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Development and Optimization of w/o/w Multiple Emulsion of Lisinopril Dihydrate Using Plackett Burman and Box-Behnken Designs

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Abstract

The aim of the present study was to develop water-in-oil-in-water (w/o/w) type Multiple Emulsion of Lisinopril dihydrate for enhancing its oral bioavailability via enhanced permeation. For primary emulsification, corn oil was used as the oil phase, Span 83 as the lipophilic surfactant and Xanthan gum was used as the viscosity enhancer. Primary emulsion was re-emulsified with aqueous phase containing Tween 20 as hydrophilic surfactant. Preliminary screening was performed using a 12-run, 8-factor, 2-level Plackett–Burman design followed by Box Behnken Design for optimization. MEs were characterized and evaluated for macroscopic and microscopic properties, globule size, entrapment efficiency, rheological properties, in vitro and ex vivo drug release and stability studies. In vitro drug diffusion study was done through dialysis bag and ex vivo permeability studies were performed in Franz diffusion cell using rat intestine. The optimized w/o/w ME showed globule size and entrapment efficiency of 15.65 + 1.967 µm and 87.35 + 3.79 % respectively. Drug flux was found to be 119.3 µg/cm2/h for drug loaded w/o/w ME and 105.1µg/cm2/h for plain drug solution. The overall results of the studies showed the potential of the w/o/w ME as promising drug delivery system for Lisinopril dihydrate.

Keywords: Lisinopril dihydrate; Water-in-Oil-in-Water Multiple emulsion; Optimization; Plackett Burman design; Box-Behnken design; Permeability; Flux

Introduction

Multiple emulsion (ME) systems are novel developments in the field of emulsion technology. MEs are complex systems, termed as emulsions of emulsions, in which globules of the dispersed phase encapsulates smaller droplets, which may normally consist of a liquid miscible with and in some cases identical with, the continuous phase. This is made possible by double emulsification; hence the systems are also called as *'double emulsion'*. Each dispersed globule in the double emulsion forms a vesicular structure with single or multiple compartments separated from the aqueous phase by a layer of oil phase compartment [1]. Inherent instability is the main problem with respect to MEs. To stabilize the system, various emulsifiers can be used. W/O emulsion can be stabilized by low HLB or oil soluble surfactants whereas O/W emulsions can be stabilized by high HLB or water soluble surfactants. Intermediate HLB can provide systems with optimal stability. MEs may be prepared by using such pairs of surfactants which will impart some degree of stability [2].

Double emulsions are helpful to maintain drug concentrations within therapeutic range in the lymph vessels. The transport of drugs to the mesenteric lymph nodes rather than into the portal system directly is due to encapsulation of the drug by the oil phase, which provides more of lipophilic environment and therefore are absorbed through lymphatic vessels. In addition, w/o/w emulsions might act as carriers for the delivery of polypeptide/protein drugs, which require both protection from the gastric fluids and delivery via the lymph nodes [3]. ME's potential biopharmaceutical applications also incorporates intestinal [4] and prolonged [5] drug delivery. Shichiri et al. developed w/o/w MEs for oral administration of insulin by protecting it from proteolytic destruction and facilitating intestinal absorption [6]. MEs are expected to allow delivery of anticancer agents by sparing normal tissues and selectively attacking tumour tissues [7]. Kim et al. studied Cytarabine-loaded w/o/w multiple emulsions using nonionic surfactants of the Tween and Span types. The release study showed that the multiple emulsion containing cytarabine in the internal aqueous phase was stable, exhibiting a prolonged release pattern [8].

Onuki et al. had successfully prepared and optimized the formulation with the highest stability and the most desirable pharmacological effect of the water-in-oil-in-water multiple emulsion for intestinal insulin delivery based on statistical methods such as the orthogonal experimental design and the response surface evaluation [9].

Our research group has previously developed w/o/w type multiple emulsions entrapping acyclovir for improving its oral bioavailability. Particle size of 33.098 ± 2.985 µm and entrapment efficiency of 85.25 ± 4.865% were obtained. Drug release from the prepared formulations showed initial rapid release followed by a much slower release. *In vivo* studies in rats indicated prolonged release and better oral bioavailability as compared to drug solution [10].

Lisinopril dihydrate (LD) a synthetic peptide derivative, is an oral long-acting angiotensin converting enzyme (ACE) inhibitor [11] widely used for the treatment of hypertension caused by increased plasma level of angiotensin II. LD is a Biopharmaceutical Classification System (BCS) class III drug [12], exhibiting variable absorption (6 to 60%) and poor bioavailability (30%) [13]. Hence, the present investigation was aimed at preparation and optimization of w/o/w Multiple Emulsion of LD with the objective of improving its oral bioavailability (via protection in gastric environment and lymphatic delivery).

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The purpose of optimization of any pharmaceutical process is to determine and evaluate independent variables that affect formulation response. Using independent variables, best response values with maximum desired characteristics can be developed with minimum batches to reduce the cost of final product [14]. The number of trials can be reduced by using combination of design tools. In this work, we employed well established statistical tools viz., Plackett-Burman for initial screening and Box Behnken Design, a sub type of response surface methodology for optimization of processing conditions and to identify multi factor interactions [15]. Plackett-Burman Design (PBD) is more practical and frequently used approach whenever a large number of variables are involved [16]. Hence to test a large number of variables with fewer experimental runs, Plackett-Burman Design (PBD) was selected for preliminary screening. Since there is limitation in PBD that it does not provide information regarding the interaction effects between different variables, it is of low utility in the main optimization stage. Therefore, Box-Behnken Design (BBD) of high resolution was used to optimize final stages of ME. Response Surface Methodology (RSM) [17] was selected to identify multi-factor interactions and optimise the processing conditions in the preparation of multiple emulsion. The Plackett–Burman screening design was constructed using Minitab version 17 (Minitab Inc., State College, PA) and RSM-Box Behnken Design using Design Expert 7.0.0 software.

Materials and Methods

Materials

Lisinopril dihydrate was received as a gift sample from Torrent Research Centre, Ahmedabad, India. Span 80, Tween 20 and Xanthan gum were obtained from S.D. Fine Chemicals Ltd., Mumbai, India. Span 83 and Corn oil were obtained from National Chemicals, Vadodara, India. Other chemical reagents and solvents used were of Analytical Reagent (A.R) or HPLC grade.

Screening of oil

Oil was selected on the basis of solubility of LD in various oils. Different oils like light liquid paraffin, Soyabean oil, Sunflower oil, Linseed oil and Corn oil were screened. 10 mL of the selected oil was added to each cap vial containing an excess of LD (100 mg). After sealing, mixtures were shaken for 15 min and were kept at 25°C for 48 h. After reaching equilibrium, the vials were centrifuged at 3000 rpm for 5 min. The supernatant was separated carefully and analyzed for LD content after appropriate dilutions with dimethylformamide (DMF) using UV-spectrophotometer (UV-1800 Shimadzu, Japan) at 595 nm [18].

Preparation of ME

Two-step emulsification technique was used to prepare ME.

- **Primary emulsification:** 4 mL of aqueous phase containing 20 mg of LD and 0.2% w/v of Xanthan gum was added to 6 mL of corn oil containing 10% w/v of Span 80 or Span 83 and emulsified at 6500 rpm for 3 min using Ultra-Turrax T-25 (IKA, India) homogenizer.
- **Secondary emulsification:** 10 mL of primary W/O emulsion was gradually added to 10 mL of distilled water containing 5% w/v of Tween 20 at 750 rpm for 10 min using High-speed Stirrer (Remi, India) to form multiple emulsion.

Experimental design

Preliminary screening using Plackett-Burman design (PBD): PBD was utilized for initial screening of significant variables affecting the particle size (PS) and entrapment efficiency (EE) of ME. The design consisted of 12 trials at 2 levels for 8 different variables using Minitab version 17 (Minitab Inc., USA, PA). The variables were (A) Concentration of lipophilic emulsifier in primary emulsion-W/O (5 and 15%w/v), (B) Phase Volume Ratio (W:O) in primary emulsion (40:60 and 60:40), (C) Speed for Primary Emulsification (6500 and 9500 rpm), (D) Time for primary emulsification (3 and 7 min), (E) Concentration of hydrophilic surfactant in secondary emulsion (3 and 7%w/v), (F) Phase Volume Ratio (W/O : W) in secondary emulsion: (40:60 and 60:40), (G) Speed for secondary emulsification (500 and 1000 rpm) and (H) Time for secondary emulsification (5 and 15 min). Relative effect of different variables were screened with the help of Pareto charts for observed responses [17].

Optimization of key variables via Box Behnken design (BBD): Based on results of the PBD, three most significant factors: Phase Volume Ratio in primary emulsion (X1), Time for primary emulsification (X2) and Concentration of hydrophilic surfactant in secondary emulsion (X3) that influenced PS (Y1) and EE (Y2) were studied using BBD. The coded and actual values of the variables in BBD are as shown in Table 1. The BBD comprised of 17 runs, 3-factors and 3-levels with five centre point trials for reproducibility. Second order polynomial models were generated with the results of BBD and were used for process optimization in preparation of ME with minimum PS and maximum EE [19].

Contour plots and response surface analysis: Two-dimensional contour plots were established between X_1 and X_2, X_1 and X_3, and X_2 and X_3 at constant level (0) of X_3, X_2 and X_1, respectively for PS and EE.

Three dimensional response surface plots were established to study the interaction of two factors keeping other factor at fixed level.

Optimization and validation: The established contour plots, response surface plots and reduced polynomial equation were confirmed by performing check point analysis. Values of independent variables were taken from three check points on contour plots and the values of PS (Y1) and EE (Y2) were calculated by substituting the values in the reduced polynomial equation. MEs were prepared experimentally as explained earlier. Each batch was prepared three times and mean values were determined. Difference in the predicted

Factors: Independent variables	Levels Used		
	-1	0	+1
X1=Phase Volume Ratio (Ratio of W: O) in primary emulsion	4	5	6
X2=Time for primary emulsification (min)	3	5	7
X3=Concentration of hydrophilic surfactant in secondary emulsion (%)	3	5	7
Responses	Constraints		
Y1=Particle size (μm)	Minimize		
Y2=Entrapment efficiency (%)	Maximize		

Table 1: Variables and their levels in the Box Behnken design.

and actual values of experimentally obtained PS and EE were checked using student's 't' test.

Simultaneous optimization technique (Desirability function) was used to simultaneously optimize the PS and EE [20]. Design Expert software (version 8.0.3, Suite, Minneapolis, USA) was used to calculate total desirability. Criteria of minimum PS and maximum EE were set to predict optimum conditions.

Characterization and Evaluation of ME

Macroscopic and microscopic evaluation

Macroscopic evaluation was performed in order to judge the homogeneity of the ME formulations. Formulations were visually observed for homogeneity, color, consistency and appearance [10]. Optical microscope (Olympus BX 40, USA) connected with a camera was used for microscopic evaluation and observations were made at 40X magnification after suitable dilutions with distilled water.

Globule size determination

The mean globule size and size distribution of the ME were measured by dynamic laser light scattering technique using particle size analyzer (Malvern Mastersizer 2000, UK) [10]. Particle size measurements were carried out at a 90° scattering angle. The samples were freshly diluted with distilled water before analysis. Each measurement was performed in triplicate.

Zeta potential

The zeta potential of the ME was determined by the laser doppler electrophoretic mobility measurement technique using Zetasizer (Malvern Nano ZS90, U.K.) at 25°C. All the measurements were carried out in triplicate.

Entrapment efficiency

5 mL of freshly prepared ME was centrifuged (Sigma Centrifuge - 3K30, Germany) at 4500 rpm for 10 min at room temperature. The supernatant was collected, suitably diluted using DMF and analysed for free drug content spectrophotometrically at 595 nm (UV – 1800, Shimadzu, Japan) [21]. Free drug content was calculated using regression equation (Y=0.0135X+0.0211) obtained in concentration range of 10-50 µg/ml. EE was calculated [10] by equation (1)

$$EE\% = \frac{\text{Total Drug Added} - \text{Free Drug}}{\text{Total Drug Added}} \times 100 \quad (1)$$

Rheological analysis

Rheological analysis was performed employing Brookfield viscometer (DV-I Prime, Brookfield, USA) using S61 spindle at 20 rpm at room temperature. All the measurements were carried out in triplicate. The rheological behaviour of each ME was evaluated by plotting the viscosity (cP) versus shear rate (1/s).

In vitro diffusion studies

In vitro drug diffusion studies were carried out using dialysis bag technique [22]. Dialysis bags with a molecular weight cut-off of 12000 Daltons and pore size 2.4 nm (Hi-media, Mumbai, India) were washed with distilled water and soaked in pH 7.4 phosphate buffer saline (PBS) overnight before use. 2 mL of freshly prepared MEs (equivalent to 1 mg/mL of Lisinopril dihydrate) was introduced into the dialysis bag and was tightly sealed at each end with thread. The dialysis bag was placed over magnetic stirrer in a beaker containing 25 mL of PBS pH-7.4 at 100 rpm and temperature was maintained at 37 ± 0.5°C. Aliquots were withdrawn at 0.25, 0.5, 1, 2, 4, 6 and 8 h and replaced with fresh buffer solution in order to maintain sink conditions [23]. Diffusion of plain drug solution (1 mg LD/mL in PBS pH-7.4 buffer) was also studied in a similar manner. The samples were analysed spectrophotometrically at 595 nm after suitable dilutions. The studies were carried out three times and cumulative percentage drug release was calculated. *In vitro* release study data was further fitted to various release models viz zero order, first order, Korsemeyer Peppas and Higuchi model to identify the mechanism and kinetics of drug release from formulated MEs. The drug release at different time points was calculated using regression equation (Y=0.012X+0.077) obtained in concentration range of 10-50 µg/ml. Regression coefficient (r^2) was calculated to identify the best-fit model [10].

Ex vivo permeability studies

The *ex vivo* permeability studies of LD loaded ME and plain drug solution were performed across rat intestine. The study protocols were approved by the Institutional Animal Ethics Committee of Pharmacy department, M S University of Baroda, Vadodara, India. For this study, non fasting male albino rats (250–300 g) were sacrificed by overdose of anaesthesia. Intestine was immediately isolated, washed properly with PBS pH 7.4 buffer solution and examined for integrity. The excised intestinal membrane was then mounted on Franz diffusion cell in such a manner that membrane did not shift from its place once the dosage form was placed onto it. The hooks were secured with rubber bands on the sides of both compartments to make it leak proof. Franz diffusion cell had a diameter of 16.9 mm and membrane with thickness of 0.2 ± 0.1 mm. The area available for diffusion was about 2.25 cm^2. The temperature of the receiver chamber containing 20 mL of diffusion media (phosphate buffer, pH 7.4) was controlled at 37 ± 0.5°C under continuous stirring with Teflon-coated magnetic bar at a constant rate, in a way that the membrane surface just flushes the diffusion fluid. 2 mL (1 mg/mL) of MEs and plain drug solution was placed in the donor compartment of Franz diffusion cell. Samples were withdrawn and replenished by the same volume of fresh buffer solution at predetermined time intervals. The drug content was determined spectrophotometrically at 595 nm for estimation of LD after suitable dilutions with PBS [10,24-26].

The formulations were studied in triplicate for permeability studies. The cumulative amount of drug permeated (µg/cm^2) was plotted as a function of time (t) for each formulation. Drug flux (permeation rate) at steady state (Jss) was calculated by dividing the slope of the linear portion of the graph by the area of the diffusion cell. The diffusion co-efficient was calculated by dividing Jss by the initial concentration of drug in the donor cell (C_0) and the mean cumulative values for %drug diffused versus time were plotted against time. The slopes of the graphs were used to calculate the diffusion coefficients and the results were subjected to one-way ANOVA [26].

Enhancement ratio (Er) was calculated [25] by Equation (2)

$$Er = \frac{\text{Jss of ME}}{\text{Jss of Plain Drug Solution}} \quad (2)$$

Where, Jss is drug flux at steady state.

Stability studies

The optimized formulations were subjected to stability testing as per International Conference on Harmonization (ICH) Q1A (R2) guidelines (ICH, 2003). Optimized formulations were sealed in Type-I transparent glass vials and stored at refrigerated condition (2–8°C),

long term condition (25 ± 2°C/60 ± 5% RH), and accelerated condition (40°C ± 2°C/75 ± 5% RH) in stability chamber (S. R. Lab Instruments, Mumbai, India) for a period of 3 months. Samples were also observed visually for phase separation and by microscopy for homogeneity (to confirm its multiple nature). At monthly intervals, samples were analysed for PS, zeta potential and EE. Each study was performed in triplicate.

Statistical Analysis

The experimental data were validated by Analysis of Variance (ANOVA) followed by F-test and Student t-test which was determined at 95% (α=0.05) significance level using Microsoft excel software (version: 2007). The co-efficient of multiple regression analysis (R^2) and the Fisher F test (Fisher variation ratio, the ratio of mean square for regression to mean square for residual) of the model were used in the statistical evaluation.

Results and Discussion

Selection of oil

Nature of oil is of great importance in case of ME since it controls the permeability of the liquid membrane, which in turn controls the release of solute across it [27]. To minimize the leaching of the drug to the external aqueous phase, the oil in which drug had minimum solubility was selected. Solubility of LD was measured in different oils to select the most appropriate oil for the preparation of MEs (Table 2). From the results obtained, it was observed that solubility of LD was minimum in corn oil when compared to other studied oils. Hence corn oil was selected to prepare the w/o/w ME of LD.

Oil	Solubility (mg/mL ± SD)
Liquid Paraffin light	0.633 ± 0.064
Sunflower oil	1.464 ± 0.096
Corn oil	0.356 ± 0.028
Linseed oil	0.426 ± 0.067
Soyabean oil	1.234 ± 0.088

Table 2: Solubility of LD in various oils.

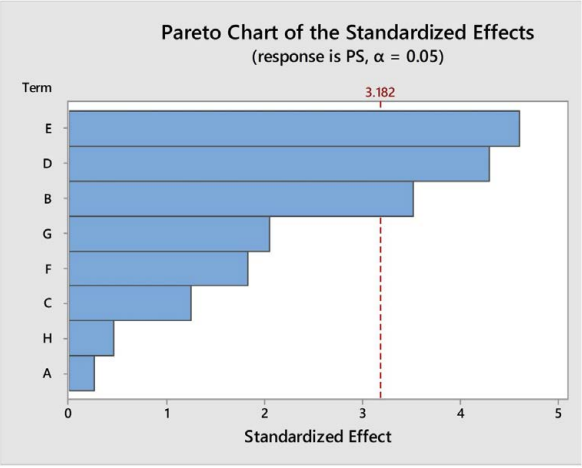

Figure 1: Pareto chart for Particle Size where A is Concentration of lipophilic emulsifier in primary emulsion-W/O, B is Phase Volume Ratio (W: O) in primary emulsion, C is Speed for Primary Emulsification, D is Time for primary emulsification, E is Concentration of hydrophilic surfactant in secondary emulsion, F is Phase Volume Ratio (W/O: W) in secondary emulsion, G is Speed for secondary Emulsification, H is Time for secondary emulsification.

Plackett-Burman design

The PBD was used for initial screening and segregation of variables that significantly influenced the PS and EE of MEs. The relative effects of each factor are shown as Pareto charts in Figures 1 and 2.

As per Pareto charts, Concentration of hydrophilic surfactant in secondary emulsion, Phase Volume Ratio (Ratio of W:O) in primary emulsion and Time for primary emulsification were the variables that significantly (P<0.05) influenced both PS and EE of MEs. The PS varied from 15.30 µm to 40.32 µm and EE varied from 62.35% to 93.54% based on various factor combinations involved. The results obtained in Pareto charts can be observed visually using half normal plots [28]. Sometimes more than one factor seems to be almost significant (variable F in Figure 2) but when visualised in half normal plots, we get actual idea that such variables only act as a pseudo significant factor. Half-normal plots (Figures 3 and 4) gave a visual indication that factors D, E and B had significant effect on PS and EE. Hence these three factors were taken for final optimization of the MEs by Box Behnken Design.

Box Behnken design (BBD)

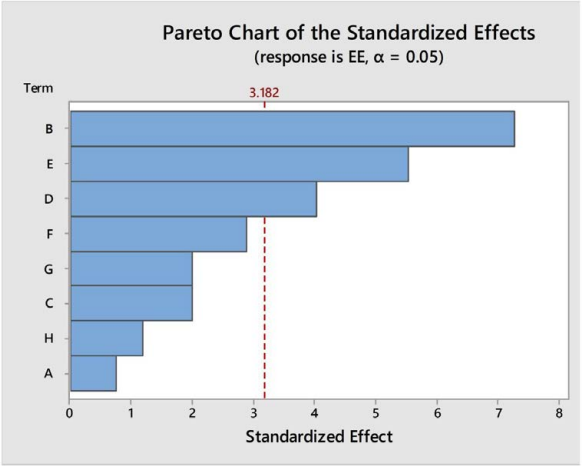

Figure 2: Pareto chart for Entrapment Efficiency where A is Concentration of lipophilic emulsifier in primary emulsion-W/O, B is Phase Volume Ratio (W: O) in primary emulsion, C is Speed for Primary Emulsification, D is Time for primary emulsification, E is Concentration of hydrophilic surfactant in secondary emulsion, F is Phase Volume Ratio (W/O: W) in secondary emulsion, G is Speed for secondary Emulsification, H is Time for secondary emulsification.

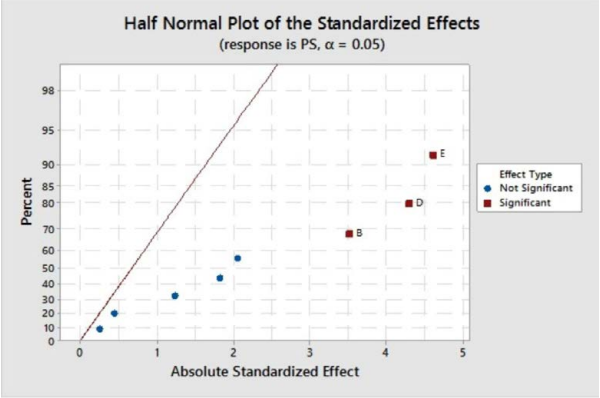

Figure 3: Half normal plot for particle size.

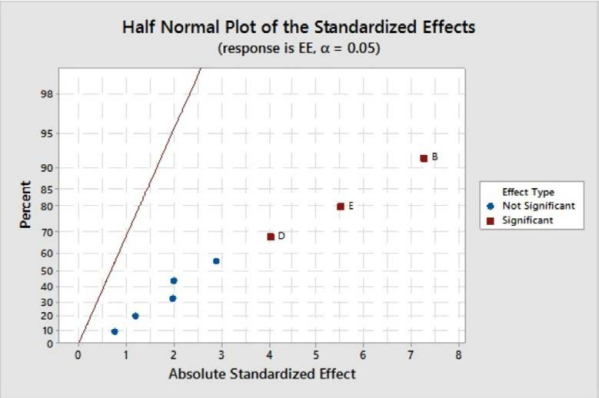

Figure 4: Half normal plot for entrapment efficiency.

Batch. No.	X_1	X_2	X_3	Y_1 (in µm)	Y_2 (in %)
BB1	0	0	0	17.74 ± 0.99	92.62 ± 4.62
BB2	1	1	0	33.98 ± 1.65	72.35 ± 1.32
BB3	-1	0	1	23.62 ± 3.42	85.25 ± 5.85
BB4	1	0	-1	25.96 ± 1.74	77.36 ± 2.47
BB5	-1	1	0	27.02 ± 2.44	84.41 ± 3.36
BB6	-1	0	-1	19.54 ± 0.95	85.87 ± 2.14
BB7	0	0	0	16.74 ± 2.12	92.62 ± 5.68
BB8	1	-1	0	33.54 ± 1.05	77.54 ± 1.42
BB9	-1	-1	0	27.56 ± 1.74	83.98 ± 5.89
BB10	0	1	1	36.56 ± 0.95	71.65 ± 3.69
BB11	0	-1	-1	32.98 ± 3.70	74.21 ± 6.84
BB12	0	-1	-1	34.68 ± 1.25	74.65 ± 5.74
BB13	0	0	0	16.74 ± 1.89	92.62 ± 2.89
BB14	0	0	0	15.54 ± 2.36	93.62 ± 4.95
BB15	1	0	1	29.24 ± 0.98	75.35 ± 3.32
BB16	0	-1	1	32.85 ± 2.11	74.84 ± 6.84
BB17	0	0	0	16.74 ± 1.65	92.62 ± 4.54

Table 3: Design matrix for BBD for Lisinopril dihydrate MEs.

Design Expert software was extensively used for designing the sequence of trials and interpreting the results [17]. BBD is rotatable or nearly rotatable second-order design based on three-level incomplete factorial design [29]. Using Box Behnken Design, seventeen batches of LD MEs were prepared by varying three independent variables, Phase Volume Ratio (W: O) in primary emulsion (X_1), Time for primary emulsification (min) (X_2), and Concentration of hydrophilic surfactant in secondary emulsion (%) (X_3). PS (Y_1) and EE (Y_2) were taken as the dependent variables and the results were recorded. The PS and EE values for the 17 batches showed a wide variation from 16.74 to 36.56 µm and 71.65 to 93.62%, respectively (Table 3).

This variation is reflected in equation 3 and equation 4 for PS and EE respectively. The p-value and t-stat demonstrated the significance of each coefficient. The corresponding coefficient is found to be more significant when smaller is the p value and larger is the magnitude of the 't' value (Table 4) [30].

$$Y_1 = 16.70 + 3.12X_1 + 0.66X_2 + 1.14X_3 + 0.24X_1X_2 - 0.20X_1X_3 + 0.50X_2X_3 + 2.07X_1^2 + 11.7X_2^2 + 5.82X_3^2 \quad (3)$$

$$Y_2 = 92.82 - 4.61X_1 - 0.94X_2 - 0.63X_3 - 1.41X_1X_2 - 0.35X_1X_3 - 0.91X_2X_3 - 3.07X_1^2 - 10.19X_2^2 - 8.80X_3^2 \quad (4)$$

The quadratic model was significant for both PS and EE with F-value of 67.37 and 632.30 respectively. Value of regression coefficient (R^2=0.9886 for PS and R^2=0.9988) signified good correlation between the responses and the chosen variables.

For PS, coefficients X_1, X_3, X_1^2, X_2^2, X_3^2 were significant while X_2, X_1X_2, X_1X_3, X_2X_3 were least contributing (Equation 3) and therefore were neglected from the full model. Since there were many insignificant terms, model reduction was used to simplify the model. Reduced polynomial equation obtained after model reduction is shown in Equation 5. After model reduction, the "Pred R-Squared" of 0.9559 was in reasonable agreement with the "Adj R-Squared" of 0.9754.

$$Y_1 = 16.70 + 3.12X_1 + 1.14X_3 + 2.07X_1^2 + 11.75X_2^2 + 5.82X_3^2 \quad (5)$$

Similarly, For EE, coefficients X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_1X_2, X_2X_3 were significant while only X_1X_3 was least contributing (Equation 4) and therefore neglected from the full model. Reduced polynomial equation obtained after model reduction was represented as Equation 6. After model reduction, the "Pred R-Squared" of 0.9894 was in reasonable agreement with the "Adj R-Squared" of 0.9969.

$$Y_2 = 92.82 - 4.61X_1 - 0.94X_2 - 0.63X_3 - 1.41X_1X_2 - 0.91X_2X_3 - 3.07X_1^2 - 10.19X_2^2 - 8.80X_3^2 \quad (6)$$

The results of ANOVA of the second order polynomial equation of PS and EE are given in Tables 5 and 6, respectively. F tabulated was found to be higher than F calculated for both PS and EE. Therefore, the neglected values did not significantly contribute for the prediction of responses selected [31]. Hence F-statistic justified the reduction of

Source	PS		t-stat	EE		t-stat
	Coefficients	p-value		Coefficients	p-value	
Model	-	*<0.0001*		-	*< 0.0001*	
Intercept	16.70	*9.73E-09*	30.8410	92.82	*4.57E-17*	478.9602
X_1	3.12	*0.0002*	7.2941	-4.61	*< 0.0001*	-30.1142
X_2	0.66	0.1650	1.5505	-0.94	*0.0005*	-6.12728
X_3	1.14	*0.0325*	2.6601	-0.63	*0.0047*	-4.07941
X_1X_2	0.24	0.6978	0.4047	-1.41	*0.0003*	-6.48454
X_1X_3	-0.20	0.7508	-0.3304	-0.35	0.1528	-1.60383
X_2X_3	0.50	0.4339	0.8300	-0.91	*0.0041*	-4.18841
X_1^2	2.07	*0.0098*	3.5144	-3.07	*< 0.0001*	-14.5135
X_2^2	11.7	*< 0.0001*	19.9150	-10.19	*< 0.0001*	-48.2283
X_3^2	5.82	*< 0.0001*	9.8569	-8.80	*< 0.0001*	-41.6582
Residual: Lack of fit	-	0.0966		-	0.5316	

Significant terms having p-value <0.05 were represented in italics.

Table 4: Regression Coefficients and their p-values for PS and EE.

		df	SS	MS	F	R^2	Adjusted R^2
Regression	FM	9	888.96	98.77	67.37	0.9886	0.9739
	RM	5	884.03	176.81	127.98	0.9831	0.9754
Residual (Error)	FM	7	10.26	1.47			
	RM	11	15.20	1.38			

Notes: SS of $Error_{(RM)}$ – SS of $Error_{(FM)}$=15.20 – 10.26=4.94.
No. of parameters omitted=4.
MS of $error_{(FM)}$=1.47.
F calculated=[(SS of $Error_{(RM)}$ – SS of $Error_{(FM)}$)/No. of parameters omitted]/ MS of $error_{(FM)}$
=(4.94/4)/1.47
=0.840
F tabulated (at p<0.05)=4.12.

Table 5: Analysis of variance (ANOVA) of full model (FM) and reduced model (RM) for PS of ME of LD.

models.

Contour plots and response surface analysis

For each PS and EE, Contour plots were generated between X_1 VS. X_2, X_1 VS. X_3, and X_2 **VS.** X_3 at fixed level (0) of third variable as shown in Figures 5 and 6, respectively. For PS (Figure 5A and 5B), the plot formed parabolic shape and showed that increase in Phase Volume Ratio (W:O) in primary emulsion (X_1), increased particle size whereas time for primary emulsification (X_2) and Concentration of hydrophilic surfactant in secondary emulsion (X_3) gave minimum particle size when these variables has mid values of 5 min and 5% respectively when plotted against X_1. On either side of mid value, particle size increased. Lowest PS of 15.65 µm was observed at lowest value of X_1. However, the Contour of Time for primary emulsification (X_2) VS. Concentration of hydrophilic surfactant in secondary emulsion (X_3) (Figure 5C) confirmed the minimum PS at the mid value.

For EE (Figure 6A), maximum entrapment was observed at low level of Phase Volume Ratio (W:O) in primary emulsion (X_1). However, Concentration of hydrophilic surfactant in secondary emulsion (X_3) should be in middle range to achieve maximum EE (Figure 6B) when plotted against X_1. Highest EE of 94.37% was observed at lowest value of X_1. The Contour of Time for primary emulsification (X_2) VS. Concentration of hydrophilic surfactant in secondary emulsion (X_3) (Figure 6C) confirmed maximum EE at the mid value. Hence, it was concluded from the Contours that low Phase Volume Ratio (W:O) in primary emulsion (40:60), mid ranges of Time for primary emulsification (5 min) and Concentration of hydrophilic surfactant in secondary emulsion (5%) were required to attain minimum PS and maximum EE.

		df	SS	MS	F	R^2	Adjusted R^2
Regression	FM	9	1068.61	118.73	632.30	0.9988	0.9972
	RM	8	1068.13	133.52	594.23	0.9983	0.9966
Residual (Error)	FM	7	1.31	0.19			
	RM	8	1.80	0.22			

Notes: SS of $Error_{(RM)}$ – SS of $Error_{(FM)}$=15.20 – 10.26=4.94.
No. of parameters omitted=4.
MS of $error_{(FM)}$=1.47.
F calculated=[(SS of $Error_{(RM)}$ – SS of $Error_{(FM)}$)/No. of parameters omitted]/ MS of $error_{(FM)}$
=(4.94/4)/1.47
=0.840
F tabulated (at p<0.05)=4.12.

Table 6: Analysis of variance (ANOVA) of full and reduced models for EE of ME of LD.

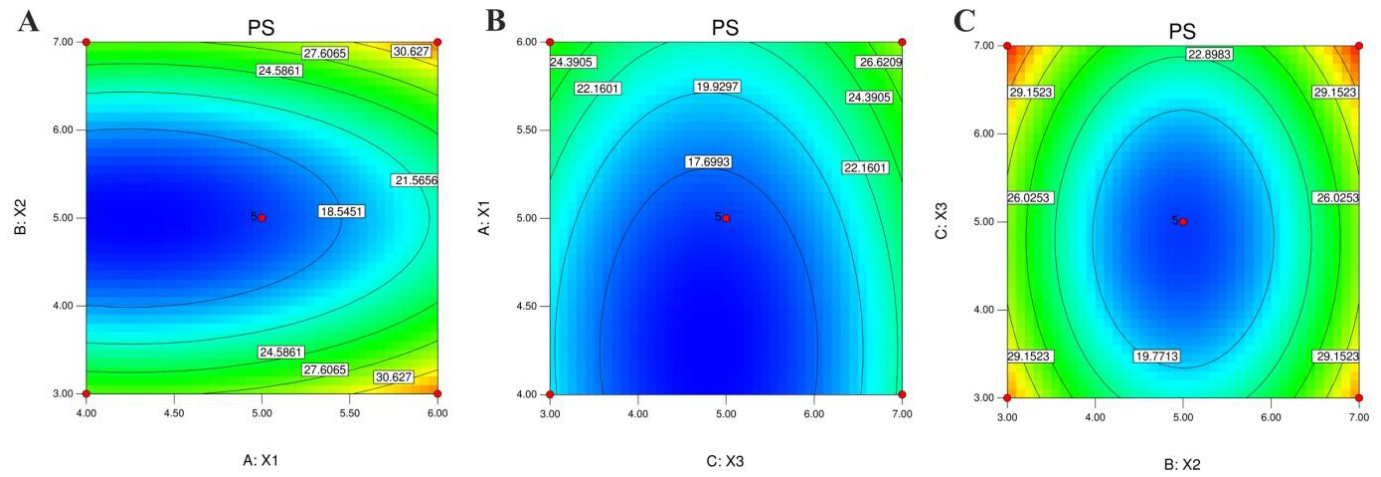

Figure 5: Contour plots showing effect of (A) X1 vs X2 (at 0 level of X3), (b) X1 vs X3 (at 0 level of X2), and (c) X2 vs X3 (at 0 level of X 1) on PS of MEs.

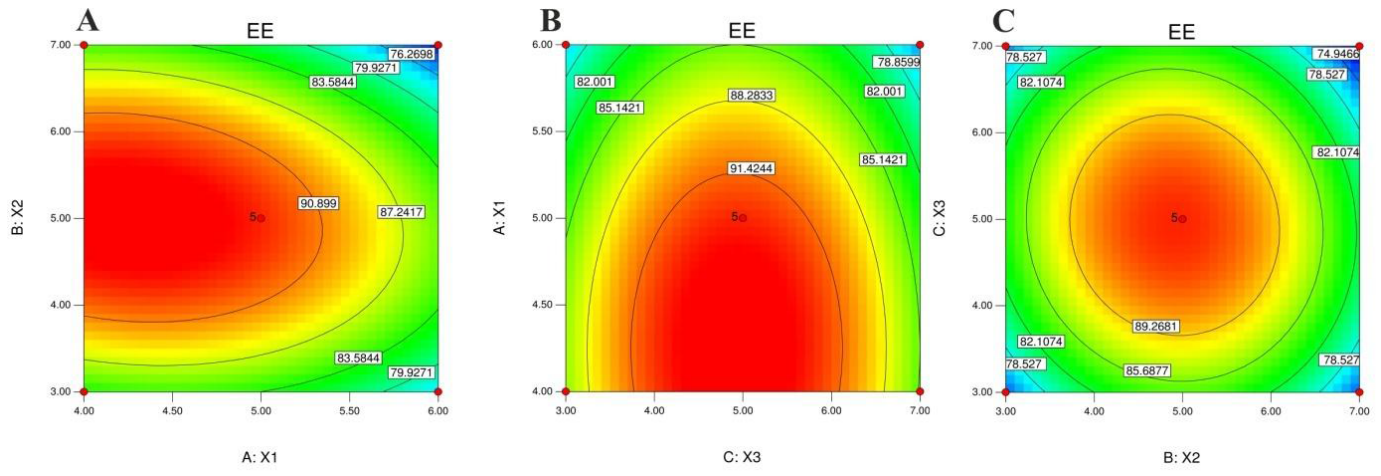

Figure 6: Contour plots showing effect of (A) X1 vs X2 (at 0 level of X3), (b) X1 vs X3 (at 0 level of X2), and (c) X2 vs X3 (at 0 level of X 1) on EE of MEs.

Response surface plots show the relationship between these variables even more clearly when plotted between X_1 and X_2, X_1 and X_3, and X_2 and X_3 at constant level (0) of X_3, X_2 and X_1, respectively for PS and EE [14]. Minimum PS and maximum EE were achieved with low level of Phase Volume Ratio (W:O) in primary emulsion, middle range of Time for primary emulsification and Concentration of hydrophilic surfactant in secondary emulsion (Figures 7 and 8).

Optimization and validation

The optimum formulation was selected based on the criteria of attaining the minimum value of PS and the maximum value of EE. Priority levels were set as '+++' for both PS and EE. The desirability function is a transformation of the response variable to a 0 to 1 scale. Response of 0 represents a completely undesirable response and 1 represents the most desirable response [32]. Based on this, thirty different solutions were predicted with the desirability of 1.

Out of them, three check point formulations were selected, and the experimental and predicted results were compared. Data analysis using student's t-test showed that there was no statistically significant difference ($p < 0.05$) between experimentally obtained values and predicted values (Table 7). Experimental values were found to be in close proximity to the predicted values and the low values of standard deviations confirmed the reproducibility of the results.

Characterization and evaluation of ME

Based on the results of optimization, Span 80 and Span 83 were tried as the lipophilic surfactant at 10% and 20% concentrations, using 5% Tween 20 as the hydrophilic surfactant. The characteristics of different MEs like%EE, droplet size, centrifugation stability and Zeta Potential were measured immediately after preparation as shown in Table 8.

Macroscopic and microscopic evaluation: In visual appearance, all formulations were homogeneous and white in color with very good consistency. Optical image (Figure 9) clearly showed the multiple nature of the ME. Microscopic analysis revealed that many small droplets were present in the internal phase of the multiple globules indicating Type B w/o/w ME, as per the Florence and Whitehill classification [2].

Globule size determination: Globule size of optimized batches were found to be in the range of 15.65 ± 1.97 µm (PDI-0.214 ± 0.098) to 20.21 ± 2.23 µm (PDI - 0.274 ± 0.052). MEs had narrow size distribution as PDI was in the range of 0.2–0.3. From the results obtained, it was found that MEs prepared with Span 80 had slightly larger size as compared to MEs prepared with Span 83. The possible reason could be the difference in HLB values of Span 80 and Span 83. The lower particle size obtained with Span 83 may be attributed to its higher lipophilicity (HLB value 3.7) and lower Critical Micelle Concentration (CMC) (0.216 mM) as compared to Span 80 (HLB value 4.3 and CMC 0.43 mM) [10]. Increase in globule size might be due to higher accumulation of the surfactant at the interface resulting in a thicker interfacial film and hence increase in the globule size [33].

Zeta potential analysis: The zeta potential is indicative of the charge on the surface of the particles and a higher zeta potential value

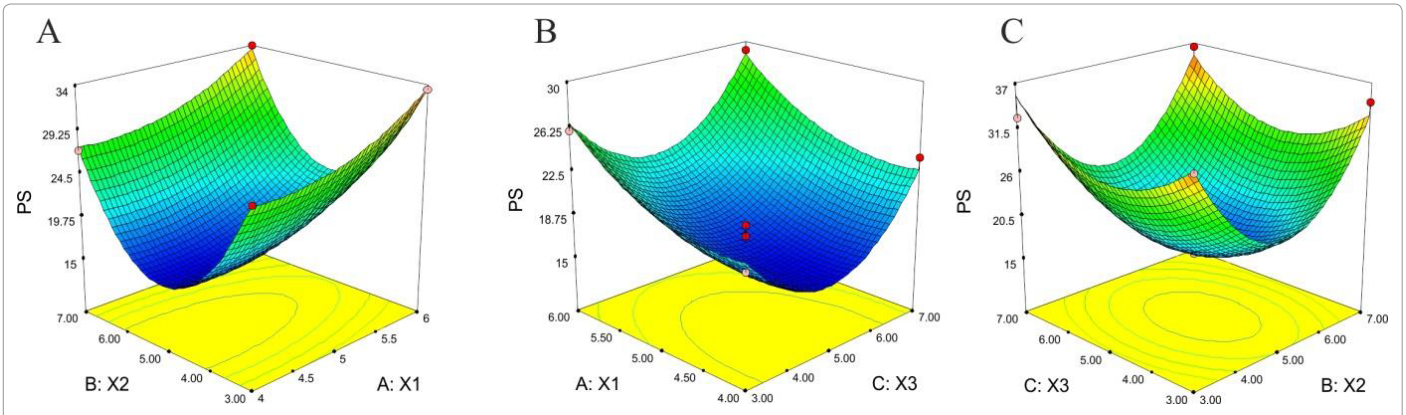

Figure 7: Response surface plot showing effect of (A) X1 vs X2 (at 0 level of X3), (b) X1 vs X3 (at 0 level of X2), and (c) X2 vs X3 (at 0 level of X 1) on PS of MEs.

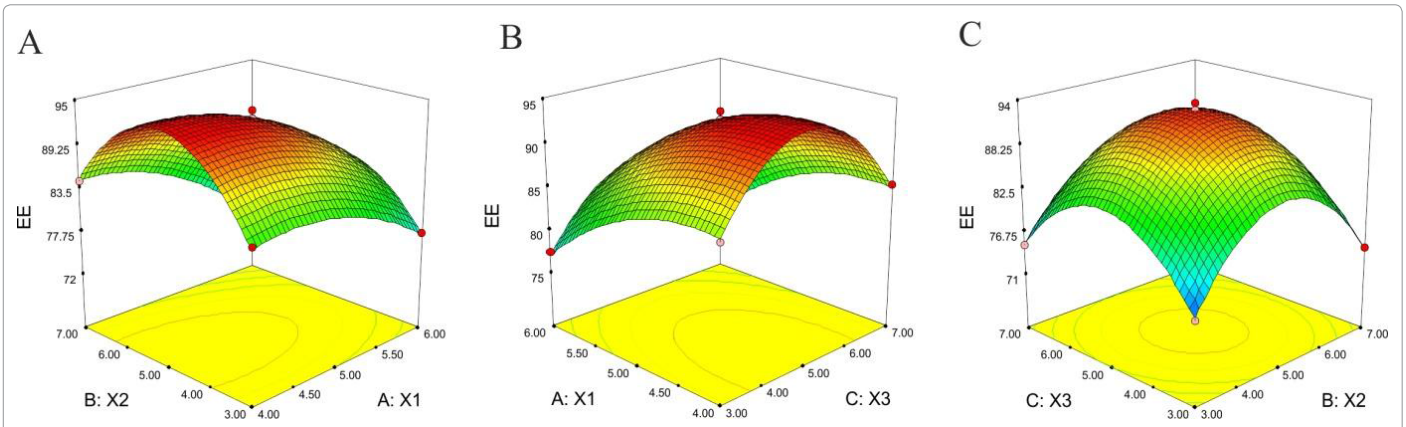

Figure 8: Response surface plot showing effect of (A) X1 vs X2 (at 0 level of X3), (b) X1 vs X3 (at 0 level of X2), and (c) X2 vs X3 (at 0 level of X 1) on EE of MEs.

Checkpoint batches with their experimental and predicted values of PS and EE							
Batch No.	X_1	X_2 (min)	X_3 (%)	PS (µm)		EE (%)	
				Experimental (Mean)	Predicted	Experimental (Mean)	Predicted
1	-0.7 (4.30)	0.055 (5.11)	-0.125 (4.75)	16.864	15.514	92.548	94.468
2	-0.75 (4.25)	-0.035 (4.93)	-0.10 (4.80)	13.987	15.485	95.264	94.509
3	-0.82 (4.18)	0.010 (5.02)	-0.45 (4.71)	14.541	15.492	93.745	94.454
$t_{calculated}$				0.7154		0.5040	
$t_{tabulated}$				4.3026		4.3026	

Table 7: Check point analysis with 't' test analysis.

Formulation	ME 1 (10% Span 80)	ME 2 (20% Span 80)	ME 3 (10% Span 83)	ME 4 (20% Span 83)
Physical Appearance	+++	+++	+++	+++
Multiple nature	+++	+++	+++	+++
EE (%)*	79.63 ± 3.15	72.35 ± 2.98	87.35 ± 3.79	80.26 ± 3.48
Droplet size* (µm)	19.25 ± 2.105	20.21 ± 2.226	15.65 ± 1.967	17.06 ± 2.112
Centrifugation Stability	Stable	Stable	Stable	Stable
Zeta Potential (mV)	-16.10 ± 1.14	-18.21 ± 2.01	-15 ± 2.33	-17.84 ± 3.58

+ Not satisfactory; ++ Good; +++ Very good.

*Value represented as the mean of three experiments ± SD.

Table 8: Characteristics of different ME formulations.

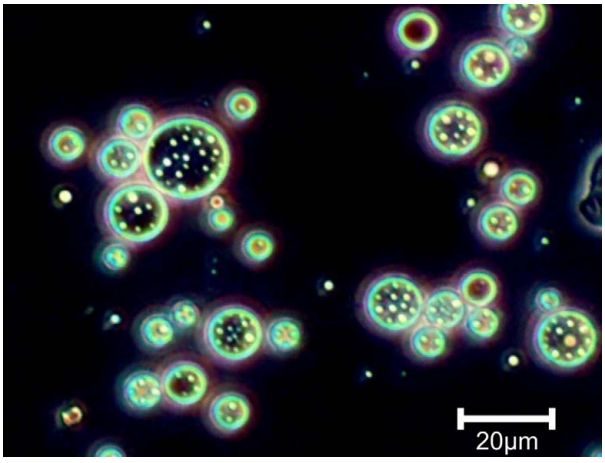

Figure 9: Optical photographs of w/o/w MEs (Under 40 X).

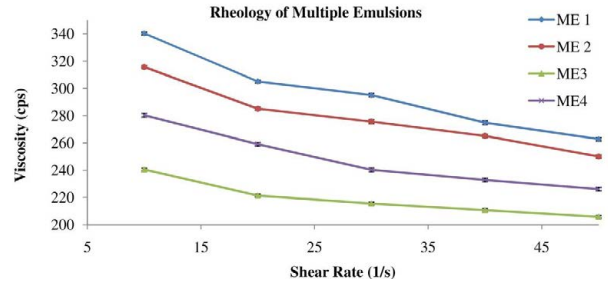

Figure 10: Graphical representation of Rheology of MEs.

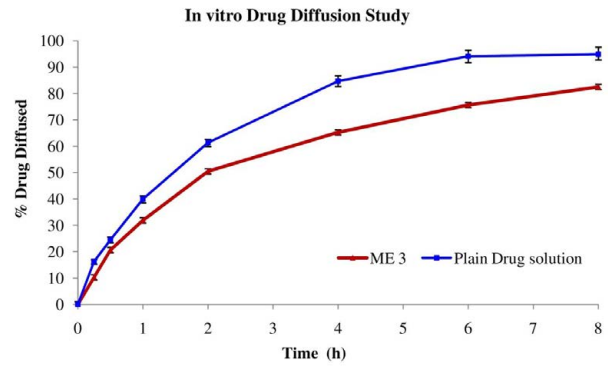

Figure 11: Release profiles of ME and plain drug solution through dialysis bag.

ensures that there is no phase separation, while a value closer to zero leads to risk of coalescence, thus suggests instability [15]. The results are shown in Table 8.

With increase in the concentration of the surfactant, there was decrease in zeta potential. This is possibly because the surfactant is non-ionic and with increase in its concentration, total charge on the globule decreases due to increased surfactant coating which also results in increased PS [34]. Negative values of the zeta potential indicate that the electrostatic repulsion between globules will prevent their aggregation and thereby stabilize the MEs [35-37].

Entrapment efficiency: From the results (Table 8), the MEs prepared with Span 83 had higher EE as compared to MEs prepared with Span 80. Lower EE could be attributed to higher solubilisation effect produced by higher concentration of Span 83. At higher concentration of surfactant, solubility of LD in the external phase may increase due to diffusion of drug from lipid into aqueous phase leading to reduced EE [38].

Rheological analysis: All the MEs exhibited Non-Newtonian, shear thinning pseudoplastic flow behaviour, with viscosity of the systems decreasing with increasing shear rate (Figure 10). Such flow behavior is characteristic of very concentrated emulsions, with a volume fraction $\Phi >> 0.74$ [39]. As a result, prepared MEs showed shear-thinning behavior [40] and the apparent viscosity decreased with increase in shear rate [41]. Furthermore, it was observed that Span 80 based MEs (ME 1 and ME 2) were more viscous as compared to Span 83 based MEs (ME 3 and ME 4). This again may be attributed to the lower HLB and greater micellization of Span 83 as compared to Span 80 [10]. Viscosity was found to increase with increase in concentration of Span.

Samples were also observed for any breakdown or loss in the integrity of multiple globules and phase inversion after viscosity

measurements by optical microscopy. There was no destruction of multiple globules or phase inversion. It was concluded that shear stress used during rheological study did not induce structural changes in MEs, such as coalescence or phase inversion [23].

***In vitro* diffusion studies:** The release profiles of LD from ME and plain drug solution through dialysis bag are presented in Figure 11. About 60% of drug was released in 2 hours and 94% of drug released in 6 hours from plain drug solution. MEs exhibited biphasic release with an initial rapid release phase (40-45%) in 1st two hours and then a slower release (60-80%) between 2nd to 8th hour. Initial release of LD might be due to presence of LD in the external aqueous phase while the second prolonged release phase can be attributed to the slow release from the inner aqueous phase governed by the interfacial barrier of the oil phase [8]. In w/o/w ME, the drug in the internal phase is forced to partition itself through two phases prior to its release into the sink solution. The rate of release from internal aqueous phase into bulk will be governed by the nature of hydrophobic barrier, its thickness and any interaction between emulsifier and drug molecules [10]. The data (Table 9) obtained from *in vitro* drug release studies was fitted to various release models. The regression coefficient value was found to be highest (r^2=0.9897) for Higuchi model [42]. The Higuchi model applied to a slab geometry permits definition of a release fraction linearly dependent on the square root of time and the reciprocal of the initial drug concentration. However, diffusion of LD through the oil film, is controlled by diffusion gradient regulation [43]. Similar work has been reported by Magdassi and Garti where they applied this model to multiple W/O/W emulsions taking into account the spherical geometry of the oil droplets [44]. Hence, it can be concluded that the release of LD from ME was by Higuchi diffusion based mechanism.

***Ex vivo* permeability studies:** The permeation of LD from ME and plain drug solution through rat intestine is presented in Figure 12. The results indicate that diffusion was faster across the intestine from the ME as compared to the drug solution.

Drug flux (permeation rate) at steady state was found to be 119.3 µg/cm^2/h for ME and 105.1 µg/cm^2/h for plain drug solution. Enhancement ratio (Er) was found to be 1.14, clearly indicating the enhancement in permeation by incorporating LD in ME, which is expected to enhance its absorption and bioavailability. LD is a BCS III drug, having high solubility but low permeability. Thus, formulation as w/o/w multiple emulsion could significantly enhance its permeability [4].

In vitro release study	Linear Correlation Coefficient (r^2) Values			
	Zero order	First order	Higuchi	Korsemeyer Peppas
	0.9310	0.9722	0.9897	0.8562

Table 9: Linear correlation coefficient values of various models for *in vitro* release study.

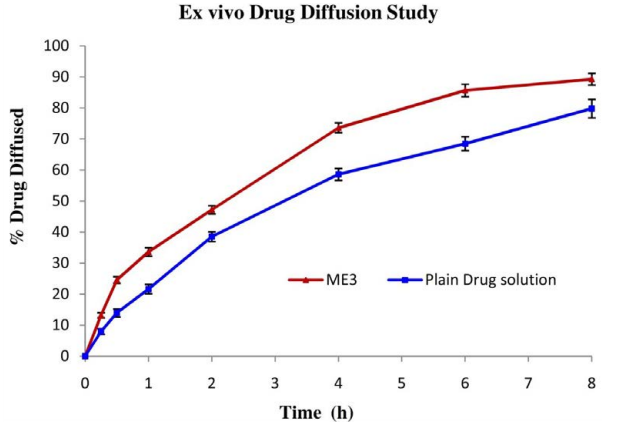

Figure 12: Permeation study of ME and plain drug solution through rat intestine.

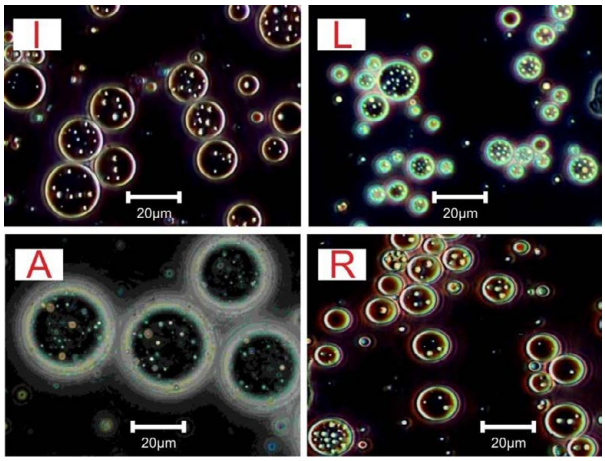

Figure 13: Optical photographs (40X) of MEs for Initial (I) and long term condition - (25 ± 2°C/60 ± 5% RH) (L), accelerated condition - (40 ± 2°C/75 ± 5% RH) (A), refrigerated condition- (2-8°C) (R) after 3 months stability.

Stability studies: Influence of different storage conditions on the stability of MEs were assessed by analyzing them microscopically, visually and for PS, zeta potential, and EE. Significant changes in the PS and EE were observed during long term stability studies. Decrease in the globule size (from 18.89 ± 2.46 to 11.09 ± 1.99 µm) and EE (from 79.18 ± 2.79 to 61.42 ± 2.23) was observed on storage at 25 ± 2°C/60 ± 5% RH. Decrease in the globule size might be due to the breaking of the ME into simple emulsion. This can also be attributed to continuous drug leakage from the MEs with time. This can also be ascribed to various phenomena such as coalescence, thinning or destruction of surfactant film or expulsion of internal aqueous phase, which are reported to be responsible for instability of MEs [10]. Drastic increase in particle size (from 18.89 ± 2.46 to 40.41 ± 3.98) and decrease in EE (from 79.18 ± 2.79 to 52.19 ± 2.47) was observed under accelerated conditions in 3 months. This increase was attributed to the coalescence of the globules. Loss of entrapped drug from ME lead to decrease in EE. This is expected as at elevated temperature, thermodynamic energy of drug molecules increases leading to a greater partitioning and diffusion of drug out of the hydrophilic layer of w/o/w emulsion causing loss of entrapped drug and reduction in EE [17]. These facts can be clearly seen from the microscopic view of the MEs as shown in Figure 13. There was major phase separation during accelerated condition, little in long term and no phase separation was seen at refrigerated conditions. Significant decrease in the viscosity of the MEs was also observed on storage. No significant change in zeta potential was observed in all three conditions.

Apparently, the formulation demonstrated acceptable physical (No Phase separation or coalesence) as well as chemical (No Drug leakage or degradation) stability [45] when stored under refrigerated conditions.

Conclusions

Stable corn oil based w/o/w MEs containing Lisinopril dihydrate were developed by two step emulsification technique using different surfactants (Span 80/Span 83/Tween 20). Important parameters were preliminary screened by Plackett-Burman design and further optimised by Box Behnken design. Phase Volume Ratio (W:O) in

primary emulsion, Time for primary emulsification and Concentration of hydrophilic surfactant in secondary emulsion, were the three key variables affecting the preparation of stable ME formulations. Good correlation was obtained between actual and predicted values for the optimised formulation. The diffusion of drug across dialysis bag showed rapid release in beginning followed by a slower rate of release in 8 hours. Permeability study showed higher permeation rate from ME than from plain drug solution. Stability studies indicated that refrigeration condition was desirable for the storage of developed ME.

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Conflict of Interest

The authors report no conflict of interest.

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