio in the preparation of IDU liposomes by REV method.

TABLE 4: CHECKPOINT TABLE

x_3 Level (molar ratio)	Values from contours		Calculated PDE	Experimentally obtained mean PDE* (±SEM)
	x_1 Level (volume)	x_2 Level (volume)		
Minimum level (-1)	0.8	0.49	40.0	38.6**(0.837)
(1:1:1)	(7.6 ml)	(1.745 ml)		
Medium level (0)	0.8	-0.2	60.0	61.5**(0.286)
(1:2:1)	(7.6 ml)	(1.4 ml)		
High level (1)	0.4	0.30	70.0	68.7**(0.388)
(1:4:1)	(6.8 ml)	(1.65 ml)		

*n=3, **difference from calculated PDE value insignificant (P>0.05), x_1- volume of organic phase (ml), x_2- volume of aqueous phase (ml),x_3- drug/PC/CHOL (molar ratio).

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