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Atorvastatin Loaded Microsponges based Emu Oil Emulgel for Faster Wound Healing

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Abstract

A micro sponge delivery system is patented, highly cross-linked, porous and polymeric in nature. Polymeric system consisting of porous microspheres that can entrap wide range of actives and then release them onto the skin over a time and in response to trigger. The fundamental appeal of the micro sponge technology overcome the problems of greasiness, stickiness associated with the conventional formulations in releasing active ingredients over an extended period of time that often result in lack of patient compliance. When applied to the skin, the MDS releases its active ingredient on a time mode and also in response to other stimuli (rubbing, temperature, pH, etc). Delivery system comprised of a polymeric bead having network of pores with an active ingredient held within was developed to provide controlled release of the active ingredients

Introduction

Micro sponge formulations have higher payload (50 to 60%), still free flowing and can be cost effective [1-3]. Microsponge scan be prepared by Emulsion solvent diffusion method and Suspension polymerization method.

Eudragits polymers are preferred to control the release of drug in formulation of microsponges by quasi emulsion solvent diffusion method. It is a copolymer of ethyl acrylate, methyl methacrylate, and a low content of Methacrylic acid ester with quaternary ammonium groups. As the polymer contains the ammonium salt groups, its permeability is pH independent [4]; Wound is defined as disruption of cellular, anatomical and functional continuity of a living tissue, produced by physical, chemical, thermal, microbial or immunological insult to the tissue [5]. A wound is colonized when growth and death of bacteria in the wound is balanced by the host. If the host is not able to keep the bacterial growth in balance, the wound will enter the infection phase (Bacterial load in excess of 10^{15}). Symptoms for an infected wound are erythema, edema, warmth, pain and exudate. Infections of chronic wounds are often polybacterial with *Staphylococcus aureus* and anaerobs being the most common in chronic wound [6].

Wound healing is the interaction of a complex cascade of cellular and biochemical actions leading to the restoration of structural and functional integrity with regain of strength of injured tissues [5]. The healing of a wound is achieved by way of the integrated phases of haemostasis, inflammation, proliferation and remodeling. These phases must occur in the proper sequence and time period, without interference and at optimalintensity, in order for a wound to heal normally [7].

However, medical therapies for wound care are limited; therefore, development of new treatment modalities to improve wound healing in diabetic patients is an essential and emerging field of investigation [8]. Numerous conservative methods, such as honey as a dressing solution [9], topical antimicrobial therapies total contact casting [10], wound dressings [11] for the treatment of diabetic wounds have been reported in the literature. Herbal therapies were also reported [12]. In this study, a well-known agent. Atorvastatin is used for the treatment of wounds. It is reported that statins may be useful in the treatment of diabetic foot ulceration (DFU) [13]. Statins are widely used for the treatment of hyperlipidaemia, has been shown to prevent cardiovascular events in patients with diabetes. In addition to preventing macrovascular diseases, statins may also be able to retard the progression of microvascular complications of diabetes [14]. Statin drugs directly enhance the ability of endothelial nitric oxide synthase to generate nitric oxide in endothelial cells independent of lipid-lowering effects [15,16]. In animal studies, the use of statins on the vascular system, such as the coronary artery, cerebral artery, small mesenteric artery, aorta, and corpus cavernosum, was shown to result in vascular relaxation by up regulating nitric oxide synthase [16-18] Indeed,

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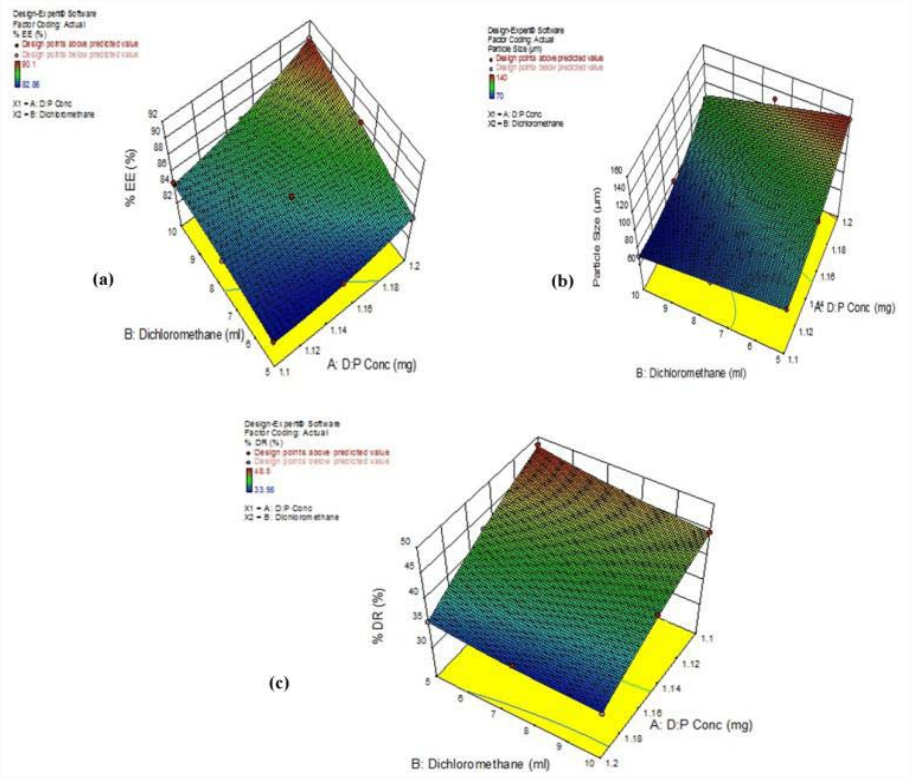

Figure 1: a) Three dimensional response surface plot for Entrapment efficiency. b) Three dimensional response surface plot for particle size. c) Three dimensional response surface plot for percentage drug release.

in addition to reducing lipid levels, these agents can improve endothelial function and reduce oxidative stress, which can improve microvascular function. In addition, statins cause a down-regulation of the preproendothelin-1mRNA level in endothelial cells, and thus reduce the synthesis of endothelin-1 [19], which is a powerful vasoconstrictor. Intensive lipid-lowering treatment has been shown to be beneficial by reducing vascular response to angiotensin-2 (AT-2) and to increase endothelium-dependent vasodilatation [20]. It was indicated that AT-2 induces T-type Ca_2channels in endothelial cells, which may play a role in the development of vascular disorders, and AT-2–induced expression of alpha 1G was inhibited by treatment with Atorvastatin [21].

The emu is a flightless bird that resembles a small ostrich. Emu oil is taken from the fat of this bird during processing. Emu oil is extracted from the subcutaneous and retroperitoneal fat of emu. Naturally Emu oil contains Omega 3, 6 and 9 Essential Fatty Acids (EFA's).May act on Cyclooxygenase (COX II), lipoxygenase, and lipoxin pathways to bring about its anti-inflammatory and other beneficial actions.

Beneficial effects of ATR in diabetic neuropathy [22] its topical effect on wounds in rats and to restore endothelial function are studied, thoroughly. Researchers developed an ointment of nitroglycerin based on the concept of NO for wound healing by using diabetes induced foot ulcer model and rat excision wound model.

Various microsponge-based topical deliveries were reported previously; EGF-impregnated collagen sponge dressing for burns mupirocin-loaded microsponge emulgel system for sustained release and enhanced drug deposition in the skin and hydroxyzine hydrochloride-loaded microsponge to control release into skin [23]. However, a need arises to treat wound at economical cost with easy

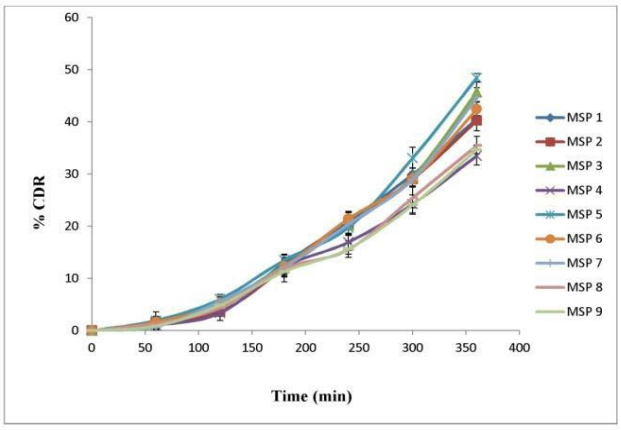

Figure 2: % Cumulative drug release from microsponge formulations.

applicability and no pain to patient. ATR an antihyperlipidaemic drug, exhibits vasodilating effects via nitric oxide pathway and reduces neuropathy and restores endothelial function in wounds. Possible mechanisms include reduction of neuropathy and ischemia restoring endothelial function through nitric oxide. Moisture regulation of wound through emulgel form, specific to the area of contact, is an important aspect and results in getting wound conditions more amenable to healing. Hence, an attempt was made to formulate ATR-loaded microsponges (ATR-M) to fulfil accessibility of drug at wound area, incorporated into emu oil emulgel base, that possess optimum moist wound management environment during later stages of wound closure. Thus, formulation provided advantage of both, microsponges that prolonged drug release due to entrapped

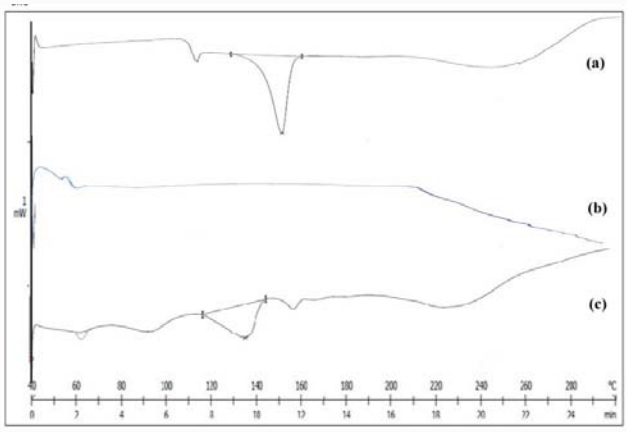

Figure 3: a) DSC thermogram of atorvastatin calcium. b) Eudragit RS 100 (b). c) atorvastatin calcium and eudragit RS 100.

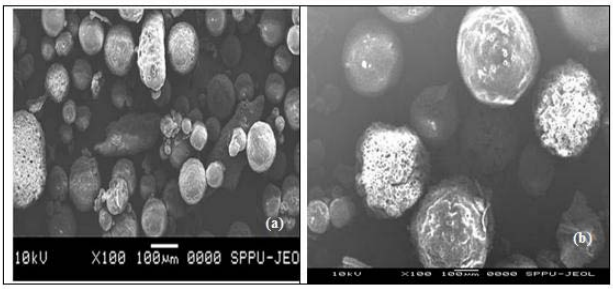

Figure 4: SEM images. a) The microsponge particle at 100x magnification. b) Microsponge particles at 100x magnification representing spherical and porous structure.

form in porous structure and emulgel for treating wounds at fast rate. Significant closure of wound on day 10 was considered as structure renewal of wound healing process.

Materials and Methods

Materials

Atorvastatin calcium was procured from Zydus Cadila Healthcare Ltd, Moraiya, Tal. Sanand (Ahmadabad), India. Eudragit RS 100 was procured from Evonik Pharma, Mumbai, India. Polyvinyl alcohol, methyl alcohol, light liquid paraffin, potassium dihydrogen phosphate, disodium hydrogen phosphate, and sodium chloride were obtained from Thermosil fine chem industries, Pune.

Preparation of ATR microsponges by quasi-emulsion solvent diffusion method

The microsponges were prepared by quasi emulsion solvent diffusion method. An accurately weighed 500 mg Eudragit RS-100 was dissolved in 5 ml of internal phase dichloromethane. This solution was then sonicated in the ultrasonic bath of 70-kHz frequency for 5 min. Once a clear solution was obtained, the 500 mg drug was added to the above solution, this solution was then sonicated in the ultrasonic bath of 70-kHz frequency for 3 min (Crest, Ultrasonic Corporation, Cortland, New York) where homogenous dispersion was obtained [24,25]. The obtained solution was then poured into PVA solution (0.750g of PVA in 100 ml distilled water i.e. external phase) which was heated upto 60c and cooled to 45 ± 0.5°C, while it was stirred by a mechanical stirrer for 75 min. During this time, the dichloromethane was completely removed by diffusion into PVA solution and evaporation through the air/liquid interface. The solidified microsponges of atorvastatin calcium were filtered, washed six times with 50 ml of n-hexane, air-dried at room temperature and stored in a desiccator for further study [26].

Optimization of Formulation Parameters and process Variables

Selection of Formulation Parameters and Process Variables: Preliminary trials were undertaken to establish physical parameters of microsponges by studying the effect of drug to polymer ratios (1:1, 1:2, and 2:1), dichloromethane (5, 10, and 15 ml), PVA (0.5% and 0.75%), and speed (500, 1000 and 1500 rpm). Effects of these various parameters were studied on entrapment efficiency, average particle size and percent drug release. Based on preliminary results, independent variables were selected for optimization process.

Experimental Design: A 3^2 full factorial design was applied to optimize microsponges using software Design Expert® (version 10.0.). Two factors, ratio of drug to polymer (X1) at 1:1, 1:1.5, and 1:2 and dichloromethane volume (X2) at 5, 7.5, and 10 ml, were selected as independent variables (Table 1). Response variables (dependent variables) were entrapment efficiency (Y1), particle size (Y2) and percent drug release (Y3). Nine formulation batches were suggested by factorial design (Table 1 and 2).

Characterization and evaluation of microsponge formulation

Determination of drug content and entrapment efficiency: Accurately weighed (50 mg) microsponge were dissolved in methanol (50 ml) and shaken for 15 min. This solution was filtered using whattman filter paper. The part of solution (0.5 ml) was withdrawn in 10 ml volumetric flask and diluted up to the mark. The quantitative determination of atorvastatin in microsponges was carried out using a linear model UV absorbance detector using double beam UV spectrophotometer (1800, Shimadzu, Japan) at 246 nm against methanol as blank [27,28] and entrapment efficiency was determined in the total amount of drug present in the product. Entrapment efficiency can be calculated based on drug content [23,27].

$$Drug\ entrapment\ efficiency = \frac{Actual\ drug\ content}{Theoretical\ drug\ content} \times 100 \ldots..(1)$$

Particle Size and production yield: Particle size analysis of drug loaded and unloaded microsponge was evaluated by optical microscope and production yield of microsponge was determined by calculating accurately the initial weight of the raw material and final weight of the microsponge obtained [27,29].

$$Percentage\ Yield = \frac{Weight\ of\ the\ microsponge\ recovered}{Weight\ (drug + polymer)} \times 100 \ldots(2)$$

***In vitro* drug release studies:** The *in vitro* release of drug from the microsponges was performed by the membrane diffusion technique using dialysis membrane 150 (LA401-1MT), Himedia, Mumbai, India. An accurately measured amount of microsponges, equivalent to 10-mg drug was suspended in the dissolution flask of the USP dissolution tester containing 200 ml phosphate buffer (pH 7.4). The apparatus was run at 50 rpm at a temperature of 37 ± 0.5°C. An aliquot of 5ml sample was collected over a period of 6 h and was assayed spectrophotometrically for the drug at λ max=246 nm. Each time sample was withdrawn and replaced with equal volume of preheated buffer solution to maintain sink condition. Experiments were done in triplicate [23].

Differential Scanning Calorimetry (DSC) analysis: Thermal

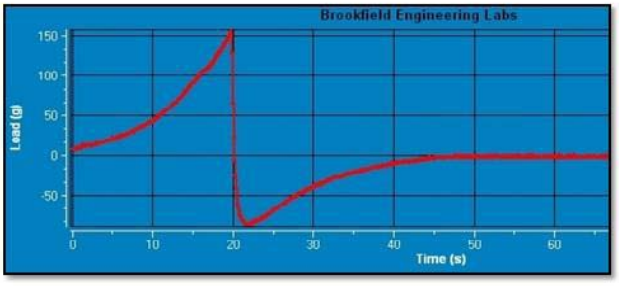

Figure 5: Texture profile analysis of emulgel.

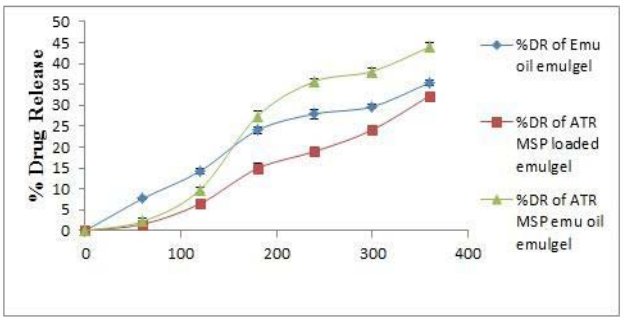

Figure 6: Comparative % drug release of emulgels.

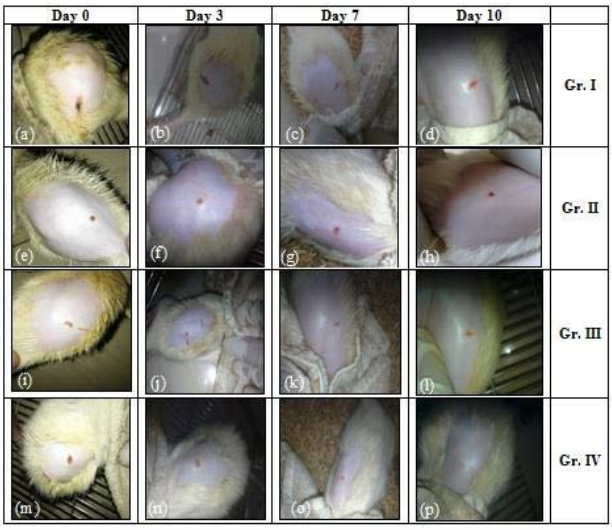

Figure 7: *In vivo* study models.

analysis of drug-loaded microsponge-based formulations was studied employing differential scanning calorimeter (DSC 1, Mettler Toledo, Switzerland). Samples (1 mg) were accurately weighed into aluminium pans and sealed. All samples were run at a heating rate of 10°C/min over a temperature range 25–300°C in atmosphere of nitrogen. DSC provides information on the physical properties of the sample and its crystalline or amorphous nature and demonstrates a possible interaction between drug and other compounds in microsponges [23].

Scanning electron microscopy: To study the morphology and surface topography, prepared microsponges were coated with gold–palladium under an argon atmosphere at room temperature and then the surface morphology of the microsponges was studied by scanning electron microscopy (JEOL oxford scanning electron microscope, Japan).

Preparation of atorvastatin microsponges loaded emu oil emulgel

Formulations of emulgel were prepared by taking three different concentrations (G_1 & G_2) of carbopol 934 (1% & 2%) respectively. Best optimized batch was selected for formulation of emulgel. Different formulations were prepared using varying amount of gelling agent and penetration enhancers. The method only differed in process of making gel in different formulation. The preparation of emulsion was same in all formulations. The gel phase in the formulations was prepared by dispersing Carbopol 934 (2g) in purified water (100 ml) (G_2) with constant stirring at a moderate speed using mechanical shaker, then the pH was adjusted to 6.5-7.4 using triethanolamine (TEA). The oil phase of the emulsion was prepared by dissolving span 20 (1g) in light liquid paraffin while the aqueous phase was prepared by dissolving tween 20 (0.5g) in purified water. Methyl paraben (0.03g) and propyl paraben (0.01g) were dissolved in propylene glycol (5g) and were mixed with the aqueous phase. Emu oil (5% w/w to D.W) was mixed in oil phase. Both the oily and aqueous phases were separately heated to 70–80^{O}C and then the oily phase was added to the aqueous phase with continuous stirring until it becomes cooled to room temperature. The obtained emulsion was mixed with the gel in 1:1 ratio with gentle stirring to obtain the emulgel [30] and in above emulgel the formulations of microsponges were mixed with continuous stirring.

Characterization of Emulgel

Physical examination: The prepared emulgel formulations were inspected visually for their color, appearance and consistency [30].

Rheological study: Viscosity measurements were done on Brookfield viscometer (LV DVE, Brookfield Engineering Corporation, USA) by selecting spindle number 64 and speed 50 rpm. Emulgel (50 g) was kept in 50 ml beaker which was set till spindle groove was dipped and dial reading was measured after three minutes. From the reading obtained, viscosity was calculated by using factor. The procedure was repeated three times and observations were recorded as mean ± SD [23].

Texture analysis profile: Texture Profile Analysis (TPA) was performed using a Brookfield CT3 Texture Analyzer in compression mode by using spreadability accessory (TA-SF). Optimized gel Formulation G2 was filled into the female probe, taking care to avoid air pocket into the samples. A conical analytical male probe (35 mm diameter of 45°) was forced down into each sample at a defined rate (1 mm/s) and to a defined depth (10 mm). At least two replicate analyses of sample were performed at temperatures of 35^{0}C. From the resulting force–time plots, the hardness (the force required to attain a given deformation), cohesiveness (the work required to deform the emulgel in down movement of probe) and adhesiveness (the work necessary to overcome the attractive forces between the surface of the sample and the surface of the probe) were derived. Spreadability was calculated from the energy required to deform the sample or from the hardness of the sample [28].

Homogeneity and grittiness: A small quantity of gel was pressed between the thumb and the index finger, the consistency of the gel was noticed (whether homogeneous or not). Also, the homogeneity can be detected when a small quantity of the gel was rubbed on the skin of the back of the hand. The grittiness of prepared gel is also

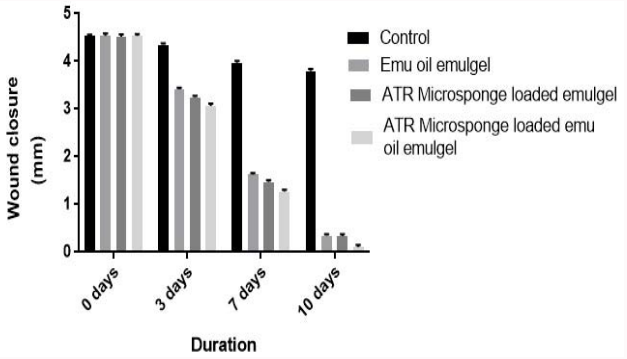

Figure 8: % Wound closure.

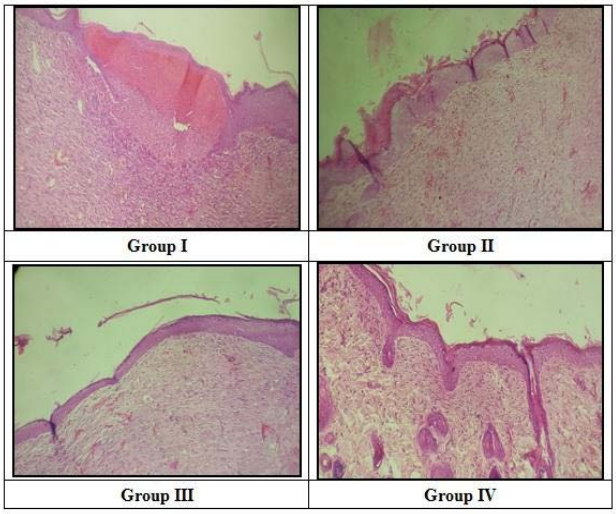

Figure 9: The finding of histopathology after 10 days.

observed in the same manner [31,32].

Drug content (emulgel): Emulgel formulations of 1.0 g was taken in 100 ml volumetric flask containing 20 ml of methanol and stirred for 30 minutes allowed to stand for 24 hours in case of microsponge loaded emulgel formulations. The resultant solution was filtered through membrane filter. The absorbance of the solution was measured spectrophotometrically at 246 nm using placebo gel solution as reference [33].

***In vitro* diffusion study:** *In vitro* release study was carried out using Franz diffusion cell with a receptor compartment (25 ml) and an effective diffusion area of 3.14 cm^2. Cellulose dialysis membrane 150 LA401-1MT (Himedia, Mumbai, India) was soaked in receptor media (phosphate buffer, pH 7.4) for 24 h before the experiment. A predetermined amount of gel containing microsponges was placed on the donor side. The receptor medium was continuously stirred at 50 rpm and thermo stated at 32 ± 0.5°C with a water jacket. At predetermined time intervals, 1 ml samples were withdrawn from the receiver compartment and replaced with an equal volume of fresh buffer. The collected samples were analyzed by UV spectrophotometer [23].

***In vivo* studies:** Healthy Sprague Dawley male rat weighing between 250-300 g were selected. All experimental procedures and protocols used in this study were reviewed and approved by the International Animal Ethics Committee of JSPM'S Rajarshi Shahu College of Pharmacy & Research, Tathawade, Pune constituted under Committee for Purpose of Control and Supervision of Experiments on Animals. Animals were divided into 4 groups with 3 rats in each group as follows: Group I: Control with no treatment, Group II: Wound treated with Emu oil emulgel, Group III: Wound treated with ATR microsponges loaded emulgel and Group IV: Wound treated with ATR microsponge loaded Emu oil emulgel.

For induction of wound, rats were anesthetized and their back hairs were shaved and the application field was outlined with marking pen just before removing skin. Wound in each rat were created by using 4 mm biopsy punch. All wounds were cleaned daily with sterile normal saline. After cleaning, gel was applied evenly in sufficient amount to cover the entire wound once a day. Wound area was measured on the days 0, 3, 7, 10 and area of each wound was calculated accurately [22].

$$Percent\ Wound\ closure = \frac{wound\ area\ on\ 0\ day - wound\ area\ on\ nth\ day}{wound\ area\ on\ 0\ day} \times 100 \quad (3)$$

where n is the number of days (0, 3, 7 and 10)

Statistical analysis: All results are presented as mean ± SEM. Statistical comparisons between control (Group I), Emu oil emulgel (Group II), ATR Microsponges loaded emulgel without emu oil (Group III) & ATR microsponges loaded emu oil emulgel (Group IV) were performed. Multiple groups were analyzed by two way analysis of variance (Two Way ANOVA) followed by appropriate Sidak's multiple comparison tests to determine statistical significance using Graph pad prism 7.0. ($P \leq 0.001$ was considered significant). *In vivo* experiments were performed at least in triplicate (n=3).

Histology of Wound Granulating Tissue: After completion of wound healing test (10 days), rats were sacrificed. Wounded area of skin containing dermis and hypodermis was isolated, carefully trimmed with cutter, and fixed in 10% neutral formalin solution. After paraffin embedding, 3 to 4 µm sections were taken and stained with haematoxylin and eosin (H and E) for study of tissue appearance and Masson's trichrome stain for the evaluation of connective tissue fibril structure and toluidine blue for the evaluation of mast cell. All the slides were observed under light microscopic [22].

Stability studies

The prepared microsponge emulgel was packed in container (20 g) and subjected to stability studies according to ICH guidelines at 40 ± 2°C/75 ± 5% RH for a period of 1& 3 month. Samples were withdrawn at 15-day& 30days time intervals and evaluated for physical appearance, pH, rheological properties & drug content [28].

Results and Discussion

Optimisation of quasi-emulsion solvent diffusion method

The preparation methods of microsponges are limited in the means of complexity and cost. Quasi-emulsion solvent diffusion method serves an alternative way for preparing microsponges [4,34]. This method seems to be promising for the preparation of atorvastatin microsponges with being easy, reproducible, rapid method and has an advantage of avoiding solvent toxicity [4].

For the optimisation of the formulations and process factors of microsponges prepared by quasi-emulsion solvent diffusion method,

Table 1: Optimum microsponge production parameters with quasi-emulsion solvent diffusion method.

Specification	Optimum values
Drug: Polymer ratio	1:01
Amount of drug (mg)	500
Amount of emulsifying agent PVA (cold)	0.75%
Inner phase solvent	Dichloromethane
Amount of inner phase solvent (mL)	5
Amount of water in the outer phase (mL)	100
Stirrer type	Mechanical stirrer
Stirring rate (rpm)	1000
Stirring time (min)	75

the effects of drug: polymer ratio (1:1, 1:2 and 2:1), internal phase solvent amount (5, 10 and 15 ml), external phase (0.50% and 0.75 %) stirrer type (mechanical stirrer) and stirring speed (500, 1000 and 1500 rpm) on the formation of microsponges were investigated for trial batches.

From above investigation Preliminary trial batches exhibited parameters suitable for the formulation of microsponges are: external phase PVA solution (750 mg PVA in 100 ml of distilled water i.e. 0.75% w/v), and stirring speed of 1000 rpm for 75 min. Drug to polymer ratio and internal phase volume (dichloromethane) were further optimized by applying factorial design (Table 1).

As the internal phase volume was increased in formulations MSP 5 (5ml), MSP 3 (7.5ml) and MSP 7 (10ml) at same polymer concentration, the entrapment efficiency of microsponge was increased was depicted in MSP 5 (82.86 ± 1.16%), MSP 3 (83.70 ± 0.81%) and MSP 7 (84.7 ± 0.91%) shown in Table 2. This is due to increased dissolution of drug and polymer in the internal phase to form more uniform dispersion. Compare to increase in internal phase, when the polymer concentration was increased (500 mg to 1000mg) this resulted in better entrapment efficiency as obtained in MSP 4 (polymer 1000 mg) 90.10 ± 0.65% which was found to be greater than MSP 7 (polymer 1000 mg) of 84.7 ± 0.91% entrapment efficiency.

The drug polymer concentration and internal phase volume (dichloromethane volume) has a leading role in particle size of the microsponge. Results showed minimum particle size of 70 ± 0.40 µm in formulation MSP 7 (polymer 500 mg, dichloromethane 10ml) which was less than the MSP 5 (polymer 500 mg, dichloromethane 5ml) having 86 ± 1.15 µm. It was observed that at same polymer concentration with increase in the internal phase volume there is decrease in the particle size of microsponge. But as we increase the polymer concentration at same internal phase volume there is increase in the particle size of microsponge like in MSP 5 (polymer 500 mg, dichloromethane 5ml) showed 86 ± 1.15 µm, MSP 6 (polymer 750, dichloromethane 5ml) showed 102 ± 1.0 µm and formulation MSP 8 (polymer 1000 mg, dichloromethane 5ml) showed 140 ± 0.57 µm. From these observations it was concluded that increased in polymer concentration at same internal phase volume (dichloromethane) resulted in increased particle size shown in table 2.

Effect of drug polymer concentration and internal phase volume on percentage drug release showed that as the polymer concentration was increased from 1 part to 2 parts, the percentage drug release was decreased. Along with polymer concentration as the internal phase volume increased (5ml to 10ml) the percentage drug release was found to be decreased. The reason for this could be decreased viscosity of the internal phase. Formulation MSP 5 having the minimum polymer concentration (500 mg) and minimum internal phase volume (5ml) was resulted in percentage drug release of 48.5 ± 0.40%. This was more comparable with formulation MSP 4 with minimum polymer concentration (500 mg) and highest internal phase volume (10ml), which showed percentage drug release of 33.56 ± 1.86%. When the polymer concentration was highest as in formulation MSP 8 (1000 mg) with minimum internal phase volume (5ml), showed 35.5 ± 0.69% percentage drug releases. Analysis of variance (ANOVA) was applied to determine significance and magnitude of effects of independent variables and their interactions on Y1, Y2, and Y3. Responses of different batches obtained by using factorial design are expressed individually in Equations. 4, 5, and 6:

$$Y1=+85.14+1.95X_1+1.74X_2+0.87X_1X_2+0.78X_1^2-0.32X_2^2 \quad (4)$$

A quadratic regression Eq. (4) obtained for Y1 demonstrated positive influence of both independent variables and their interaction with significant F value 140.22. Positive influence of both the independent variables on entrapment efficiency was due to high solubility of drug and polymer in dichloromethane to form uniform dispersion, creating porous structure to entrap drug shown in Figure 1-a.

$$Y2 =+88.22+23.67X_1-12.33X_2-5.00X_1X_2+13.67X_1^2-0.33X_2^2 \quad (5)$$

A quadratic regression Eq. (5) generated for Y2 (Particle size)

Table 2: Characterization of atorvastatin microsponges (MSP 1-MSP 9).

Batch	Drug: Polymer	DCM	PVA Solution (0.75% w/v) (ml)	Drug content (%)	Production yield (%)	EE (%)	Particle Size (µm)	CDR (%)
MSP 1	1:1.5	7.5	100	41.21±1.01	89.49±0.93	85.21± 0.49	82±0.77	40.60 ± 1.13
MSP 2	1:1.5	10	100	37.05±0.97	87.21±0.90	86.43± 0.97	80±0.98	40.26 ± 2.02
MSP 3	1:1	7.5	100	43.22±0.96	88.26±0.87	83.70± 0.81	78±0.96	45.73 ± 1.87
MSP 4	1:2	10	100	41.41±1.13	88.09±1.06	90.10± 0.65	104±1.2	33.56 ± 1.86
MSP 5	1:1	5	100	45.97±1.02	90.58±0.86	82.86± 1.16	86±1.15	48.50 ± 0.40
MSP 6	1:1.5	5	100	44.18±1.13	92.04±0.82	83.13± 0.75	102±1.0	42.49 ± 1.24
MSP 7	1:1	10	100	42.17±1.21	86.20±1.00	84.70± 0.84	70±0.40	44.68 ± 1.86
MSP 8	1:2	5	100	43.83±0.96	92.23±1.07	84.79± 0.91	140±0.57	35.50 ± 0.69
MSP 9	1:2	7.5	100	42.32±1.27	90.07±1.00	88.05± 0.90	132±0.98	34.72 ± 0.81

Table 3: Comparative drug release data of emulgels.

Time (min)	%DR of Emu oil emulgel	%DR of ATR MSP loaded emulgel	%DR of ATR MSP emu oil emulgel
0	0	0	0
60	7.66±0.11	1.54±0.33	2.25±0.66
120	14.22±0.66	6.42±0.41	9.76±0.67
180	24.01±0.79	14.88±1.13	27.37±1.20
240	27.93±1.07	18.97±0.50	35.71±0.65
300	29.67±0.51	24.09±0.81	38.09±0.71
360	35.38±0.78	32.25±0.70	43.92±0.96

Table 4: Comparative study results of in *vivo* study in different animal groups.

Day	Group I (Control)		Group II (Emu oil emulgel)		Group III (ATR microspongesemulgel)		Group IV (ATR Microsponges loaded emu oil emulgel)	
	Wound area (mm)	Wound closure (%)	Wound area (mm)	Wound closure (%)	Wound area (mm)	Wound closure (%)	Wound area (mm)	Wound closure (%)
0	4.52±0.017 (NS)	0	4.53±0.029 (NS)	0	4.52±0.029 (NS)	0	4.53±0.023 (NS)	0
3	4.34± 0.0.17 (***)	3.98	3.41±0.017 (***)	24.72	3.24±0.017 (***)	28.31	3.02±0.029 (***)	33.18
7	3.97±0.023 (***)	12.16	1.62±0.017 (***)	63.23	1.50±0.020 (***)	66.81	1.21±0.024 (***)	73.23
10	3.80±0.023 (***)	15.92	0.39±0.017 (***)	91.62	0.34±0.020 (***)	92.47	0.08±0.023 (****)	98.23

was increased with increase in the polymer concentration (X_1), this was represented in equation showing the positive sign for X_1, dichloromethane volume (X_2) have the negative effect as showed in equation, as the dichloromethane volume increased the particle size decreased and having significant F value 22.65. The individual and combined effects of factors on the particle size are further explained with the help of 3D response surface plots which are showed in Figure 1-b suggested the correlation between two variables. The (Figure 1-b) showed the increase in the particle size with increase in the polymer concentration, and decrease in the particle size with increase in the dichloromethane volume. The combine effect of the polymer concentration and dichloromethane volume on particle size is shown in Figure 1-b. It was observed that with minimum dichloromethane volume and highest polymer concentration resulted in the formation of particles with high particle size, and as the dichloromethane volume and polymer concentration was decreased the particle size was found to be decreased.

$$Y3 = +40.77 - 5.85X_1 - 1.33X_2 + 0.47X_1X_2 - 0.66X_1^2 + 0.49X_2^2 \quad (6)$$

The quadratic regression Eq. (6) for Y3 showed Percentage drug Release (Y_3) was decreased with increase in concentration of polymer(X_1), this was represented in equation showing the negative sign for X_1, dichloromethane volume (X_2) have the negative effect, as the dichloromethane volume increased the percentage drug release decreased with significant with F value 214.72.The individual and combined effects of factors on the percentage drug release are further explained with the help of 3D response surface plots as showed in Figure 1-c. The (Figure 1-c) showed the combined effect of the polymer concentration and dichloromethane volume on the percentage drug release the low polymer concentration and low dichloromethane volume resulted in maximum percentage drug release of formulation, when compared with the high polymer concentration and low dichloromethane volume.

In Vitro Drug Release Studies

Drug release was calculated by performing the *in vitro* drug release study. Formulation MSP 4, MSP 8 and MSP 9 having the higher concentration of polymer resulted in release of 3.40 ± 1.48%, 4.38 ± 0.70% and 4.92 ± 1.31% of drug, respectively in first 120 min, followed by 16.97 ± 1.29%, 15.57 ± 1.56% and 15.68 ± 1.06%, respectively in 240min. and finally 33.56 ± 1.86%, 35.5 ± 71% and 34.72 ± 0.81%, respectively after 360 minute.

Formulation MSP 1, MSP 2 and MSP 6 showed 4.29 ± 1.02%, 3.64 ± 0.95%, 5.24 ± 1.15% of drug release, respectively in first 120 min followed by 21.03 ± 1.61%, 20.42 ± 1.18% and 21.46 ± 1.36% after 240 min and at the completion of 360 min resulted in 40.60 ± 1.13%, 40.26 ± 2.02% and 42.49 ± 1.24% release respectively.

Formulation MSP 3, MSP 5, MSP 7 having lower concentration of polymer resulted in release of drug compare to the other formulations showed 5.38 ± 0.90%, 6.07 ± 0.82%, 5.65 ± 0.87% of drug release in first 120 min followed by 20.23 ± 1.19%, 19.79 ± 1.24% and 20.43 ± 0.98% after 240 min and 45.73 ± 1.87%, 48.50 ± 1.36% and 44.68 ± 1.86% of drug release respectively after 360 min due to of low polymer concentration in the formulation. Form this it was observed that increasing polymer concentration decrease the drug release from the microsponges and resulted in sustained release of drug form (Figure 2).

Table 5: Stability analysis of formulated emulgel G2 at 40°C ±2°C/75% RH ± 5% RH for 30 Days.

Parameter	Optimized ATR microsponges loaded emulgel		
	40°C ±2°C/75% RH ± 5% RH		
	0 Day	15 Day	30 Day
Color	White Creamy	White Creamy	White Creamy
Odour	NO	NO	NO
pH measurement	6.98±1.11	6.96±1.03	6.96±0.96
Viscosity	10530±1.32	10518±1.26	10498±1.41
% Drug Content	96.20±0.83	96.14±0.82	96.11±0.89
Skin Irritation	NO	NO	NO

Characterization and Evaluation of Micro Sponges

Drug content: Less amount of dichloromethane created more porous structure to accommodate more amount of drug, therefore more drug content in MSP 5 than MSP 7. Drug content was found within 37-46% (Table 2).

Percentage yield: Effect of drug: polymer concentration and internal phase volume on percentage yield showed that as the polymer concentration was increased from 1 part to 2 parts the percentage yield was also increased. But along with polymer concentration as the internal phase volume (dichloromethane) increased (5-10 ml) the percentage yield was found to be decreased. The reason for this could be decreased viscosity of the internal phase. Formulation MSP 5 having the minimum polymer concentration (500 mg) and minimum internal phase volume (5 ml) resulted in percentage yield of 90.58 ± 0.86%. This was more comparable with formulation MSP 7 with minimum polymer concentration (500 mg) and highest internal phase volume (10 ml), which showed percentage yield of 86.20 ± 1.00%. When the polymer concentration was highest as in formulation MSP 4 (1000 mg) with minimum internal phase volume (5 ml), showed 88.09 ± 1.06% production yield. From these results, it was concluded that with increase in polymer concentration the production yield increases but it is decreased with increase in the internal phase volume (Table 2).

Differential Scanning Calorimetry (DSC) and Scanning Electron Microscopy (SEM): DSC is a basic method to determine crystallanity and amorphous state of the drug in compound. Figure (3-a) shows DSC thermogram with sharp endothermic peak at 147.5°C for ATR, corresponding to its melting point, Figure (3-b) shows thermogram with sharp endothermic peak at 61.60°C for Eudragit RS 100. Figure (3-c) shows the sharp DSC peaks of ATR and Eudragit RS 100 transform to a broad endothermic peak with shift in melting points toward lower value at 143.5°C for ATR-MSP. This indicates that crystalline form of drug was converted into amorphous form with increased in solubility. SEM of ATR-MSP revealed uniform, spherical shape, and porous nature (Figure 4a-b).

Optimization: Optimization Based on result of Y1, Y2, and Y3 optimized formula was achieved good entrapment efficiency, minimum particle size and Cumulative drug release having desirability 1.0. Formulation MSP 5 was considered to be optimized formulation with entrapment efficiency 82.86 ± 1.16%, particle size of 86 ± 1.15µm and % cumulative drug release 48.5 ± 0.40%.

Characterization of Micro sponges Loaded Emulgel

Physical study, pH, viscosity and drug content measurement: The developed emulgel formulations were white creamy, viscous preparations with smooth and homogeneous appearance. The pH and viscosity values of prepared emulgels were found to be of 6.98 ± 1.10 and 10231 ± 1.23 cps (50 rpm using spindle no. 63) respectively. The drug content was 96.24 ± 0.97%. The drug content uniformity test for all the emulgels indicated that the drug was uniformly dispersed in emulgel formulations.

Texture profile analysis: Texture profile analysis of emulgel shows the spreadability in term of cohesiveness and adhesiveness. Spreadability denotes the extent of area to which the emulgel readily spreads on its application. The greater the viscosity, lesser the spreadability [35] and more is the retention of emulgel on the skin. Texture profile analysis of the formulated emulgel (MSP 5) showed the hardness of 154 g which was the maximum force value in the graph. The area under the positive curve is the energy required to deform the sample is 5.3 mJ (hardness work done). The hardness work done and firmness show the spreadability of sample. Higher value of firmness and hardness work indicated less spreadable sample conversely the less value indicate more spreadable sample. The maximum negative force (87 g) on the graph indicated sample adhesive force; the more the negative value the more 'sticky' the sample. The area under the negative part of the graph is known as adhesiveness (4.6 mJ) which is the energy required for breaking probe sample contact shows in Figure 5. These results expressed the retention time of the emulgel on the site of application [36].

***In vitro* diffusion study:** The release of the drugs from its emulsified gel formulation can be ranked in the following descending order: ATR microsponges loaded emu oil emulgel > emu oil emulgel>ATR microsponges loaded emulgel without emu oil where the amounts of the drug released after 360 min were 43.92 ± 0.96, 35.38 ± 0.78 and 32.25 ± 0.70 respectively (Table 3). From above results, it can be concluded that emu oil helps to increased drug diffusion from ATR microsponges loaded emu oil emulgel compared with ATR microsponges loaded emulgel without emu oil and emu oil emulgel (Figure 6).

***In vivo* study:** The wound area and percentage wound closure was calculated for all animal groups such as control (Group I), emu oil emulgel (Group II), ATR microsponges loaded emulgel (without emu oil) (Group III) and ATR microsponges loaded emu oil emulgel (Group IV).

The percentage wound closure is defined as progressive reduction in wound area. It was observed that, among all the groups, group IV showed 33.18% wound closure on day 3 compared to group I, group II and group III which showed 3.98%, 24.72% and 28.31%, respectively.

It was observed that from day 3 to day 7, group IV which received only ATR microsponges loaded emu oil emulgel application showed 40.05% faster wound closure rate and after day 7 showed 73.23% wound closure. While group III which received application of ATR microsponges emulgel (without emu oil) showed 38.5% wound closure rate from day 3 to day 7 and after day 7 showed 66.81% wound closure. Group II which receives application of emu oil emulgel showed wound closure rate of 38% from day 3 to day 7, while this rate was decreased to 27 % from day 7 to day 10. This indicates that, group IV showed rapid wound healing activity compared to the other groups. The study was continued upto 10 days, where processes of scar formation have been initiated shown in Table 4.

It was illustrated that ATR microsponges loaded emu oil emulgel shows good healing properties as well as more wound closing as compared to the ATR microsponge emulgel without emu oil and emu oil emulgel. So, from above study we said that emu oil helps in enhanced wound healing in animal model when combined with ATR microsponges loaded emulgel (Figure 7). Further histology was carried out to confirm the result.

Statistical analysis: All results are presented as mean ± SEM. Statistical comparisons between control (Group I), Emu oil emulgel (Group II), ATR Microsponges loaded emulgel without emu oil (Group III) & ATR microsponges loaded emu oil emulgel (Group IV) were performed. Multiple groups were analyzed by two way analysis

of variance (Two Way ANOVA) followed by appropriate Sidak's multiple comparison tests to determine statistical significance. When all four groups were compared with each other on 0 day its shows statistically non-significant ($P>0.99$) activity because on 0 day wound created and no treatment should be given to the animal model. Then treatment should be carried out from 1st day to 10th day. When Group I, Group II, Group III and Group IV compared with each other on 3rd day, 7th day and 10th day shows statistically significant ($P \leq 0.001$). ATR microsponges loaded emu oil emulgel (Group IV) results were more statistically significant than control (Group I), Emu oil emulgel (Group II) and ATR microsponges loaded emulgel without emu oil (Group III) compared on 3rd day, 7th day and 10th day. Group IV 10th day results shows more significant wound closure ($P \leq 0.001$) as compared with other three groups on 3rd day and 7th day results respectively which are shown in Table 4 and 8.

Histopathology: Histopathology of wound tissue of control (Group I) showed large number of inflammatory infiltrate and granulation tissue compared to animal models treated with ATR microsponges loaded emu oil emulgel (Group IV) which showed mild inflammatory infiltrate, while animal models treated with emu oil emulgel (Group II) and ATR microsponges loaded emulgel (Group III) showed moderate inflammatory infiltrate. Complete epithelisation was observed in group II, III and IV as shown in Figure 9. Control group showed formation of blood vessels which indicated that neovascularization is in progress. Haemorrhages were seen in group II and III, indicated that neovascularization is still in process, while haemorrhages were not observed in group IV, indicating that process of neovascularization is nearly complete. However, in group IV, formation of hair follicles can be seen. Collagen arrangement was seen to scattered in control, while irregular arrangement was seen in group IV. However, regular arrangement was seen in group II and III animals.

Stability Studies

Stability studies were performed as per ICH guidelines. The results indicate that there was no evident change in the physical appearance and drug content of formulations after subjecting them to stability studies. Optimized formulation G2 was chosen for stability studies. At fixed time interval drug content determination of these formulations showed that there were no significant changes in the values when compared to the initial formulations. Thus we may conclude that the drug does not undergo degradation on storage (Table 5).

Conclusion

The study was focused to formulate microsponge loaded emulgel of atorvastatin calcium and emu oil to treat wound and release the drug in epidermis in controlled manner with assurance that the drug remains primarily localized. Atorvastatin calcium reduces the neuropathy and ischemia restoring endothelial function through nitric oxide, so atorvastatin calcium loaded microsponges were emu oil as penetration enhancer, formulation showed that its prohealing action. In the excision wound model, atorvastatin calcium microsponges loaded emulgel containing emu oil shows a significant increase in the percentage wound closure of wounds. The formulated microsponge loaded gel showed extended drug release hence, it can be better supportive drug delivery in treatment of wound. On the basis of obtained data, it can be concluded that 5% emu oil emulgel of Atorvastatin calcium loaded microsponges has excellent wound healing activity.

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