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Quality by Design Approach for Development of W/O Type Microemulsion Based Transdermal Systems for Atenolol

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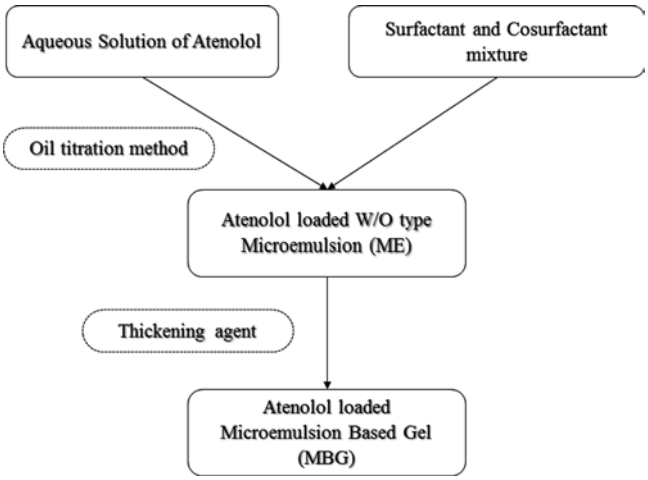
Abstract

The objective of the present investigation was to develop microemulsion (ME) based transdermal systems of highly water soluble drug, Atenolol by quality by design technique. Atenolol loaded W/O MEs were optimized using D-optimal design with concentrations of oil, surfactants mixture and water as independent variables which was converted into microemulsion based gel (MBG). The results of *in vitro* permeation of the optimized batch of Atenolol loaded MBG revealed significant increase in permeability parameters as compared to its convention gel. All these results suggested suitability of W/O type MEs as carriers for transdermal delivery of highly water soluble drug, Atenolol.



KEYWORDS: atenolol, D-optimal design, microemulsion, principal component analysis, quality by design, thickening agents, transdermal drug delivery

1. INTRODUCTION

Atenolol (AT) is a β adrenergic receptor blocking agent without sympathomimetic properties or membrane-stabilizing activity which is used for the treatment of hypertension and cardiac arrhythmia. AT endures extensive first-pass hepatic metabolism and has an absolute oral bioavailability of about 50–60%. Further, short biological half-life (2.8-7.4 h), low value of log P (0.23) and lower value of molecular weight (266 gm/mole) renders AT as an ideal candidate for the transdermal drug delivery systems (TDDS) in order to achieve its therapeutic levels alongwith patient compliance.[1–3] TDDSis a well-accepted route for systemic delivery of drugs but only a few drugs could be effectively delivered through this route due to barrier properties of stratum corneum (SC). To overcome this limitation, novel drug delivery systems like microemulsion, nanoparticles, dendrimers, carbon nanotubes and vesicular systems are currently being used by the researchers to facilitate drug transportation through the skin.[4,5] Microemulsions (MEs) are translucent and thermodynamically stable mixtures of oil, surfactant, cosurfactant and water with either the oil droplets are dispersed in water (O/W) or water droplet are dispersed in oils (W/O) with a droplet size typically in the range of 10-100 nm.[6,7] During the past years, MEs have received increasing attention because of the advantages including enhanced drug solubility, thermodynamic stability and increased drug permeation rate.[8] A number of mechanisms have been anticipated to

elucidate the advantages of MEs for TDDS.These included an increase in thermodynamics towards the skin with a large amount of a drug incorporated in the formulation which may favor drug partitioning into skin. Further, the ingredients of MEs are supposed to reduce the diffusional barrier of SC and increase the permeation rate of drug via skin. Aqueous phase titration method had been successfully explored for the preparation of O/W type MEs of many lipophilic drugs[9,10] but with respect to the hydrophilic nature of AT, oil phase titration method was employed for preparation of AT loaded W/O type MEs in the present investigation.[11–13]The Quality by Design (QbD) paradigm underlying pharmaceutical drug product development relies on multivariate data, both from formulation and the process in order to explain the multi-factorial relationship between formulation variables, process variables and drug product attributes.[14] Design of experiments (DoE), risk assessment, principal component analysis (PCA) and process analytical technology (PAT) are the major tools that can be used in QbD process as and when necessary.[15] The majority of scientists now routinely use DoE as a part of scientific approach in order to reduce costs and improve quality within timelines to obtain robust products and processes. In light of these, the aim of present investigation was to design and develop W/O type MEs for transdermal delivery AT using QbD approaches.

2. MATERIALS AND METHDS

2.1. Materials

AT was obtained as a gift sample from Torrent Research Center, Gandhinagar, India. The materials like; Capmul MCM, Capmul PG8, Captex 355, Acconon CC-6, Capmul GMO50, Capmul PGE 860, Caprol ET and Capmul MCM C8 were generously donated by Abitec Corporation, USA. Miglyol 812 and Imwitor 742 were kindly gifted from Sasol GmbH, Witten, Germany. Capryol 90, Labrafac CC, Labrafac Lipophile WL1349, Labrafil M 2125CS, Maisine 35-1 and Paceol were gifted from Gettefosse Saint-Priest Cedex, France. Sefsol 218 was obtained as a gift sample from Nikko Chemicals, Tokyo, Japan. Cremophor RH40, Gelucire 44/14, Lauroglycol 90 and Solutol HS 15 were donated from BASF Corporation, USA. Other Chemicals like Tween 20, Tween 40, Tween 60, Tween 80, Polyethylene Glycol (PEG) 400, Propylene Glycol (PG) and Sodium alginate were purchased from Himedia Labs, Mumbai, India whereas Span 40, Span 60, Span 80, Isopropyl alcohol (IPA), n-butanol, Ethanol, White wax, Xanthan gum and Cetosteryl alcohol were procured from SD Fine Chem, Mumbai, India. Isopropyl Myristate (IPM), Olive oil, Oleic acid, Castor oil, Magnesium stearate, Titanium dioxide, Zinc oxide and Colloidal silica were procured from Loba Chem, Mumbai, India. Double distilled water was used throughput the study.

2.2. Selection Of Microemulsion Components

2.2.1. Selection Of Oil (Solubility Studies)

The solubility of AT was measured in numerous oils and distilled water by shake flask method. An excess amount of drug was introduced into 2 mL of each oil and these mixtures were sealed in glass vials. Each of the samples was vortexed (GeNei, Bangalore,

India) for 5 min in order to facilitate initial mixing. Further, vials were charged on an environmental shaker bath (Tempo Instruments and Equipments Pvt. Ltd., Mumbai, India) for a period of 72 h at 37°C with 300 rpm speed. After an equilibrium for additional 72 h at 25°C temperature, each vial was centrifuged at 10000 rpm for 10 min using a centrifuge (Remi Laboratory Instruments, Mumbai, India). The supernant of each sample was filtered through a membrane filter (0.45 μm) to remove any undissolved drug if present. The amount of drug in all samples was determined by their subsequent dilution with pH 7.4 phosphate buffer using double beam UV Visible spectrophotometer (UV-1700, Shimadzu Corporation, Tokyo, Japan) against blank. The study was repeated in triplicate and their mean values were documented.[11–13]The solubility study was also conducted for selected surfactants and cosurfactants in order to evaluate their solubilization potential.

2.2.2. Selection Of Surfactant (Emulsification Study)

For each of drug, eight lipophilic nonionic surfactants (Acconon CC-6, Capmul GMO50, Caprol ET, Lauroglycol 90, Capmul MCM C8, Span 40, Span 60 and Span 80) were screened to evaluate their propensity for emulsification of aqueous phase. For each surfactant 10 mL of 10% w/v solution was prepared in preselected oil phase and subsequently distilled water was added to each of these solutions with an increment of 10 μL alongwith vortexing until the system becomes cloudy. The study was performed in triplicates and the average values of amount of water emulsified were documented.[16,17]

2.2.3. Selection Of Cosurfactant (Emulsification Study)

The optimized blend of surfactant was combined with five different cosurfactants, namely, PEG 400, PG, IPA, n-butanol and Ethanol at a fixed surfactant to cosurfactant ratio (S_{mix}) of 1:1. Different combinations of water and S_{mix} in weight ratios (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:3, 3:7, 3:2, 7:3, 4:1 and 9:1) were titrated by optimized oil phase in order to delineate all the boundaries of phases precisely formed. At the end of titration, the concentrations of each components were calculated and pseudo-ternary phase diagrams were constructed with apex representing oil, S_{mix} and water using Sigma Plot® software (Stat-Ease. Inc. Minneapolis, USA). The microemulsifying phase was identified as the region in phase diagram where clear and transparent formulations were obtained by visual observation. The cosurfactant with highest microemulsification area in pseudo-ternary phase diagram was optimized for all further trials.[16,17]

2.3. Selection Of Surfactant And Cosurfactant Ratio (K_m)

The K_m ratio was optimized by constructing pseudo-ternary phase diagrams for various ratios (1:3, 1:2, 1:1, 2:1, 3:1) by oil titration method as mentioned earlier for cosurfactant selection. The pseudo-ternary phase diagram with highest microemulsion area was selected as optimized ratio of K_m for all further trials.[16–18]

2.4. Preparation Of AT Loaded W/O Type Mes

AT loaded MEs were prepared by dissolving a fixed amount of AT (2.5% w/w) in distilled water with subsequent addition of optimized surfactant and cosurfactant (S_{mix}).

The resultant mixtures were continuous stirred for a period of 2 min on vortex mixer. The optimized amount of oil phase was further added slowly with continuous stirring using a high speed homogenizer (Remi Laboratory Instruments, Mumbai, India). All formulations were vortexed for 10 min on vortexer and sonicated for 5 min in an ultrasonicator bath (Frontline FS-4, Mumbai, India). The resultant MEs were sealed in a glass vial and stored at room temperature before further evaluations.[11–13]

2.5. Optimization Of AT Loaded Mes

The optimization of AT loaded MEs was conducted using DoE and PCA techniques as a part of QbD paradigm. On the basis of pseudo-ternary phase diagrams the levels of oil, surfactant cosurfactant and water were decided in terms of their maximum possibility of microemulsification. A three factor D - optimal mixture design was employed for systemic study of joint influence of the effect of independent variables [concentration of oil (X_1), concentration of surfactant mixture (surfactant and cosurfactant - X_2) and concentration of water (X_3)] on critical dependent variables. The design consisted total 16 runs (AT-ME-F1 to AT-ME-F16) (Table 1) and each of them was formulated in triplicates in order to estimate reproducibility of model [19,20]. Critical responses were identified amongst all restrained evaluation parameters by PCA using a trial version of Unscrambler® 10.2 (CAMO AS, Norway, Switzerland). The data of evaluation parameters for all batches of experimental design of AT loaded MEs were utilized to construct loading plot, scoring plot, agglomerative hierarchy cluster analysis (AHCA) plot, correlation loading plot and scree plot by PCA.[21–23] A second order quadratic

model incorporating interactive and polynomial terms was exercised to evaluate the responses.

$$Yi = b_1X_1+b_2X_2+b_3X_3+b_{12}X_{12}+b_{13}X_{13}+b_{23}X_{23}+ b_{123}X_1X_2X_3 (1)$$

where Yi was dependent variable, b_i was the expected coefficient for factor X_i. The main effects (X_1, X_2 and X_3) highlighted average result of altering one factor at a time from its lowest value to highest value. The interaction terms (X_{12}, X_{13} and X_{23}) depicted change in responses when two factors were simultaneously altered.[19,20] Data were further analyzed by Microsoft Excel® version 2010 (Microsoft corporation, Washington, USA) for regression analysis. Analysis of variance (ANOVA) study was executed to assure nonsignificant difference between developed full model and reduced model. Contour and response surface plots were generated to study response variations against independent variables using Sigma Plot® and Design Expert® softwares (Stat-Ease. Inc. Minneapolis, USA). Additionally the composition of optimized (check point) batch was derived by constructing overlay plots. The percentage relative error of each response was calculated using following equation in order to judge validity of the model.[24,25]

$$\% \text{ Relative Error} = \frac{(\text{Predicted value} - \text{Experimental value})}{\text{Predicted value}} \times 100 \quad (2)$$

2.6. Evaluation Parameters Of AT Loaded W/O Type Mes

2.6.1. Globule Size And Size Distribution

All the batches of AT loaded MEs were subjected to measurement of globule size and size distribution immediately after preparation. The sample was subjected to a brief period of sonication (15–30 sec) in order to minimize any aggregation if present. The

samples were analyzed by particle size analyzer (Zetatrac, U2552, New York, USA) at 25°C with an angle of 90°. The study was repeated in triplicates for confirmation of reproducibility.[11–13]

2.6.2. Zeta Potential (Z)

The zeta potential (ζ) of all the batches of AT loaded MEs was determined by the particle size analyzer. The analysis was performed with purified water adjusted to a standardized conductivity of 50 μS/cm with sodium chloride solution in order to avoid changes in ζ values due to day-to-day variations occurring in the conductivity of water. The mean values of ζ for three independent samples were documented.[26,27]

2.6.3. Refractive Index (Ri)

The RI values of all the batches of AT loaded MEs were determined using refractometer (Bausch and Lomb Optical Company, Rochester, NY, USA). One drop of the sample was placed on the sample holder and the values of RI were recorded in triplicates against distilled water as blank.[26,27]

2.6.4. Percentage Transmittance (%T)

The percent transmittance of all the batches of AT loaded MEs was measured by subjecting each sample to UV spectrophotometer at 650 nm using distilled water as a blank.[11–13]

2.6.5. Percentage Drug Content (% Dc)

All the experimental design batches of AT loaded MEs were subjected to assay analysis in order to determine their percentage drug content. Accurately weighed samples were dissolved individually in 10 mL of methanol and stirred by vortex mixer for a period of 10 min. Each of the solutions was filtered, using membrane filter (0.45 µm) and the drug content of each filtrate was estimated spectrophotometrically against blank using a Double beam UV Visible Spectrophotometer. The study was repeated for three independent samples in order to confirm reproducibility of the results.[11–13]

2.6.6. Viscosity

The viscosity of all the batches of AT loaded MEs was determined by using rheometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) with S62 spindle and 25°C temperature in triplicates.[26,27]

2.6.7. In Vitro Permeation Study

2.6.7.1. Preparation of skin.

The *in vitro* skin permeation study was carried out under the guideline compiled by Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA, Ministry of Culture, Government of India) and all the study protocols were approved by the Local Institutional Animal Ethics Committee of Atmiya Institute of Pharmacy, Rajkot, Gujarat, India (CPCSEA No.:1004/9O/Q/06//CPCSEA/PG-1005, Dated: 10/01/2012). The abdominal skin of goat was obtained from local slaughter house

within 1 h of scarification in order to analyze *in vitro* permeation of developed MEs. After hair was shaved carefully with an electric clipper, the skin was subjected for removal of subcutaneous fat and other extraneous tissues without damaging the epidermal surface. The excised skins were washed and examined for integrity, and then stored at 4°C for 24 h in phosphate buffer pH 7.4 before to be used for permeation experiments.[28–31]

2.6.7.2. Permeation Study

Goat abdominal skin was mounted with the SC facing opposite to the receptor compartment on the Franz diffusion cell (Orchid scientific, Nasik, India) containing a diffusion area of 1.77 cm^2. The receptor compartment was filled with 16 mL of pH 7.4 phosphate buffer and the content was magnetically stirred at 300 rpm to prevent stagnant layer formation. The temperature of the system was maintained at 32°C. The donor compartment was filled with 1 mL of AT loaded MEs to achieve desired drug concentration at the site. Aliquots of 0.5 mL was withdrawn at predetermined intervals (0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h) from receptor medium and replaced immediately with an equal volume of receptor solution to maintain the volume constant. The amount of drug permeated across abdominal skin was measured after suitable dilution using UV visible spectrophotometer against blank. In order to estimate extent of enhancement by ME formulations, an aqueous solution of AT with same concentration was also subjected to *in vitro* permeation under the same circumstances. The study was repeated in triplicates and the average values were used for the calculation.[28–31]

2.6.7.3. Data Analysis

Average cumulative amount of drug permeated per unit surface area of the skin was plotted against time. The steady state flux (J_{ss}) was calculated from the slope of the linear portion of the plot and expressed as:

$$J_{ss} = dM / dt \quad (3)$$

$$Kp = \frac{Jss}{Cdonor} \quad (4)$$

$$ER = \text{Flux from microemulsion formulation / Flux from drug solution} \quad (5)$$

where, M was the cumulative amount of AT permeated (mg) through skin per unit area (cm^2) within experimental time t (h), J_{ss} was the flux and C_{donor} was the total amount of AT in donor compartment.[28–31]Other parameters such as, permeability coefficient (K_p) and enhancement ratio (ER) were also calculated according to the equations illustrated.Further, in order to quantify the drug concentration in the skin (drug retained in skin) after permeation study, donor solutions were removed and the skin was washed twice with distilled water before unclamping from diffusion cells. The skin pieces were transferred in 10 mL of ethanol (95% v/v) and subsequently mixed with a mechanical stirrer. After 2 h stirring, the contents were centrifuged at 5000 rpm for 15 min in a centrifuge. The supernants were analyzed for amount of drug present after adequate dilution with methanol using UV visible spectrophotometer against blank.[32]

2.6.8. Thermodynamic Stability

The optimized batch of AT loaded ME was subjected to different thermodynamic stability tests in order to assess their physical stability. All samples were evaluated in terms of phase separation at the end of analysis.[11–13] Six cycles between refrigerator temperature (2 - 8°C) and 45°C with storage at each temperature not less than 48 h were conducted. Additionally, optimized formulation was centrifuged at 10000 rpm for a period of 10 min using a centrifuge.

2.6.9. Electrical Conductivity

Electrical conductivity of the optimized batch of AT loaded MEwas measured with conductometer (CM 180, Elico, Hyderabad, India) by inserting the probe in 10 mL of prepared sample in a beaker. The study was repeated thrice and their average values were documented.[11–13]

2.6.10. Ph

The pH of the optimized batch of AT loaded MEwas measured by digital pH meter (Systronics, Mumbai, India)which was previously standardized using pH 4.0 and 7.0 standard buffers. The study was repeated in triplicates and the average values were summarized.[11–13]

2.6.11. Cloud Point

The cloud point of the optimized batch of AT loaded ME was measured in a temperature controlled water bath by visual inspection of opacity developed in the ME with increase in temperature.[18]

2.6.12. Specific Gravity

The specific gravity of optimized batch of AT loaded ME was measured with specific gravity bottle. Accurately measured, 25 mL of drug loaded ME was transferred into a specific gravity bottle at 25 ± 1°C and calculated for its density. The experiment was repeated thrice to estimate reproducibility of results.[11–13]

2.6.13. Transmission Electron Microscopy (Tem)

The optimized batch of AT loaded ME was subjected to transmission electron microscope (H-7000, Hitachi, Ibaraki, Japan) in order to estimate globule morphology. Briefly, each of drug loaded ME was plunged for 10 – 15 min on a coated carbon grid stained with 2% uranyl acetate solution. The samples were subsequently washed with fresh distilled water before analysis. Radiation generated at 200 kV was utilized as X-Ray source with camera length of 100 cm. Two dimensions of X-Ray patterns were photographed for each sample studied.[11,26]

2.7. Preparation Of AT Loaded Microemulsion Based Gels (Mbgs)

An amount of drug representing 2.5% w/w for AT loaded ME was added to distilled water, consisting of the optimized quantities of surfactant and cosurfactant. This aqueous

part of optimized ME was vortexed for 10 min on vortexer and sonicated in an ultrasonicator bath until the drug was completely dissolved. Pre-optimized amount of thickening agent was dispersed in optimized amounts of selected oil phase by using high speed homogenizer at 1,000 rpm. This dispersion was kept in dark for 24 h for complete swelling of selected thickening agent. The previously prepared aqueous part loaded with AT was added slowly to this non-aqueous dispersion under magnetic stirring. All the batches of MBGs were allowed to stand for 24 h for complete gelation with subsequent sealing in glass vials and storage at room temperature until further evaluations.[33,34]

2.8. Selection Of Thickening Agent

Various thickening agents, namely, white wax, cetostearyl alcohol, magnesium stearate, titanium dioxide, zinc oxide and colloidal silica were evaluated for their ability to thicken optimized batch of AT loaded ME. The optimized thickening agent was further evaluated for the effect of its concentrations.[35,36]

2.9. Preparation Of Conventional Gel

Conventional gel of AT was prepared by adding similar amount of drug (2.5% w/w for AT) to the previously soaked oil dispersion of thickening agent containing optimized amount of preselected thickening agent.[35,36]

2.10. Evaluation Parameters Of AT Loaded W/O Type Mbgs

2.10.1. Appearance

All the batches of AT loaded MBGs were evaluated visually for their color, homogeneity, consistency and phase separation.[34–36]

2.10.2. Globule Size And Size Distribution

The optimized batchof AT loaded MBG was diluted (100 times) in respective optimized oil phase and endangered to measurement of globule size and size distribution immediately after preparation. The sample was subjected to a brief period of sonication (15–30 sec) in order to minimize any aggregation if present. The samples were analyzed by particle size analyzer at 25°C with an angle of 90°. The study was repeated in triplicates for confirmation of reproducibility.[34–36]

2.10.3. Zeta Potential (Z)

The zeta potential (ζ) of optimized batch of AT loaded MBG was determined by the particle size analyzer after their dilution (100 times) with optimized oil phase. The analysis was performed similar to as with MEs. The mean values of ζ for three independent samples were documented.[34–36]

2.10.4. Ph

The pH of the optimized batch of AT loaded MBG was measured by digital pH meter. Each of samples was subjected to 10 times dilution by optimized oil phase before analysis. The study was repeated in triplicates and the average values were summarized.[34,35]

2.10.5. Viscosity

The viscosity of the optimized batch of AT loaded MBG was determined by using rheometer with S61 spindle and 25°C temperature in triplicates.[34,35]

2.10.6. Spreadability

The measurement of spreadability of the optimized batch of MBG was done by placing the formulation (0.5 gm) within a circle of 1-cm diameter premarked on a glass plate. Over which a second glass plate was placed and a weight of 500 gm was allowed to rest on the upper glass plate for 5 min. The increase in the diameter due to spreading of the gel was noted.[34]For comparison purpose spreadability of drug loaded conventional gel was also performed by the similar method.

2.10.7. Percentage Drug Content (% Dc)

Accurately weighed amount (1 gm) of the optimized batch of AT loaded MBG was transferred in a 100 mL volumetric flask and the volume was made up to the mark with methanol. The formulations were vortexed for 10 min on vortexer and sonicated in an ultrasonicator bath until the drug was completely dissolved. The solution was filtered, using membrane filter (0.45 μm) and the drug content of filtrate was estimated spectrophotometrically against blank using double beam UV visible spectrophotometer. The study was repeated for three independent samples in order to confirm reproducibility of the results.[34]

2.10.8. In Vitro Permeation Study

The *in vitro* skin permeation study of AT loaded MBGs was carried out under the guideline compiled by CPCSEA, Ministry of Culture, Government of India and all the study protocols were approved by the Local Institutional Animal Ethics Committee of Atmiya Institute of Pharmacy, Rajkot, Gujarat, India (CPCSEA No.:1004/9O/Q/06//CPCSEA/PG-1005, Dated: 10/01/2012). Goat abdominal skin was mounted with the SC facing opposite to the receptor compartment on the Franz diffusion cell containing a diffusion area of 1.77 cm^2. The receptor compartment was filled with 16 mL of pH 7.4 phosphate buffer and the content was magnetically stirred at 300 rpm to prevent stagnant layer formation. The temperature of the system was maintained at 32°C. The donor compartment was filled with 1 gm of optimized batch of AT loaded MBGto achieve desired drug concentration at the site. An aliquots of 0.5 mL was withdrawn at predetermined intervals from receptor medium and replaced immediately with an equal volume of receptor solution to maintain the volume constant. The amount of AT permeated across goat abdominal skin was measured after suitable dilution using UV visible spectrophotometer against blank. In order to estimate extent of enhancement by MBG formulations, a conventional gel of AT with same concentration was subjected to *in vitro* permeation under the same circumstances. The study was repeated in triplicates and the average value were used for the calculation.[33–36]

2.10.9. Skin Irritation Study

Twelve different wister rats were randomly divided into two groups as test and reference for skin irritation study of optimized batch of AT loaded MBGformulation. The dorsal surface of each of rat was cleaned and the hairs were removed by shaving using an electrical clipper. The optimized batch of each AT loaded MBG was applied to the dorsal surface of one group of rats as test and 0.8% v/v aqueous solution of formaldehyde was applied to another group of rats as reference. Each of rat was evaluated for for any sign of erythema or edema over a period of 48 h according to Draize scoring method[37]. A 5-point scale was used for scoring as; no erythema/edema = 0, very slight erythema/edema = 1, slight erythema/edema = 2, moderate erythema/edema = 3, severe erythema/edema = 4. The primary dermal irritation index (PDII) was calculated by adding the average erythema and edema scores for the 1 h, 12 h, 24 h and 48 h scoring intervals and dividing by the number of evaluation intervals. The test sample was considered as non-irritating, slightly irritating, moderately irritating and severely irritating based on the values of PDII.[13,38]

2.10.10. Stability Study

The optimized batch of AT loaded MBG was subjected to stability study as per International Conference on Harmonization (ICH) guidelines. The sample was filled in a 10 gm collapsible aluminum tubes and stored at 40 ± 2°C/75 ± 5% RH over a period of 6 months in a stability chamber (Remi Electrotechnik Ltd. Mumbai, India). At predetermined time intervals samples were evaluated statistically.[33,35,39]

3. RESULTS AND DISCUSSION

3.1. Selection Of Microemulsion Components

3.1.1. Screening Of Oil

Development of MEs formulation depends on physicochemical properties of drugs. Lipophilic drugs are encapsulated in oil phase of O/W MEs, whereas hydrophilic drugs are encapsulated in aqueous phase of W/O MEs. Solubility of the lipophilic drugs in the oil phase and hydrophilic drugs in aqueous phase is an important criterion for the selection of ME components.[6,26] Because AT is a hydrophilic drug, its solubility in water is more important than oil phase. The solubility of AT in different oils as well as in distilled water were determined and it was found to be highest in Sefsol 218 (121.92 mg/mL) as compared to other oils (Table 2). The data illustrated high solubilization capacity of novel synthetic oils as compared to edible oils. Therefore Sefsol 218 was selected as the optimized oil phase for the development of W/O type ME formulation of AT. The solubility of AT in distilled water was found to be 144.79 mg/mL which was higher compared to selected oil phase. To observe the part of surfactants and cosurfactant in drug solubilization the solubility studies were accompanied in different surfactants and cosurfactants for AT. Highest solubility for AT was observed with Capmul GMO50 (200.65 mg/mL) from surfactant category (Table 2) whereas from numerous cosurfactants selected, ethanol exhibited highest solubility (356.43 mg/mL) for AT (Table 2). However, the selection of surfactant and cosurfactant for MEs was not done on the basis of solubility studies since it was strongly believed that both of them play a crucial role in emulsification. Good solubility of drugs in surfactant and cosurfactant was

considered as an additional advantage as this feature may prevent drug precipitation during storage.[38,39]

3.1.2. Screening Of Surfactant

The surfactant selected must be able to lower the interfacial tension to a very small value and should be of the appropriate lipophilic character to provide the correct curvature at the interfacial region of W/O type MEs.[6–8]In addition to this, the selected surfactant should be pharmaceutically acceptable and nonirritant to the skin. In order to prepare W/O type MEs lipophilic surfactants with low HLB (less than 8) are required. In the present study, nonionic surfactants were selected since they are known to be less affected by pH change, generally regarded as safe and are biocompatible.[16,17] The surfactant screening was done on the basis of their emulsification potential which was measured in terms of amount of water emulsified by each surfactant. The results of emulsification study by lipophilic surfactant depicted highest emulsification potential of Span 80 than all other lipophilic surfactants. Thus, Span 80 was selected as surfactants for further optimization of AT loaded MEs. The results of emulsification studies of surfactants; Caprol GMO 50 and Capmul MCM C8 demonstrated poor emulsification potential, even though they had very good solubility for AT. Hence, it has been resolved that it was not necessary that surfactants with good drug solubility also provides good emulsification. The superior performance of Span 80 might be due to their higher affinity for the aqueous phase.[38,39]

3.1.3. Screening Of Cosurfactant

Cosurfactants provide a flexible film that can readily deform around droplets of MEs and be of the appropriate character to provide the correct curvature at the interfacial region. It also penetrates into surfactant monolayer providing additional fluidity to interfacial film and thus disrupting the liquid crystalline phases.[38,39] Pseudo-ternary phase diagrams of different cosurfactants are illustrated in Figure 1. The results depicted highest microemulsification region with ethanol as cosurfactant. Further, it was noteworthy that pseudo-ternary phase diagram with ethanol as cosurfactant covered 25-60% w/w of oil, 30-55% w/w of S_{mix} and 5-10% w/w of water for AT loaded MEs. Moreover, as depicted in the result of solubility studies for cosurfactant, AT revealed excellent solubility in ethanol which was considered to be an added advantage in terms of providing higher drug loading to the final formulations. Hence, for all further trials, ethanol was selected as cosurfactant.

3.2. Optimization Of Surfactant And Cosurfactant Ratio (K_m)

The surfactant to cosurfactant ratio plays a crucial role in the characteristics of MEs and hence it was optimized by constructing pseudo-ternary phase diagrams. Pseudo-ternary phase diagrams of optimized surfactant (Span 80) and cosurfactant (ethanol) blend with different ratios (K_m) (3:1, 2:1, 1:1, 1:2, 1:3) are shown in Figure 2. These S_{mix} ratios were chosen to reflect increasing concentrations of cosurfactant with respect to surfactant and increasing concentrations of surfactant with respect to cosurfactant for detailed study of the phase diagrams. The data depicted decrease in microemulsification region with

increase in cosurfactant concentrations with respect to surfactant and hence the study was limited to 1:3 K_m ratio.[38,39] Similarly there was a significant increase in the microemulsification region with increase in the amount of surfactant upto 2:1 K_m ratio. Moreover, ethanol at higher concentration has been reported to possess skin irritation effect and hence 2:1 K_m ratio was selected as optimized for all further trials. The optimized phase diagram of AT loaded MEs had largest microemulsification region with oil concentration 25-55% w/w, S_{mix} concentration 40-70% w/w and water concentration 5-15% w/w.

3.3. Optimization Of AT Loaded Microemulsion

The levels of experimental design could not be chosen arbitrarily, where the composition is a factor of interest, because the sum of all the fractions of components equals to unity. Classical experimental designs do not consider specific experimental constraints, and thus they will not have the better prediction power. For instance, the possible experimental runs are displayed by an equilateral triangle in a three component mixture design, where the real value of the responses could be then represented as distance orthogonal to factor space. Moreover, the range covering the components is limited in the design space, which could be represented by irregular polyhedron delimited by extreme vertices. In such cases, D - optimal design would be appropriate, as maximum prediction power could be obtained in selected set of experimental runs, minimizing the variance associated with the estimates of coefficients in the model. To simplify the calculations, the actual levels of oil, S_{mix} and water were transformed on the basis of the D-optimal

design to the simpler levels such that the maximum level corresponds to one while minimum level corresponds to zero.[19,20] The actual values of each of selected factor has been summarized against their respective coded values in Table 1. The results of responses like globule size, polydispersibility index (PI), zeta potential (ζ), refractive index (RI), percentage transmittance (%T), percentage drug content (%DC), viscosity (□), cumulative amount of drug permeated after 24 h (Q_{24}), flux (J_{ss}), lag time (t_L), enhancement ratio (ER), permeability coefficient (K_p) and drug retained in skin (DRS) for all experimental design batches of AT loaded MEs have been summarized in Table 3.

As depicted in Figure 3(A), first principal component (PC1) was responsible for 63% of the total variance in the data set and second principal component (PC2) was responsible for a further 26%.[40] The results of all 16 batches were further treated with agglomerative hierarchy cluster analysis (AHCA).[41,42] A graphical display of the result of AHCA is shown in Figure 3(B) as dendrogram. The results demonstrated clustering of all formulations into five major groups; group I (F12, F15, F16 and F4), group II (F10, F9, F7 and F11), group III (F13 and F2), group IV (F14 and F5) and group V (F3, F8, F16 and F1). Further, all the five groups were found to be relatively distant and substantially different from one another. Clusters of formulations were correlated by PCA score plot similarly (Figure 3(C)).Correlation loading plot was constructed to decide most important variables for further optimization. The results scrutinized globule size, Q_{24}, J_{ss} and t_L as four critical responses on the basis of their retention between two eclipses (Figure 3(D)). Further, globule size - t_L and Q_{24} – J_{ss} were plotted on the same side of PC1 which

suggested positive correlation between them. Similarly, t_L – Q_{24}, globule size – J_{ss}, globule size – Q_{24} and t_L – J_{ss} were plotted on opposite side of PC1 which suggested negative correlation between them. These results implies that if the globule size of ME was increased, the t_L would increase whereas Q_{24} and J_{ss} would decrease. Moreover, all other variables were plotted on correlation loading plot near to origin and hence, they were not discussed further. The scree plot for AT loaded MEs (Figure 3(E)) illustrates that the eigenvalues for each component were in descending order. There was one large gap/break in the data between components 1 and 2 and then the eigenvalues begins to flatten out with component 3 which indicated significance of first two components (PC1 and PC2). All other components (PC3 to PC7) which appeared after the break were assumed to be trivial and hence removed from the study. This separation was further supported by the calculation of %CV for all components. The data for %CV of PC3 to PC7 accounted for almost 100% variation which justified removal of these terms. At the end, it was speculated that globule size, cumulative amount of drug permeated in 24 h (Q_{24}), flux (J_{ss}) and lag time (t_L) were most important variables in the preparation of AT loaded MEs and hence, they were further selected for the optimization. For all 16 batches dependent variables, globule size (Y_1), cumulative amount of drug permeated in 24 h (Q_{24} – Y_2), flux (J_{ss} – Y_3) and lag time (t_L – Y_4) exhibited wide variations from 41.14 to 78.35 nm, 2036.27 to 3324.15 μg/cm^2, 97.36 to 158.25 μg/cm^{2}h and 0.30 to 0.58 h, respectively (Table 3). The data clearly indicated strong influence of selected factors (X_1, X_2 and X_3) on all four responses (Y_1, Y_2, Y_3 and Y_4) (Table 3). A stepwise multivariate linear regression was performed to evaluate the observations. The fitted polynomial

equations (full and reduced model) relating the responses to the transformed factors are shown in Table 4. The polynomial equations could be used to draw conclusions after considering the magnitude of coefficients and their mathematical sign. For all four responses [globule size (Y_1), Q_{24} (Y_2), J_{ss} (Y_3) and lag time (Y_4)] coefficients b_{12} and b_{123} were found to be insignificant ($P > 0.05$) and hence, they were separated from full model to develop a reduced model.[43,44] The removal of insignificant terms was further justified by executing ANOVA test (Table 5). The high value of correlation coefficients for globule size (Y_1), Q_{24} (Y_2), J_{ss} (Y_3) and lag time (Y_4) illustrated goodness of fit. The critical values of F for Y_1, Y_2, Y_3 and Y_4 were found to be 4.26 (df = 2, 9). For all four responses, calculated F values [1.88 (Y_1), 1.18 (Y_2), 0.20 (Y_3) and 1.01 (Y_4)] were less than their respective critical values which supported nonsignificant difference between full and reduced models.[43,44] The data of all the 16 batches of experimental design were used to generate interpolated values with the assistance of response surface and contour plots.

3.3.1. Influence Of Formulation Factors On Globule Size (Y_1)

For a ME based systems globule size will always be one of the critical parameter. The decrease in size of MEs will results into decrease in diffusional distance which ultimately leads to increase in skin penetration.[6,7] A lowest value of globule size (41.14 nm) was observed with batch AT-ME-F15(Table 3). Moreover, response surface and contour plots (Figure 4) for Y_1 also illustrated strong influence of all three factors (oil, S_{mix} and water) studied. The data of regression analysis revealed negative values for coefficients b_1, b_2

and b_3 which indicated that globule size was decreased with increasing oil, S_{mix} and/or water concentration. This might be attributed solubilization potential of selected oil, surfactant, cosurfactant and water for AT.

3.3.2. Influence Of Formulation Factors On Q_{24} (Y_2)

The major aim of the present investigation was to improve therapeutic efficacy of AT by enhancing its skin permeation. The higher aqueous solubility of AT would be opposed by hydrophobic layer of skin.[10–13] A highest value of Q_{24} (3324.15 µg/cm^2) was observed with batch AT-ME-F15(Table 3). Moreover, response surface and contour plots (Figure 5) for Y_2 also illustrated strong influence of all three factors (oil, S_{mix} and water) studied. The data of regression analysis revealed positive value for coefficients b_1, b_2 and b_3 which indicated that Q_{24} was increased with increasing oil, S_{mix} and/or water concentration. This might be attributed to reduction in globule size of MEs with increase in amount of water, membrane disturbing potential of surfactant (Span 80) and cosurfactant (ethanol) alongwith enhanced lipophilicity of the water soluble drug by lipid phase.[6,7]

3.3.3. Influence Of Formulation Composition Factors On J_{ss} (Y_3)

The flux (J_{ss}) of all experimental design batches was strongly influenced by all three independent variables (oil, S_{mix} and water) with a highest value of 158.25 µg/cm^{2}h for batch AT-ME-F4 (Table 3). In addition to these, response surface and contour plots (Figure 6) for Y_3 also exemplified strong influence of all three variables studied. The data of regression analysis revealed positive value of b_1, b_2 and b_3 coefficients which indicated

that J_{ss} was increased with increasing oil, S_{mix} and/or water concentration similar to as discussed earlier for Q_{24}.[6,7]

3.3.4. Influence Of Formulation Factors On T_l (Y_4)

Lag time (t_L) was highly influenced by all three independent variables studied with lowest t_L value of 0.30 h for batch AT-ME-F4 (Table 3). Moreover, response surface and contour plots (Figure 7) for Y_4 also illustrated strong influence of three factors (oil, S_{mix} and water) analyzed. The data of regression analysis revealed negative value of b_1, b_2 and b_3 coefficients which indicated that t_L was decreased with increasing concentration of oil, S_{mix} and/or water. This might be attributed to reduction in globule size with increase in concentration of water, alteration of diffusivity with increase in surfactant and cosurfactant concentrations alongwith enhanced lipophilicity of formulation with an increase in oil concentration.[6,7]

3.3.5. Model Validation And Selection Of Optimized Batch

The criterias for selection of optimum formulation of AT loaded ME was arbitrarily selected as lowest values of globule size and lag time with maximum value of Q_{24} and J_{ss}. On the basis of these criteria the check point/optimized batch of AT loaded ME was constructed practically according to the levels of factors illustrated in Table 6. The predicted as well as observed values of all four responses for optimized batch of AT loaded ME have been summarized in Table 6. The results depicted nonsignificant ($P>0.05$) difference and lower % relative error between experimentally obtained and

theoretically computed data of all four responses (globule size, Q_{24}, J_{ss} and t_L) which suggested suitability of design applied.[43,44]

3.4. Evaluation Parameters AT Loaded W/O Type Mes

3.4.1. Globule Size And Size Distribution

Globule size of AT loaded MEs for all the batches of experimental design was probed as one of the crucial response in course of their optimization based on PCA studies. The values of all the batches of experimental design have been summarized in Table 3. The globule size of the optimized batch of AT loaded ME was found to be 46 nm which confirmed nanometer size of developed formulation. The estimation of globule size distribution for MEs was done in terms of polydispersibility index (PI)[29] and their values are exemplified in Table 3. The PI of the optimized batch of AT loaded ME was found to be 0.17 which illustrated narrow size distribution of developed formulations.[6,7]

3.4.2. Zeta Potential (Z)

Zeta values of all experimental design batches of AT loaded MEs are summarized in Table 3. The negative values of ζ for all batches were solely attributed to the presence of free fatty acids in the oil phase of MEs since both surfactant and cosurfactant used were nonionic in nature. The ζ value of the optimized batch of AT loaded ME was found to be - 36.79 mV. All the values of zeta potential were higher than □30 mV□ which supported stability of dispersed systems.[26,27]

3.4.3. Refractive Index (Ri)

The results of RI for all batches of experimental design for AT loaded MEs (Table 3) confirmed isotropic nature of the systems.[26,27] The RI values of all formulations were in the range of 1.4 – 1.6. The RI value of the optimized batch of AT loaded ME was found to be 1.47.

3.4.4. Percentage Transmittance (%T)

In order to characterize isotropic nature of MEs, transmittance study was conducted and the results of %T for all batches have been summarized in Table 3.[6,7] The %T value of the optimized batch of AT loaded ME was found to be 99.94.

3.4.5. Percentage Drug Content

The percentage drug contents of all batches of experimental design are summarized in Table 3. The values of % drug content were almost 100% alongwith very low standard deviations, suggested uniform dispersion of drug in developed formulations.[26,27] The value of % drug content for the optimized batch of AT loaded ME was found to be 99.56.

3.4.6. Viscosity

The viscosity of the optimized batch of AT loaded ME was found to be 34.26 cps at 25°C.

3.4.7. In Vitro Permeation

The effect of formulation components (oil, S_{mix} and water) on skin permeation of all experimental batches were studied by *in vitro* permeation study using goat abdominal skin. The results of *in vitro* skin permeation data for all experimental design batches exhibited significant enhancement in *in vitro* permeation by MEs compared to its aqueous solution (control) ($P<0.05$). All permeability parameters (Q_{24}, J_{ss}, K_p and ER) were significantly increased with ME as compared to its aqueous solution ($P<0.05$). These could be due to the difference in mean size of internal phase droplets of ME as compared to itsaqueous formulation. Moreover, different mechanisms have been proposed to explain the enhanced transdermal delivery of drugs using MEs which includes; increased thermodynamic activity of the drug due to increase in its solubilization, through the action of MEs ingredients (surfactants and cosurfactants) as permeation enhancers and increased skin lipophilicity by presence of oil. All these possibilities were supported in our results as values of Q_{24} and J_{ss} were increased with increasing oil, surfactant, cosurfactant and water contents.[28–32] The optimized batch of AT loaded ME also exhibited significant enhancement ($P<0.05$) in all studied permeability parameters as compared to aqueous solution of drug . The diffusion pattern of the optimized batch of AT loaded MEs depicted 368.60 μg/cm^2 of AT permeated with respect to only 35.58 μg/cm^2 for pure AT within 1 h. The significantly higher values of Q_{24}, J_{ss}, K_p and lower values of t_L for the optimized batch of drug loaded ME as compared to pure drug suggested marked improvement in diffusion rate AT by the developed micro sized formulations. Moreover, parameters like difference factor (f_1) and similarity factor (f_2) confirmed nonsimilarity of diffusion profiles of AT loaded ME and pure drug as f_1 value

was higher than 15 and f_2 value was lower than 50.[45]The increase in diffusion velocity of drug loaded MEs could be attributed to reduction in globule size and decrease in diffusion distance.[6,7]

3.4.8. Thermodynamic Stability

The objective of thermodynamic stability study revealed excellent stability of optimized batch of AT loaded ME with no signs of phase separation or precipitation at various stress conditions studied.[6,7]

3.4.9. Electrical Conductivity

The value of conductivity was found to be 14.16 μs/cm for AT loaded ME which proposed W/O type microemulsions formation.[11–13]

3.4.10. Ph

The value of pH of the optimized batch of AT loaded ME was found to be 6.69 which was within the acceptable limits for transdermal formulation and non-irritant to skin.[11–13]

3.4.11. Cloud Point

The cloud point of the optimized batch of AT loaded ME was found to be 58.58°C, which revealed stability of systems at physiological temperature *in vivo*.[18]

3.4.12. Specific Gravity

The specific gravity of optimized batch of AT loaded ME was found to be 0.98 gm/mL.[11]

3.4.13. Transmission Electron Microscopy (Tem)

TEM images illustrated formation of spherical micelles with size range of 10 - 100 nm (Figure 8). These results were in accordance to that of globule size analysis.[11,26]

3.5. Selection Of Thickening Agent

Various gelling agents such as white wax, cetostearyl alcohol, magnesium stearate, titanium dioxide, zinc oxide and colloidal silica were evaluated for the gelling of optimized batch of drug loaded MEs.[33,34] It was observed that white wax and cetosteryl alcohol affected the structure of the ME and resulted in macroemulsion formation. Titanium dioxide, zinc oxide and magnesium stearate were unable to yield desirable viscosity for the gel formulation and had resulted into sedimentation. Only colloidal silica could yield gel without disturbing the microstructure of the optimized batch of ME. The selected thickening agent was further evaluated for the effect of its concentration on optimized batch of drug loaded ME. The results revealed that only 5% w/v of colloidal silica concentration provided suitable viscosity to AT loaded MBG formulations.

3.6. Evaluation Parameters Of AT Loaded W/O Type Mbgs

3.6.1. Appearance

The optimized batch of AT loaded MBG was almost transparent, homogeneous, consistent alongwith no signs of phase separation for 24 h.[33,34]

3.6.2. Globule Size And Size Distribution

The results of globule size and its distribution of the optimized batch of AT loaded MBG have been summarized in Table 7. The data illustrated no significant difference ($P<0.05$) in the values of globule size and PI which conformed that inspite of conversation of liquid formulation (ME) into a semisolid form (MBG) the globule size and its distribution remains same.[33,34]

3.6.3. Zeta Potential (Z)

The results of zeta potential of the optimized batch of drug loaded MBG have been summarized in Table 7. The data illustrated no significant difference ($P<0.05$) in the values of ζ which conformed stability of final formulation inspite of conversation of liquid formulation (ME) into a semisolid form (MBG).[33,34]

3.6.4. Ph

The value of pH for the optimized batch was found to be 6.7 for AT loaded MBG, which was in the acceptable range for a transdermal formulations.[34]

3.6.5. Viscosity

The optimized batch of AT loaded MBG showed enhancement in the values of viscosity (203.47 cps) as compared to its ME. This enhancement in viscosity was attributed to the presence of colloidal silica as thickening agent. This enhancement might be predicted for the convenience in application of final formulations.[35,38]

3.6.7. Spreadability

The diameter of spreading for drug loaded MBG was found to be 10.47 cm compared to 5.56 cm for its conventional gel. High spreadability value of MBG compared to conventional gel indicated better spreading ability at the site of application alongwith reduction in application time.[35]

3.6.8. Percentage Drug Content (% Dc)

The percentage drug contents of the optimized batch of AT loaded MBG was found to be 99.37. The values of % drug content were almost 100% alongwith very low standard deviations, suggested uniform dispersion of drug in developed formulations.[35]

3.6.9. In Vitro Permeation

The optimized batch of AT loaded MBG exhibited significant enhancement ($P<0.05$) in all studied permeability parameters as compared to its conventional gel (Figure 9). The diffusion pattern of the optimized batch of AT loaded MBG depicted 387.58 μg/cm^2 of AT permeation within 2 h compared to only 80.52 μg/cm^2 for AT loaded conventional gel. The significantly higher values of Q_{24}, J_{ss}, K_p and lower values of t_L for the optimized

batch of AT loaded MBG as compared to its conventional gel suggested marked improvement in diffusion rate. Moreover, parameters like f_1 and f_2 confirmed nonsimilarity of diffusion profiles of AT loaded MBG and its conventional gel as f_1 value was higher than 15 and f_2 value was lower than 50.[45]The results of permeation parameters depicted no significant difference between diffusion profiles of AT loaded MBG and ME. Further, marginally higher values of Q_{24}, J_{ss}, K_p and somewhat lower values of t_L for the optimized batch of AT loaded ME as compared to its MBG might be attributed to the addition of thickening agent (Table 7). The desired input rate of AT was decided by calculating drug concentrations required to elicit the pharmacological effect as per following equation.

$$\text{Desired Input Rate} = C_{ss} \times C_L \times \text{B.W.} \quad (6)$$

where, C_{ss} was drug concentration at therapeutic level; BW was the standard human body weight (70 kg) and C_L was the total body clearance of the drug. The desired values of drug input rate was found to be 1008 µg/h whereas the practical value of J_{ss} for optimized batch of AT loaded MBG was found to be 140.69 µg/cm^{2}h which revealed an application area of 7.16 cm^2 in order to match the desired input rate. Since this area was highly manageable for a transdermal formulation it fulfills the criterias for patient compliance.

3.6.10. Skin Irritation Study

Primary skin irritation study was performed for optimized batch of AT loaded MBG to exclude any possibility of potential dermal irritation. A PDII of 0.1275 for AT loaded

MBG revealed that the optimized formulation was non-irritant, free from skin sensitization and safe for use.[13,37,38]

3.6.11. Stability Study

In the present work, accelerated stability study was carried out for optimized batch of MBG at 40 ± 2°C and 75 ± 5% RH for six months and their results are summarized in. Results of the stability study of optimized batch of AT loaded MBG showed no remarkable change in the all selected responses at the end of 6 months.[35,39]

4. CONCLUSIONS

The present investigation revealed that both rate and extend of AT transport across animal skin were highly dependent on the concentration of oil, surfactant, cosurfactant and water of developed formulations. The AT loaded ME; composed with 32.27% w/w of Sefsol 218 as oil phase, 35.25% w/w of Span 80 as surfactant phase, 17.67% w/w of ethanol as cosurfactant phase and 14.86% w/w of water as aqueous phase was found to be optimum with a mean globule size of 46 nm. The AT loaded ME was successfully converted into MBG by using colloidal silica as thickening agent. The optimized batch of AT loaded MBG delivered AT with a flux value of 140.69 µg/cm^{2}h in the *in vitro* permeation study. Thus, it could be concluded from the present investigation that a W/O type microemulsion could be an excellent approach for successful transdermal delivery of highly water soluble drugs like, AT. However further, *in vivo* investigations are required to confirm improved antihypertensive efficacy of AT.

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Table 1 Design layout of Box-Behnken design batches for AT loaded MEs.

Batch Code	Transformed Values		
	X_1[a]	X_2[b]	X_3[c]
AT-ME-F1	-1	-1	0
AT-ME-F2	1	-1	0
AT-ME-F3	-1	1	0
AT-ME-F4	1	1	0
AT-ME-F5	-1	0	-1
AT-ME-F6	1	0	-1
AT-ME-F7	-1	0	1
AT-ME-F8	1	0	1
AT-ME-F9	0	-1	-1
AT-ME-F10	0	1	-1
AT-ME-F11	0	-1	1
AT-ME-F12	0	1	1
AT-ME-F13	0	0	0
Coded Values	*Actual Values (%w/w)*		
	X_1[a]	X_2[b]	X_3[c]
0	25	40	5
1	55	70	15

[a] X_1: Amount of oil (Capmul MCM), [b] X_2:Amount of surfactant mixture [(Span 80: Ethanol (1:1)], [c] X_3:Amount of water

Table 2 Solubility of AT in various oils, surfactants, cosurfactants and distilled water

Oils	*Solubility (mg/mL)*	*Surfactants and Cosurfactants*	*Solubility (mg/mL)*
Capmul MCM	95.56 ± 9.43	Acconon CC-6	20.65 ± 2.09
Capmul PG8	63.56 ± 4.76	Capmul GMO50	200.65 ± 14.65
Captex 355	12.49 ± 1.65	Caprol ET	138.67 ± 9.33
Capryol 90	83.47 ± 5.53	Lauroglycol 90	24.65 ± 3.10
Labrafac CC	55.45 ± 4.65	Capmul MCM C8	148.45 ± 9.09
Labrafac Lipophile WL 1349	50.46 ± 3.65	Span 40	132.46 ± 7.67
Labrafil M 2125 CS	33.69 ± 4.15	Span 60	125.42 ±6.16
Maisine 35-1	17.67 ± 2.18	Span 80	110.88 ± 5.55
Imwitor 742	63.24 ± 5.57	Polyethylene Glycol (PEG) 400	177.15 ± 8.14
Miglyol 812	78.15 ± 7.01	Propylene Glycol (PG)	135.65 ± 9.15
Isopropyl Myristate	59.78 ± 4.62	Isopropyl Alcohol (IPA)	222.14 ± 15.26
Paceol	94.46 ± 9.54	n-Butanol	167.56 ± 11.87
Sefsol 218	121.92 ± 12.45	Ethanol	356.43 ± 20.14
Oleic Acid	25.35 ± 3.45		
Olive Oil	7.82 ± 1.46		
Castor Oil	9.14 ± 1.16		

Distilled Water	144.79 ± 13.57	

The results are of mean ± SD (n=3)

Table 3(a) Results of D-optimal design batches of AT loaded MEs.

Batch Code	Globule Size (nm)	PI[a]	ζ [b] (mV)	RI [c]	%T [d]	%DC [e]	□ [f] (cps)
AT-ME-F1	50.65 ± 3.65	0.12 ± 0.04	- 34.25 ± 1.45	1.45 ± 0.04	100.56 ± 0.33	99.46 ± 0.35	45.25 ± 2.57
AT-ME-F2	78.35 ± 5.67	0.18 ± 0.07	- 38.1 ± 2.25	1.47 ± 0.04	97.90 ± 1.06	100.05 ± 0.65	30.13 ± 1.50
AT-ME-F3	72.09 ± 4.45	0.19 ± 0.03	- 45.13 ± 3.09	1.39 ± 0.06	98.35 ± 0.67	98.46 ± 0.76	56.36 ± 4.65
AT-ME-F4	42.14 ± 2.33	0.17 ± 0.06	- 44.24 ± 4.09	1.50 ± 0.07	100.03 ± 1.04	96.26 ± 0.36	19.46 ± 1.21
AT-ME-F5	75.24 ± 4.67	0.21 ± 0.04	- 32.35 ± 3.13	1.49 ± 0.05	98.98 ± 0.15	99.46 ± 0.76	47.78 ± 2.09
AT-ME-F6	53.13 ± 4.13	0.23 ± 0.03	- 37.65 ± 2.18	1.43 ± 0.04	98.46 ± 0.36	98.98 ± 1.09	23.25 ± 1.15
AT-ME-F7	67.35 ± 4.89	0.11 ± 0.05	- 46.00 ± 3.23	1.38 ± 0.07	99.25 ± 0.90	97.26 ± 1.56	35.37 ± 1.65
AT-ME-F8	61.00 ± 3.35	0.16 ± 0.04	- 41.13 ± 2.04	1.48 ± 0.04	99.65 ± 0.56	98.21 ± 1.30	53.14 ± 3.57
AT-ME-F9	56.36 ± 4.09	0.17 ± 0.05	- 40.65 ± 4.24	1.45 ± 0.05	97.90 ± 0.76	99.90 ± 0.33	40.14 ± 1.09
AT-ME-	46.21 ±	0.19 ±	- 39.15 ±	1.48 ±	101.03 ±	99.46 ±	34.25 ±

F10	3.65	0.03	3.45	0.08	0.46	0.67	1.10
AT-ME-F11	65.12 ± 4.76	0.12 ± 0.05	- 38.46 ± 2.03	1.51 ± 0.03	100.34 ± 0.47	97.25 ± 1.56	42.14 ± 1.36
AT-ME-F12	52.78 ± 3.98	0.13 ± 0.04	- 36.65 ± 2.23	1.52 ± 0.04	100.43 ± 0.67	96.66 ± 2.78	21.00 ± 1.03
AT-ME-F13	77.89 ± 3.19	0.15 ± 0.02	- 35.56 ± 1.85	1.45 ± 0.05	101.23 ± 0.90	97.36 ± 1.98	27.74 ± 1.26
AT-ME-F14	71.11 ± 4.65	0.16 ± 0.05	- 33.46 ± 1.05	1.47 ± 0.02	99.98 ± 0.58	98.57 ± 0.39	54.26 ± 2.35
AT-ME-F15	41.14 ± 2.78	0.17 ± 0.06	- 38.09 ± 1.67	1.48 ± 0.04	97.98 ± 0.66	99.13 ± 0.67	16.36 ± 0.98
AT-ME-F16	49.78 ± 3.05	0.18 ± 0.03	- 35.25 ± 1.24	1.40 ± 0.05	99.90 ± 0.79	98.25 ± 1.14	50.00 ± 3.56

The results are of mean ± SD (n=3), [a] PI: Polydispersibility index, [b] ζ: Zeta potential, [c] RI: Refractive index, [d] %T: Percentage transmittance, [e] %DC: Percentage drug content, [f] □: Viscosity

Table 3 (b) Results of D-optimal design batches of AT loaded MEs.

Batch Code	Q_{24}[a] (mg/cm^2)	J_{ss}[b] (mg/cm^2h)	t_L[c] (h)	ER[d]	K_p[e] X 10^{-2}	%DRS[f]
AT-ME-F1	3091.30 ± 345.47	145.20 ± 32.46	0.34 ± 0.04	2.40 ± 0.23	0.5808 ± 0.0556	134.36 ± 12.46
AT-ME-F2	2036.27 ± 210.46	99.25 ± 17.65	0.57 ± 0.05	1.64 ± 0.35	0.3970 ± 0.0477	145.57 ± 14.50
AT-ME-F3	2392.14 ± 313.10	111.35 ± 19.04	0.51 ± 0.03	1.84 ± 0.33	0.4454 ± 0.0357	134.26 ± 11.56
AT-ME-F4	3232.35 ± 350.16	158.25 ± 23.46	0.30 ± 0.07	2.61 ± 0.29	0.6330 ± 0.0678	144.36 ± 13.15
AT-ME-F5	2183.14 ± 289.14	104.26 ± 13.56	0.53 ± 0.04	1.72 ± 0.31	0.4170 ± 0.0246	125.47 ± 9.45
AT-ME-F6	2931.09 ± 405.04	134.26 ± 17.05	0.35 ± 0.08	2.21 ± 0.38	0.5370 ± 0.0473	146.37 ± 9.25
AT-ME-F7	2413.10 ± 325.05	115.47 ± 14.34	0.47 ± 0.03	1.91 ± 0.25	0.4618 ± 0.0376	110.65 ± 12.43
AT-ME-F8	2514.14 ± 316.04	124.00 ± 15.15	0.41 ± 0.02	2.05 ± 0.17	0.4960 ± 0.0473	124.68 ± 12.12
AT-ME-F9	2715.46 ± 335.33	130.15 ± 17.55	0.40 ± 0.07	2.15 ± 0.13	0.5206 ± 0.0498	110.56 ± 8.54
AT-ME-	3109.46 ±	153.25 ±	0.34 ±	2.53 ±	0.6130 ±	96.47 ±

F10	395.14	23.15	0.09	0.19	0.0657	7.78
AT-ME-F11	2509.09 ± 298.33	117.47 ± 17.34	0.45 ± 0.02	1.94 ± 0.25	0.4698 ± 0.0573	145.47 ± 13.12
AT-ME-F12	2846.36 ± 256.56	141.36 ± 22.06	0.37 ± 0.03	2.33 ± 0.26	0.5654 ± 0.0468	167.36 ± 14.56
AT-ME-F13	2036.43 ± 305.90	97.36 ± 13.54	0.58 ± 0.04	1.61 ± 0.14	0.3894 ± 0.0466	130.00 ± 11.33
AT-ME-F14	2313.54 ± 389.14	110.34 ± 12.45	0.50 ± 0.06	1.82 ± 0.26	0.4413 ± 0.0356	113.65 ± 8.89
AT-ME-F15	3324.15 ± 278.34	157.36 ± 23.65	0.31 ± 0.07	2.60 ± 0.27	0.6294 ± 0.0763	125.57 ± 9.24
AT-ME-F16	3087.14 ± 340.14	148.15 ± 25.45	0.33 ± 0.09	2.45 ± 0.23	0.5926 ± 0.0903	130.26 ± 9.84

The results are of mean ± SD (n=3), [a] Q_{24}: Cumulative amount of drug permeated, [b] J_{ss}: Flux, [c] t_L: Lag time, [d] ER: Enhancement ratio, [e] K_p: Permeability coefficient, [f] DRS: Drug retained in skin

Table 4 Regression analysis of Box-Behnken design batches of AT loaded MEs.

Coefficients	Globule Size (Y_1)		$Q_{24}(Y_2)$	
	FM[a]	RM[b]	FM[a]	RM[b]
b_1	-78.28	-78.16	2029.77	2031.49
b_2	-71.27	-71.15	2345.57	2337.28
b_3	-41.70	-41.79	3278.47	3282.12
b_{12}[c, d]	1.38	----	-19.08	----
b_{13}	-27.32	-29.28	928.00	865.11
b_{23}	-26.22	-28.18	1075.62	1012.72
b_{123}[c, d]	-45.30	----	-1390.64	----
Coefficients	J_{ss} (Y_3)		t_L (Y_4)	
	FM[a]	*RM*[b]	*FM*[a]	*RM*[b]
b_1	98.17	97.84	-0.5749	-0.5708
b_2	110.92	110.60	-0.5007	-0.4966
b_3	158.24	158.32	-0.3085	-0.3091
b_{12}[c, d]	-3.17	----	-0.0676	----
b_{13}	39.77	38.85	-0.3127	0.3078
b_{23}	50.57	49.65	-0.2812	-0.2763
b_{123}[c, d]	-17.72	----	0.1746	----

[a] FM: Full model, [b] RM: Reduced model, [c] Nonsignificant (P>0.05) coefficients for Y_1, [d] Nonsignificant (P>0.05) coefficients for Y_2, [e] Nonsignificant (P>0.05) coefficients for Y_3, [f] Nonsignificant (P>0.05) coefficients for Y_4

Table 5 Calculation for testing the model in portions for AT loaded MEs.

Globule Size (Y_1)				
	DF[c]	SSR[d]	MS[e]	R^2 = 0.9998
Regression				Fcal = 1.88
FM[a]	7	60042.17	8577.45	Fcritical = 4.26
RM[b]	5	60038.91	12007.78	DF = (2, 9)
Residual				
FM	9	7.79	0.8655	
RM	11	11.05	1.0045	
Cumulative Amount of Drug Permeated – Q_{24} (Y_3)				
	DF	*SSR*	*MS*	R^2 = 0.9998
Regression				Fcal = 1.18
FM	7	1.17×10^8	16696877	Fcritical = 4.26
RM	5	1.2×10^8	23374798	DF = (2, 9)
Residual				
FM	9	15833.32	1759.25	
RM	11	19984.50	1816.77	
Flux - J_{ss} (Y_3)				
	DF[c]	*SSR*[d]	*MS*[e]	R^2 = 0.9998
Regression				Fcal = 0.20
FM[a]	7	268502.20	38357.45	Fcritical = 4.26

RM[b]	5	268499.97	53699.99	DF = (2, 9)
Residual				
FM	9	49.46	5.4958	
RM	11	51.64	4.6951	
Lag Time - t_L (Y_4)				
	DF[c]	*SSR*[d]	*MS*[e]	R^2 = 0.9994
Regression				Fcal = 1.01
FM[a]	7	2.9876	0.4268	Fcritical = 4.26
RM[b]	5	2.9874	0.5974	DF = (2, 9)
Residual				
FM	9	0.0017	0.0001	
RM	11	0.0019	0.0001	

[a] FM: Full model, [b] RM: Reduced model, [c] DF: Degree of freedom, [d] SSR: Sum of square residuals, [e] MS: Mean of squares

Table 6 Formulation composition and results of check point batch for AT loaded MEs.

Type of Component		Name of Component	Concentration (%w/w)
Oil (X_1)		Sefsol 218	32.27
Surfactant Mixture (X_2)	Surfactant	Span 80	35.25
	Cosurfactant	Ethanol	17.62
Water (X_3)		Distilled water	14.86
Responses	*Predicted Value*	*Experimental Value* [a]	*% Relative Error*
Globule Size (Y_1)	44.09 nm	46.00 ± 2.68 nm	4.33
Q_{24} (Y_2)	3207.22 µg/cm^2	3025.4 ± 325.58 µg/cm^2	5.65
J_{ss} (Y_3)	154.27 µg/cm^{2}h	147.65 ± 14.57 µg/cm^{2}h	4.29
t_L (Y_4)	0.3077 h	0.3235 ± 0.05 h	5.13

[a] The results are of mean ± SD (n=3)

Table 7 Comparison of evaluation parameters of optimized batch of AT loaded MBG and ME.

Parameters	MBG	ME
Globule Size (nm)	52.15	46.00
PI[a]	0.16	0.17
Zeta Potential (mV)	- 33.48	- 36.79
Viscosity (cps)	203.47	34.26
Q_{24}[b]	3025.4 µg/cm^2	1003.35 µg/cm^2
J_{ss}[c]	147.65 µg/cm^{2}h	40.23 µg/cm^{2}h
t_L[d] (h)	0.32	1.00
K_p[e] x 10^{-2} (cm/h)	0.59	0.16
ER[f]	3.67	-
%DRS[g] (µg/cm^2)	154.26	25.69
f_1[h]	80.28	-
f_2[i]	0.30	-

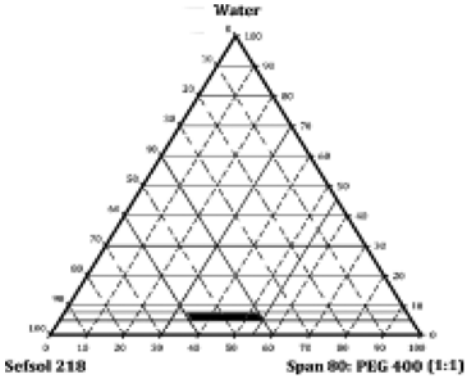
The results are mean ± SD (n=3), [a] PI: Polydispersibility Index, [b] Q_{24}: Cumulative amount of drug permeated at the end of 24 h, [c] J_{ss} : Flux (h), [d] t_L:Lag time, [e] K_p: Permeability coefficient, [f] ER: Enhancement ratio, [g] DRS: Drug retained in skin, [h] f_1: Difference factor, [i] f_2: Similarity factor

Table 8 Comparison of diffusion parameters of optimized batches of AT loaded MBGs and their conventional gel.

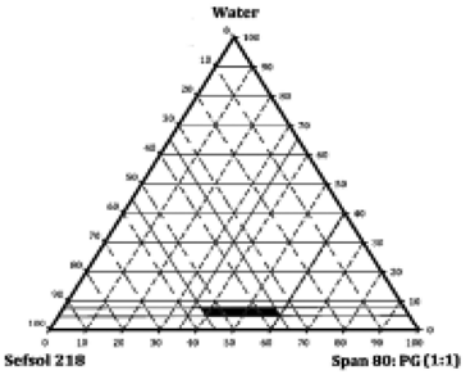
Parameters	MBGs	Conventional Gel
Q_{24}[a]	2893.54 µg/cm^2	905.35 µg/cm^2
J_{ss}[b]	140.69 µg/cm^{2}h	35.90 µg/cm^{2}h
t_L[c] (h)	0.5	1.5
K_p[d] x 10^{-2} (cm/h)	0.56	0.14
ER[e]	3.92	-
%DRS[f] (µg/cm^2)	145.37	20.37
f_1[g]	74.58	-
f_2[h]	2.47	-

The results are mean ± SD (n=3), [a] Q_{24}: Cumulative amount of drug permeated at the end of 24 h, [b] J_{ss} : Flux (h), [c] t_L:Lag time, [d] K_p: Permeability coefficient, [e] ER: Enhancement ratio, [f] DRS: Drug retained in skin, [g] f_1: Difference factor, [h] f_2: Similarity factor

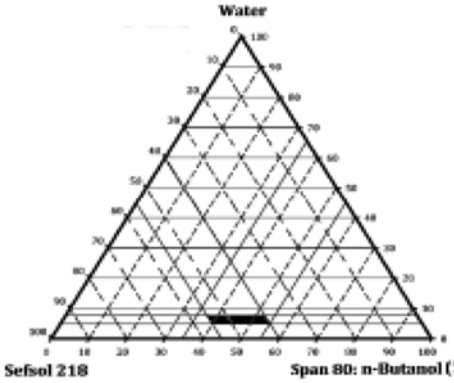
Figure. 1. Pseudo-ternary phase diagrams with different cosurfactant (A) PEG 400, (B) PG, (C) n-Butanol, (D) IPA (E) ethanol for AT loaded MEs.



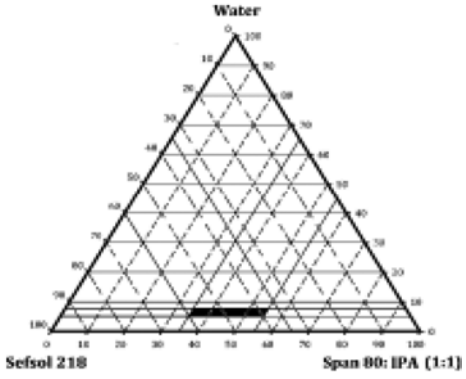
(a)



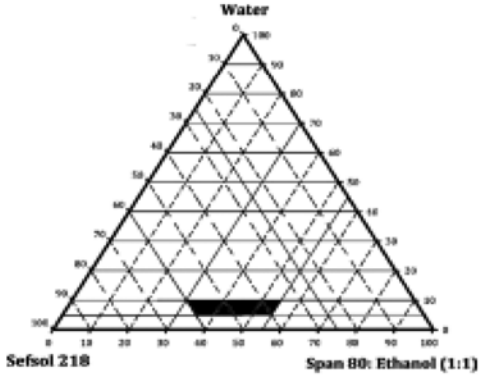
(b)



(c)



(d)



(e)

Figure. 2. Pseudo-ternary phase diagrams with different ratios of S_{mix} (A) 3:1, (B) 2:1, (C)) 1:1, (D) 1:2, (E) 1:3 for AT loaded MEs.

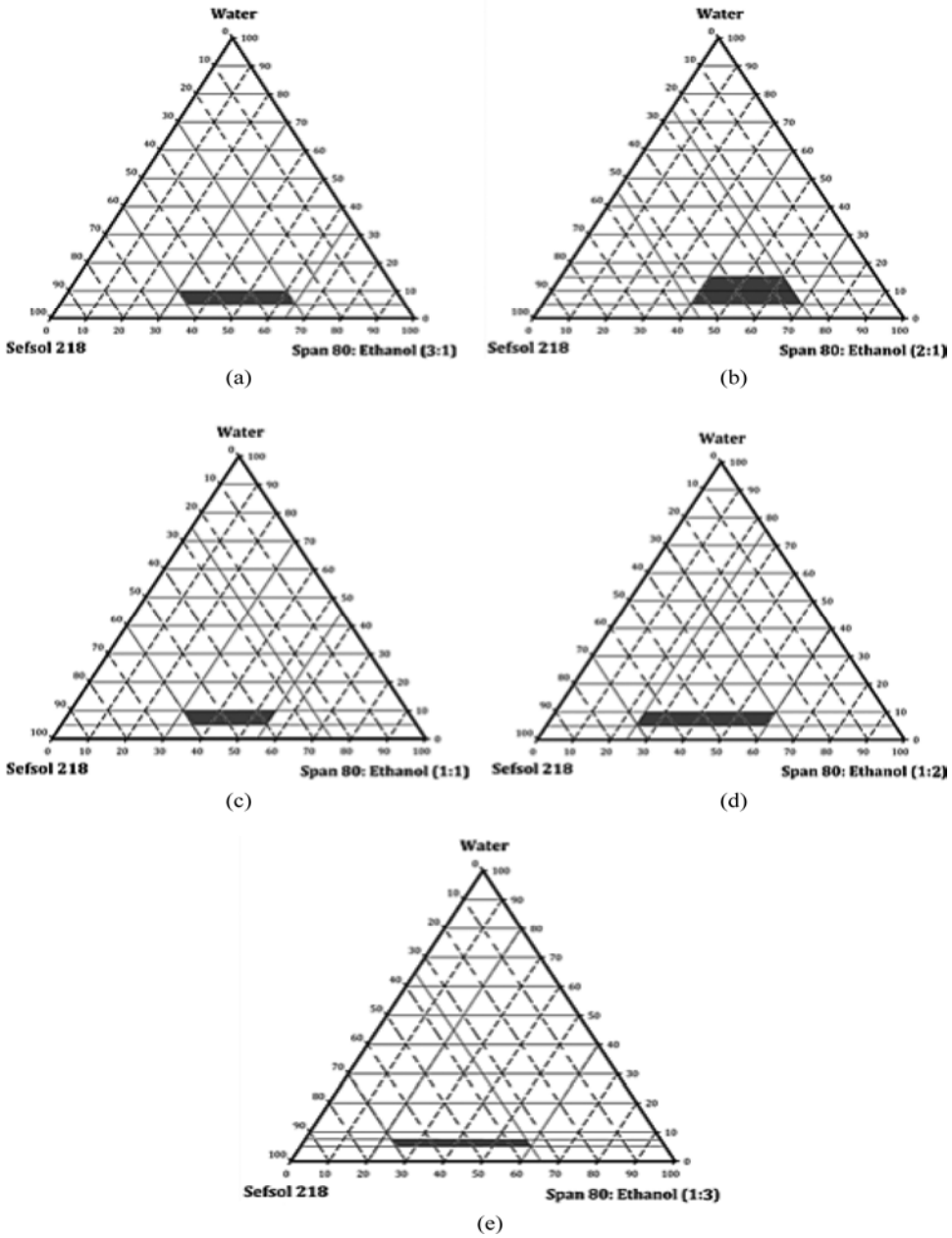


Figure. 3. PCA study for AT loaded MEs showing (A) Loading plot (B) Dendrogram from AHCA (C) Scoring plot (D) Correlation loading plot and (E) Scree plot Loading plot from

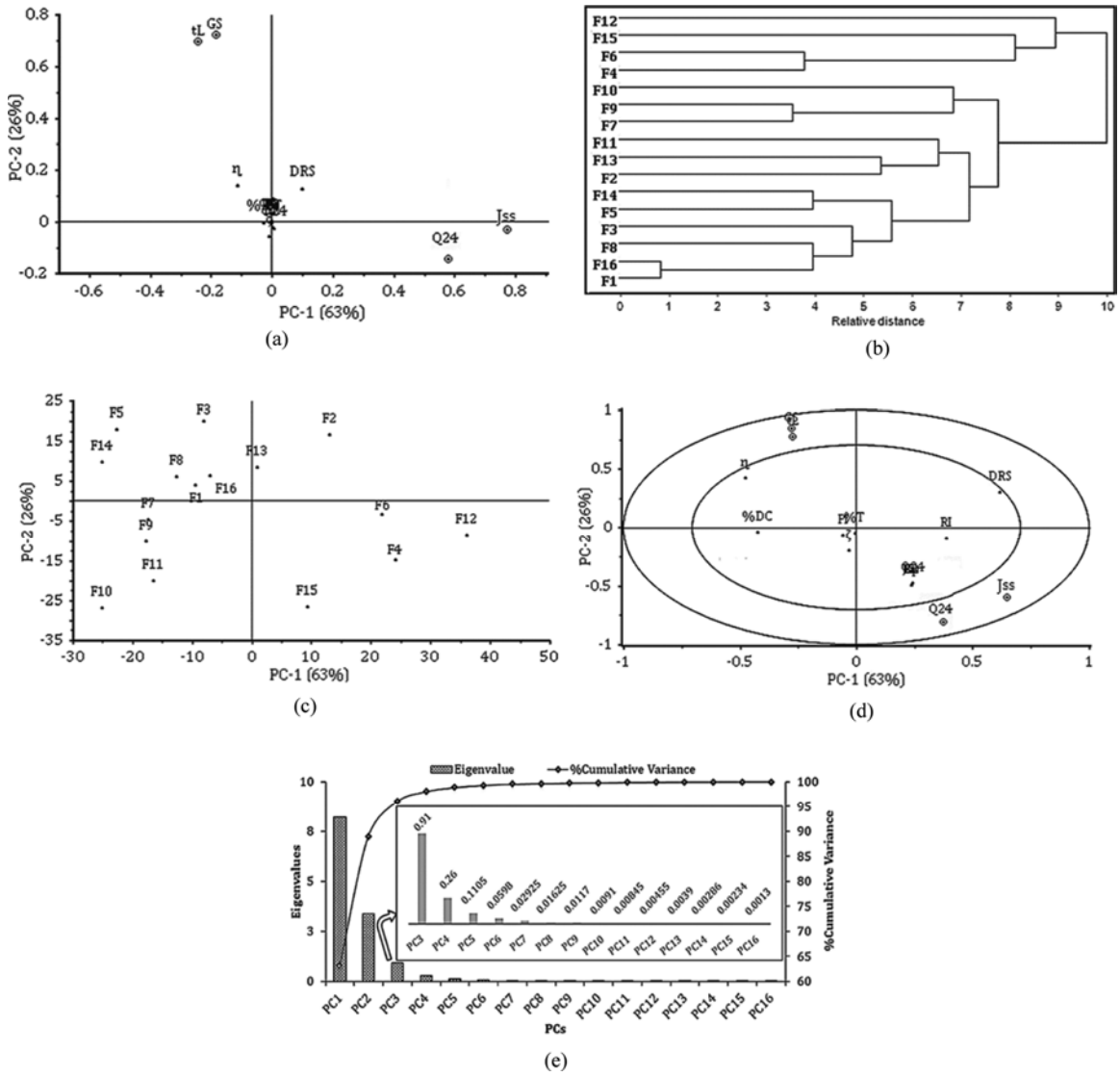


Figure. 4. Influence of formulation factors on globule size (Y_1) of AT loaded MEs by response surface plots (A) Effect of X_1 and X_2, (B) Effect of X_1 and X_3, (C) Effect of X_2 and X_3 and contour plots (D) Effect of X_1 and X_2, (E) Effect of X_1 and X_3, (F) Effect of X_2 and X_3.

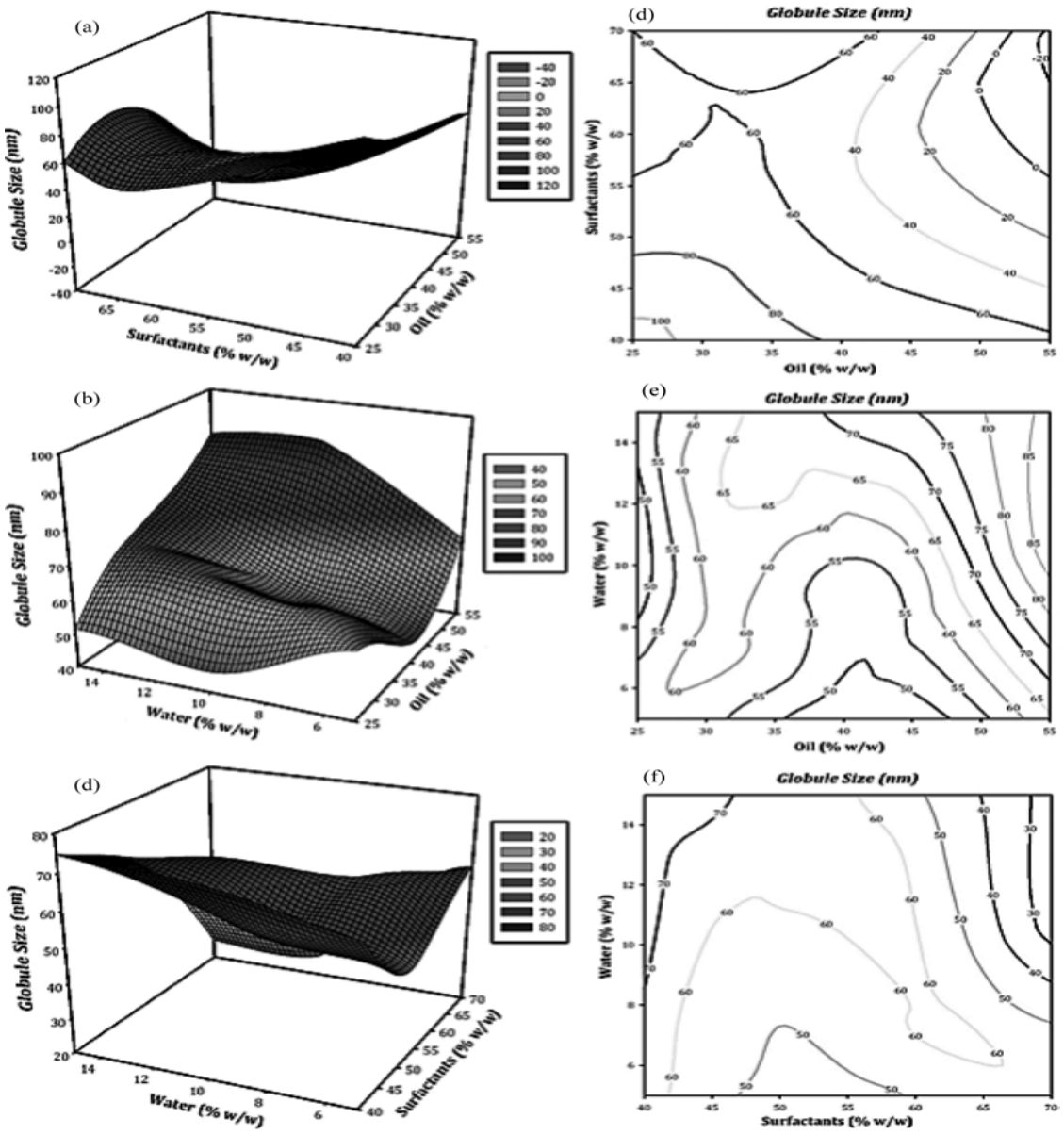


Figure. 5. Influence of formulation factors on Q_{24} (Y_2) of AT loaded MEs by response surface plots (A) Effect of X_1 and X_2, (B) Effect of X_1 and X_3, (C) Effect of X_2 and X_3 and contour plots (D) Effect of X_1 and X_2, (E) Effect of X_1 and X_3, (F) Effect of X_2 and X_3.

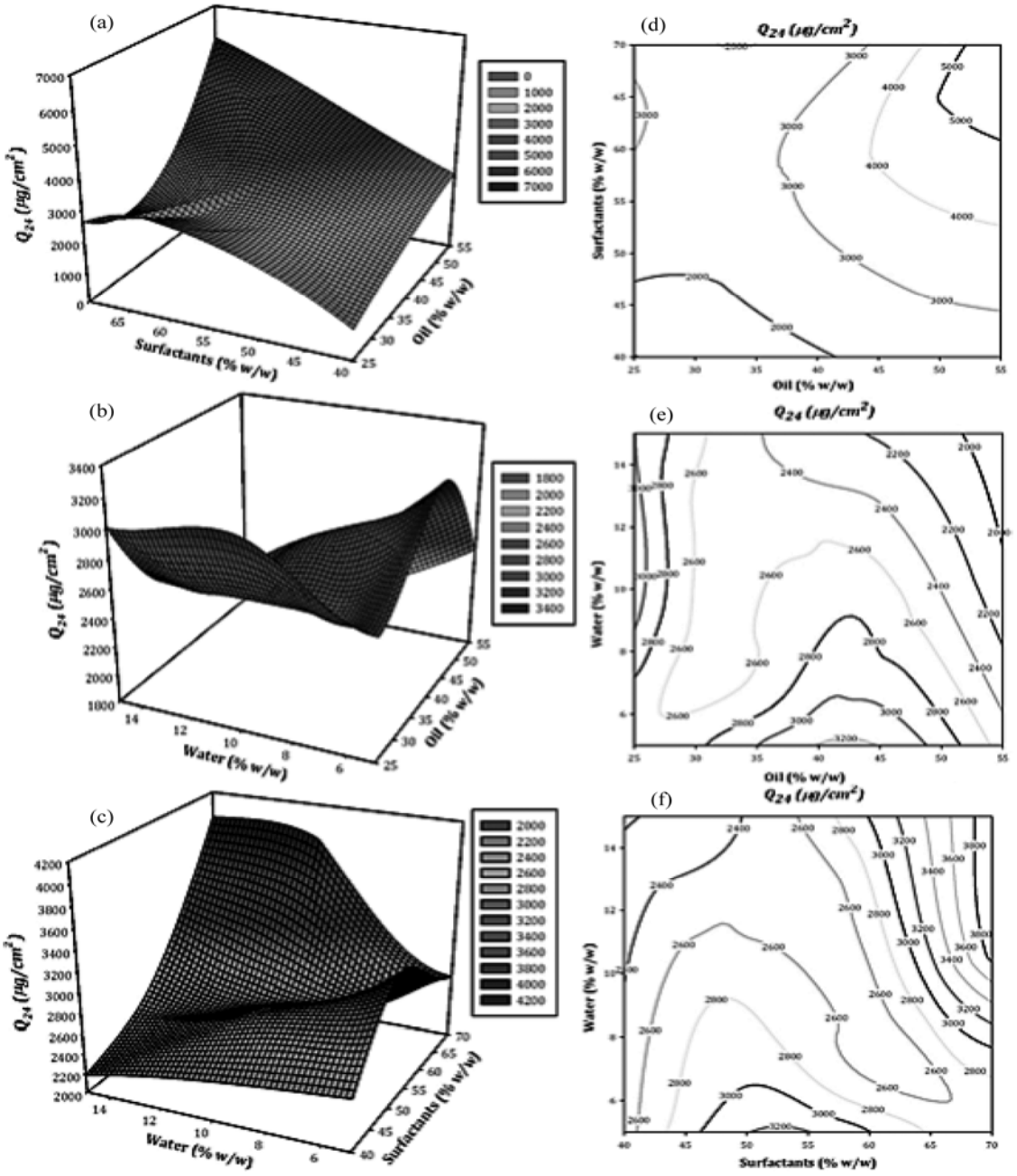


Figure. 6. Influence of formulation factors on J_{ss} (Y_3) of AT loaded MEs by response surface plots (A) Effect of X_1 and X_2, (B) Effect of X_1 and X_3, (C) Effect of X_2 and X_3 and contour plots (D) Effect of X_1 and X_2, (E) Effect of X_1 and X_3, (F) Effect of X_2 and X_3.

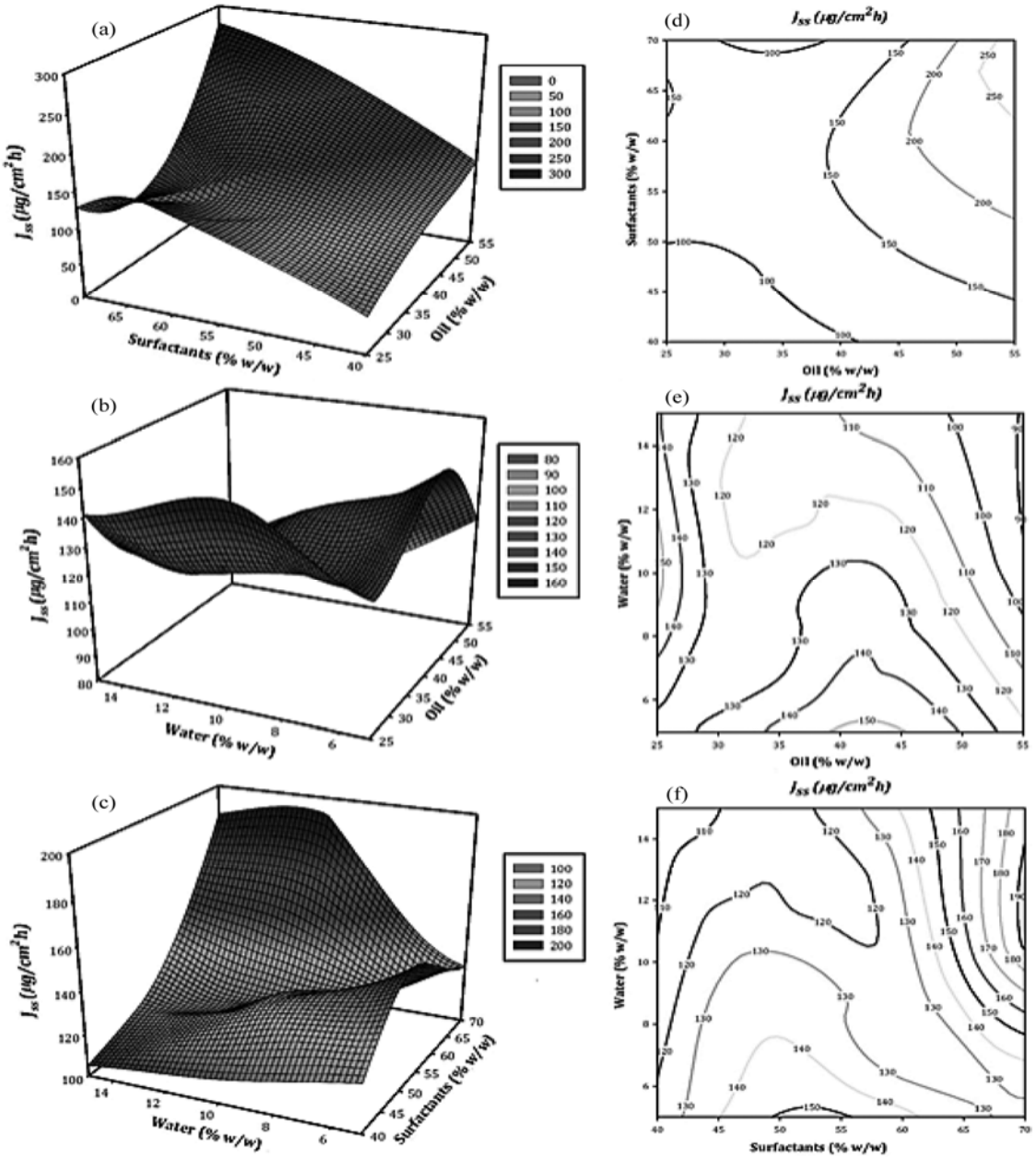


Figure. 7. Influence of formulation factors on t_L (Y_4) of AT loaded MEs by response surface plots (A) Effect of X_1 and X_2, (B) Effect of X_1 and X_3, (C) Effect of X_2 and X_3 and contour plots (D) Effect of X_1 and X_2, (E) Effect of X_1 and X_3, (F) Effect of X_2 and X_3.

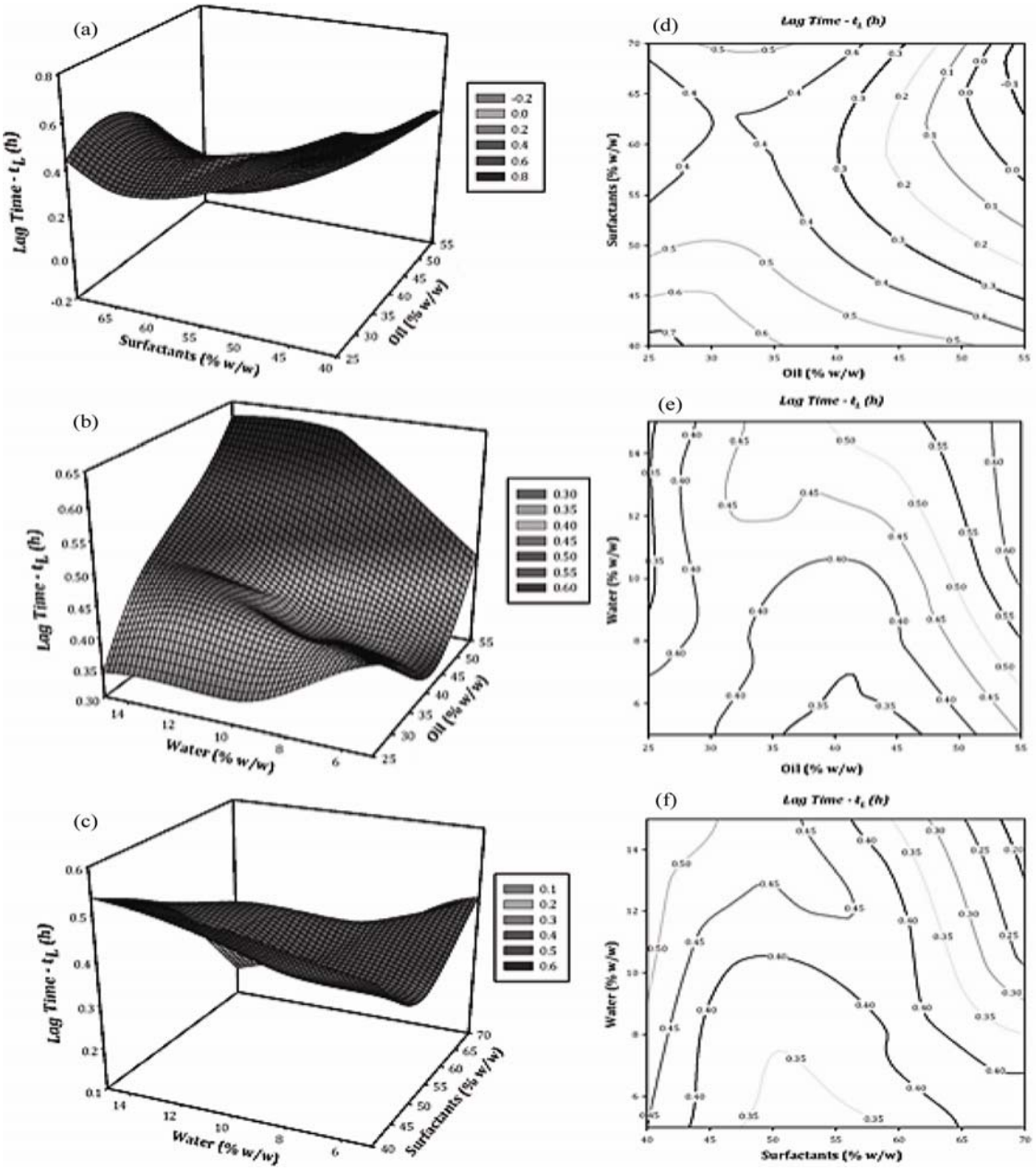


Figure. 8. TEM images of optimized batch of AT loaded MEs.

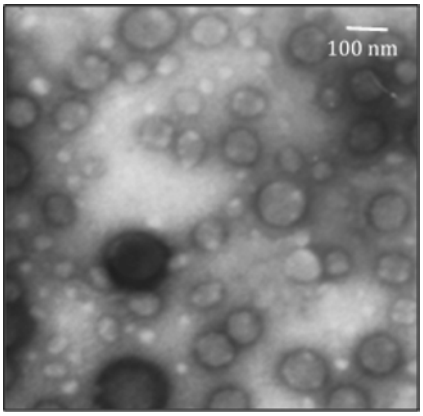


Figure. 9. Comparison of *in vitro* drug permeation profiles of optimized batch of AT loaded MBG against their respective conventional gel, Error bar represents SD (n=3).