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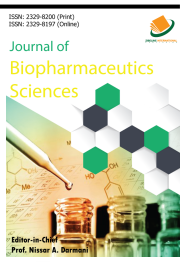

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OPTIMIZATION OF GEL BASED SYSTEM OF LERCANIDIPINE BY STATISTICAL DESIGN FOR TRANSDERMAL DELIVERY; HISTOPATHOLOGICAL EXAMINATION AND RHEOLOGICAL CHARACTERIZATION

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ABSTRACT

Our goal was to develop a transdermal gel formulation of lercanidipine by applying statistical approach for enhanced permeation and suitable application over skin. A 3-factors, 3-level Box-Behnken design were used to optimize the formulation. Ex vivo skin permeation studies were carried out to evaluate the responses such as flux, cumulative amount release in 24 hour and lag time using Franz-type diffusion cell. Confocal laser scanning microscopy (CLSM) of treated rat skin were used to assess the penetration ability and histopathological study was carried out in a quest to evaluate any skin irritation potential In vivo on rat skin and permeation mechanism of optimized Lercanidipine gel formulation after application over skin. Rheological characterization was carried out in order to clarify the suitability of optimized gel formulation for application over skin. The optimized formulation was having a composition of carbopol 934P (0.725%), soya lecithin (0.1%), and propylene glycol (15%).The skin permeation rate of lercanidipine significantly increased in proportion to the concentration of propylene glycol and lecithin while the gelling agent lay to decrease in skin permeation at higher level of it. Lag time has inverse relation with gelling agent and propylene glycol concentration. The result of CLSM study demonstrated significant penetration (>90µm) across rat skin while histopathological exa mination revealed the possible permeation mechanism by increasing cell gaps and rupturing of normal cell junction. This finding suggested a transdermal gel formulation of lercanidipine is having a good perspective to find effective formulation for transdermal drug release.

KEYWORDS

Solar Energy, Solar Cooling, Adsorption Cooling, Parabolic Through Collector

1. BACKGROUND OF THE STUDY

Recently, it has been demonstrated that transdermal route is an effective delivery mode as approximately one third of drugs involve delivery into or across the skin: this estimate includes all the drugs having their action site in the skin as well as those drugs that has to be taken up by the systemic circulation [1]. In addition, transdermal route has several advantages over other route: as it avoids the pain, possibility of infection, patient compliance issues associated with injections, provide control drug release over long period of time and eludes hepatic first-pass metabolism via the conventional route that can lead to loss in larger fraction of administered dose of drug [2,3].

The permeation ability of a drug molecule across skin and its quantitative release to achieve effective concentration in biological tissue has direct influence over the quantity of drug that can be transported into or across the skin (dermal or transdermal delivery. There are so many factors involved in this process such as the skin permeability, properties of the selected drug candidate and the characteristics of the excipients [4-7]. LER, a third generation calcium channel blocker belonging to dihydropyridine group is having properties such as extensively high first pass metabolism, smaller dose, short biological half life, poor oral bioavailability (10%), that make it an ideal moiety to be developed as transdermal dosage form [8-11].

The designing of a dosage form with minimum number of trials and possibly in lesser time are very critical for formulation scientists [12]. In this study, Box-Behnken statistical design has been explored for optimization the gel formulation of LER as it requires lesser experimental runs with less time consumption as compared to other statistical designs. Hence it is more efficient and economical technique over conventional process of formulation optimization. Also, Box–Behnken design present more efficient matrices with increased number of published works in recent years [13]. The objectives of the study was to optimize a novel gel formulation of LER by applying Box–Behnken design by observing the effect of three factors (gelling agent, amount of lecithin and propylene glycol) over permeation of LER with optimizing the level of these factors to obtain the targeted skin permeation profiles (high flux, maximum release in 24 hours and short lag time) across rat skin as the response surface methodology (RSM) with polynomial equations is a useful statistical tool to investigate the effect of independent variables on dependent variables (responses) based on a limited number of trials [14-17].

2. MATERIALS AND METHODS

2.1 Materials

The following materials were used as received, without further purification. Carbopol 934P was obtained from Ranbaxy Research Laboratories (Gurgaon, Haryana, India). Soya lecithin (99 %) was received from Lipoid, Germany: Propylene glycol (PG) was supplied by Loba Chemicals Pvt. Ltd (Mumbai, Maharashtra, India).triethanolamine (Junsei Chemical Co., Japan) were of analytical grade. All other materials used in the study were of analytical grade. Double distilled water was used throughout the study.

2.2 Methods

2.2.1 Preparation of drug formulation using the factorial designs

All the selected independent and dependent variables along with their levels are presented in Table 1. Gel formulation of LER was prepared as per experimental runs suggested by Box-Behnken design (Table 1). After complete hydration of polymer in double distilled water over night, the drug (0.1% W/W) dissolved in mixture of lecithin in propylene glycol and triethanolamine was added and mixed completely, and then water was added to give a total weight of 100 gm. pH was adjusted if necessary.

2.2.2 Determination of pH

pH of all prepared gel formulations were recorded (Table 1) in triplicate after appropriate dilution with a glass microelectrode (Mettler Instruments, Giessen, Germany) by bringing it in contact with the gel and allowing it to equilibrate for 1min [18].

2.2.3 Spreadability

The spreadability of gel formulation was determined by placing 0.5 gm of formulation within a circle of 2 cm diameter premarked on a glass plate over which a second glass plate were placed. A weight of 500 gm were allowed to rest on upper glass plate for 5 minute [19]. The increase in diameter due to spreading of formulation was noted (n=3), and the percent spread by area were calculated as follows:

$$\% A = A_2 / A_1 * 100 \quad (1)$$

Where, A is % spread by area, A1 represents total area before spreading (cm^2) and A_2 is final area after spreading.

2.2.4 Drug Content Estimation

A 0.1% w/v solution of all prepared experimental gel formulations were prepared by dispersing in distilled water and subjected to magnetic stirring (400 rpm) for 5 min. The dispersion was then filtered to remove undissolved residue. Exactly 1 ml of the filtrate was diluted to 5 ml and absorbance was measured at 242 nm. An unloaded gel was also subjected to a similar determination to observe the effect of excipeints on the absorbance. Using the standard curve of LER in distilled water, the drug content in gel was finally estimated.

2.2.5 Ex vivo skin permeation studies of lercanidipine across excised hairless rat skin

All the procedures were carried out according to protocol approved by Institutional Animal Ethics Committee of Jamia Hamdard for preparation of full thickness rat skin (Approval no. 808). After sacrificing with excess ether inhalation, hair on abdominal surface was removed with hair clipper taking extreme precaution not to damage the skin of male albino wistar rat (weighing between 200-250 grams). The shaved skin was then excised, subcutaneous tissue was surgically removed and dermal-side was wiped with cotton swab dipped in isopropyl alcohol to remove adhering fat [20]. The prepared full thickness skin was washed immediately with phosphate buffered saline (PBS), wrapped in aluminium foil and stored at -20 ºC till further use (used wi thin two weeks of preparation).

Ex vivo skin permeation studies were carried out by using Franz diffusion cell with a surface area of 0.785 cm2. Excised hairless rat skin was mounted between donor and receptor compartments of the diffusion cell such that stratum corneum (SC) faces towards the donor compartment. 1 gram gel was placed in the donor compartment and 10 ml of phosphate buffer saline (PBS, pH 7.4) containing 40% (v/v) of polyethylene glycol (PEG) 400 was placed in receptor compartment containing 0.02% w/v of sodium azide to retard microbial growth. PEG 400 was incorporated to maintain sink conditions and the contents of receptor compartment were agitated at 400rpm over a multi-magnetic stirrer (Cintex, Mumbai, India) at 37±2 ◦C. Samples of 1ml were collected at predetermined time points and replenished with PBS (pH 7.4) containing 40% (v/v) PEG 400. The cumulative amount of LER permeated was analyzed with UV spectrophotomet at 240 nm and concentration was corrected for sampling effects according to following equation [21-22]:

$$C_n^1 = Cn\left(\frac{V_t}{V_t - V_s}\right)\left(\frac{C_{n1-1}}{C_{n-1}}\right) \quad (2)$$

Where C^1 was the corrected concentration of the nth sample, Cn represented the measured concentration of LER in the nth sample; Cn^{-1} 1 depicted the corrected concentration in the $(n-1)^{th}$ sample. Cn^{-1} defined the measured concentration of the LER in the $(n-1)^{th}$ sample, Vt described the total volume of the receiver fluid and Vs was the volume of the sample drawn.

A plot between amounts of LER permeated across excised rat skinVs time was drawn and permeation parameters were calculated [22]. The target flux was calculated using the following Equation [22],

$$T = \frac{C_{SS} * C_{Lt} * BW}{A} \quad (3)$$

Where, Css represented the LER concentration at therapeutic level (21.25 µg/L), Clt depicted the total body clearance of LER: 0.97 L/h/kg (calculated from volume of distribution, 1.120 L/kg and half life of 4.3 h) [23], BW was the standard body weight of rats which were used in the study (0.250 kg), and A represented the surface area of the franz diffusion cell (i.e. 0.785 cm^2). The calculated target flux (T) value for LER was 6.56 µg cm^{-2} h^{-1}.

2.2.6 Design of experiment

A 3-factor 3-level Box- Behnken statistical design expert software (Version 8.0.7.1, Stat-Ease Inc, Minneapolis, MN) was used in a quest to explore quadratic response surfaces and construct second-order polynomial equations for optimization of gel system. The dependent and independent variables selected were shown in Table 1 along with their low, medium and high levels. Statistical evaluation of main effects, interaction effects and quadratic effects of different independent variables on the amount of LER permeated in 24 h (Q24), flux and lag time was made. A design matrix comprising of 17 experimental runs was constructed. The non-linear computer generated quadratic model was given as:

$$Y = b_0 + b_2X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_2X_2^2 + b_{33}X_3 \quad (4)$$

Where, Y was the measured response associated with each factor level combination; b0 represented an intercept; b1 to b33 were regression coefficients computed from the observed experimental values of Y; and X_1, X_2 and X_3 were the coded levels of independent variables. The terms X_1, X_2 and X_3 (i = 1, 2 or 3) represented the interaction and quadratic terms, respectively [24,25]. Statistical validity 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 R2 values.

2.2.7 Rheological Measurements

Rheological characterization of optimized gel formulation (0.5 gm) was performed at 80 rpm using R/S CPS Plus Rheometer (Version:9.00, Serial number: #303169, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) at 25±0.5°C with Spindle C 50-1 (diameter=50mm).The software used was Rheo3000. Controlled stress rate study was conducted to get important information about flow behavior with change in speed of spindle (rpm) and shear strain in two steps (step 1; 1.528-80 rpm, step 2;80-1.528 rpm). Wait time for the operation was 180 second. Flow behavior of optimized gel formulation sample was analyzed by fitting the experimentally generated equation of shear stress–shear rate data to different common models such as Power law, Equation (5) and Herschel–Bulkley model, (Equation (6)):

$$T = kv^n \quad (5)$$

$$T = T_0 + kv^n \quad (6)$$

Where, y was the shear rate (1/second, 1/s), T represented the shear stress (Pascal, Pa), K depicted the consistency coefficient (Pa s^n), n described the flow behavior index (dimensionless), and T0 was the yield stress (Pa) [26].

Histopathology of Treated rat skin with gel and untreated rat skin (control)

Histopathological study entailed to evaluate any in vivo skin irritation and permeation mechanism when optimized LER gel applied over rat skin. For the application of formulation, male wistar rats were prepared one day

before [27]. The rats were sacrificed 24 h after applying the gel formulation over the abdominal skin and specimens of the exposed portion of skin and adjacent untreated skin area were collected for study. The skin pieces were immediately fixed in 10% formalin. Subsequently, each tissue was rinsed with running water, dehydrated using a graded series of alcohols and embedded in paraffin wax and sections of 5 µm thickness were cut from each sample. These sections were then stained with haematoxylin–eosin for microscopic observation (Motic, Tokyo,Japan). Adjacent skin not treated with the formulation served as a control.

2.2.8 Confocal laser scanning microscopy study (CLSM)

CLSM study was done for optimized gel formulation in a quest to assess its penetration ability in terms of depth of penetration with Fluoview software. After application of gel over rat abdominal skin, the experiment was run in same manner in Franz diffusion cell described under ex vivo skin permeation studies. After 8 hours skin was removed and washed thoroughly with distilled water. The treated area was excised and examined for probe penetration [28]. The excised rat skin was positioned on microscope slide so that SC side faces towards cover glass. The CLSM (Laser confocal microscopy with fluorescence correlation spectroscopy-Olympus FluoviewTM FV1000) was carried out with an organ laser beam with excitation at 488 nm and emission at 590 nm. Each skin sample sliced in section of 6- 10µm through the z axis of CLSM.

2.2.9 Stability Studies

The optimized gel formulation was subjected to stability studies and accelerated stability studies as per ICH Q1A (R2) guidelines. at different temperatures and ambient humidity conditions. Stability study was done to check the stability of developed formulation at refrigerated temperature (8 ±2 oC), and room temperature (25±2 oC). Accelerated stability study was done at 30°C/65% RH, 40°C/65%RH and 50°C/75% RH to predict the shelf life at room temperature. The formulation samples were packed in 10 gm collapsible tubes and charged to stability chambers (Thermo lab, Mumbai, India) with humidity and temperature control. They were withdrawn at specified time intervals of 0, 15, 30, 45, 60, 75, and 90 days for clarity, pH, drug content, and viscosity analysis. The drug content analysis was carried out by UV spectrophotometric analysis at 242 nm wavelength over a period of 3 months under accelerated conditions. The shelf life was calculated according to method of Shakeel et al, 2008.

3. RESULT AND DISCUSSION

All the prepared gel formulations were inspected for their cosmetic qualities such as colour, smell, texture and consistency. They were observed as slightly yellow in colour with a pleasant smooth homogeneous appearance and texture. The pH value of gel formulation was ranged from 6.09 to 6.98 (Table 1): pH near to 7.0 is quite acceptable to avoid the risk of irritation upon application to the skin [29].

Table 1: Variables and observed responses in box-behnken design for gel formulations.

Formulation Code	Independent Variables			Dependent Variables (Actual)			Dependent Variables (Predicted)			Drug content (%)	pH of gel formulation
	X1 (%w/w)	X2 (%w/w)	X3 (%w/w)	Y1 (µg/h/cm^2)	Y2 (µg)	Y3 (h)	Y1 (µg/h/cm^2)	Y2 (µg)	Y3 (h)		
BBD 1	-1.00	-1.00	0.00	9.64	332.73	3.27	10.63	346.65	3.81	96.73±0.05774	6.13
BBD 2	1.00	-1.00	0.00	14.45	368.05	0.74	14.90	386.76	1.04	92.13±0.05774	6.25
BBD 3	-1.00	1.00	0.00	8.12	496.58	7.63	7.67	477.87	5.06	94.26±0.05774	6.75
BBD 4	1.00	1.00	0.00	12.29	223.23	1.96	11.30	209.31	2.30	89.73±0.05774	6.53
BBD 5	-1.00	0.00	-1.00	6.92	313.53	3.55	5.49	300.38	4.77	89.73±0.05774	6.09
BBD 6	1.00	0.00	-1.00	14.92	338.18	2.48	14.03	320.25	2.00	95.26±0.05774	6.86
BBD 7	-1.00	0.00	1.00	11.36	498.71	3.54	12.25	516.64	4.10	94.96±0.05774	6.96
BBD 8	1.00	0.00	1.00	10.18	255.18	2.43	11.61	268.32	1.34	97.33±0.05774	6.90
BBD 9	0.00	-1.00	-1.00	6.91	227.04	3.85	7.35	226.26	2.76	97.26±0.05774	6.19
BBD 10	0.00	1.00	-1.00	12.92	504.45	3.23	14.80	536.31	4.01	96.00±0.00000	6.55
BBD 11	0.00	-1.00	1.00	22.13	673.45	1.88	20.25	641.59	2.09	96.73±0.05774	6.34
BBD 12	0.00	1.00	1.00	6.70	284.53	2.61	6.25	285.31	3.35	98.93±0.05774	6.74
BBD 13	0.00	0.00	0.00	14.14	567.13	3.10	14.13	567.08	3.05	98.13±0.05774	6.28
BBD 14	0.00	0.00	0.00	14.11	567.25	3.06	14.13	567.08	3.05	94.33±0.05774	6.88
BBD 15	0.00	0.00	0.00	14.19	567.05	3.06	14.13	567.08	3.05	93.5±0.00000	6.17
BBD 16	0.00	0.00	0.00	14.14	567.15	3.09	14.13	567.08	3.05	97.5±0.000000	6.74
BBD 17	0.00	0.00	0.00	14.09	567.01	3.07	14.13	567.08	3.05	95.73±0.05774	6.98

Independent variables	Level used, actual			Dependent variables
	Low (-1)	Medium (0)	High (+1)	
X_1= gelling agent	**0.5%**	**1.0%**	**1.5%**	Y1=Flux (µg/cm^2/h)
X_2= Concentration of lecithin (%w/w)	**0.1%**	**0.3%**	**1.0%**	Y2= Release, 24 h (µg)
X_3= Concentration of PG (%w/w)	**5.0%**	**10.0%**	**15.0%**	Y3= Lag time (hours)

Spreadability is one of the important criteria for the formulation that has to be applied over skin as therapeutic efficacy of such formulation depends on its spreading ability. Also, the spreadability is a key factor to patient compliance and helps in uniform application of gel to the skin. A good gel formulation takes less time to spread and spreadability value of 6.25 g cm/s indicated that the gel was easily spreadable by small amount of shear and possessed acceptable bioadhesion.

LER content of all gel formulations were estimated by withdrawing samples at random from three different sampling points in a single batch in similar manner. Estimations were made spectrophotometrically, after dispersion of the gel in distilled water. The content of LER in gels was found to be within limits (> 90%). Samples within a batch were uniform as evident from the low standard deviation value (≤5.77%).

3.1 Ex vivo skin permeation studies

Ex vivo permeation study of lercanidipine across excised rat abdominal skin was carried out for all the prepared gel formulation and permeation parameters were calculated (Table 1).

3.2 Effect of Factors over Responses : One factor analysis

One factor plot and two domensional contour plots were suggested by design expert software to anlyze the effect of selected independent varriables over the responses as depicted in Fig. 1 & 2. These plots were drawn to analyze effect of one factor as well as two factors at a time over the selected responses respectively.

From the one factor plot of gelling agent for permeation flux (Figure 1a), it can be seen that the permeation rate of LER increased in proportion up to the mid level of gelling agent then it started to decreases i.e. at higher level of gelling agent. The result of Box-Behenken design analysis showed that the effect of the gelling agent over all the three responses Y1, Y2, and Y3 were highly significant ($p < 0.01$, Table 3).

Effects of lecithin over permeation profile of LER was investigated since lecithin is reported as a significant skin penetration enhancer, and in support of this our study results showed clearly that as the lecithin concentration raised from its low level to high level, skin permeation flux of LER also increases in proportion (Figure 1b) [30]. The result of Box-Behnken design analysis showed that the effect of the lecithin concentration over responses Y1, Y2 were significant ($p < 0.05$) while it was not significant for response Y3 (Table 3).

The basis behind selection of PG was its ability to solubilise poorly soluble drug as vehicle, can readily permeate across skin along with drug molecule and potential to alter the skin structure, thereby it may modify the permeation across skin [31,32]. Also, PG has wide acceptability as solvents in transdermal formulations for its permeation enhancing influence over several drugs and synergistic action with some terpenes in permeation enhancement [33-35]. In addition, it is systemically safe and locally highly tolerable when applied over skin [36]. Solubility of the permeant moiety is crucial in transdermal drug delivery as it affects partitioning of the drug molecule between the formulation and the skin, which in turn influences the permeation rate of the drugs [37,38]. In our case, LER was having good solubility in PG, as the proportion used in the formulation led to increase the drug partitioning towards the skin. PG used in the formulation may enhance intracellular drug mobility by salvation of alpha keratin in corneocyte and allowing alteration in skin structure which led to enhanced percutaneous absorptions of the drug molecule, therefore permeability of the LER increased due to raised thermodynamic activity and enhanced in transcellular diffusion [39,40]. Figure 1c depicted that flux of LER increased with increase in the concentration of PG from its low level to high level, because PG has co- solvent effect and penetration enhancing property for permeant moiety [41].

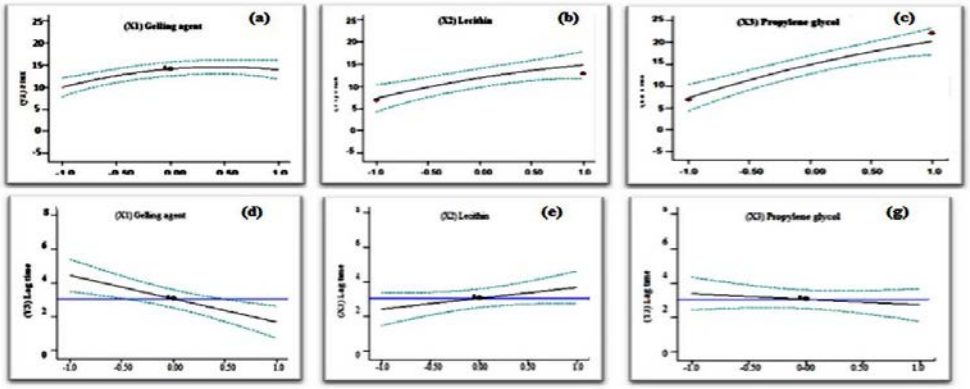

Figure 1: One factor effect plot of gelling agent, lecithin, and propylene glycol concentration for flux (a-c) and lag time (d-f) as analyzed by Box-Behnken design ($p \leq 0.05$)

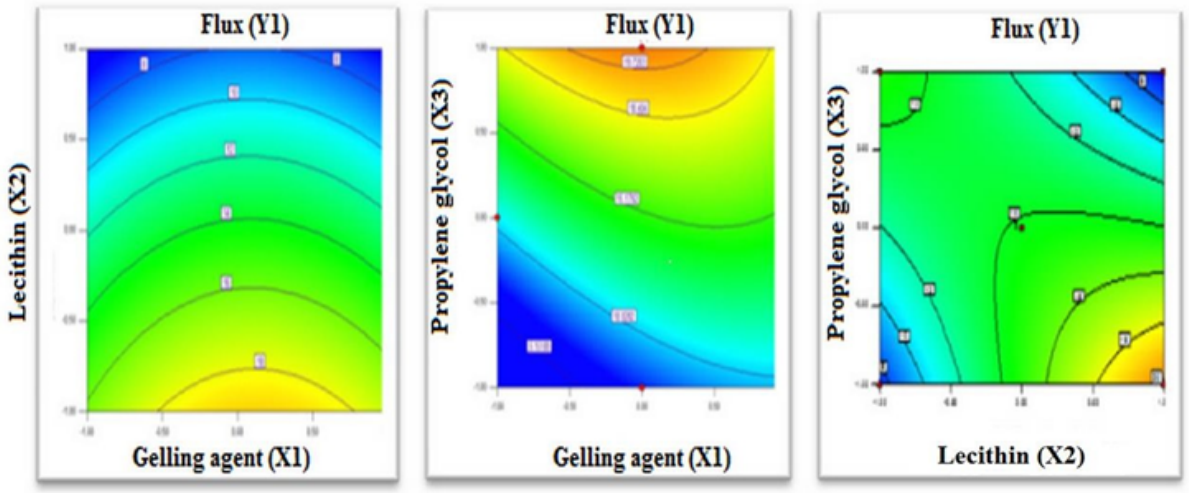

Figure 2: Contour plots as analyzed by Box-Behnken design: showing effect of gelling agent and lecithin, gelling agent and propylene glycol, lecithin and propylene glycol concentration over flux of lercanidipine across rat skin from developed gel formulation (left-right)

Table 2: Coefficient table showing values of interaction term along with their p value

Response	Intercept (b_0)	X_1	X_2	X_3	X_1X_2	X_1X_3	X_2X_3	X_1^2	X_2^2	X_3^2
Y1, Flux	14.134	1.975 p = 0.0072	-1.6375 p=0.0171	1.0875 p=0.0777	-0.16 p=0.8360	-2.295 p=0.0177	-5.36 p=0.0002	2.1645 p=0.0204	-0.8445 p=0.2826	-1.1245 p=0.1651
Y2,Release	567.078	- 57.113 p = 0.0003	-11.56 p=0.2191	41.0838 p = 0.0020	-77.167 p=0.0004	-67.04 p=0.0009	-166.58 p<0.0001	-141.44 p<0.0001	-70.481 p=0.0006	-74.229 p=0.0004
Y3, lag time	3.052	-1.3817 p = 0.0021	0.6275 p=0.1063	-0.3317 p=0.3756						
Legend	p <.01, Highly significant : .01<= <.05, significant : .05<= p <.10, moderately significant : p >=.10: not significant									

Table 3: Summary of results of regression analysis for responses y1, y2 and y3 for fitting to different models

S No.	Model	Responses	R^2	Prob > F	Model F value	Adequate Precision	SD	%CV
1	Quadratic	Response(Y1)	0.9365	<0.0500	11.47	12.927	1.49	12.22
2	Quadratic	Response(Y2)	0.9879	<0.0500	63.37	23.268	5.60	24.22
3	Linear	Response(Y3)	0.5867	<0.0500	6.15	8.100	33.51	1.02

3.3 Model evaluation

A 3-factor, 3-level Box-Behnken statistical design which requires 17 experimental runs was used to optimize the gel formulation. The observed ranges of responses i.e. Y1, Y2, and Y3 for all experimental runs were found as 6.70-22.13 µg cm2 h-1, 223.23-673.45 µg and 0.74 -7.63 h respectively. All the responses observed for 17 prepared formulations were simultaneously fitted to first order, second order and quadratic models and it was observed that the best-fitted model was quadratic for response Y1 and Y2 while there was linear relationship for Y3 among factors on basis of comparative values of R2, SD, and %CV as shown in Table 3 along with the regression equation generated for each response.

The required flux to attain therapeutic level was calculated as about 6.56 µg cm -2 h-1. Hence, the permeation rate of optimal formulations in the optimization process was set at above 6.56 µg cm -2 h-1.

3.4 Contour plots and response surface analysis:

Regression equation generated for each response by design expert software is as follows

$$(Y1) = +14.13 + 1.98X_1 - 1.64X_2 + 1.09X_3 - 0.16X_1X_2 - 2.30X_1X_3 - 5.36X_2X_3 - 2.16X_1^2 - 0.84X_2^2 - 1.12X_3^2 \quad (7)$$

$$(Y2) = +567.08 - 57.11X_1 - 11.56X_2 + 41.08X_3 - 77.17X_1X_2 - 67.05X_1X_3 - 166.58X_2X_3 - 141.45X_1^2 - 70.48X_2^2 - 74.23X_3^2 \quad (8)$$

$$(Y3) = +3.05 - 1.38X_1 + 0.63X_2 - 0.33X_3 \quad (9)$$

The value of R2 for Equation (7) was found to be 0.9365; it indicated goodness of fit to applied model (Table 3). The model F value of 11.47 (p < 0.005) and Values of Prob > F less that 0.0500 implied that the model was significant (Table 3).

In this case X_1X_2, X_1X_3, X_2X_3,$X1^2$ were significant mod el terms as depicted from coefficients (Table 2). Adequate precision measures the signal to noise ratio. A ratio greater than 4 was desirable. The ratio of 12.927 indicated an adequate signal. This model can be used to navigate the design space. In our finding, we found that the flux of LER increased slightly but later on it decreased as concentration of the polymer increased as at higher concentration the gel network of the polymer become stronger leading to slow release of drug remains entrapped in it [42]. Also, the polymer has interstitial micro voids forming channels inside the polymer matrix which is influenced by polymer content and the degree of swelling. Thus, an increased amount of polymer led to reduce channels size due to increase in degree of swelling thereby decreases the permeation rate of drug [43].

The value of R2 for Equation (8) was found to be 0.9879; it indicated goodness of fit to applied model (Table 3). The model F value of 63.37 (p < 0.005) and Values of "Prob > F " less than 0.0500 implied that the model was significant (Table 3). In this case X_1X_2, X_1X_3, X_2X_3,X^2 , X^2, X^2 were significant model terms as depicted from coefficients (Table 3). The value of adequate precision of 23.628 indicated an adequate signal. In our finding, we found a higher permeation rate (flux) of LER at lower level of the lecithin in presence of gelling agent and PG. The explanation is that the transdermal permeation of phospholipids (main constituent of lecithin) illustrated that fluidity of the intercellular lipids of the SC increased in presence of phospholipids that led to the enhanced permeation of drug [44].

3.5 Analysis of data and optimization of Lercanidipine gel formulation

Statistical optimization technique based upon response surface methodology proved to be a very useful approach for selecting optimized pharmaceutical formulations [45]. Optimization of the formulation was done with an aim to achieve maximum flux, maximum release of the drug and lag time in an appropriate range of the formulation by applying constraints for the responses; 18.0 ≤ Y1 ≥ 25.0 µg cm-2 h-1, 550.0 ≤Y2 ≥ 680.0 µg and 2.0 ≤ Y3 ≥ 5.0 hour. The trading of various response variables and comprehensive evaluation of feasibility and exhaustive grid search gave the optimized gel formulation of LER with composition and predicted values of all selected responses (Table 4).

The suggested optimized formulation was formulated as per composition (Table 4) and evaluated for the ex vivo skin permeation profile by using Franz diffusion cell and permeation parameters were calculated. The validation of applied statistical design involve that the parameters of optimized formulation should be in limit as per prediction and percentage prediction error must be less (<5%). Percentage prediction error calculation used to validate generated equations and thus depicts the applicability of RSM model.

3.6 Rheological Measurements:

The optimized gel exhibited a non-Newtonian (non linear relationship between shear stress and shear rate, Figure 3a), pseudo plastic behavior, which is typical of hydrophilic polymeric systems. This behavior was considered important, as it will facilitate spreading upon application over biological surface. Usually, hydrophilic polymeric systems exhibits non-Newtonian, pseudoplastic behavior, which contributes to their spreadability on application over a biologic surface; a higher degree of pseudoplasticity leads to easy spreadability of formulation [46].

Table 4: Composition of optimized ler gel formulation along with their characterization parameters

Composition of optimized gel (%w/w)			Viscosity (Pa s)	Eta at Dmax=480.06 1/s (Pas)	Eta at Dmin=9.06 1/s (Pas)	Thixotropy (Pa/s)	Yieldstress (Pa)	Depth of Penetration (µm)	Responses					
									Experimental			Predicted		
X1	X2	X3							Y1	Y2	Y3	Y1	Y2	Y3
0.725	0.1	15	9.898 ± 14.874	2.313	53.563	762.552	534.027	96.95	20.89	613.19	2.66	19.88	633.86	2.72
Percent prediction error(%)				Responses			Y1					4.83		
							Y2					-3.37		
							Y3					-2.25		

As non-Newtonian behavior was observed with gel formulation, the viscosity was decreased progressively by increasing the shear rate indicated a shear-thinning system (an ideal pre-requisite for semisolid formulations) i.e. the consistency of such system will reduce in proportion to applied shear as in case of application over skin. Also, in case of semisolid preparation they must have high consistency during storage but the consistency should reduce for easy evacuation from storage tubes when shear is applied.

Results of rheological study demonstrated that viscosity value ranged from 2.313 Pas up to 53.563 Pas at decreasing down the shear rate from 480.06 1/s to 9.06 1/s with an apparent viscosity (Eta mean) of 9.898 ± 14.8743 Pa s for optimized gel formulation as shown in Table 4. This higher value could ensure the stability of formulation.

Yield stress, a controversial rheological parameter, is the minimum shear stress above which flow can be observed, has recognizable influence over semisolids spreadability as lower values of yield stress increases spread ability but decrease retention over applied surface [47]. In our experiment we found a higher value of yieldstress (Table 4) that could lead to a good retention of formulation over applied surface as spreadability will not be too high due to significantly higher value of yield stress.

Thixotropy (ability of gel to recover to its original microstructure), a kind of behavior that simply reflects deformation of solid structure of gel with applied shear rate [48]. A higher value of thixotropy for optimized gel formulation i.e. 762.552 Pa/s (Table 4) indicated an excellent ability to recover from deformation to original structure upon application over biological surface when it exposed to different rate of shear.

Finally, after fitting studied rheological parameters to different common models we found that Herschel– Bulkley model was the best fit model to explain the flow behavior of optimized formulation (Table 5), because the confidence of fit was almost perfect (R2 =0.9948) for this model.

3.7 Histopathology of Treated rat skin with gel and untreated rat skin (control):

Effect of LER gel on skin was investigated by observing the section photomicrographs of normal untreated skin which served as control (Figure 4a) and skin treated with gel formulation (Figure 4b) at 24 h after application.

The photomicrographs of untreated rat skin (control) showed normal tightly multilayered structure of skin with well defined SC, epidermis and dermis (Figure 4a). In contrast to this, definite changes were observed in the skin morphology as it showed scattered, loose SC when it was treated with gel formulation (Figure 4b). Also, the image of skin treated with gel showed significant modification of the skin surface as it appeared rougher than that of skin treated as control. Cell gaps further increased and normal cell junction was broken, furthermore, the phenomenon of the skin flake desquamating from the SC could be clearly observed. It could be inferred that LER gel had significantly changed the structure of SC, and this change could be beneficial to the permeation of drugs through skins. The conclusion was consistent with the results obtained from ex vivo skin permeation experiments.

So the possible mechanism of permeation enhancement of gel preparations for LER may occur via widening of cell gaps and rupturing of normal cell junction led to a weak barrier function of SC. As a result, drug molecules could permeate through the SC by passing the numerous cavities presented on the surface of SC after the treatment of LER gel preparations.

Also, there were no indication of skin irritation (erythema or edema) on visual inspection of applied skin surface with gel formulation and it was predicted that developed gel formulation might be safe with perspective of skin irritation.

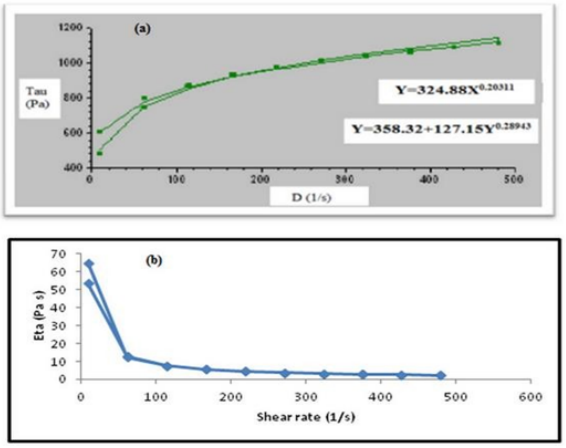

Figure 3: Rheogram of lercanidipine gel system showing (a) influence of shear rate over viscosity, (b) non linear relationship between shear stress and rate of shear (non-Newtonian behavior

Table 5: Rheological parameters of gel fitted to different rheological models

Model	K (Pa s^n)	n	τ_0 (Pa)	Confidence of fit
Herschel–Bulkley	127.1494	0.28943	358.32	0.99489
Power law	324.88	0.20311	-	0.98269

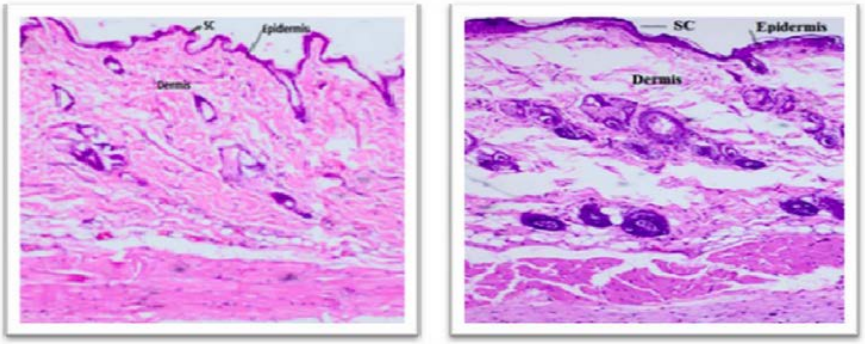

Figure 4: Photomicrographs of rat skin sample: (A) control group showing normal epidermis, dermis and subcutaneous tissues at10x (B) skin sample from lercanidipine gel treated animal at 10x.

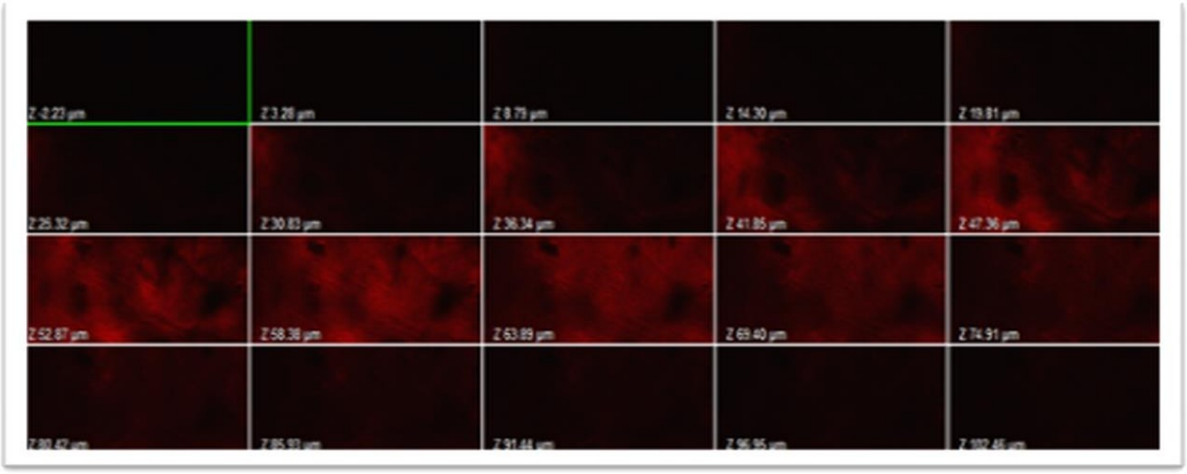

Figure 5: CLSM images of sections of excised rat skin incubated for 8 h with rhodamine RR loaded lercanidipine gel showing the layer by layer penetration of red fluorescence into stratum corneum (SC), epidermis, and dermis.

Table 6: Thickness of different layer of skin in rats and human [47]

Species	Thickness (in µm)		
	SC	Epidermis	Whole skin
Rat	18	32	2.09
Human	17	47	2.97

3.8 Confocal laser scanning microscopy study (CLSM) The thickness for SC of rat or humans skin were reported as 18µm and 17µm respectively (Table 6) and usually the action site for most of the therapeutic complication associated with skin lies below SC, so a drug moiety must have to penetrate atleast to a depth of 20-200 µm across skin in such conditions [49]. CLSM studies were conducted to clarify the percutaneous penetration as skin permeation studies on full thickness rat abdominal skin was carried out with optimized gel formulation for 8 h to accomplish an adequate penetration. A typical native and the corresponding CLSM images (Fig ure 5) of the excised rat skin sample treated with formulation clearly depicted the fluorescence emitted by rhodamine red RR (0.03%) labeled gel formulation and delineated the trans dermal potential of developed gel formulation across rat skin in term of depth of penetration (>90µm, Figure 5). As previous finding suggested that rat skin showed structural resemblance (Table 6) and comparable skin permeation kinetics with human skin [50]. Hence the prominent efficient delivery of LER through optimized gel systems across rat skin as evident from CLSM study (Figure 5) demonstrated that the permeation enhancement in the depth of skin (>90µm) provide a good perspective for transdermal delivery.

3.9 Stability studies

The stability of formulation during storage is undoubtedly another important prerequisite for its successful clinical application. Stability facet is the major constraint in development as marketed preparation. During stability studies, pH, viscosity, drug content were determined at specified time interval as depicted in table 6. Although these parameters were slightly varied with respect to time but the changes in the observed parameters were not found to be statistically significant ($p > 0.05$) which indicated that optimized formulation were stable.

During accelerated stability study the degradation of LER was very slow at each temperature which indicated the chemical stability of LER in the developed gel formulation. The shelf-life of developed formulation was found to be 1.572 years at room temperature. These results indicated that both physical as well as chemical stability of LER can be enhanced in developed gel formulation.

4. CONCLUSION

Gel based system of LER with powerful permeation ability was investigated for effective transdermal delivery.The present study conclusively illustrated the use of Box–Behnken statistical design for predicting the Q24, flux, and lag time in optimization of gel formulations. The derived polynomial equations and contour plots aid in predicting the values of selected independent variables for optimum gel formulations with desired properties.

Skin permeation of drug which has to be delivered across skin is influenced by so many factors, among them are the release of the drug from the formulation, drug penetration into the stratum corneum and the drug diffusion into the skin layers up to the dermis to reach the systemic circulation has prime importance to improve the therapeutic efficacy of formulation.

Phospholipids (main constituent of lecithin) and propylene glycol favors the transdermal drug permeation in various extents. The result of

histopathological examination and CLSM study clearly demonstrated the clear evidence of penetration ability of optimized gel formulation in term of structural changes and depth of penetration across excised rat skin. Furthermore, rheological characterization showed a non-Newtonian, pseudoplastic and shear thinning system which is typical of hydrophilic polymer system and suitable for application over biological surface.

Stability study showed that changes in observed parameters were not observed to be statistically significant (P>0.05) which signified that optimized formulation was stable both physically and chemically, and the shelf life of developed formulation was calculated as 1.572 years at room temperature

DECLARATION OF INTEREST

The authors report no conflicts of interest

REFERENCES

[1] Robinson, J.R., Lee, H.L. 1987. Controlled Drug Delivery Fundamentals and Applications. 2nd Edition, Marcel Decker Inc., New York, 524-552.

[2] Joshi, A., Raje, J. 2002. Sonicated transdermal drug transport. Journal of Controlled Release, 83, (1), 13-22.

[3] Roberts, M.S., Cross, S.E., Pellett, M.A. 2002. Dermatological and Transdermal Formulations, Skin transport. In, K.A. Walters (Ed.), Marcel Dekker, New York, 97-216.

[4] Touitou, E., 2002. Drug delivery across the skin, Expert Opinion on Biological Therapy, 2, (7), 723-33.

[5] Hadgraft, J. 2003. Dermal and transdermal delivery, Modified Release Drug Delivery Technology. In, M.J. Rathbone, Journal, Hadgraft, M.S., Roberts (Ed.), Marcel Dekker Inc., New Yark, NY, 471– 480.

[6] Brown, M.B., Martin, G.P., Jones, S.A., Akomeah, F.K., 2006. Dermal and transdermal drug delivery systems: current and future prospects. Drug Delivery, 13, (3), 175–187.

[7] Trommer, H., Neubert, R.H. 2006. Overcoming the stratum corneum: the modulation of skin penetration. A review. Skin Pharmacology and Physiology, 19, (2), 106–121.

[8] Matthews, E.A., Dickenson, A.H. 2001. Effects of ethosuximide, a T- type Ca (2+) channel blocker, on dorsal horn neuronal responses in rats. European Journal of Pharmacology, 415, (2-3), 141-149.

[9] Gasser, R., Köppel, H., Klein, W. 1999. Lercanidipine, a new third generation Ca-antagonist in the treatment of hypertension. Journal of Clinical and Basic Cardiology, 2, (2), 169-174.

[10] Kallakunta, V.R., Bandari, S., Jukanti, R., Veerareddy, P.R. 2012. Oral self emulsifying powder of lercanidipine hydrochloride: Formulation and evaluation. Powder Technology, 221, 375–382.

[11] Barchielli, M., Dolfini, E., Farina, P., Leoni, B., Targa, G., Vinnacia V., Tajana, A. 1997. Clinical pharmacokinetics of lercanidipine. Journal of Cardiovascular Pharmacology, 29, S1–S15.

[12] Hamed, E., Sakr, A. 2001. Application of multiple response optimization technique to extended release formulations design. Journal of Controlled Release, 73, (2-3), 329–338

[13] Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., lia Escaleira L.A. 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, 76, 965–977.

[14] Heurtault, B., Saulnier, P., Pech, B., Venier-Julienne, M.C., Proust, J.E., Phan-Tan-Luu, R., Benoit, J.P. 2003. The influence of lipid nanocapsule composition on their size Distribution. European Journal of Pharmaceutical Sciences, 18, (1), 55–61.

[15] Huang, Y.B., Tsai, Y.H., Yang, W.C., Chang, J.S., Wu, P.C., Takayama, K., 2004. Once-daily propranolol extended-release tablet dosage form: Formulation design and in vitro/in vivo investigation. European Journal of Pharmaceutics and Biopharmaceutics, 58, (3), 607–614.

[16] Shah, R.B., Zidan, A.S., Funck, T., Tawakkul, M.A., Nguyenpho, A., Khan, M.A. 2007. Quality by design, Characterization of selfnano- emulsified drug delivery systems (SNEDDs) using ultrasonic resonator technology. International Journal of Pharmaceutics, 341, (1–2), 189–194.

[17] Zidan, A.S., Sammour, O.A., Hammad, M.A., Megrab, N.A., Habib J.M., Khan, M.A., 2007. Quality by design, Understanding the product variability of a self-nanoemulsified drug delivery system of cyclosporine A. Journal of Pharmaceutical Sciences, 96, (9), 2409–2423.

[18] Chaudhary, H., Kohli, K., Amin, S., Rathee, P., Kumar, V. 2011. Optimization and Formulation Design of Gels of Diclofenac and Curcumin for Transdermal Drug Delivery by Box-Behnken Statistical Design. Journal of Pharmaceutical Sciences, 100, (2), 580-593.

[19] Bachhav, Y.G., Patravale, V.B. 2009. Microemulsion based vaginal gel of fluconazole: formulation; in vitro and in vivo evaluation. International Journal of Pharmaceutics, 365, (1-2), 175-179.

[20] Azeem, A., Talegaonkar, S., Negi, L.M., Ahmad, F.J., Khar, R.K., Iqbal, Z. 2012. Oil based nanocarrier system for transdermal delivery of ropinirole, A mechanistic, pharmacokinetic and biochemical investigation. International Journal of Pharmaceutics, 422, (1-2), 436-444.

[21] Hayton, W.L., Chen, T. 1982. Correction of perfusate concentration for sample removal. Journal of Pharmaceutical Sciences, 71, (7), 820–821.

[22] Gannu, R., Palem, C.R., Yamsani, V.V., Yamsani, S.K., Yamsani, M.R. 2010. Enhanced bioavailability of lacidipine via microemulsion based transdermal gels, Formulation optimization, ex vivo and in vivo characterization. International Journal of Pharmaceutics, 388, (1-2), 231-241.

[23] Farina, P., Targa, G., Leoni, B., Tajana, A. 1997. Pharmacokinetics of lercanidipine in animals. Journol of cardiovascular pharmacology, 29, (1), 586-596.

[24] Govender, S., Pillay, V., Chetty, D.J., Essack, S.K., Dangor, C.M., Govender, T. 2005. Optimisation and characterisation of bioadhesive controlled release tetracycline microspheres. International Journal of Pharmaceutics, 306, (1-2), 24–40.

[25] Chopra, S., Pati, G.V., Motwani, S.K. 2007. Release modulating hydrophilic matrix systems of losartan potassium, optimisation of formulation using statistical experimental design. European Journal of Pharmaceutics and Biopharmaceutics, 66, (1), 73–82.

[26] Jafari, S.M., Beheshti, P., Assadpoor, E. 2012. Rheological behavior and stability of D-limonene emulsions made by a novel hydrocolloid (Angum gum) compared with Arabic gum. Journal of Food Engineering, 109, (1), 1–8.

[27] Touitou, E., Dayan, N., Bergelson, L., Godin, B., Eliaz, M. 2000. Ethosomes - novel vesicular carriers for enhanced delivery, characterization and skin penetration properties. Journal of Controlled Release, 65, (3), 403-418.

[28] Bentley, M., Marchetti, J.M., Nágila, R., Ali-Ziad, A., Collet, J.H., 1999. Influence of lecithin on some physical chemical properties of poloxamer gels, rheological, microscopic and in vitro permeation studies. International Journal of Pharmaceutics, 193, (1), 49-55.

[29] Larrucea, E., Arellano, A., Santoyo, S., Ygartua, P. 2001. Combined effect of oleic acid and propylene glycol on the percutaneous penetration of tenoxicam and its retention in the skin. European Journal of Pharmaceutics and Biopharmaceutics, 52, (2), 113-119.

[30] Squillante, E., Needham, T., Maniar, A., Kislalioglu, S., Zia, H. 1998. Codiffusion of propylene glycol and dimethyl isosorbide in hairless mouse skin. European Journal of Pharmaceutics and Biopharmaceutics, 46, (3), 265-71.

[31] Levang, A.K., Zhao, K., Singh, J. 1999. Effect of ethanol/propylene glycol on the in vitro percutaneous absorption of aspirin, biophysical changes and macroscopic barrier properties of the skin. International Journal of Pharmaceutics, 181, (2), 255–263.

[32] Levison, K.K., Takayama, K., Isowa, K., Okabe, K., Nagai, T. 1994. Formulation optimization of indomethacin gels containing a combination of three kinds of cyclic monoterpenes as percutaneous penetration enhancers. Journal of Pharmaceutical Sciences, 83, (9), 1367–1372.

[33] Cornwell, P.A., Barry, B.W., Stoddart, C.P., Bouwstra, J.A. 1994. Wide-angle X-ray diffraction of human stratum corneum, Effects of hydration

and terpene enhancer treatment. The Journal of Pharmacy and Pharmacology, 46, (12), 938–950.

[34] Krishnaiah, Y.S.R., Satyanarayana, V., Karthikeyan, R.S. 2002. Effect of the solvent system on the in vitro permeability of nicardipine hydrochloride through excised rat epidermis. Journal of Pharmaceutical Sciences, 5, (2), 123–130.

[35] Goodman, M., Barry, B.W. 1989. Action of penetration enhancers on human stratum corneum as assessed by differential scanning calorimetry, Percutaneous absorption, mechanisms, methodology, drug delivery, 567–593.

[36] Abd El-Bary, A., Tayel, S., Amin, S.Y., Osman, A. 1992. Bioavailability of salbutamol sulphate from different suppository formulations. Journal of Pharmaceutical Sciences, 33, 1031–1043.

[37] Ebdon, J.R., Jones, M.S. 1996. Flame retardants (Overview), Polymeric materials encyclopedia. In, Salomone JC (Ed.), Boca Raton, London, CRC Press, 2397–2411.

[38] Changez, M., Varshney, M., Chander, J., Dinda, A.K. 2006. Effect of the composition of lecithin/n propanol/isopropyl myristate/water microemulsions on barrier properties of mice skin for transdermal permeation of tetracaine hydrochloride, In vitro. Colloids and Surfaces B Biointerfaces., 50, (1), 18-25.

[39] Giannakou, S.A., Dallas, P.P., Rekkas, D.M., Choulis, H. 1995. Development and in vitro evaluation of nitrendipine transdermal formulations using experimental design techniques. International Journal of Pharmaceutics, 125, (1), 7–15.

[40] Agyralides, G.G., Dallas, P.P., Rekkas, D.M. 2004. Development and in vitro evaluation of furosemide transdermal formulations using experimental design techniques. International Journal of Pharmaceutics, 281, (1-2), 35–43.

[41] Li, W., Nadig, D., Rasmussen, H.T., Patel, K., Shah, T. 2005. Sample preparation optimization for assay of active pharmaceutical ingredients in a transdermal drug delivery system using experimental designs. Journal of Pharmaceutical and Biomedical Analysis, 37, (3), 493–498.

[42] Rhee, Y.S., Chang, S.Y., Park, C.W., Chi, S.C., Park, E.S. 2008. Optimization of ibuprofen gel formulations using experimental design technique for enhanced transdermal penetration. International Journal of Pharmaceutics, 364, (1), 14–20.

[43] Garg, A., Aggarwal, D., Garg, S., Singla, A.K., 2002. Spreading of semisolid formulations, An update Pharmaceutic Technology, 26, 84-105.

[44 Barnes, H.A., Walters, K. 1985. The yield stress myth?, Rheologica Acta, 24, (4), 323-326.

[45] Kieweg, S.L., Geonnotti, A.R., Katz, D.F. 2004. Gravity-induced coating flows of vaginal gel formulations: in vitro experimental analysis. Journal of Pharmaceutical Sciences, 93, (12), 2941-2952.

[46] Kieweg, S.L., Katz, D.F. 2007. Interpreting properties of microbicide drug delivery gels: analyzing deployment kinetics due to squeezing Journal of Pharmaceutical Sciences, 96, (4), 835-850.

[47] Barnes, H.A. 1997. Thixotropy-a review, Journal of Non-Newtonian Fluid Mechanics, 70, 1-33.

[48] Godin, B., Touitou, E. 2007. Transdermal skin delivery, Predictions for humans from in vivo, ex vivo and animal models. Advanced Drug Delivery Reviews, 59, (11), 1152-1161.

[49] Venuganti, V.V., Perumal, O.P. 2009. Nanosystems for dermal and transdermal drug delivery, drug delivery nanoparticles formulation and characterization. in Pathak Y. (Ed.), Informa Healthcare USA, 126-155.

[50] Saino, V., Monti, D., Burgalassi, S., Tampucci, S., Palma, S., Allemandi, D., Chetoni, P. 2010. Optimization of skin permeation and distribution of ibuprofen by using nanostructures (coagels) based on alkyl vitamin C derivatives. European Journal of Pharmaceutics and Biopharmaceutics, 76, (3), 443-449.