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Article

In-Vitro and Ex-Vivo Evaluation of Transfersomal Gel of Methotrexate

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Methotrexate on its oral and intravenous administration results in unwanted adverse effects. This drawback can be overcome by transdermal delivery because of its painless objective for systemic drug administration. Transfersomes are ultra-deformable vesicles with the flexibility to reach deeper tissues of the skin. The objective of this research work was to develop methotrexate transfersomal gel by thin film hydration technique, evaluated for entrapment efficiency, deformability, mean vesicle size, and stability, and incorporated into carbopol gel for ease of handling and skin applicability for a longer period of retention on skin. MTX-TFS gel & conventional gel were characterized for consistency, transparency, viscosity, and pH. *Ex-vivo* skin permeation studies were performed using abdominal goat skin and drug release kinetic parameters and transdermal flux were calculated using mathematical models. The results indicate that MTX was successfully entrapped (84.77 ± 2.35 %w/w) in transfersomes having 240±1.6 nm vesicle sizes and 27.13±0.7 deformability index. The gel was permeated through the skin at a rate of 28.12±2.58 µg/cm^2/hr as compared to the conventional gel (10.35±2.14 µg/cm^2/hr). From the study, it was concluded that the MTX-TFS gel can be used as a possible substitute for the conventional formulation for transdermal drug delivery due to 3 times improvement in transdermal flux.

Keywords: Methotrexate. Transfesomes. Transdermal flux. Skin permeation. Ex-vivo.

ABBREVIATIONS

MTX-TFS (Methotrexate Transfersomes), Rheumatoid arthritis (RA), Phospholipon 90 G (PC), Edge activator (EA), phosphate buffer saline (PBS), large multilamellar vesicles (LMLVs)

INTRODUCTION

Rheumatoid arthritis (RA) is the autoimmune disorder caused when the immune system of the body is not working properly. Pain and swelling in the wrist and small joints of the hand and feet are the most common symptoms of RA (Luqmani, Cox, 2016; Mehaneesha *et al.*, 2020).

Methotrexate (MTX) is a folic acid antagonist and it is used in treatment of cancer, psoriasis and rheumatoid arthritis. Now a days, MTX is a cornerstone therapy for RA. It would be preferable to deliver MTX by the transdermal route to circumvent the systemic use of MTX which causes the hepatic toxicity with rarely bone marrow, lung, or liver toxicity (Sandhya Lekshmi *et al.*, 2017). Additionally its capacity for passive diffusion is limited because of water solubility and ionised form at physiological pH (dos Santos *et al.*, 2017).

A Transfersome (TFS) is an artificial vesicle designed to be like a cell engaged in exocytosis, and thus suitable for controlled and, potentially targeted drug delivery. TFS is self-regulating and self-optimizing vesicle. Thus it enables to cross various transport barriers efficiently, and act as a drug carrier for non-invasive targeted drug delivery and sustained release of therapeutic agents (Pandey, Misra, Sharma, 2017; Shivanand *et al.*, 2009).

The survey declared that the transdermal gels were helpful to control rising healthcare costs in many western countries and also will place a greater emphasis on home

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health care and self-administration of drug therapies for chronic conditions such as arthritis and pain management (Gupta, Singhal, Nimisha, 2022; Jana *et al*., 2014; Marwah *et al*., 2016; Sarwa *et al*., 2015). According to clinical research, ample of phase-I, Phase-II and Phase-III clinical trials are going with transfersomal products of ointment and creams. This will focus on upcoming demand of transfersomes for transdermal drug delivery.

Drug release from transdermal gel can be explained by Fick's law which is about the steady-state flux of drug that occurs when this rate becomes constant. The flux occurs after passage of a lag time in skin diffusion studies, which is a function of the drug 'loading' the stratum corneum and dermis, diffusivity, and thickness of the skin (Merine, Merine, 2015).

The presented research work focuses on preparation and physicochemical characterization of MTX-TFS gel and its *in-vitro* and *ex-vivo* performance. Objective of the research is to minimize adverse effects associated oral MTX drug, to enhance drug permeations through skin by incorporation of MTX in transfersomes and to target the concentration of MTX at the site of arthritis and to provide continuous sustained action.

MATERIAL AND METHODS

Material

MTX was procured as gift sample from West Coast Pharmaceutical Pvt. Ltd. Ahmedabad, (India). Phospholipon 90 G (lipid) was received as a gift sample from Lipoid AG, Switzerland. Double distilled water was prepared in laboratory for study. All materials used for study confirmed to USP 24 standards and purchased from ACS chemical Pvt. Ltd. Ahmedabad (India).

Formulation of Methotrexate Transfersomes (MTX TFS)

Transfersomes were prepared by conventional rotary evaporation sonication method (Modi, Patel, Bharadia, 2017). Precisely, Phospholipon 90 G (PC) mixed with Edge activator (EA) Span 80 (1:1) were taken in a clean, dry, round bottom flask and then mixture was dissolved in Methanol: Chloroform (1:2). The organic solvents were evaporated by rotary evaporator above the lipid transition (40^0C). Final traces of solvent were removed under vacuum overnight. The deposited lipid film was hydrated with phosphate buffer saline PBS (pH 7.4) containing MTX (2%w/v) to furnish the desired concentration in the final preparation by rotation at 50 rpm for 1 hr at 50^0C temperatures. The resulting vesicles were swollen for 2 hr at 25^0C to get large multilamellar vesicles (LMLVs). The thick suspension was broken by sonication for 30 min at 4^0C at a frequency of 53 kHz to achieve desired vesicle size (200-300 nm).

Formulation and optimization of Vesicular Gel

Transfersomal suspension was dispersed in gel to achieve the desired rheological characteristics as well as texture for applicability of transdermal application. Carbopol 934P was selected as the gelling agent based on the compatibility with nanoparticulate dispersions, feel, aesthetic appeal and ease of spreadability. For preparation of gel, Carbopol 934P was dispersed in demineralized water by stirring at 800 RPM for 60 minutes. Then propylene glycol was added and the mixture was neutralized by dropwise addition of triethanolamine. pH of gel was adjusted to 6.5. MTX-TFS suspension was mixed into the carbopol hydrogel by an electrical mixer at 25 rpm for 5 minutes to get uniform gel (Saraf *et al*., 2011). Concentrations of carbopol were varied from 1-3% for optimization of gelling agent (Table I). MTX-TFS gel was also compared with conventional gel of methotrexate equivalent to 0.25%w/v.

TABLE I - Composition and Physicochemical properties of different transfeosomal gels of MTX

Code	PSMTX	CGMTX	TGMTX-I	TGMTX-II	TGMTX-III
Formulation	Plain Methotrexate solution	Conventional gel	Transfersomal gel		
Concentration of MTX	0.25%W/V	0.25% w/v	Transfersomal suspension equivalent to 0.25% w/v		
Carbopol 934P	-	2%	1%	2%	3%
Propylene glycol		10%	10%	10%	10%
Triethanolamine		1.5%	1.5%	1.5%	1.5%
Distilled water Qs	100 ml	100 ml	100 ml	100 ml	100 ml
Consistency		++	+	++	+++
Transperancy		+++	+++	+++	++
pH		7.2±0.4	6.9±0.5	6.8±0.2	7.0±0.3
Viscosity		12700 cps	10600 cps	13500 cps	17300 cps

+ shows consistency level and transperancy level

Characterization of methotrexate transfersomes

Methotrexate transfersomes were characterized for vesicle size, %Entrapment Efficiency and Deformability Index (Vesicle Elasticity Measurement) (Patel *et al*., 2009; Duangjit *et al*., 2011).

Vesicle size of MTX TFS was determined *by* diluting 1 ml of suspension with deionized water and examined by a Malvern Particle sizer and Zeta Potential Analyzer (Malvern Instruments Ltd., UK) at room temperature.

%Entrapment efficiency of MTX TFS was estimated by centrifugation method. Supernatant was collected by centrifugation of MTX TFS carried out at 14000 rpm for 30 minute and diluted with PBS (pH 7.4). Supernatant was analyzed to determine unentrapped Methotrexate by UV spectrophotometer at 303 nm. Encapsulation efficiency is calculated using equation (1).

$$\%Entrapment\ Efficiency = \frac{Total\ drug - Unentrapped\ drug}{Total\ drug} X100 \quad (1)$$

The deformability study was done by using a home-built device by extrusion of transfersomes formulation through filter membrane (pore size diameter 100 nm), using a stainless steel filter holder (50 mm diameter), by applying a pressure of 2.5 bar. The quantity of vesicles suspension extruded in 5 minutes was measured and calculated using equation (2).

$$E = JX\left[\frac{r_v}{r_p}\right]^2 \quad (2)$$

Where, E is elasticity of vesicles membrane, J is amount of suspension extruded in 5 minutes, r_v = vesicles size, r_p = pore diameter.

Characterization of methotrexate transferosomal gel

TFS enriched hydrogel were characterized using physicochemical properties, in vitro drug diffusion study, ex vivo skin permeation study and skin deposition study (Sarwa *et al*., 2015; Surini *et al.,* 2020).

Physicochemical properties of gel

The conventional gel of MTX and TFS enriched hydrogels were characterized for their physicochemical properties such as color, odor, viscosity and pH.

In vitro drug diffusion study

Membrane diffusion technique was used to determine the permeation rate of drug from gel (Opatha, Titapiwatanakun, Chutoprapat, 2020; Sana *et al.*, 2021). The efficient diffusion area of the cell was 2.83 $cm^2$and the diffusion medium was 50 ml of freshly prepared phosphate buffered saline pH 7.4 (PBS) equilibrated at 37±0.5^{0}C temperature. The prepared MTX TFS gel (equivalent to 2.5 mg) was placed on one side of the dialysis membrane. The receptor fluid was stirred by a Teflon-coated magnetic bead operated to a magnetic stirrer and rubbing action was continued initially by brush manually. Samples (2 ml) were withdrawn through the sampling port at regular intervals (1, 2, 4, 8, 12, and 24 hours) and replaced with equal amount of fresh diffusion medium. Sink condition was maintained throughout the experiment. The samples were analyzed spectrophotometrically at 303 nm for release of MTX in receiver media from formulation. Study was carried out in triplicate. For reference purpose plain solution of MTX was also taken in account for in-vitro release study (Kapoor, Pandit, Nagaich, 2021).

Preparation of skin

Fresh abdominal goat skin was collected from slaughter house and was used after peeling the skin from underlying cartilage. Skin hairs were shaved and the preliminary wash of skin was done with normal saline. Skin was dried between two filter papers and was used directly in study without storage. Modified Franz diffusion cell having receptor compartment volume of 50 ml of PBS pH 7.4 was used for study. Experiments were performed in two stages.

Ex vivo Skin Permeation Study

The first stage was used in determination of the vesicle permeating the skin. Skin membrane was mounted, with stratum corneum side up and donor compartment dry and open to atmosphere. The skin was floated on receiver solution for 24 h for equilibrium and pre hydration. This approach was suggested to maintain transepidermal hydration gradient which has been proposed to generate the driving force for skin permeation of transfersomes. All formulations bearing equivalent to 2.5 mg MTX, were placed into the donor compartment over skin. Both the compartments were maintained at 37±0.5^{0}C and the receptor compartment was stirred using a magnetic stirrer. Samples were withdrawn (2 ml) through the sampling port at regular intervals (1, 2, 4, 8, 12, 18 and 24 hours) and replaced with equal amount of fresh diffusion medium. Sink condition was maintained throughout the experiment. The samples were filtered through a 0.45 µm membrane filter and analyzed for drug content by UV. Study was carried out in triplicate(Jana *et al.*, 2014; Samanthula, Satla, Bairi, 2019).

Skin Deposition study

At the end of first stage, the donor compartment and skin surface were washed five times with warm receptor medium. The second stage drug deposition study was employed to determine skin deposition.

Skin mounted on the diffusion cell was removed carefully after 24 hr and skin surface was washed five times with warm (45 ^{0}C) receptor medium. Amount of drug deposited on the skin was determined by cutting the cleaned skin into small pieces and then mashing it with 10 ml of PBS pH 7.4. It was then mechanically shaken in a water shaker bath at 37^{0}C for 2 hour for complete extraction of drug. The above dispersion was first filtered through Whatman filter paper no. 1 and then further filtered through 0.45 µ membrane filter. After suitable dilution, the MTX in the filtrate was determined by UV (Kapoor, Pandit, Nagaich, 2021).

Calculation of Skin Permeation Parameters

Skin permeation parameters such as steady-state permeation rate (J_{ss}) and lag time (LT, hrs) were calculated by plotting cumulative amount of drug permeated per unit area Vs time (Khan *et al.*, 2015; Marwah *et al.*, 2016). From the slope and X-intercept of the linear portion, respectively, the permeability coefficient (K_p) and Enhancement ratio (E_r) was calculated by following equations (3) and (4).

$$K_p = \frac{J_{ss}}{c_0} \quad (3)$$

$$E_r = \frac{J_{ss}\ of\ formulation}{J_{ss}\ of\ plain\ drug} \quad (4)$$

Determination of drug release kinetics

To determine the mechanism of drug release from gel formulations, the data were treated according to first-order (log cumulative percentage of drug remaining vs time), Higuchi's (cumulative percentage of drug released vs square root of time), zero-order (cumulative amount of drug released vs time) and korsmeyer peppas' (log cumulative percentage of drug released vs log time) pattern (Arpitha, Dhurke, 2019; Marwah *et al*., 2016).

Physical stability of hydrogel

Carbopol gel as a vehicle has good thermal stability. The ability of hydrogel to maintain its consistency on storage was determined by keeping it at 22±3^{0}C (room temperature, RT) and 5±2^{0}C for 30 days(Marwah *et al*., 2016; Roy, Saha, Saha, 2013; Surini *et al.,* 2020).

RESULTS

MTX TFS suspension was milky yellow colored liquid having 240±1.6 nm vesicle size (Figure 1), 84.77 ± 2.35 %w/w entrapment efficiency and 27.13±0.7 deformability index.

Physicochemical properties of conventional gel of MTX (CGMTX) and transfersomal gels of MTX (TGMTX I, II, III) were shown in Table I.

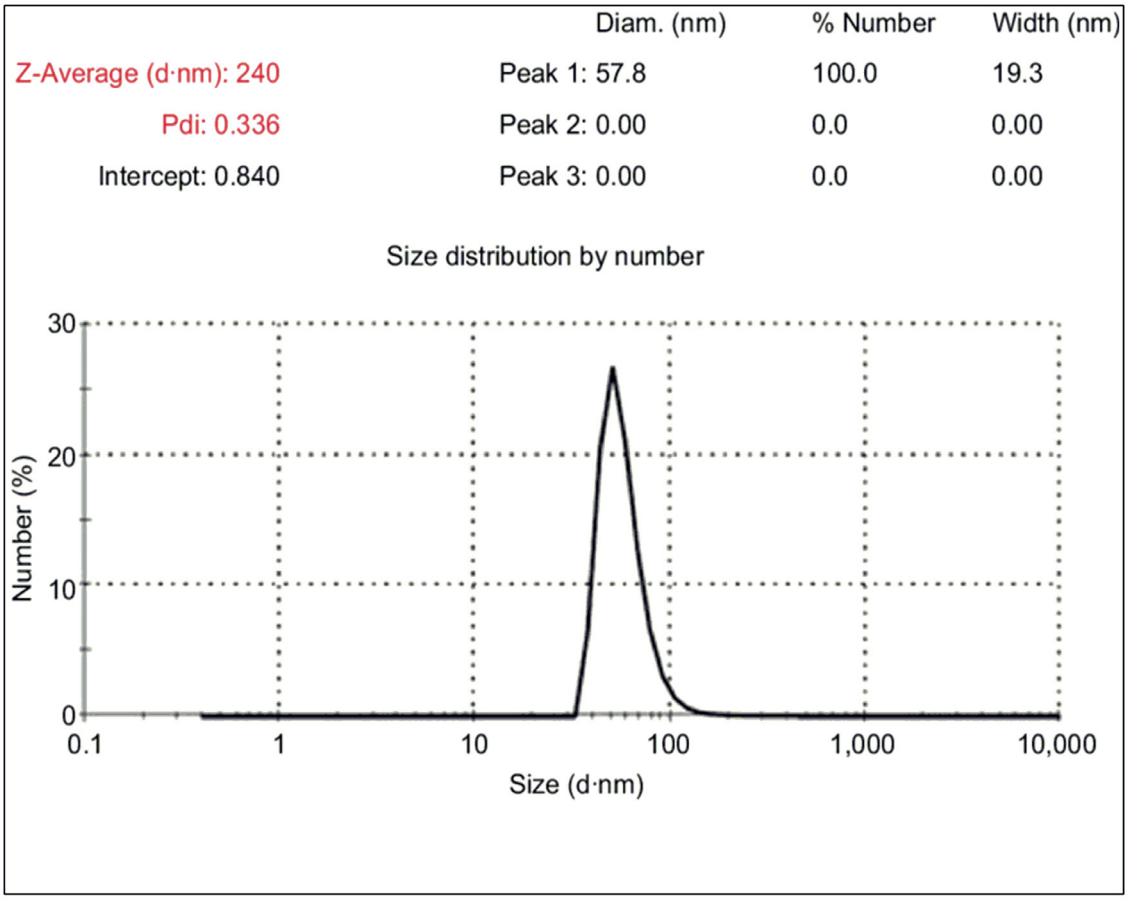

FIGURE 1 - Vesicle Size of MTX TFS suspension.

In-vitro drug release study

Figure 2 describes % cumulative drug release from different formulations. It describes 28.54±0.36% cumulative drug was released from CGMTX after 8 hrs. TGMTX gels were given from 63.22±0.48% to 69.46±0.28% drug release after 8 hrs, which was 3 times greater than the release from conventional gel. Almost 91.1267±0.4119% drug was released from TGMTX I after 24 hrs.

Drug diffusion parameters from In-Vitro drug release study

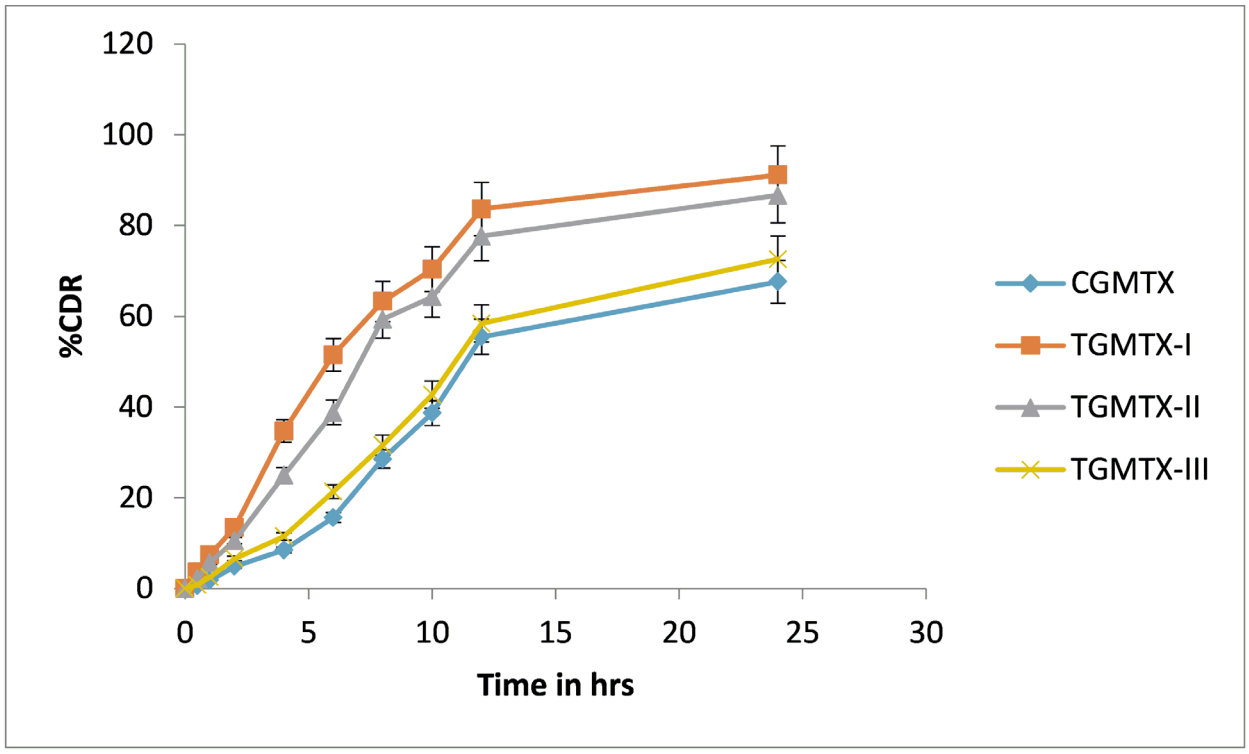

FIGURE 2 - In-vitro %Cumulative drug release Vs time of different MTX transdermal preparations.

Transdermal flux calculation was the measurement of the ability of the drug to transport through skin membrane. Flux is also function of molecular weight of compound and smaller as well as hydrophilic compounds have faster value of it (Alkilani, McCrudden, Donnelly, 2015). Transdermal flux value was 37.38±2.86 µg/cm^2/hr for TGMTX-I revealed that approximately 805.01±3.64 µg/cm^2 drug was permeated after 24 hrs (Table II).

TABLE II - Flux, Permeability and Kinetic assessment of diffusion data of MTX from different transdermal preparations

Type of Study	Formulation code	Permeated amount at 24 h (µg/cm2)	Flux (µg/cm2/hr)	Permeability coefficient (Kp)(cm2/h)	Enhancement ratio (Er)	Amount of drug retained in skin	R2				n
							Zero	First	Higuchi	Korsemeyer peppas	
In-Vitro	**PSMTX**	881.01±3.01	13.53±0.53	0.009412	1	NA	NA	NA	NA	NA	NA
	CGMTX	597.23±2.22	18.12±6.42	0.011248	1.194895	NA	0.914	0.668	0.887	0.982	1.392
	TGMTX-I	805.01±3.64	37.38±2.86	0.014952	1.588589	NA	0.806	0.833	**0.937**	0.826	1.104
	TGMTX-II	765.08±2.35	36.19±1.76	0.014476	1.537538	NA	0.835	0.818	**0.930**	0.882	1.176
	TGMTX-III	640.87±2.70	29.78±3.65	0.011912	1.265465	NA	0.923	0.961	**0.914**	0.962	1.277
Ex-Vivo	**CGMTX**	252.9152±2.65	10.35±2.14	0.00414	0.321928	6.32±1.25%	0.976	0.962	0.807	0.979	1.007
	TGMTX-I	597.2323±2.52	28.12±2.58	0.011248	0.87465	32.53±3.61%	0.914	0.943	0.887	0.975	1.312

R2: *correlation coefficient,* ***n:*** *diffusional release exponent*

Drug release kinetics from In-Vitro drug release study

Release kinetic study of all formulation was studied (Table II) for different mathematical models (zero order, first order, Higuchi equation and Korsemeyer Peppas model). Calculations are performed using Microsoft excel 2007. The best fit with higher correlation nearby (R^2> 0.99) was found with the zero order for CGMTX, which indicated constant drug release from the formulation irrespective of drug content present in formulation.

Ex-vivo skin permeation study

Ex-vivo skin permeation was performed on TGMTX-I only because of in-vitro good results with that batch only. Amount of MTX permeated through excised goat skin over 24 hours plotted versus time (Figure 3). It was observed that TGMTX-I was able to permeate 597.23±3.64 µg/cm^2 MTX after 24 hrs from skin whereas CGMTX permeate only 252.92±2.22 µg/cm^2 only.

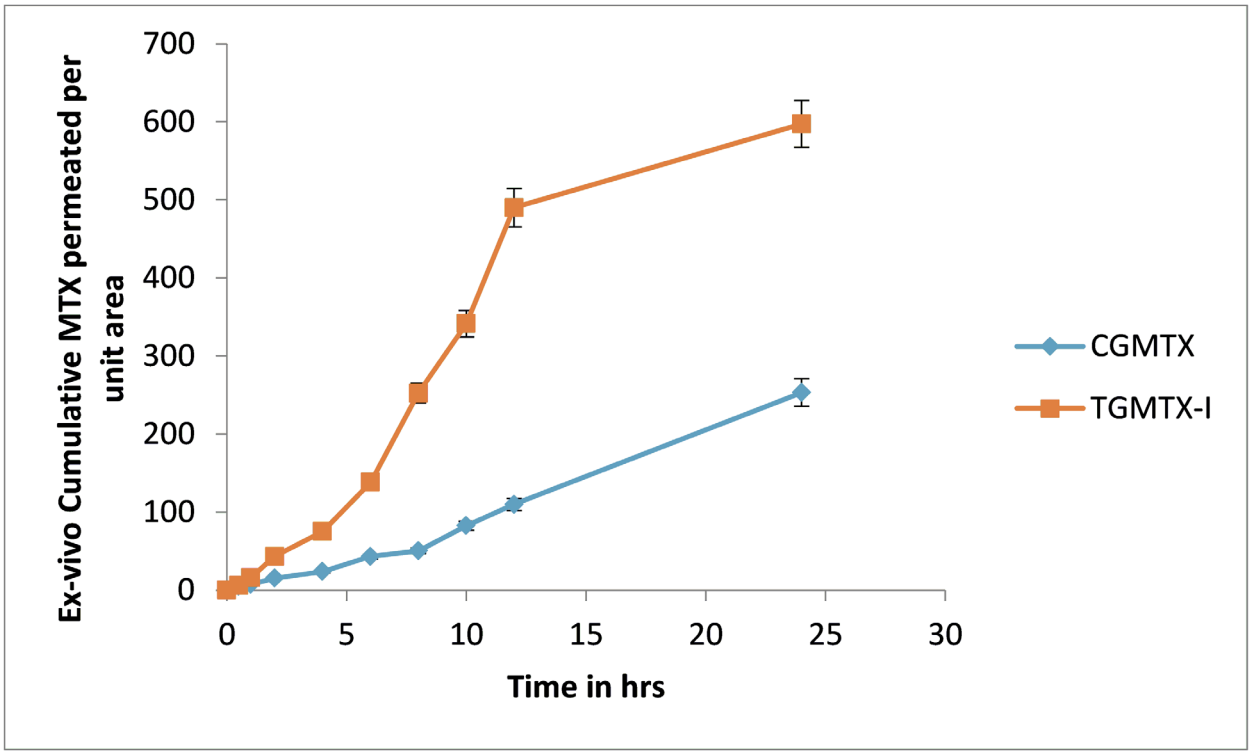

FIGURE 3 - Ex-vivo Cumulative MTX permeated per unit area from different topical preparations (µg/cm^2).

Skin permeation parameters from Ex-Vivo Skin permeation study

Skin permeation study is considered as gold standard for drug delivery assessment from transdermal formulations. Due to number of layers in skin composition, it was necessary to check skin permeation of drug from formulation (Malakar *et al.*, 2012). Skin permeation parameters were calculated by plotting a curve between cumulative amounts of drug permeated per unit area (µg/cm^2) Vs time (Figure 3). The flux was obtained from the slope of the linear portion of the graph.

Flux was decreased from 37.38±2.86 to 28.12±2.58 µg/cm^2/hr (Table II) due to accumulation of drug and vesicles in skin layers. But after 24 hrs excised skin contains approximately 6.32±1.25% and 32.53±3.61% for CGMTX and TGMTX-I respectively. So it is said that TGMTX formulation will be effective in the treatment of deep localized arthritis infections.

Drug release kinetics from Ex-Vivo skin permeation study

Table II describes Ex-vivo Drug release Kinetic assessment of diffusion data of MTX TFS gel formulations. The best fit with higher correlation nearby (R^2> 0.99) was found with the zero order for the transfersomal formulation reveals that release of MTX from the transfersomes vesicles were due to diffusion.

Physical stability of Transfersomal gel of MTX

TGMTX-I was optimized batch which shows good transdermal flux and permeability comparable to other ones. Table III shows that carpobopl gels provide improved thermal stability.

TABLE III - Physical stability of TGMTX-I

Time	Temp.	Consistency	Transperancy	pH	Viscosity
Initially	25±2 ^{0}C	++	+++	6.9±0.5	10600 cps
	4±2 ^{0}C	++	+++	6.8±0.2	10500 cps
After 30 days	25±2 ^{0}C	++	+++	7.1±0.3	9800 cps
	4±2 ^{0}C	+++	+++	6.8±0.5	11300 cps

DISCUSSION

Methotrexate loaded tranfersomal suspension was successfully prepared by thin film hydration method. All gel formulations are yellow to pale yellow in color representing drug. Gels are odorless with good consistency. pH of gels were neutral reveals that they were applicable on the skin and no irritation will persists (Luki´c, Panteli´c, Savi´c, 2021).

Results of in-vitro diffusion study showed the sustained action of MTX transfersomal gels during 24 hrs with good permeation as compared to CGMTX from dialysis membrane. It is essential for the treatment of rheumatoid arthritis to prolong 24 hrs drug releases. TGMTX-I were permeated 2278.17±32.64 µg from in-vitro dialysis membrane after 24 hours.

Comparing TGMTX gels, drug release was higher from TGMTX-I as compared to TGMTX-II (86.60±0.26%) and TGMTX-III (72.54±0.30%) after 24 hrs because of higher concentration of carbopol in gels. This was due to effect of concentration of Carbopol 934P in preparation of gel. Carbopol 934P was evaluated as bioadhesive polymer and since drug release rate was slow from its matrix (Nakanishi, Kaiho, Hayashi, 1998). According to study, increment in carbopol concentration leads to higher mucoadhesion force and that followed slower drug release. So from TGMTX-I to TGMTX-III, drug release after 24 ours was decreased.

Dosage required to treat rheumatoid arthritis for methotrexate is 7.5 mg orally weekly. So, it was clear from flux that around 7.5 mg of drug could be possible to penetrate through skin for treatment of rheumatoid arthritis. Results also indicated that the flux of TGMTX were 2 to 3-fold higher than conventional formulation of drug (CGMTX). This was due to surfactant presence in walls of transfersomes, that provides flexibility to deform and them reform through skin pores (Opatha, Titapiwatanakun, Chutoprapat, 2020). In 2011, Reshmy and their co-authors reviewed that improvement in transdermal flux with transfersomes were because of "transdermal osmotic gradient" mechanism. The transfersomal gel acted as skin penetration barrier and prevent water loss from the skin. So, lipid layers resist dehydration process and lipid molecules loaded with drug reloacate from dry part of epidermis to moist part of stratum corneum and so on higher content of drug was transferred into skin (Rajan *et al*., 2011).

As per drug release kinetics for in-vitro diffusion study, higuchi model was followed by all the transfersomal formulations, that represented drug diffusion from matrix and was time dependent process (Merine, Merine, 2015). Here $n > 0.89$ for all formulations, which leading to super case II transport, concludes drug release from swelling of system matrix and time independent.

From results of ex-vivo diffusion study, acceptable release profile of TGMTX-I reveals sustain drug delivery from carbopol hydrogel as well as higher amount of drug permeation comparable to CGMTX. MTX TFS gel has the advantages that they enhance the skin retention of drug and TFS can also serve as a drug reservoir that provides a localized and controlled drug delivery and it is also possible to deliver sufficient amount of drugs into skin. Results indicated that the flux and permeability coefficient of TFS were 3 fold higher than conventional gel. It can be concluded from the results that TFS in vesicular gel forms

could penetrate and deposit MTX 5 times more than conventional formulations.

According to drug release kinetics of ex-vivo study, the mechanism of release kinetics was evaluated by fitting the permeation data to the zero-order and Higuchi diffusion models. All permeation profiles of gels fit well into the Higuchi diffusion model (RSD > 0.99), and a linear relationship was found between the amount of drug released and the square root of time. It could be concluded that the vesicles acted as reservoir systems for continuous delivery of the encapsulated drug.

It has been demonstrated that as transfersomes due to their elasticity and high deformable structure could reach deeper dermal tissues and even the systemic circulation, they ensure higher skin permeation than conventional gel. Therefore, elastic vesicles have superior characteristics compared to rigid conventional formulations (Lee, Kim, Nam, 2020; Maurya *et al*., 2010).

Gel was pale yellow in colour and odourless during its physical stability study. No significant change in results of stability at both tested temperatures because of Carbopol 934 P.

CONCLUSION

The results findings confirmed that Methotrexate loaded vesicular formulations were successfully deformed and permeated through in-vitro membrane and from excised goat skin as per in-vitro and ex-vivo study respectively. They lead to retention of drug in various strata of skin following transdermal application. Incorporation of TFS suspension into gel improves stability of transfersomes. Carbopol hydrogel of MTX-TFS provide sustain action for 24 hrs and permeate 90-95% (2.5 mg) of applied drug which is essential dosage of methotrexate in arthritis.

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