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Development, characterization and optimization of methotrexate-olive oil nano-emulsion for topical application

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Abstract: The chronic inflammatory conditions like psoriasis has an increased prevalence and is linked with various associated life threatening disease conditions. The main objective of this project was to developed a methotrexate-olive loaded nano emulsion. The formulation was assessed for various parameters including Thermodynamic Stability, physico-chemically characterization, drug release kinetics and entrapment efficiency and *in vitro*/ *in vivo* skin permeation analysis. Final optimized formulation had a particle size 18.27±5.78 nm with a PDI of 0.25±0.01, whereas the average entrapment efficiency of formulation was 74.68±2.1%. The release kinetics suggested 97.72% drug release at pH 5 after 20 hrs. The FTIR data confirmed that the chemical structure of drug is retained with efficient loading into the formulation. Permeation data showed that an average of 79.23±3.6µg/cm^2 of methotrexate was permeated from the nano emulsion with an average flux of 2.326±0.45µg/cm^2/h after 24 hrs. Finally *in vivo* studies on rabbit skin confirmed that the structural changes of intercellular lipid layers in the stratum corneum are not responsible for enhanced skin permeation of methotrexate loaded nano emulsion. It was concluded that olive oil based MTX-NE is suitable for topical application and can be used for management of psoriasis.

Keywords: Psoriasis, topical delivery, skin permeation, *in vivo* analysis.

INTRODUCTION

Psoriasis is a chronic inflammatory disease affecting about 1-3% a world population. This life-long disease has an equal gender distribution with incidence rate upto 50 - 140 new cases per one lac people per year (Pinto *et al.*, 2014). Although, psoriasis itself is not a life threatening condition, however, it may be allied with various life threatening disease conditions (Chandran and Raychaudhuri, 2010). It is linked with high levels of morbidity and ailment leading to decrease patient quality of life.

The management protocols of the psoriasis differ based on severity index of disease. Topical agents represents first line typically sufficient active treatments for psoriasis to combat mild to moderate type of psoriasis(Chong *et al*., 2013). Mild to moderate psoriasis is named if disease affected the surface area of body <10% (Mitra and Wu, 2010). The Dithranol, coal tar, analogs of vitamin, corticosteroids, retinoid and caritolytic agents like salicylic acid (Montaudié *et al.*, 2011; Rahman *et al.*, 2012; Pinto *et al.*, 2014) constitutes topical agents commonly used for management of psoriasis. Likewise, phototherapy and systemic therapy is needed when, the effects of topical therapy are sub optimal or when the intensity of psoriasis limits the use of topical agents (Laws and Young, 2012). Systemic therapies in treatment of psoriasis includes biological and non-biological treatment strategies as mono therapies or drug combinations (Chong *et al.*, 2013).

Methotrexate, orally administered retinoid and cyclosporine are widely accepted systemic agents from non-biological categories. Amongst all, the methotrexate administered via oral and parental routes has shown significant results in treatment of psoriasis owing to its anti inflammatory as well as inhibition of epidermal cell proliferative nature (Shen *et al.*, 2012; Micha *et al.*, 2011). However, large number of toxicities have been reported including liver toxicity, GIT related side effects including, vomiting, diarrhea and stomatitis. However concomitant supply of folic acid to the patient could minimize the occurrence of some side effects (Montaudié *et al.*, 2011).

Nano dermatology is an area of augmented interest that can be considered as nicer solution for management and treatment of psoriasis (Saraceno *et al.*, 2013). The development of nano scale systems offer sustained drug

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release over extended period of time thus shielding it from degradation. This is advantageous by virtue of attaining maximum therapeutic effect and minimizing drug related toxicities based on clearance and over dose (Papakostas *et al.*, 2011). Moreover such therapeutical strategies help to reduce drug administration frequency thus increasing patient compliance (Gupta *et al*., 2012). The nano emulsions are thermodynamically stabilized dispersion of o/w that facilitate drug delivery across the skin primarily by increasing the concentration gradient. They also alter the skin barrier function due to the presence of surfactants that are well known penetration enhancers (Kreilgaard, 2002). Further, the nano emulsions present numerous advantages over other carriers for the colloidal delivery of drugs in terms of ease of preparation, high solubilization capacity for lipophilic and hydrophilic drugs, long term stability as well as improved dermal drug delivery.

During current project, a stable oil in water methotrexate loaded nano emulsion was developed to enhance the local effect.

MATERIALS AND METHODS

Chemicals

All chemicals used during current investigation were of analytical grade and purchased from Sigma Aldrich (USA), Merck (Germany) and Acros Organics (Belgium). Methotrexate was kindly gifted by Wilson Pharmaceuticals, Islamabad. The olive oil was purchased commercially from local Market (Marhaba Industries, Pakistan)

Pre formulation studies

Methotrexate solubility determination of. Λmax

The solubility studies of drug and other constituents were determined by method developed by Sobhani *et al.*, (2015). Briefly the methotrexate was added in excess into 2ml of olive oil, surfactant, co-surfactant and water contained in capped vial. The mixture was thoroughly mixed for 5min by means of vortex mixer and then incubated at 37±10^{O}C for 72hrs using shaking water bath in order to obtain equilibrium for the formation of uniform dispersion. Centrifugation was done at 10,000 rpm for 10min and then supernatant was collected. Methotrexate concentration was determined by UV-Vis spectrophotometer at 303nm wavelength. For the determination of λmax of methotrexate, weighed quantity of drug was dissolved in phosphate buffer of pH 7.4.

Preparation of methotrexate loaded nano emulsion (MTX-NE)

A high shear homogenization technique was employed to formulate methotrexate loaded nano emulsion. The formulation was comprised of olive oil as oil phase whereas Tween 80 as well as PEG 400 was used as surfactant and co-surfactant respectively. To constitute nano emulsion, oily phase was prepared by subjecting olive oil, PEG 400 and methotrexate to magnetic stirring at 700 rpm for 1hr at 70^{O}C. The aqueous phase was then prepared by adding Tween 80 by continuous stirring to distilled water for 1hr at 70^{O}C. There was drop wise addition of oily phase into aqueous phase. This process was subjected to continuous magnetic stirring to get final formulation. Formulation optimization was attempted by altering concentration of both surfactant and co-surfactant against clarity and homogeneity. To check the phase separation of the formed nano emulsion, the formulation was sonicated for 15min followed by its placement at room temperature (Mahdi *et al.*, 2011). Same procedure was used to formulate blank nano emulsion formulation (Table 1).

Investigation of thermodynamic stability of methotrexate loaded nano emulsions

Dispersion stability of nano emulsions at various conditions was checked in order to determine the reconciliation of nano emulsions claim to be stable in thermodynamic and kinetic systems.

Freeze thaw cycle

The optimized preparation was stored at -20^{O}C for 24hrs followed by its placement at room temperature. Formulation returned to the original state within 2-3 min showed thermodynamic stability. Triplicates were examined (Mahdi *et al.*, 2011).

Centrifugation studies

The protocol of centrifugation studies involves subjecting nano emulsion formulation to centrifugation at 3500 rpm for duration of 30min. This was done to note any turbidity formation and phase separation.

Heating cooling cycle

To check thermal stability, nano emulsion formulation was subjected to alternate heating and cooling cycle. Temperature was set at 4^{O}C in the refrigerator for cooling cycle whereas heating cycle employed 45^{O}C temperature. Duration of this study was 48hrs. Triplicates were examined (Bali *et al*., 2011).

Characterization of optimized methotrexate nano emulsion

The optimized formulation of methotrexate loaded nano emulsion was physico-chemically characterized for size and size distribution, charge, Poly Dispersity Index (PDI), zeta potential, pH, entrapment efficiency, drug content, morphology, drug release, drug permeation and retention respectively (Czajkowska-Košnik *et al.*, 2015).

Size and size distribution

Prepared formulation was subjected to particle size and particle size distribution determination by means of

dynamic light scattering techniques using zetasizer Nano ZS 90 (Malvern Instruments; Worcestershire, UK). It is provided with a software of version 6.34, laser having wavelength of 635 nm possessing detect angle of 90^{O}. A mixture of 10µl of methotrexate loaded nano emulsion and deionized water was subjected to vortexing for 2 minutes to form uniform dispersion. The diluted formulation was then observed in triplicates at temperature of 25±0.1^{O}C. Final results were expressed in mean±SD (Din *et al.*, 2017).

Zeta potential and poly dispersity index (PDI)

These two parameters of Methotrexate loaded nano emulsions were determined by Nano zetasizer 90 (Malvern Instruments; Worcestershire, UK) at 25±1^{O}C by loading 700µl quantity of the formulation in capillary tube folded and integrated with gold electrode. The process was conducted in triplicates and results in mean±SD were expressed.

pH

The pH of the prepared formulation was determined by pH meter (Accumet meter 21039, Denver Instruments, USA) at 25±1^{O}C. Firstly calibration of the instrument was done by buffer solutions of known pH 3, 7 and 9. Results were evaluated as mean ± SD of triplicate results.

Entrapment Efficiency

The methotrexate loaded nano emulsion formulation was subjected to centrifugation at 20°C to obtain complete separation between the nano emulsion formulations that occupied the filter unit and the aqueous phase corresponding to filtrate. After complete separation, the obtained filtrate was used to quantify non incorporated Methotrexate by using UV–Vis spectrophotometer (UV-1601, Shimadzu, Japan) at λmax 303nm. A standard curve of Methotrexate in phosphate buffer solution of PH 7.4 was used for the quantification of methotrexate and the results expressed as mean ± SD (n=3). The results were compared to blank formulation used as control. Taking in to account the drug initially added to the nano emulsion formulation and subtracting the free methotrexate remaining in the filtrate it was possible to determine the amount of drug incorporated in the nano emulsion formulation, thus the entrapment efficiency by the following equation (Agnihotri, *et al,*, 2006).

Drug entrapment efficiency (DEE)=

$$[(addeddrug - freedrug)/(drugadded)] * 100$$

Drug content analysis

The drug content of prepared methotrexate loaded nano emulsions was determined using UV-spectrophotometer. About 2ml of methotrexate loaded nano emulsion was incorporated in Eppendorf tube (Cat no 037108; SCILOGEX, USA). Centrifugation (D3024, SCILOGEX, USA) at 13000 rpm for duration of 15 minutes was done. The supernatant was collected. Approximately 0.5 ml of supernatant was taken and diluted with pH 7.4 phosphate buffer. The sample was stirred at 1000 rpm for 10 minutes using magnetic stirrer. Absorbance was determined at λmax of 303 nm using UV-Vis spectrophotometer (UV-1601, SHIMADZU, Japan). To extract entrapped drug in methotrexate loaded nano emulsion, 1 ml of methanol and sediment was subjected to vortexing for 5 min. The mixture was diluted with phosphate buffer of pH 7.4. It was then stirred for 10 minutes. Again the absorbance was calculated at λmax of 303 nm by using UV-Vis spectrophotometer (UV-1601, SHIMADZU, Japan). Drug loads of both supernatant and sediment were employed to calculate the drug content (Nawaz and Wong, 2017).

Drug Content = Drug in supernatant + Drug in sediment

Morphological parameters

To determine the detailed structure of methotrexate loaded nano emulsion, scanning electron microscope (SEM, JSM 910, JEOL, Japan) was used. The prepared formulation was centrifuged at 12000 rpm for 5 minutes for the removal of aqueous phase from the formulation. About 03 drops of osmium tetra oxide was mixed with sediment as fixation medium and kept at 8^{O}C for 2 hrs. Dilution of the sample was achieved by means of washing medium composed of 0.1 M phosphate buffer. These two processes (centrifugation and washing) were repeated twice. Dehydration of the sample was done with acetone. An aliquot of methotrexate loaded nano emulsion was placed on the carbon film with copper grid 400 mesh size in the liquid sample holder. Then imaging of selected areas was accomplished at accelerated voltage of 8.0 kV and level of magnification 10000X (Sood *et al*., 2014).

Drug release analysis

The release profile of methotrexate loaded nano emulsion and Methotrexate solution was determined using Tuffryn membrane. This membrane with pore size of 0.45µm acts as partitioning media between the donor and recipient compartments of Franz diffusion cell (Perme gear, Inc. No: 4G-01-00-15-12; India: area of diffusion = 1.767 cm^2). The receptor compartment was filled with 7.0 ml fresh phosphate buffer of pH 5.5 as simulated skin fluid with 32±2^{O}C temperature. 1 ml samples were withdrawn from receptor compartment at regular time intervals. This was replaced with same quantity of phosphate buffer. The samples were analyzed using UV-Vis spectrophotometer (UV-1601 SHIMADZU, Japan). The result triplicates were shown as mean± SD. The data was plotted in graph as time versus concentration. The drug release mechanism was incorporated into power law kinetic model and shown in the following equation (Yin and Li, 2011).

Power Law

$Mt/M_{\infty} = Kt^{n}$

Where

Mt and M_∞ = fraction of drug released after time t.
"K = rate constant.
n= exponential release value.
When n = 0.5, then it is Quasi-Fickian diffusion mechanism.
When n > 0.5, then release of drug occurs by anomalous, non-Fickian, case II or zero order release mechanism
When n = 0, it is zero order release mechanism or case II

Invitro skin permeation of methotrexate loaded nano emulsion formulation

Animal Skin Harvesting

Healthy male rabbit weighing 2.0 to 2.5kg were purchased from local market. All procedures relating to experimental animal use were performed by adapting the international guidelines (OECD Environment, Health and Safety). They were kept on normal diet for 7 days for the purpose of acclimatization. All animals were sacrificed by overdose of sodium pentobarbital ketamine intravenously followed by cervical dislocation. The abdomen was shaved using shaving blades and skin excised carefully. The skin was washed by using normal saline (0.9 % NaCl solution). The adhered fats were removed by scalpel. Skin was covered in aluminium foil and stored at -20ºC in deep freezer till further use. Frozen skin was thawed at room temperature for 3 hrs before use.

Skin permeation study

Franz diffusion cell assembly having vertical diffusion cell (Perme gear, Inc. No: 4G-01-00-15-12; India: diffusion area = 1.767 cm^2) was employed for performing skin permeation studies. The Franz diffusion cell was equipped with magnetic stirring as well as system of heating circulation having programmable temperature control device .Skin permeation study was done to determine the rate of drug transport across the skin as well as to predict the amount of drug localized in different layers of skin. The pre-conditioning of the rabbit skin was accomplished by skin hydration before experiment with phosphate buffer solution for 30 minutes. The receptor fluid was stirred gently by using magnetic stirrer. This stirring is done for the prevention of boundary layer effects. Receptor compartment was filled with 7 ml PBS (pH 7.4) followed by constant stirring at 500 rpm. Heating circulation system was employed to maintain 37ºC±0.5ºC. Between donor and receptor compartments, the rabbit skin was mounted such that the epidermis faced the formulation while dermis layer was towards the receptor compartment. For 1 hr mounted skin upper surface was left dry and open. Skin dermal side and the receptor medium were in contact with each other. About 1ml of the formulations was loaded in the donor compartment. The aliquots (1ml) of the sample were taken out through the sampling port at predetermined time intervals. Equal volume of fresh solution was replenished immediately to the receptor phase maintained at the same temperature. Sink environment was maintained till the end of experiment (till 24hrs). Analysis of the collected samples was done for methotrexate contents after dilution with PBS (pH 7.4) using UV-Vis spectrophotometer. Results were expressed as mean ± SD. The cumulative amount of methotrexate permeated through rabbit skin was plotted as a function of time.

The cumulative amount of methotrexate permeated per unit area was calculated by using the following equation (Zeb *et al*., 2016);

$$Qn = CnVr + \sum_{i=0}^{n-1} CiVs/A$$

Where
Qn = cumulative amount of drug permeated per unit area ($\mu g/cm^2$) corresponding to nth sample time, C_n = receptor fluid concentration of drug at nth sample, C_I = receptor fluid drug concentration at ith (n-1) sample, V_r = receptor solution volume (7 ml), V_s = withdrawn volume of sample (0.5ml), A = effective permeation area of diffusion cell ($1.767cm^2$)

The steady state flux (J_{ss})was expressed as $\mu g/cm^2$ /h. Lag time (t_{lag}) were determined from slope and x- Intercept of linear portion of graph, respectively.

Permeability coefficient (K_p) was obtained by dividing the steady state flux (J_{ss}) by the initial Methotrexate amount in donor compartment. Enhancement ration was determined by dividing the flux of the test formulation by that of the control formulation.

$$\text{Enhancement ratio} = \frac{\text{Jss of Methotrexate loaded nano emulsion}}{\text{Jss of Methotrexate solution}}$$

Permeation study results were compared with Methotrexate solution (Ioele *et al.*, 2015).

Skin drug retention studies

After completion of permeation experiment, skin was carefully removed from Franz diffusion cell, cleaned with phosphate buffer solution, tap dried with soft tissue paper, cut into small pieces of $1cm^2$ and stirred in phosphate buffer solution of pH 7.4 for 24hrs to extract retained drug from the skin. Following day, the samples were filtered through cellulose acetate filter 0.45 µ size and analyzed on UV visible spectrophotometer at 303 nm wavelengths(Panonnummal *et al*., 2017). Triplicates were carried out and results averaged.

Mechanistic studies for drug retention

For FTIR the skin samples treated with nano emulsion, gel base, and nano emulsion constituents were subjected to vibrational spectroscopic analysis by placing the skin on zinc selenide crystal using a pressure clamp to ensure high sensitivity and close contact. Both epidermis and dermis were subjected to scanning over a wave number range of 675 - 4000 cm-1. The characteristic peaks were recorded. At least triplicates were carried out and results averaged.

For DSC analysis, 2-3mg of skin samples treated with nano emulsion, gel and nano emulsion constituents were crimped in aluminum pan and heated from 30-200 ºC with continues nitrogen gas purging at 40 ml/min at heating rate of 10ºC/min. The characteristic peak temperature and enthalpy values were recorded. At least triplicates were carried out and results averaged (Nawaz and Wong, 2018).

STATISTICAL ANALYSIS

All experiments were performed in triplicates. The results were shown as mean value ± SD. Analysis of statistical data was carried out using SPSS software version 18 (SPSS Inc., Chicago).

RESULTS

Solubility determination

Methotrexate solubility observed in olive oil is 66.43±4.47µg/ml. The relative solubility of Tween 80, PEG 400 and water are 811.33±3.46µg/ml, 781.07±2.41µg/ml and 0.0399±0.17µg/ml respectively. Tween 80 having high HLB (hydrophilic lipophilic balance) value and being non ionic can enhance the drug solubility in medium chain mono, di or triglycerides.

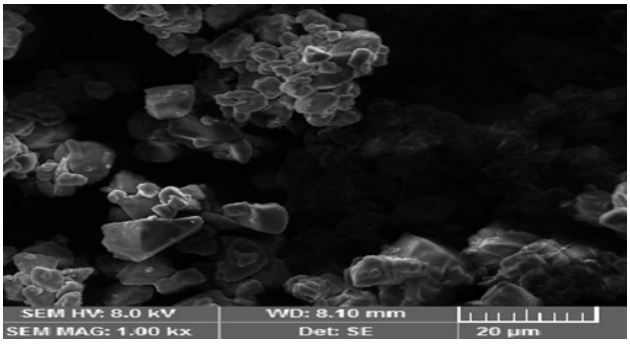

Fig. 1: SEM image of Methotrexate loaded nano emulsion optimized formulation

Formulation of methotrexate loaded nano emulsion

High speed homogenizer was used in this study for the preparation of Methotrexate loaded nano emulsion using Tween 80 as a surfactant. Formulation ingredients along with solubility profiles are shown in table 2 and 3.

Thermodynamic stability studies

Methotrexate loaded nano emulsion was exposed to different thermodynamically stressed conditions. A change in physical features was compared to blank nano emulsion formulation. The prepared formulation passed the requisite thermodynamic tests (table 4).

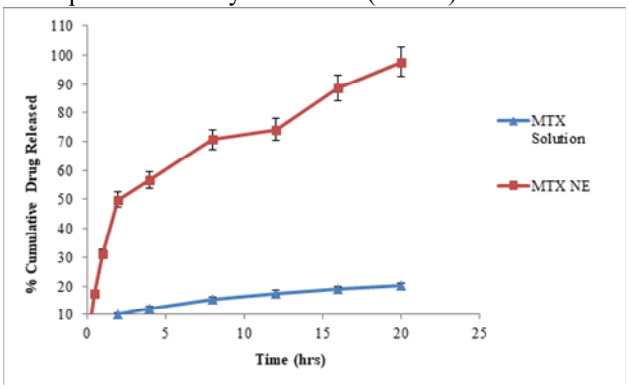

Fig. 2: Cumulative %age drug release from MTX solution, optimized MTX nano emulsion

Characterization of methotrexate loaded nano emulsion

Size, Size Distribution and Zeta Potential

The particle size, PDI and Zeta potential values of both blank and drug loaded formulations are presented in table 5. The observed globule size of Methotrexate loaded nano emulsion is 18.27±5.78nm with a PDI of 0.25±0.01.

pH values of nano emulsion formulations

The pH of Methotrexate loaded nano emulsion determined was 5.81±0.22. This is acceptable for application as transdermal systems. pH values for plain formulation as well as nano gel formulations are also expressed in the table 6. All formulations exhibited pH values within the established range scale of physiological pH of the skin.

Table 1: Quantitative compositional formula for 0.25% w/w methotrexate nano emulsion formulation

Formulation Code	Drug (Methotrexate)	Oil phase (olive oil)	Surfactant	Co surfactant	Water
M 1 (Blank)	_____	7.5 ml	0.25 gm (Tween 80)	0.25 gm (PEG 400)	q.s
M 2	0.25 gm	7.5 ml	0.5 gm (Tween 80)	0.5 gm (PEG 400)	q.s
M 3	0.25 gm	6.5 ml	0.25 gm (Tween 20)	0.25 gm (PEG 400)	q.s
M 4	0.25 gm	5.5 ml	0.25 gm (Span 80)	0.25 gm (PEG 400)	q.s
M 5	0.25 gm	7.5 ml	0.25 gm (Span 20)	0.25 gm (PEG 400)	q.s
M 6	0.25 gm	5.5 ml	0.25 gm (Tween 80)	0.25 gm (PEG 400)	q.s
M 7	0.25 gm	6.5 ml	0.25 gm (Tween 20)	0.25 gm (PEG 400)	q.s
M 8	0.25 gm	7.5 ml	0.25 gm (Span 80)	0.25 gm (PEG 400)	q.s

M 2 = optimized nano emulsion formulation

Table 2: Chief ingredients of nanoemulsion formulation with respective solubility profiles

Ingredients	Oil	Surfactant	Co-surfactant	Water
Concentration	66.43±4.47 µg/ml	811.33±3.46 µg/ml	781.07±2.41 µg/ml	0.399±0.17 µg/ml

Table 3: Quantitative compositional formulae for active formulations and their respective blank formulations for Methotrexate loaded nano emulsion

(A) O/W Nano emulsion Formulation		
Ingredients	Blank Formulation	Optimized Nano emulsion
Oily phase		
Olive oil	7.5	7.5
Polyethylene glycol 400	0.5	0.5
Methotrexate	-------	0.25
Aqueous phase		
Tween 80	0.5	0.5
Methyl paraben	0.03	0.03
Propyl paraben	0.01	0.01
Distilled water	q.s (quantity sufficient)	q.s (quantity sufficient)

Table 4: Physical characterization of blank and methotrexate loaded nano emulsion at 4ºC, 25ºC & 45ºC

Observed Characteristics	Temp	At 0 hr		After 24 hrs		After 48 hrs	
		Blank	NE	Blank	NE	Blank	NE
Color	X	W	PY	W	PY	W	PY
	Y	W	PY	W	PY	W	PY
	Z	W	PY	W	PY	W	PY
Odor change	X	___	N	N	N	N	N
	Y	___	N	N	N	N	N
	Z	___	N	N	N	N	N
Phase separation	X	___	N	N	N	N	N
	Y	___	N	N	N	N	N
	Z	___	N	N	N	N	N
Centrifugation stability (3500rpm for 30 min)	X	St	St	St	St	St	St
	Y	St	St	St	St	St	St
	Z	St	St	St	St	St	St
Thermodynamic test	X	P	P	P	P	P	P
	Y	P	P	P	P	P	P
	Z	P	P	P	P	P	P

Where Temp=Temperature, X=at 4ºC, Y=at 25ºC, Z=at 45ºC, NEG=Nano emulsion gel, W=Whitish, PY=Pale yellow, St=Stable, N=No change, P=Passed

Table 5: Size distribution, zeta potential & entrapment efficiency of blank and optimized methotrexatenano emulsion formulation

Formulation	Particle size(nm)	PDI	Zeta potential	EE%
Blank NE	24.78± 5.78	0.231± 0.51	-4.10± 4.87	---------
MTX NE	18.27± 5.78	0.25± 0.01	- 3.30± 5.10	74.68± 2.1

Note = Data are expressed as mean ± SD (n = 3).

Entrapment efficiency and drug content determination

The nano emulsion preparation exhibited an average entrapment efficiency of 74.68±2.1%. The entrapment efficiency as well as high homogeneity of a system is dependent on high drug solubility in connection with selected oil phase as well as drug compatibility with other ingredients. The percentage of drug contents of optimized formulations of MTX NE found to be 94.6±0.62% and 92.35±0.6% respectively (table 6).

Table 6: Physico chemical characteristics of methotrexate nano emulsion

Character	MTX NE
Physical Appearance	Transparent
Clarity	Yes
pH	5.81± 0.22
Homogeneity	Excellent
Drug Content (%)	94.6± 0.62

Note =Data are expressed as mean± SD (n= 3).

Table 7: Release Kinetics of prepared formulations

Formulations	Power Law Kinetic Model			
	K± SD	R^2	N	Release Mechanism
MTX SOL	0.30± 0.155	0.8708	0.413	Fickian Diffusion
MTX NE	0.001± 0.00189	0.9268	0.427	Fickian Diffusion

Table 8: Skin permeation parameters of MTX NE & MTX solution

Formulation	Cumulative amount permeated for24 hours ($\mu g/cm^2$)	Permeation flux($\mu g/cm^2/h$)	Enhancement ratio
MTX NE	79.23± 3.6	2.326± 0.45	2.134
MTX SOL	40.31± 5.34	1.092± 0.046	1.00

Note = Data are expressed as mean± SD (n= 3)

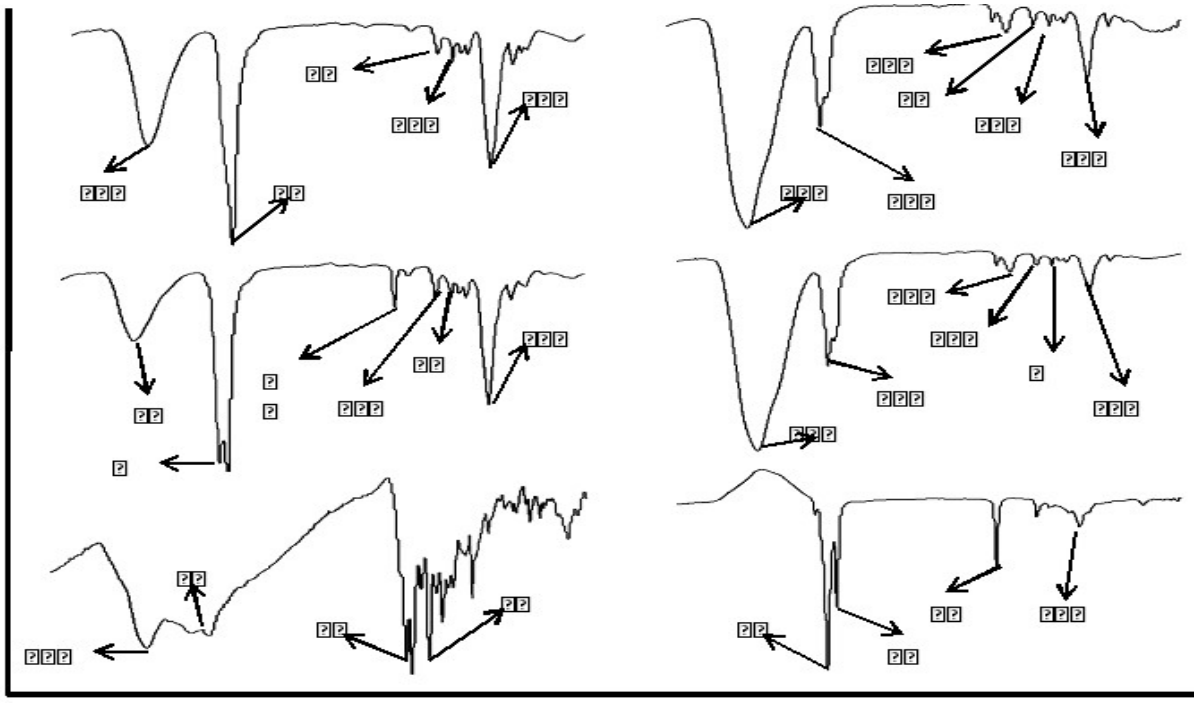

Fig. 3: ATR-FTIR spectra of (A) Methotrexatenano emulsion (B) Tween 80 (C) PEG 400(D) Olive oil (E) Methotrexate (F) Blank Nano emulsion.

Scanning electron microscopy

The SEM morphology showed uniformly distributed particles having well defined spherical shape (fig 1) that provides greater permeability to the system.

In vitro drug release studies

In vitro release profiles for the simulated physiological condition of the skin were recorded (fig. 2). This experiment employed a receptor medium having phosphate buffer solution pH 5.5 maintained at a temperature of 32±0.5^{O}C. Methotrexate loaded nano emulsion displayed rapid initial phase of drug release in the initial 2 hours with further sustained release upto 20 hours. After 20 hours, about 97.72% drug is released from methotrexate loaded nano emulsion at pH 5.5 (table 7). The nano size of the formulation enhanced the dissolution

rate of MTX into aqueous phase with a controlled profile and no burst release was recorded.

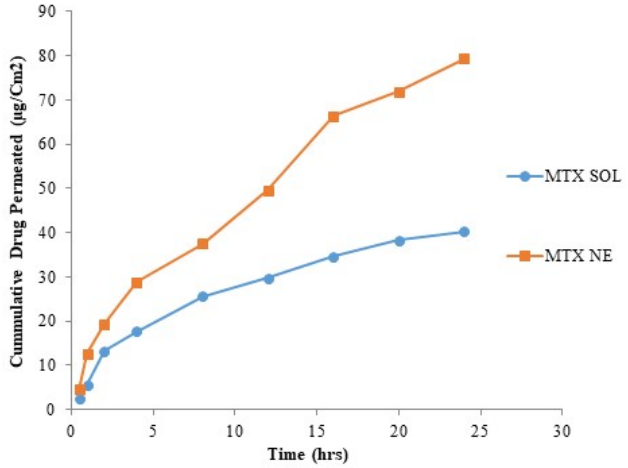

Fig. 4: Skin permeation profile of MTX sol& MTX NE across rabbit skin for 24hrs. Data are expressed as mean ±SD (n=3).

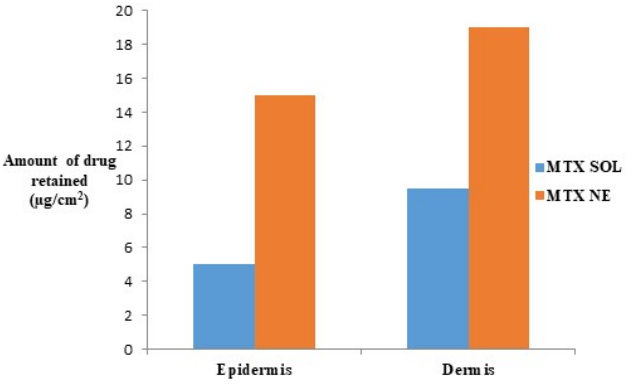

Fig. 5: Amount of drug retained (µg/cm^2) in epidermis & dermis layers of skin

ATR - FTIR analysis

ATR-FTIR was performed to check the compatibility between prepared formulation and various ingredients used to accomplish nano emulsion formulation (fig. 3). It was observed that formulation as well as free drug, the peak at 1645cm $^{-1}$is because of -C=O stretching of -COOH group. Peaks at 3414.63, 1490.2 and 1645 cm^{-1} relates to N-H stretching, C=C stretching and C-N stretching respectively.

In vitro skin permeation studies

The in vitro skin penetration was assessed using Franz diffusion cell. Using rabbit skin, a significant difference for MTX-NE was recorded compared to MTX solution (table 8). Permeation data showed that an average of 79.23±3.6µg/cm^2 of Methotrexate was permeated from the nano emulsion with an average flux of 2.326±0.45µg/cm^2/h after 24 hrs. Average amount of drug permeated from Methotrexate solution was 40.31±5.34µg/cm^2 with a flux of 1.092±0.046 µg/cm^2/h after 24 hrs. methotrexate exhibited permeation from nano emulsion which is almost double with respect to its permeation from solution (fig. 4).

Drug retention studies

The results of drug retention studies showed that the drug is retained more in deeper epidermis and dermis layers in case of MTX-NE formulations as compared to control drug solution (ANOVA P<0.05) (fig. 5).

Methotrexate loaded nano emulsion formulations impact on skin structure (Study of drug retention mechanisms)

During DSC study, the untreated skin showed endothermic transition at temperatures of 65.1ºC, 82.5ºC and 115.4ºC for the melting of intercellular lipids, denatured proteins as well as protein associated lipids in stratum corneum, respectively. The observed endothermic transitions at 64.9^{o}C, 82.3^{o}C and 115.6ºC, after treatment with optimized formulations indicating the marked lipid organization changes absent.

DISCUSSION

Amongst all tested carriers, the nano-emulsion favored greater solubility of methotrexate in olive oil that was an indication of greater stability (Lawrence and Rees, 2000). There was no visible indication of creaming, turbidity or phase separation. Sediment or creaming might occur during centrifugation process and O/W emulsions are good candidates because oil is less dense than aqueous phase. Prepared formulation passed centrifugation test which shows that the overall system possessed greater homogeneity as well as stability. Tween 80 employed as surfactant as well as emulsifying agent generally resists the mobility of oil globules with respect to centrifugal forces. This leads to hampered aggregation, phase separation or creaming. Freeze thaw cycle test was passed by the formulation indicating system stability due to greater homogeneity. If the system is non-homogenous, then the oil globules segregate by crystallized particles of ice upon freezing. There will be disruption of lipid film covering the droplets. At thawing, melting and coalescence of droplets occur and result in phase separation or creaming. The surfactants reduce the association of oil globules and creaming and maintain the homogeneity of the system. The homogeneity of the system is shown by heating and cooling cycle stability studies at high temperature conditions. Both kinetic energy as well as Brownian motion of the oil particles increase at high temperature but the system is still stable may be due to ordered and well organized structures formed within nano emulsion system by using surfactant as well as co surfactant (Ngan *et al*. 2014; Ali *et al*., 2014).

A small particle size yields large surface area therefore release of the drug into the aqueous medium is greater resulting in greater absorption of drug. It has been suggested that incorporated drug particles also associated with microstructure of the system, reducing the globule size particularly, if the drug is amphiphilic. PDI ensures globule size uniformity within the formulation. If PDI is less than 0.5, then it depicts good homogeneity and uniformity of globule size in the formulation. The methotrexate loaded nano emulsion had optimal value of zeta potential -3.30±5.10 mV confirming that the system is physically stable. Zeta value represents electrostatic repulsion between particles. Preparations having zeta potential values greater than ±30mV are considered stable without aggregation (Sabitha *et al.*, 2013). Greater zeta potential values confirmed the electrostatic repulsive forces among oil globules, preventing their coalescence and resulting in uniform dispersion having sufficient stability.

The case when drug is insoluble in water then it can be entrapped by oil globules which can further be stabilized by using surfactant and co-surfactant. The concentration of surfactant is also a very crucial factor known to affect drug encapsulation as it exhibits an inverse relationship with drug encapsulation. This might be due to the fact that an increased concentration of surfactant results in decreased particle size leading to lower drug entrapment in the nano emulsion formulation (Liu *et al.*, 2010). Moreover, drug partitioning also resulted in augmented drug solubilization from hydrophobic to aqueous phase.

Methotrexate loaded nano emulsion displayed rapid initial phase of drug release in the initial 2 hours with further sustained release upto 20 hours. The possible reason of initial burst release from Methotrexate loaded nano emulsion might be due to superficial drug entrapment. This is favored by cooling resulting from high temperature to room temperature transition during nano emulsionformulation (Zur Mühlen and Mehnert, 1998). It can be deduced that this kind of drug release pattern is the foundation of intended topical application. Thus the initial rapid and later on gradual release of Methotrexate would minimize the loss of active moiety that might occur due to daily routine. This favors an improved administration through the skin with respect to the hydrating / occlusive characteristics of the nano emulsions followed by adequately maintained gradual and prolonged drug release. After 20 hours, about 97.72% drug is released from Methotrexate loaded nano emulsion at pH 5.5. The nano size of the formulation enhances the dissolution rate of MTX into aqueous phase. Also solubility and drug release is enhanced. Release is through controlled fashion. No burst release mechanism exists except initial rapid release during first 2 hours in case of Methotrexate loaded nano emulsion formulation. In chronic condition, like psoriasis, slow release pattern is beneficial whereas rapid or burst release is not useful. Also burst release causes toxicity due to greater drug release and faster absorption of the drug in inflamed psoriatic lesions (Divya *et al.*, 2016).

The FTIR spectra revealed several peaks in the final formulation which confirmed the chemical structure retained within the drug with efficient loading into the formulation. The peaks at 3502 and 2923cm^{-1} in ATR - FTIR of tween 80 is because of the characteristic stretching vibrations of -OH and symmetric stretching vibrations of methylene -CH_2 groups respectively (Panonnummal and Sabitha, 2018).

The skin permeation experiments conclude that MTX-NE formulations play significant role in controlling methotrexate release as well as drug targeting to the skin. Studies have shown that the diffusion rate is affected due to physicochemical characteristics of the formulation. These include hydrogen bonding ability, drug loading capability, surface charge and mode of drug application (Pinto *et al.*, 2014). Tween 80 employed as surfactant in the formulations. It causes lipid packing fluidization. It also increased water contents of stratum corneum by skin lipid extraction mechanism (López *et al.*, 2000). Polyethylene glycol which is employed as a solvent also affects the penetration of drug from MTX-NE. These summative effects are responsible to reduce barrier functions of the epidermis leading to augmented transdermal drug transport. The order of skin permeation parameters increased from MTX-NE >MTX solution. MTX-NE exhibit better skin permeation due to the ability of these formulations to squeeze themselves through relatively smaller pores of the skin. On the other hand, lower permeation potential is exhibited by MTX solution when compared to MTX-NE. This might be attributed and explained due to the inherent low permeability of Methotrexate. Low partition coefficient and hydro solubility tendency of methotrexate at physiological conditions represent the responsible factors (Zeb *et al.*, 2017).

During skin retention studies, greater retention was due to strong interaction between keratinocytes and nano sized formulation. In addition MTX deposition in both epidermis and dermis is also improved by the addition of Tween 80 (Lopes, 2014). Anatomy of the skin revealed that the thickness of dermis is more as compared to epidermis; therefore drug retention will be more in dermis than epidermis. The greater retention of drug in both of these layers is considered significant as these layers are mainly affected by psoriasis.

The stratum corneum corresponding to lipids and protein molecular vibrations produces bands at different wave numbers. These peaks are of major importance. The asymmetric and symmetric stretching vibrations of CH_2

around 2920 cm^{-1}, 2850 cm^{-1} appeared respectively. These were derived from the lipoid hydrocarbon chains. Moreover , bands around 1650 cm^{-1} and 1550 cm^{-1} are attributed to the stretching vibrations of amide 1 (C=O stretching) and amide 11 bonds (C-N stretching and C-H bending) in stratum corneum proteins, respectively (Obata *et al.*, 2010). There is radical drift of CH_2 stretching vibrations to higher wave number. This is the consequence of changes in the stratum corneum. These changes include structural changes as well as lipid bilayers fluidization(Vaddi *et al.*, 2002). ATR-FTIR spectra of the untreated epidermis showed peak at 3340.31 cm^{-1} & 1640.46 cm^{-1}. These peaks are assigned to both asymmetric stretching vibration of CH_2 and stretching vibration of amide 1 bond respectively. Furthermore, treatment of the epidermis with methotrexate loaded nano emulsions did not show significant changes upon vibrations and bands appeared at 3340.99 cm^{-1} & 1640.17 cm^{-1}. Almost same observations were found in the treatment of dermis with Methotrexate nano emulsion. The enhanced permeation of Methotrexate loaded nano emulsion shown by the results of DSC and ATR - FTIR suggest that it is attributed to shape transformation, elasticity with no obvious structural changes.

CONCLUSION

It was concluded that olive oil based MTX-NE is suitable for topical application in the management of psoriasis. Prepared formulation exhibits ideal properties for efficient topical application of methotrexate with increased skin permeation as well as improved skin retention and therefore an excellent alternative for oral methotrexate tablet.

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