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Enhanced bioavailability of lacidipine via microemulsion based transdermal gels: Formulation optimization, *ex vivo* and *in vivo* characterization

Ramesh Gannu, Chinna Reddy Palem, Vamshi Vishnu Yamsani, Shravan Kumar Yamsani, Madhusudan Rao Yamsani*

National Facilities in Engineering and Technology with Industrial Collaboration (NAFETIC) Centre, University College of Pharmaceutical Sciences, Kakatiya University, Warangal 506 009, Andhra Pradesh, India

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ABSTRACT

The purpose of the present study was to develop and optimize the microemulsion based transdermal therapeutic system for lacidipine (LCDP), a poorly water soluble and low bioavailable drug. The pseudo-ternary phase diagrams were developed for various microemulsion formulations composed of isopropyl myristate, Tween 80 and Labrasol. The microemulsion was optimized using a three-factor, three-level Box–Behnken design, the independent variables selected were isopropyl myristate, surfactant mixture (Tween 80 and Labrasol) and water; dependent variables (responses) were cumulative amount permeated across rat abdominal skin in 24 h (Q_{24}; Y_1), flux (Y_2), and lag time (Y_3). Mathematical equations and response surface plots were used to relate the dependent and independent variables. The regression equations were generated for responses Y_1, Y_2 and Y_3. The statistical validity of the polynomials was established, and optimized formulation factors were selected by feasibility and grid search. Validation of the optimization study with 10 confirmatory runs indicated high degree of prognostic ability of response surface methodology. The gel of optimized formulation (ME-OPT) showed a flux of 43.7 $\mu g\,cm^{-2}\,h^{-1}$, which could meet the target flux (12.16 $\mu g\,cm^{-2}\,h^{-1}$). The bioavailability studies in rabbits showed that about 3.5 times statistically significant ($p < 0.05$) improvement in bioavailability, after transdermal administration of microemulsion gel compared to oral suspension. The *ex vivo–in vivo* correlation was found to have biphasic pattern and followed type A correlation. Microemulsion based transdermal therapeutic system of LCDP was developed and optimized using Box–Behnken statistical design and could provide an effective treatment in the management of hypertension.

1. Introduction

Lacidipine (LCDP) is a calcium channel blocker used in the treatment of hypertension and atherosclerosis. It also possesses antioxidant effect and is one of the most vascular selective of the dihydropyridines (Lee and Bryson, 1994; Mc Cormack and Wagstaff, 2003). LCDP undergoes extensive first-pass hepatic metabolism and has a mean absolute bioavailability of about 10% (range 4–52%). LCDP is completely metabolized in the liver by cytochrome P450 3A4 to pharmacologically inactive metabolites (Mc Cormack and Wagstaff, 2003). In addition, its limited aqueous solubility contributes to its limited bioavailability. However the low oral bioavailability restricts its use, therefore alternative mode of delivery system is desirable, to deliver the drug at effective concentrations to treat hypertension. There are no reports on transdermal delivery of LCDP. In this investigation, an attempt has been made to deliver LCDP transdermally across skin using microemulsions.

Microemulsion (ME) is defined as an O/W or W/O emulsion producing a transparent product that has a droplet size from 10 to 100 nm and does not have the tendency to coalesce (Kreilgaard, 2002; Lawrence and Rees, 2000). MEs are composed of oil phase, surfactant, cosurfactant and aqueous phase at appropriate ratios (Mohammed and Manoj, 2000). MEs have several specific physicochemical properties such as transparency, optical isotropy, low viscosity and thermodynamic stability (Lawrence and Rees, 2000; Mohammed and Manoj, 2000; Baroli et al., 2000). It is promising for both transdermal and dermal delivery of drugs as an efficient route of drug administration (Kreilgaard, 2002; Rhee et al., 2001; Kreilgaard et al., 2000; Baboota et al., 2007; Kamal et al., 2007; Chen et al., 2007). Several mechanisms have been proposed to explain the advantages of microemulsions for the transdermal and dermal delivery of drugs (Chen et al., 2006; Zhao et al., 2006). First, the thermodynamics towards the skin is increased due to large amount of a drug incorporated in the formulation. Second, the increased thermodynamic activity of the drug may favor its partitioning into

* Corresponding author. Tel.: +91 870 2438844; fax: +91 870 2453508.
E-mail address: ymrao123@yahoo.com (M.R. Yamsani).

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the skin. Third, the ingredients of microemulsion may reduce the diffusional barrier of the stratum corneum and increase the permeation rate of drug via skin by acting as permeation enhancers. Also, the hydration effect of microemulsion on the stratum corneum may influence the permeation ability of formulations (Mohammed and Manoj, 2000). Although many drugs have been incorporated in microemulsion for transdermal and dermal delivery, LCDP has not been evaluated. In this study, O/W microemulsions containing LCDP have been developed after screening oils and surfactants. Pseudo-ternary phase diagrams were constructed to obtain the components and their concentration ranges, and the microemulsion formulations varied according to a Box–Behnken statistical design to find out the most suitable components ratio for the optimized formulation. The formulation was also evaluated for *in vivo* performance in rabbits.

2. Materials and methods

2.1. Materials

Lacidipine and Labrasol (Gatteffose, France) were gift samples from Dr. Reddys Laboratories, Hyderabad, India. Liquid paraffin, Tween 80, isopropyl myristate, polyethylene glycol 400 were purchased from Merck, Mumbai, India. Oleic acid was purchased from SD Fine Chemicals, Mumbai, India. Double distilled water was used for the preparation of microemulsions. All other chemicals and solvents were of analytical reagent grade.

2.2. Solubility studies

2.2.1. Screening of oils and surfactants for microemulsions

To find out appropriate oil phase in microemulsions, the solubility of LCDP in various oils such as oleic acid, isopropyl myristate (IPM), liquid paraffin, eucalyptus oil and sesame oil and each oil containing 10% (v/v) of dimethyl formamide were measured. An excess amount of LCDP was added to 10 mL of oil and the mixture was shaken at 25 °C for 72 h. The resulting suspension was then centrifuged (Mikro 220R, Tuttlingen, Germany) for 10 min at 10,000 rpm. The supernatant was filtered through a membrane filter (0.45 μm) and the drug concentration in filtrate was determined by high performance liquid chromatography (HPLC) analysis after appropriate dilution. The oil phase that showed high solubility of LCDP was used in the preparation of microemulsions containing 0.2% LCDP.

2.3. Construction of phase diagrams and formulation of LCDP loaded microemulsions

To find out appropriate components in the formation of microemulsions, two safe and compatible nonionic surfactants, polysorbate 80 and a cosurfactant, Labrasol, were chosen. Pseudo-ternary phase diagrams were constructed using water titration method in order to obtain the concentration range of components for the existing range of microemulsions. The weight ratio of surfactant to cosurfactant (S_{Mix}) varied as 1:1, 1:2, 1:3, 2:1 and 3:1. For each pseudo-ternary phase diagram at a specific surfactant/cosurfactant weight ratio was mixed with oil at a ratio of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9 (w/w). Water was added drop by drop to each oily-surfactant mixture under magnetic stirring at room temperature, until the mixture became clear at a certain point. The concentrations of components were recorded in order to complete the pseudo-ternary phase diagrams, and then the contents of oil, surfactant, cosurfactant and water at appropriate weight ratios were selected based on these results. LCDP loaded microemulsions were prepared by dissolving LCDP in DMF:IPM (1:9) and surfactant mixture, adding the required quantity of water, and stirring to form a clear and transparent liquid. The resulting microemulsions were tightly sealed and stored at ambient temperature, and their physical stability was measured by observing periodically the occurrence of phase separation.

2.4. Rheological measurements

The rheological properties of MEs were measured using Brookfield Programmable DVIII+ Digital Rheometer (Brookfield Engineering Laboratories Inc., MA, USA). The rheological measurements were performed using a controlled stress rheometer with the cone (24 mm) and plate geometry. The viscosity was determined by torque sweep from 10 to 110%. All the measurements were performed in triplicate at 25 °C. The equilibrium time before every measurement was 5 min and the sample volume used was approximately 0.5 mL. Calculation of rheological properties were performed using Rheocalc 32 software (Brookfield Engineering Laboratories Inc., USA). The data was analyzed using "Power Law" (Bonacucina et al., 2004) as expressed by Eq. (1).

$$\tau = KD^n \quad (1)$$

where τ is shear stress; K is gel index (GI) or consistency index; D is shear rate; and n is flow index. 'Rheocalc 32' software was used to automatically apply the model to generated data, and the value of GI was recorded.

2.5. Preparation of rat abdominal skin

The animal study was conducted in accordance with the approval of the Animal Ethical Committee, Kakatiya University, India. Wistar rats weighing 150–200 g were sacrificed using anaesthetic ether. The hair of test animals was carefully trimmed with electrical clippers and the full thickness skin was removed from the abdominal region. The epidermis was prepared surgically by heat separation technique (Levang et al., 1999), which involved soaking the entire abdominal skin in water at 60 °C for 45 s, followed by careful removal of the epidermis. The epidermis was washed with water and used for *ex vivo* permeability studies.

2.6. Ex vivo permeation studies

Franz diffusion cell with a surface area of 3.56 cm^2 was used for *ex vivo* permeation studies. The rat skin was mounted between donor and receptor compartments of the diffusion cell with stratum corneum facing the donor compartment. Microemulsion (4 mL) was placed in the donor compartment and 13 mL of phosphate buffer saline (PBS, pH 7.4) containing 40% (v/v) of polyethylene glycol (PEG) 400 was placed in receptor compartment. PEG 400 was incorporated to maintain sink conditions and the contents of receptor compartment were agitated at 400 rpm and was placed over a multi-magnetic stirrer (Cintex, Mumbai, India). The study was conducted at 37 °C and samples of 1 mL were collected at predetermined time points and replenished with PBS (pH 7.4) containing 40% (v/v) PEG 400. The cumulative amount of LCDP permeated was determined using HPLC (Gannu et al., 2009) and concentration was corrected for sampling effects according to Eq. (2) (Hayton and Chen, 1982):

$$C_n^1 = C_n \left(\frac{V_T}{V_T - V_S}\right)\left(\frac{C_{n-1}^1}{C_{n-1}}\right) \quad (2)$$

where C_n^1 is the corrected concentration of the nth sample, C_n is the measured concentration of LCDP in the nth sample, C_{n-1}^1 is the corrected concentration in the $(n-1)$th sample. C_{n-1} is the measured concentration of the LCDP in the $(n-1)$th sample, V_T is the

total volume of the receiver fluid and V_S is the volume of the sample drawn.

The steady state flux (J_{ss}) was calculated from the slope of the steady state portion of the line in the plot of drug amount permeated Vs time (h). Permeability coefficient (K_p) was calculated by dividing the flux with concentration of the drug in microemulsion. The lag time was calculated from the intercept on the time axis in the plot of cumulative amount permeated Vs time. The target flux was calculated using the following Eq. (3). The enhancement ratio was calculated as the ratio of flux obtained with microemulsion and control (solution).

$$\text{Target flux} = \frac{Css \times CLt \times BW}{A} \quad (3)$$

Css, the LCDP concentration at therapeutic level (8.6 μg L^{-1}) and *CLt* the total body clearance, 83.9 mL h^{-1} (calculated from volume of distribustion, 2300 mL kg^{-1} and half life 19 h) (Clarke's, 2007), *BW* the standard human body weight of 60 kg, *A* represents the surface area of the diffusion cell (i.e. 3.56 cm^2). The calculated target flux value for LCDP was 12.16 μg cm^{-2} h^{-1}.

2.7. Experimental design

Box–Behnken statistical design was used to statistically optimize the formulation factors and evaluate main effects, interaction effects and quadratic effects on the amount of LCDP permeated in 24 h (Q_{24}), flux and lag time. A 3-factor, 3-level Box–Behnken design was used to explore quadratic response surfaces and constructing second-order polynomial models with Design Expert (Version 7.1, Stat-Ease Inc., Minneapolis, MN). The Box–Behnken design was specifically selected since it requires fewer runs than a central composite design (CCD) in cases of three or four variables. This cubic design is characterized by set of points lying at the midpoint of each edge and center point of the multidimensional cube (Box and Behnken, 1960). A design matrix comprising of 17 experimental runs was constructed. The non-linear computer generated quadratic model is given as $Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$ where Y is the measured response associated with each factor level combination; b_0 is an intercept; b_1 to b_{33} are regression coefficients computed from the observed experimental values of Y; and X_1, X_2 and X_3 are the coded levels of independent variables. The terms X_1X_2 and X^2 (i = 1, 2 or 3) represent the interaction and quadratic terms, respectively (Govender et al., 2005; Chopra et al., 2007). The dependent and independent variables selected were shown in Table 2 along with their low, medium and high levels, which were selected based on the results from psuodo-ternary phase diagrams. The proportion of oil (X_1), S_{Mix} (X_2) and water (X_3) used to prepare the 17 experimental trials and the respective observed responses are given in Table 2.

2.8. Check point analysis and optimization model validation

Statistical validation of the polynomial equations generated by Design Expert was established on the basis of ANOVA provision in the software. The models were evaluated in terms of statistically significant coefficients and R^2 values. Various feasibility and grid searches were conducted to find the optimum parameters. Various 3D response surface graphs were provided by the Design Expert software. Ten optimum checkpoint formulations were selected by intensive grid search over the whole experiment region to validate the experimental model and polynomial equations. The optimized checkpoint formulation factors were evaluated for various response properties. The resultant experimental values of the responses were quantitatively compared with the predicted values to calculate the percentage prediction error.

2.9. Droplet size determination

The droplet size analysis of optimized microemulsion was determined using dynamic light scattering method employing a Zeta Sizer (Malvern Instruments Ltd., Worcestershire WR 14 1XZ, UK).

2.10. Stability studies

The stability study was conducted for formulation ME4 and ME gel. Sufficient samples were placed in amber colour containers and were further placed at 40 ± 2 °C/75 ± 5% R.H. (Skylab Instruments & Engineering Pvt Ltd., Thane, India) for 6 months. Samples were withdrawn at time intervals of 1, 2, 3 and 6 months. The *ex vivo* permeation study was conducted according to the procedure described in Section 2.6 and drug content in the formulations was estimated using HPLC (Gannu et al., 2009).

2.11. Skin irritation studies

The skin irritation study was performed using six rabbits. The hair of rabbits on dorsal side was shaved with electrical shaver and ME gel (about 4 g) formulation was applied. The development of erythema was monitored for 7 days.

2.12. In vivo bioavailability study in rabbits

The animal study protocol was reviewed and approved by the Institutional Animal Ethical Committee, University College of Pharmaceutical Sciences, Kakatiya University, India. White New Zealand rabbits weighing 3.30 ± 0.35 kg were selected for the study. The bioavailability of LCDP (8 mg in 4 g of ME gel) from microemulsion based gel was compared with an oral suspension. The suspension was prepared by suspending 8 mg of LCDP in 5 mL of water containing 0.5% (w/v) of sodium carboxy methyl cellulose. They were allowed free access to food and water, until the night prior to dosing and were fasted for 10 h. Latin square cross over design was followed; the animals were divided into two groups each consisting of three rabbits. The rabbits to be used for application of ME gel were shaved carefully with the help of electrical shaver before application of ME gel followed by cleaning with water. To one group, oral suspension (8 mg/5 mL) was administered through feeding tube followed by rinsing with 10 mL of water and ME gel to another group in first phase. In second phase vice versa was followed and was conducted after 15 days of wash out period. The ME gel was applied over a surface area of 4 cm^2 and was covered with a water impermeable back up membrane and was further fixed with the help of adhesive membrane. Blood samples (2.5 mL) from marginal ear vein were collected at preset intervals of 0.0, 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 72 h; 0.0, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, 18.0, 24.0, 36.0, 48.0 and 72.0 h respectively, after administration of oral suspension and application of ME gel. All blood samples were allowed to clot and centrifuged for 10 min at 4000 rpm. The serum was separated and transferred into clean micro-centrifuge tubes and stored at −20 °C until HPLC analysis. The amount of LCDP in the samples was estimated using HPLC (Gannu et al., 2009).

2.13. Pharmacokinetic analysis

Pharmacokinetic parameters of LCDP after administration of LCDP ME gel and oral suspension were estimated for each rabbit by using a computer program, KINETICA 2000 (Version 3.0, Innaphase Corporation, Philadelphia, USA). Noncompartmental analysis was used to calculate the pharmacokinetic parameters, C_{Max}, T_{Max} and area under the curve (AUC). C_{Max} (ng mL^{-1}) and T_{Max} (h) were the observed maximal drug concentration and its time, respectively. The relative bioavailability *F*

for transdermal drug delivery system was calculated using Eq. (4):

$$\text{Relative bioavailability} = \frac{[\text{AUC}]\text{ME gel}}{[\text{AUC}]\text{Oral suspension}} \quad (4)$$

2.14. Statistical analysis

Statistical comparisons were made using Student's *t*-test using Sigmastat software package (Jandel Corp., CA, USA). Results were considered significant at 95% confidence interval ($p < 0.05$) and results were expressed as mean ± SD.

2.15. Ex vivo–in vivo correlation

The cumulative amount of LCDP permeated across rat abdominal skin *ex vivo* from ME gel was compared against the extent of absorption i.e., cumulative AUC values for a possible *ex vivo–in vivo* correlation.

3. Results and discussion

3.1. Selection of components for microemulsions

The solubility of LCDP in various oils was analyzed in order to select components for microemulsions. The solubility of LCDP was

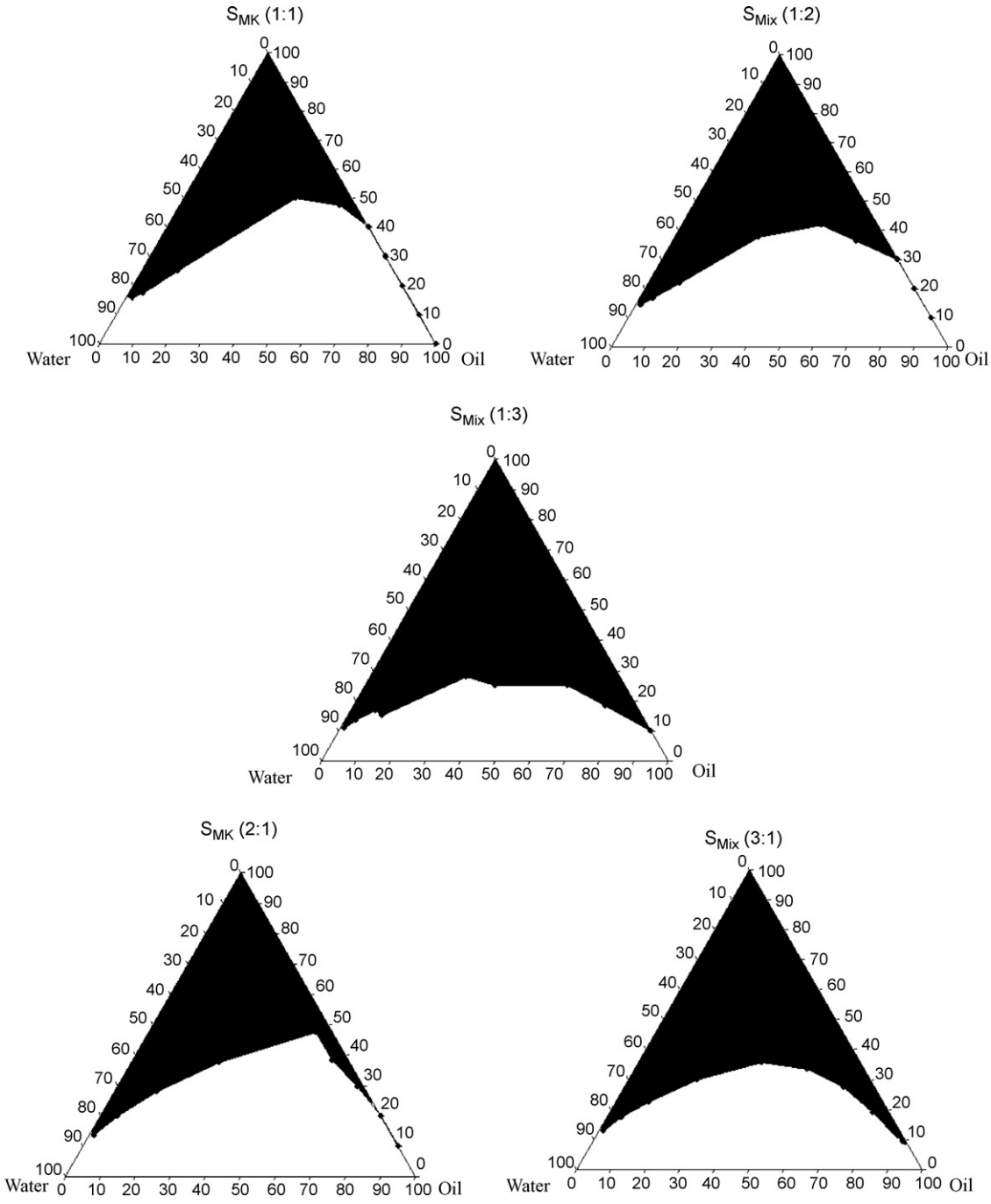

Fig. 1. Pseudo-ternary phase diagrams of microemulsions composed of oil phase (IPM:DMF, 9:1), surfactant (Tween 80), cosurfactant (Labrasol) and water.

Table 1
Solubility of LCDP in oils.

Oil phase	Solubility (mg)
Oleic acid	2.16 ± 0.115
Liquid paraffin	0.02 ± 0.001
Sesame oil	2.46 ± 0.130
IPM	3.81 ± 0.202
Eucalyptus oil	3.17 ± 0.168
Oleic acid + DMF	18.82 ± 0.999
Liquid paraffin + DMF	0.11 ± 0.005
Sesam oil + DMF	22.79 ± 1.209
IPM + DMF	42.83 ± 2.215
Eucalyptus oil + DMF	33.81 ± 1.794

found to be 42.83, 33.81, 22.79, 18.82 and 0.11 mg mL^{-1} respectively in isopropyl myristate (IPM), eucalyptus oil, sesame oil, oleic acid and liquid paraffin each containing 10% (v/v) of dimethyl formamide (DMF). Plain oils showed low solubility (Table 1), to solubilize LCDP large amounts of oil phase is needed, and therefore a solubilizer DMF was incorporated. IPM containing 10% (v/v) DMF was selected as oil phase to solubilize LCDP that could further maintain large concentration gradient towards skin. Previous reports indicated that the superior dermal flux appeared mainly due to the large solubilizing capacity of the microemulsions, which led to larger concentration gradient towards the skin (Kreilgaard et al., 2000; Sintov and Shapiro, 2004).

3.2. Construction of pseudo-ternary diagrams

Microemulsions were made up of IPM containing 10% (v/v) of DMF as oil phase, a surfactant of Tween 80 and an aqueous phase in the presence of cosurfactant (Labrasol). Fig. 1 presents the pseudo-ternary phase diagrams with various weight ratios of Tween 80/Labrasol. The transparent microemulsion region is presented in phase diagrams. The rest of the region on the phase diagram represents the turbid and conventional emulsions based on visual observation. From Fig. 1, it was found that the area region of microemulsions isotropic region became enlarged as the S_{Mix} decreased, reaching the maximum point at S_{Mix} of 1:3.

3.3. Rheological measurements

CP-40 spindle was used for the viscometric characterization of MEs. The decrease in viscosity of the MEs observed with an increasing shear rates, can be described well by an exponential function and hence the obtained data was analyzed using "Power Law" (Bonacucina et al., 2004) as expressed by Eq. (1). The GI value for different formulations is presented in Table 2. The gel index was found to be ranging from 2.0 to 163.2.

3.4. Ex vivo skin permeation experiments

The permeation profiles of LCDP microemulsions through rat skin are shown in Fig. 2. The permeation parameters including the cumulative amount at 24 h, flux and lag time (Table 2) for all experimental formulations were calculated and the permeation was found to follow zero-order model ($R^2 > 0.974$). The cumulative amount ranged from 3640.5 to 7704.5 μg, flux ranged from 29.7 to 64.7 μg cm^{-2} h^{-1} and lag time ranged from 2.16 to 2.48 h indicating that the permeation parameters of LCDP from microemulsions were markedly influenced by the composition of microemulsions. The oil phase, IPM selected in the present work also act as an effective penetration enhancer (Goldberg-Cettina et al., 1995). The surfactant and cosurfactant in the microemulsions may reduce the diffusional barrier of the stratum corneum by acting as permeation enhancers (Peltola et al., 2003). Formulation ME4 showed

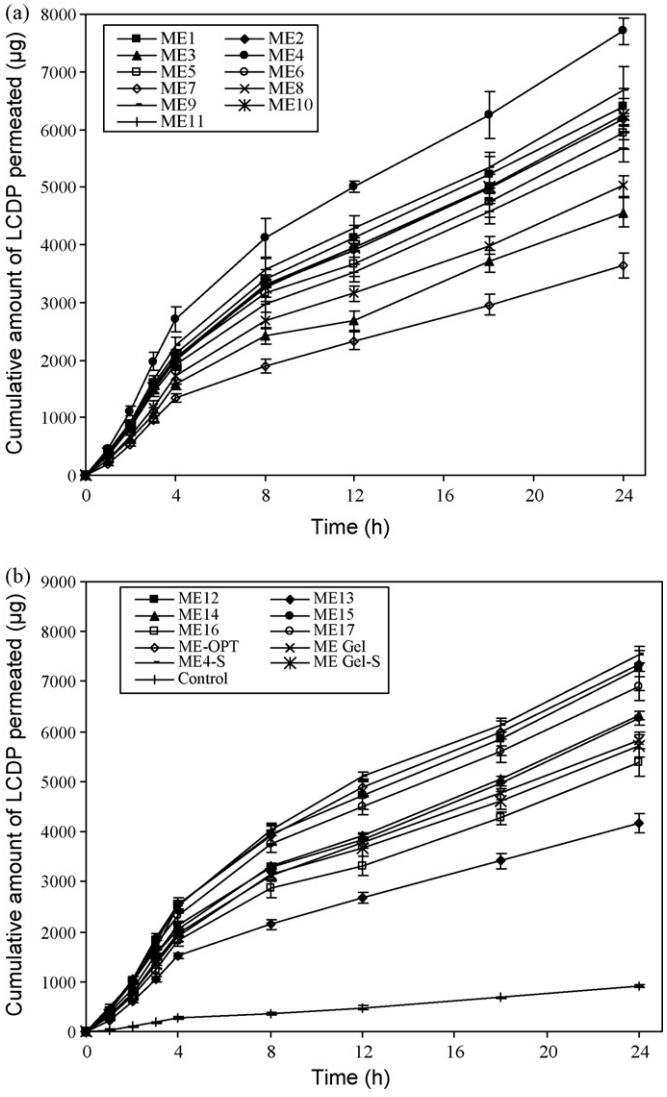

Fig. 2. *Ex vivo* permeation profiles of LCDP from microemulsions, ME gel and control, values represented are mean ± SD ($n = 3$).

the higher amount of LCDP permeated (7704.5 μg) with a flux of 64.7 μg cm^{-2} h^{-1} and a lag time (2.17 h). LCDP solution (control) showed a cumulative amount of 910.3 μg permeated with a flux of 8.0 μg cm^{-2} h^{-1} and a lag time of 2.83 h. The enhancement ratios of microemulsions were 3.7–8.1-fold higher than the control (LCDP was dissolved in water containing 5% (v/v) of DMF and 40% (v/v) of PEG 400). These results demonstrated that microemulsion had a potent enhancement effect for transdermal delivery.

It was observed that the amount of LCDP permeated and flux was decreased and prolonging lag time with increase in the amount of surfactant. The result might be due to a decreased thermodynamic activity of drug in microemulsion at higher concentrations of surfactant. The drug permeation was found to decrease with increase in the G.I. At higher levels of oil the G.I. was found to be more and the result is in accordance with earlier studies (Gallarate et al., 1990; Gasco et al., 1991) and might be attributed to the gel formation in the microemulsion that will increase its viscosity and further decrease the permeation in the skin. The diffusion through the double layer microemulsion might be a rate-determining step, as the viscosity plays an important role in controlling the release of the drug into the receptor compartment (Ho et al., 1996).

The high proportion of cosurfactant in S_{Mix} could lower the interfacial tension of the surfactant in microemulsions, resulting

Table 2
Variables and observed responses in Box–Behnken design for microemulsions.

Formulation	Independent variables			Dependent variables			G I	Kp × 10^{-3} (/cm)
	X_1 (g)	X_2 (g)	X_3 (mL)	Y_1 (μg)	Y_2 (μg/h/cm^2)	Y_3 (h)		
ME1	0	−1	1	6398.3	55.8	2.24	11.6	7.0
ME 2	0	0	0	6170.9	52.6	2.26	20.8	6.6
ME 3	1	−1	0	4561.1	38.6	2.16	80.4	4.8
ME 4	−1	0	1	7704.5	64.7	2.17	2.0	8.1
ME 5	0	−1	−1	5931.4	48.7	2.34	23.5	6.1
ME 6	0	0	0	6183.6	53.3	2.16	18.1	6.7
ME 7	1	1	0	3640.5	29.7	2.48	163.2	3.7
ME 8	1	0	1	5024.5	41.7	2.23	57.5	5.2
ME 9	−1	1	0	6683.4	55.9	2.30	3.1	7.0
ME 10	0	0	0	6255.3	53.3	2.18	16.4	6.7
ME 11	0	1	−1	5681.8	47.2	2.29	28.7	5.9
ME 12	−1	0	−1	7260.6	61.1	2.41	3.5	7.6
ME 13	1	0	−1	4175.8	35.2	2.39	92.5	4.4
ME 14	0	0	0	6323.5	53.4	2.26	17.5	6.7
ME 15	0	0	0	6270.4	51.4	2.27	19.6	6.4
ME 16	0	1	1	5381.4	44.9	2.16	49.4	5.6
ME 17	−1	−1	0	6914.6	59.2	2.37	4.8	7.4

Independent variables	Levels used, actual (coded)		
	Low (−1)	Medium (0)	High (+1)
X_1 = oil (g)	6	11	16
X_2 = S_{Mix} (g)	10	13	16
X_3 = water (mL)	68	76	84

in a more flexible and dynamic layer (Trotta et al., 1999; Wu et al., 2001). The drug in this energy-rich system can diffuse across the flexible interfacial surfactant film between the phases; a thermodynamic process that increases partitioning and diffusion into the stratum corneum. However at high levels of oil and surfactant mixture the drug permeation and flux were found to be decreased, this was due to the affinity of LCDP towards oil phase and surfactant system.

3.5. Formulation optimization by experimental design

A three-factor, three-level Box–Behnken statistical experimental design was used to optimize the formulation variables as the response surface methodology requires 17 experiments. The independent variables and the responses for all 17 experimental runs are given in Table 2. The contour plots and 3D response surface plots drawn using Design Expert software are shown in Fig. 3. Based on the results of pseudo-ternary phase diagrams, appropriate ranges of the components were chosen. The oil phase concentration that could form microemulsion was found to be 6–16% and was selected as oil concentration to identify the optimum proportion of oil. Previous reports revealed that there was a really tight relationship between the hydration effect of the stratum corneum and the dermal permeation (Mohammed and Manoj, 2000), and the thermodynamic activity of drug in microemulsions was a significant driving force for the release and penetration of drug into skin (Mohammed and Manoj, 2000). Based on pseudo-ternary phase diagrams, the water content was determined between 68 and 84%. The surfactant mixture (surfactant, cosurfactant, S_{Mix} 1:3), that could form clear microemulsion with large area was selected as variable and was found to be 10–16%. Design Expert software was used to optimize the formulation and to develop the mathematical (quadratic) equations are shown in Eqs. (5)–(7).

The responses, Q_{24} (Y_1) and flux (Y_2) were found to be significantly higher (Y_1, 5381.4–7704.5 μg; Y_2, 44.9–64.7 μg cm^{-2} h^{-1}) only when the oil and S_{Mix} were used at 6 or 11% (v/v) and 10 or 13% (w/v) concentration level respectively. The lag time (Y_3) was found to be ranging from 2.16 to 2.48 h at low to high levels of S_{Mix}. The ranges of other responses, Y_1 and Y_2 were 3640.5–7704.5 μg and 29.7–64.7 μg cm^{-2} h^{-1}, respectively.

The responses of these formulations ranged from a low drug penetration of 3640.5 μg (ME7, high level of oil and S_{Mix} and medium level of water) to a higher penetration of 7704.5 μg (ME4, low level of oil, medium level of S_{Mix} and high level of water). For estimation of quantitative effects of the different combination of factors and factor levels on Q_{24}, flux and lag time, the response surface models were calculated with Design Expert software by applying coded values of factor levels. The model described could be represented as:

$$Y_1(Q_{24}) = 6240.74 - 1395.15X_1 - 302.29X_2 + 182.39X_3 - 172.35X_1X_2 - 101.20X_1X_3 - 191.83X_2X_3 - 298.86X_1^2 - 491.98X_2^2 + 99.47X_3^2 \quad (5)$$

$$Y_2(\text{Flux}) = 52.8 - 11.96X_1 - 3.08X_2 + 1.86X_3 - 1.40X_1X_2 - 0.73X_1X_3 - 2.35X_2X_3 - 2.71X_1^2 - 4.24X_2^2 + 0.59X_3^2 \quad (6)$$

$$Y_3(\text{lag time}) = 2.22 - 0.034X_1 + 0.02X_2 - 0.044X_3 - 0.098X_1X_2 - 0.05X_1X_3 - 0.01\,X_2X_3 + 0.11X_1^2 - 0.005X_2^2 + 0.04X_3^2 \quad (7)$$

3.6. Fitting of data to the model

Formulation ME4 showed a significantly higher amount of drug permeation (Y_1, Q_{24}) and higher flux (Y_2) among the formulations. The responses observed for 17 formulations prepared were simultaneously fit to first order, second order and quadratic models using Design Expert 7.1.5. It was observed that the best fit model was quadratic model and the comparative values of R^2, standard deviation and % coefficient of variation are given in Table 3 along with the

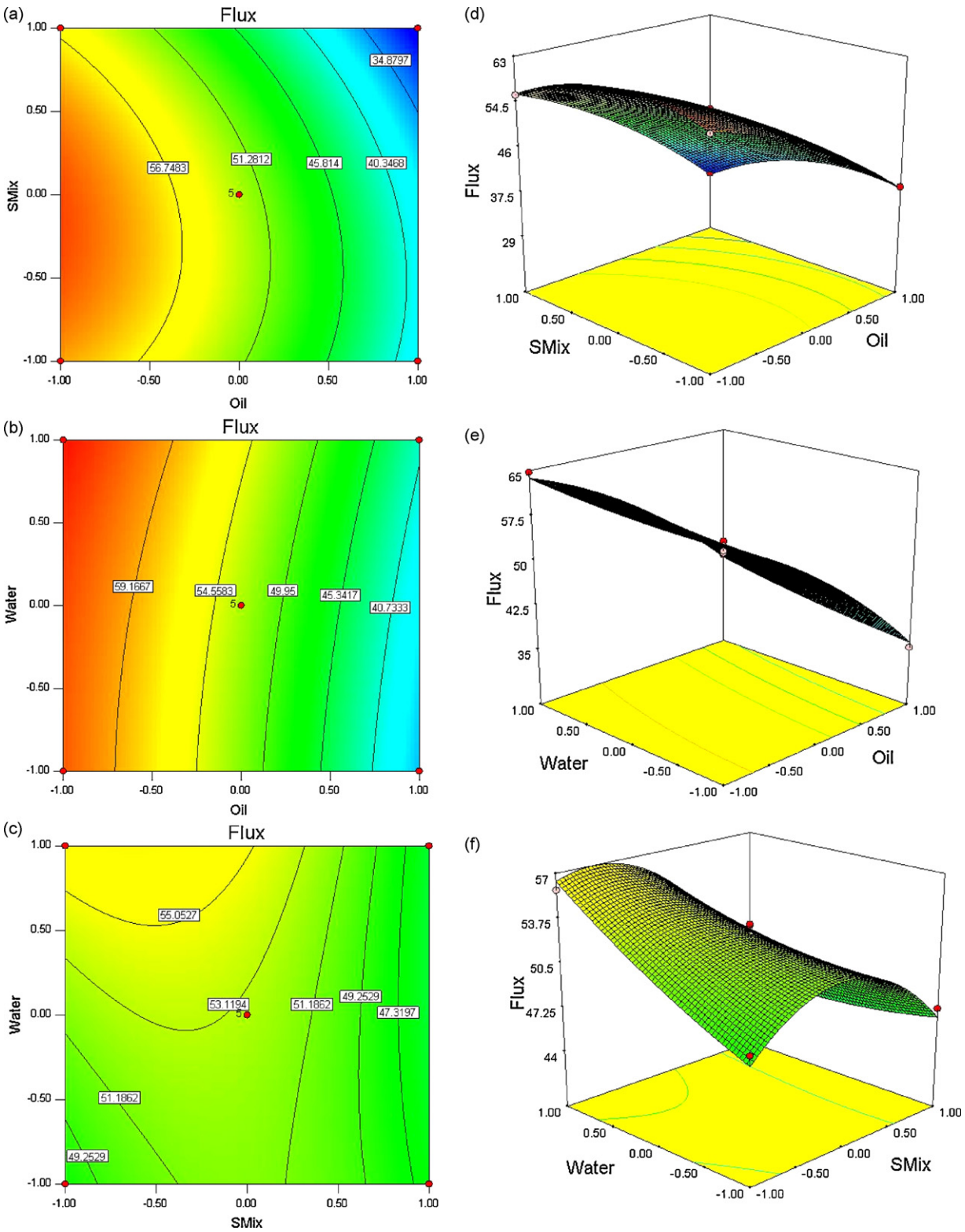

Fig. 3. Contour plot showing effect of (a) oil (X_1) and S_{Mix} (X_2), (b) oil (X_1) and water (X_3), (c) S_{Mix} (X_2) and water (X_3) on response Y_2 (flux), corresponding response surface plots (d–f).

regression equation generated for each response. A positive value represents an effect that favors the optimization, while a negative value indicates an inverse relationship between the factor and the response. It is evident that the independent variable X_3(water) is having positive effect on the responses, Q_{24} (Y_1) and flux (Y_2). The independent variable X_2 is having positive effect on the response lag time (Y_3).

The three-dimensional response surface plots (Fig. 3d–f) were drawn to estimate the effects of the independent variables on response and to select the optimal formulation. The required

Table 3
Summary of results of regression analysis for responses Y_1, Y_2 and Y_3 for fitting to quadratic model.

Quadratic model	R^2	Adjusted R^2	Predicted R^2	Adeq Precision	SD	% CV
Response (Y_1)	0.9896	0.9761	0.8458	30.90	166.10	2.81
Response (Y_2)	0.9950	0.9887	0.9502	45.04	0.99	2.00
Response (Y_3)	0.7798	0.4966	−1.488	4.69	0.07	3.18

flux to reach therapeutic concentration calculated was found to be about 12.16 μg cm^{-2} h^{-1}. Hence, the penetration rate of optimal formulations in the optimization process was set at above 12.16 μg cm^{-2} h^{-1}. Formulation, ME4 showed maximum flux of 64.7 μg cm^{-2} h^{-1} and could meet the target flux, calculated from the pharmacokinetic parameters of LCDP indicating that the concentrations may be enough to elicit the pharmacological effect.

3.7. Data analysis

Formulations ME4, ME9, ME12 and ME17 had the higher Q_{24} and flux. Table 4 shows the observed and predicted values with residuals and percent error of responses for all the formulations. The Q_{24} and flux obtained at various levels of the 3 independent variables (X_1, X_2 and X_3) was subjected to multiple regression to yield a second-order polynomial equation. The value of the correlation coefficient (R^2) of Eq. (5) was found to be 0.9896, indicating good fit (Table 3). The "Pred *R*-Squared" of 0.8458 is in reasonable agreement with the "Adj *R*-Squared" of 0.9761. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable, the ratio of 30.90 (Table 3) indicates an adequate signal. The Q_{24} values measured for the different formulations showed wide variation (i.e., values ranged from a minimum of 3640.5 μg in ME7 to a maximum of 7704.5 μg ME4). The results clearly indicate that the Q_{24} value is strongly affected by the variables selected for the study. 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 level to its high level. The interaction terms (X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2) show how the Q_{24} changes when two variables are simultaneously changed. The negative coefficients for all 3 independent variables indicate an unfavorable effect on the Q_{24}, while the positive coefficients for the interactions between 2 variables indicate a favorable effect on Q_{24}. Among the 3 independent variables, the lowest coefficient value is for X_1 (−1395.15), indicating that this variable is insignificant in prediction of Q_{24}.

The value of R^2 of Eq. (6) was found to be 0.9950, indicating good fit (Table 3). The "Pred *R*-Squared" of 0.9502 is in reasonable agreement with the "Adj *R*-Squared" of 0.9887. "Adeq Precision" a measure for the signal to noise ratio was found to be 45.04, indicating an adequate signal. The flux values of ME1, ME4, ME9, ME12 and ME17 were found to be more among the formulations. The flux values were found to be increased from medium to low levels of X_1; low to medium levels of variable X_2 and low to high levels of X_3. The flux values measured for the different formulations showed wide variation (i.e., values ranged from a minimum of 29.7 μg cm^{-2} h^{-1} in ME7 to a maximum of 64.7 μg cm^{-2} h^{-1} in ME4). The interaction terms (X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2) show how the flux changes when 2 variables are simultaneously changed. The positive coefficients X_1 and X_3^2 for the interactions between 2 variables indicate a favorable effect on flux. Among the 3 independent variables, the lowest coefficient value is for X_1 (−11.96), indicating that this variable is insignificant in prediction of flux.

The value of R^2 of Eq. (7) was found to be 0.7798 (Table 3). The signal to noise ratio was found to be 4.695, indicating an adequate

Table 4
Composition of checkpoint formulations, the predicted and experimental values of response variables and percentage prediction error.

Optimized formulation composition (X_1:X_2:X_3)	Response variable	Experimental value	Predicted value	Percentage prediction error
7.0:10.2:70.6	*Y*1	7185.4	7244.5	−0.82
	*Y*2	60.8	62.03	−2.02
	*Y*3	2.41	2.38	1.24
6.7:12.7:73.7	*Y*1	7310.5	7265.8	0.61
	*Y*2	61.8	61.4	0.65
	*Y*3	2.37	2.36	−4.42
8.0:13.4:83.0	*Y*1	6987.6	7163.7	−2.52
	*Y*2	59.4	60.4	−1.68
	*Y*3	2.36	2.31	2.12
10.0:12.4:72.5	*Y*1	6445.5	6536.2	−1.41
	*Y*2	53.8	55.1	−2.42
	*Y*3	2.30	2.26	1.74
9.6:10.9:81.5	*Y*1	6910.1	6838.4	1.04
	*Y*2	58.3	58.7	−0.69
	*Y*3	2.28	2.26	2.59
9.5:12.8:69.5	*Y*1	6740.3	6658.9	1.21
	*Y*2	54.8	55.9	−2.01
	*Y*3	2.34	2.30	1.71
10.5:12.7:73.0	*Y*1	6487.6	6399.5	1.36
	*Y*2	53.1	54.0	−1.69
	*Y*3	2.28	2.25	3.43
10.4:14.8:71.7	*Y*1	5983.8	6141.4	−2.63
	*Y*2	50.1	51.6	−2.99
	*Y*3	2.30	2.26	2.59
9.1:15.3:74.3	*Y*1	6520.8	6452.1	2.59
	*Y*2	53.8	53.3	1.66
	*Y*3	2.25	2.24	−1.36
12.5:10.9:83.0	*Y*1	6115.3	6225.7	−1.81
	*Y*2	52.4	53.5	−2.10
	*Y*3	2.20	2.17	1.36

signal. The lag time values of ME3, ME6, ME10 and ME16 were found to be less among the formulations, however the difference was insignificant ($p > 0.05$). The lag time values were found to be increased from low to high levels of X_1; high to low levels of X_2 and medium to low levels of variable X_3. The results attributed to that the deposition of drug from MEs within the layers of stratum corneum, might increase the lag time. The solubilizing capacity and affinity of drug in variable X_2 could result in increased lag time. The interaction terms (X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2) show how the lag time changes when 2 variables are simultaneously changed. The positive coefficients (X_2, X_2X_3, X_1^2 and X_3^2) for the interactions between 2 variables indicate a favorable effect on lag time. Among the 3 independent variables, the lowest coefficient value is for X_3 (−0.044), indicating that this variable is insignificant in prediction of lag time.

3.8. Contour plots and response surface analysis

Two-dimensional contour plots and three-dimensional response surface plots are shown in Fig. 3, which are very useful to study the interaction effects of the factors on the responses. These types of plots are useful in study of the effects of two factors on the response at one time. In all the presented figures, the third factor was kept at a constant level. All the relationships among the three variables are non-linear, although exhibit a nearly linear relationship of factor X_2 with factors X_1 and X_3, in the form of almost straight lines up to the medium level of S_{Mix} (Fig. 3). At higher concentrations of S_{Mix} these become curvilinear or non-linear.

Factors X_2 and X_3 have curvilinear relationship at all levels of the two variables on the response Y_2. Response surface plots show the relationship between these factors even more clearly. The Q_{24} and flux were found to be increased with increasing concentrations of either S_{Mix} (up to medium level) or water at constant concentration of oil phase.

3.9. Optimization

The optimum formulation was selected based on the criteria of attaining the maximum value of Q_{24}, maximum flux and low value of lag time by applying constraints on Y_1 ($5800 \leq Y \leq 8000$), Y_2 ($50 \leq Y \leq 65$) and Y_3 ($2.00 \leq Y \leq 3.00$). Upon trading of various response variables and comprehensive evaluation of feasibility and exhaustive grid search, the formulation composition with oil concentration of 6.5%, S_{Mix} 11.4% and water 82.0% was found to fulfill the maximum requisite of an optimum formulation because of maximum Q_{24} (7347.2 μg), flux (62.9 $\mu g\,cm^{-2}\,h^{-1}$) and low lag time (2.16 h) values. The optimized formulation was formulated as microemulsion gel using hydroxy propyl methyl cellulose (HPMC K 4 M) at 4% (w/v) in the microemulsion. The microemulsion gel showed about 5820.8 μg of drug permeated in 24 h, flux 46.1 $\mu g\,cm^{-2}\,h^{-1}$ and with lag time of 2.22 h. The flux of microemulsion gel was found to meet the target flux (12.16 $\mu g\,cm^{-2}\,h^{-1}$).

3.10. Validation of response surface methodology

Ten checkpoint formulations were obtained from the RSM, the composition and predicted responses of which are listed in Table 4. To confirm the validity of the calculated optimal parameters and predicted responses, the optimum formulations were prepared according to the above values of the factors and subjected to *ex vivo* permeation studies. From the results presented in Table 4, the predicted error was below 5%, indicating that the observed responses were very close to the predicted values. Percentage prediction error is helpful in establishing the validity of generated equations and to describe the domain of applicability of RSM model. Linear correlation plots between the actual and the predicted response variables were shown in Fig. 4. The linear correlation plots drawn between the predicted and experimental values demonstrated high values of R^2 (Q_{24}, 0.9421; flux, 0.9698; lag time, 0.9539) indicating goodness of fit.

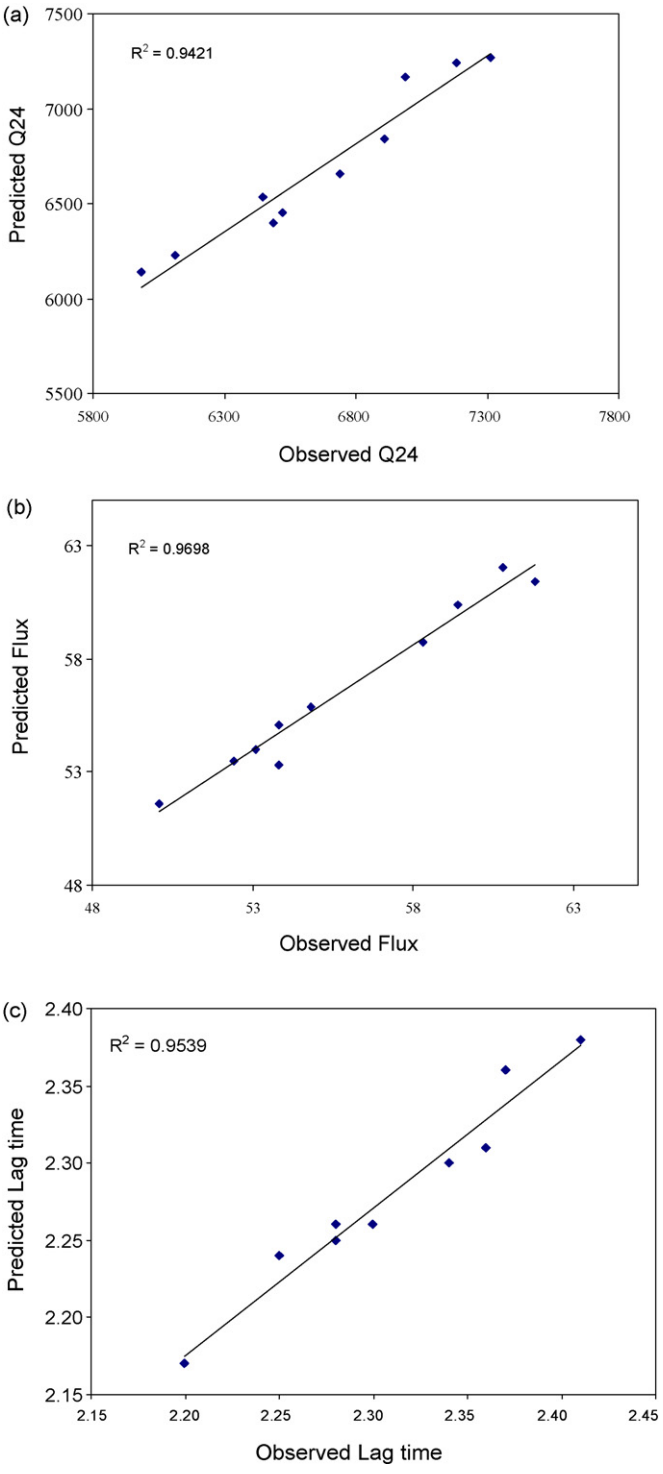

Fig. 4. Linear correlation plots (a, b, c) between actual and predicted values.

3.11. Determination of droplet size

The parameters for physicochemical characters of the optimized formulation (ME-OPT) were as follows: 30.5 nm for mean particle

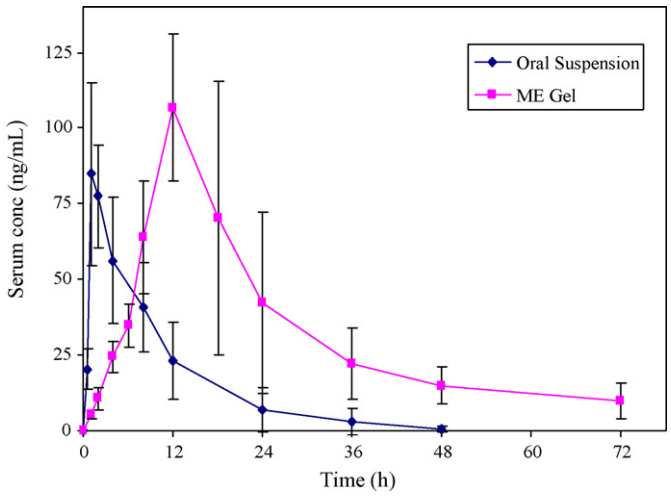

Fig. 5. Serum profiles of LCDP in Rabbits, after administration of oral suspension and ME gel, each containing 8 mg of LCDP, values represented are mean ± SD ($n = 6$).

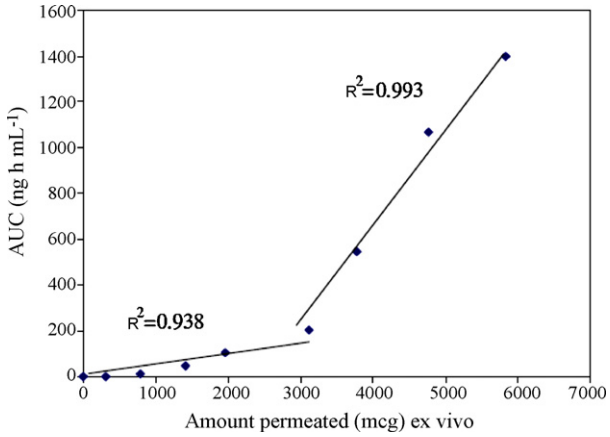

Fig. 6. *Ex vivo–in vivo* correlation of cumulative amount permeated *ex vivo* Vs AUC.

size with a poly dispersity index of 0.11 and 2.31 cP of gel index. All microemulsion formulations were stable at ambient temperature in the presence or absence of LCDP. No changes of particle size, phase separation and degradation of LCDP were observed during 6 months.

3.12. Stability studies

The LCDP content in the stability samples after 6 months was found to be 98.6 and 98.8% in ME4 and ME gel respectively. The results reveal that LCDP was stable during the study. The *ex vivo* permeation profiles are shown in Fig. 2. Formulation ME4 and ME gel showed 7540.0 and 5715.8 μg of LCDP permeated in 24 h. The flux values were found to be 46.10 and 43.7 $\mu g\,cm^{-2}\,h^{-1}$, respectively. The results suggested that the formulations did not show significant difference ($p > 0.05$) in permeation profiles compared to that of initial permeation profiles indicating that both the formulations are stable.

3.13. Skin irritation study

The skin irritation studies could not find any irritation, erythyma indicating that the ME gel is non-irritant.

3.14. In vivo bioavailability studies

The results from the bioavailability study (Table 5, Fig. 5) reveal that LCDP is released and permeated well from microemulsion gel by transdermal route, as compared to the oral suspension. The C_{Max} of LCDP was found to be 97.4 and 110.0 $ng\,mL^{-1}$ after administration of oral suspension and ME gel respectively. The C_{Max}, T_{Max} and AUC profiles were compared. In four rabbits, C_{Max} was higher for transdermal route than oral route and in the remaining rabbits C_{Max} for oral route was higher than the transdermal route. The T_{Max} values in all rabbits were higher for transdermal administration than the oral administration and the difference was statistically significant ($p < 0.05$). This difference was because of stratum corneum that could delay the permeation of LCDP from microemulsion gels in contrast, suspension administered by oral route is an immediate release dosage form. The overall mean value of AUC_{0-t} by transdermal route was 3.5 times higher than that of oral route, and the difference was found to be statistically significant ($p < 0.05$) demonstrating improved bioavailability of LCDP from microemulsion gel. This could be due to avoidance of first-pass hepatic metabolism by transdermal route. The reported oral bioavailability of lacidipine was 10% (Mc Cormack and Wagstaff, 2003), because of first-pass metabolism. In the present study the bioavailability of LCDP by transdermal route was found to be 35.1%. Therefore for the effective management of chronic hypertension LCDP in the form of microemulsion gel could provide an effective treatment.

3.15. Ex vivo–in vivo correlation

Ex vivo–in vivo correlation between the cumulative % of drug permeated across rat abdominal skin and AUC showed a biphasic curve pattern (Fig. 6), which can be distinguished into two regions for ME gel. Good linear correlation was observed with correlation coefficients, $R^2 = 0.938$ during lag phase and $R^2 = 0.993$ during absorption phase. Point to point correlation of *ex vivo* permeation of drug to *in vivo* performance was observed, indicating that it follows type A correlation (Emami, 2006). The slow permeation of LCDP through skin in initial stages is explained as follows; in first phase LCDP was released and permeated through skin and deposition of LCDP took place in skin layers and concentration build up was maintained. Permeation and concentration buildup at the skin is the lag phase observed in the first region. Concentration built up resulted in the flux establishment and AUC increased at a rapid rate in the second phase. This indicates that initially drug permeated into medium rapidly but it takes some time for permeation and absorption. Once the necessary flux is established, absorption was rapid as large amount of drug is deposited in the layers of skin.

4. Conclusions

The present study conclusively demonstrates the use of a Box–Behnken statistical design is valid for predicting the Q_{24}, flux, and lag time in optimization of microemulsion formulations. The

Table 5
Pharmacokinetic parameters of LCDP in rabbits after administration of oral suspension and ME Gel each containing 8 mg of LCDP, values represented are mean ± SD ($n = 6$).

Formulation	C_{Max} ($ng\,mL^{-1}$)	T_{Max} (h)	AUC_{0-t} ($ng\,h\,mL^{-1}$)	$AUC_{0-\infty}$ ($ng\,h\,mL^{-1}$)
Oral suspension	97.4 ± 9.25	1.2 ± 0.41	782.4 ± 360.76	804.8 ± 372.82
ME gel	110.0 ± 28.55	13.0 ± 2.45	2272.0 ± 982.51	2702.2 ± 1226.55

derived polynomial equations and contour plots aid in predicting the values of selected independent variables for preparation of optimum microemulsion formulations with desired properties. The developed microemulsion gel formulation was efficacious for the delivery of lipophilc and poorly soluble drugs such as lacidipine. The results demonstrated that the formulation was nonirritating and did not cause any erythyma upon transdermal administration. Results of bioavailability study showed improved permeation of the drug from the microemulsion gel compared to oral suspension. Good *ex vivo–in vivo* correlation was obtained with correlation coefficients of 0.938 and 0.993 during lag and permeation phase respectively.

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