

Enhancement of Transdermal Delivery of Tamoxifen Citrate using Nanoemulsion Vehicle

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Abstract: The main objective of this study was to develop a potential of nanoemulsion formulation for transdermal delivery of tamoxifene citrate for breast cancer. Of the oils tested, arachis oil was chosen as the oil phase of the nanoemulsion, as it showed a good solubilizing capacity and excellent skin penetration rate of drug. The nanoemulsion area was identified by constructing pseudoternary phase diagrams. The prepared nanoemulsion was subjected to different thermodynamic stability tests. The nanoemulsion formulation that passed thermodynamic stability tests were characterized for droplet size, transmission electron microscopy and refractive index. The nanoemulsions were characterized by DSC and FTIR to ensure the compatibility among its ingredients. Transdermal permeation of tamoxifene citrate through rat skin was determined by Keshary-Chien diffusion cell. A significant increase in permeability parameter such as steady-state flux (J_{ss}) was observed in optimized nanoemulsion formulation A1, which consist of 5% wt/wt of drug, 4.12 % wt/wt of oil phase, 37.15 % wt/wt of surfactant (mix) and 58.73 % wt/wt of distilled water. It possessed a mean globule size of 68 nm. Transmission electron microscopy demonstrated spherical particle morphology and DSC and FTIR study revealed the compatibility among the ingredient. These results proposed that the prepared system could be promising to improve transdermal efficacy of the tamoxifen citrate.

Key Word: Transdermal drug delivery; nanoemulsion; in vitro permeation.

INTRODUCTION:

Tamoxifen citrate (TAM), an estrogen receptor antagonist is known to be a drug of choice for hormone sensitive breast cancer¹. Tamoxifen citrate is also indicated for treatment of estrogen receptor-positive tumors in the premenopausal population². Tamoxifen is generally administered through oral and parenteral route. Tamoxifen citrate undergoes extensive hepatic metabolism after oral administration in humans³. Despite being quite effective on oral administration, tamoxifen citrate exhibits certain side effects like distaste for food, abdominal cramps, nausea and vomiting. However, its other infrequent side effects include endometrial carcinoma, ocular problems, thromboembolic disorders and acquired

drug resistance on long-term therapy⁴⁻⁶. Therefore, developing a therapeutic system to provide a transdermal delivery is beneficial. Transdermal drug delivery may offer an alternative for the delivery of drug because it avoids the problems of gastrointestinal intolerance, avoid first pass liver metabolism and eliminates the need for intravenous access⁷. In recent years, much attention has been focused on lipid-based formulations to improve oral bioavailability of lipophilic drugs. In fact, the most popular approach is the incorporation of the drug compound into inert lipid vehicles such as oils, surfactant dispersions⁸, liposomes⁹, microemulsions, nanoemulsions, with particular emphasis on self-emulsifying and self-nanoemulsifying drug delivery systems

(SNEDDS)¹⁰. One of the most promising techniques for enhancement of transdermal permeation of drugs is the microemulsion or nanoemulsion technique¹¹⁻¹². Nanoemulsion are thermodynamically stable transparent (translucent) dispersions of oil and water stabilized by an interfacial film of surfactant and cosurfactant molecules having average droplet size of 10 to 140nm¹³⁻¹⁵. Many studies have shown that nanoemulsion formulations possess improved transdermal and dermal delivery properties both in vitro¹⁶⁻²⁸, as well as in vivo²⁹⁻³³. In transdermal delivery, the goal of dosage design is to maximize the flux through the skin into the systemic circulation. The nanoemulsion system is a promising vehicle due to powerful ability to deliver drug through skin²⁴. Therefore the present study focus potential of the nanoemulsion system in transdermal delivery of tamoxifen citrate (TAM).

MATERIAL AND METHODS:

Materials:

Tamoxifen citrate was a gift sample from Biochem Pharmaceutical Ltd (Mumbai, India). Arachis oil, Jojoba oil, Sesame oil, Coconut oil, Castor oil was purchased from S.D Fine Chemical(Mumbai, India). Labrafil M 1944CS was gift sample from Gattefosse. Cremophore EL, Tween 80 was gift sample from Cadila Health Care Ltd (Ahemdabad, India). Distilled water was purchased freshly from Chetak Distillery Ltd Rahuri, India. All other chemical and reagent used in the study were of analytical reagent grade.

Methods

Screening of Excipients:

The solubility of tamoxifen citrate in various oils (Arachis oil, jojoba oil, Coconut oil, Sesame oil, Castor oil) surfactants (Cremophore EL, Labrafil M 1944CS, Tween-80) and cosurfactants (Ethanol, Butanol, Propanol) was determined by dissolving an excess amount of tamoxifen citrate in 2 mL of each of the selected oils, surfactants and cosurfactants in 5-mL stoppered vials. Excess amount of tamoxifen citrate was added to each 5-mL stoppered vial and mixed using a vortex mixer. The vials were then kept at 37 ± 1.0 °C in an isothermal shaker (Nirmal International, India) for 72 hours to get to equilibrium. The equilibrated samples were removed from the shaker and centrifuged at 3000 rpm for 15 min. The supernatant was taken and filtered through a 0.45-µm membrane filter. The concentration of tamoxifen citrate was determined in each solution by UV spectrophotometer at 272 nm.

Pseudo-ternary phase diagram:

On the basis of the solubility studies, arachis oil was selected as the oil phase. Cremophore EL and ethanol were selected as surfactant and cosurfactant, respectively. Distilled water was used as an aqueous phase. Surfactant and cosurfactant (S_{mix}) were mixed at different mass ratios (1:1, 2:1). These ratios were chosen in increasing concentration of surfactant with respect to cosurfactant for a detailed study of the phase diagrams. For each phase diagram, oil and S_{mix} at a specific ratio was mixed thoroughly at different mass ratios from 1:9 to 9:1 in different glass vials. Nine different combinations of oil and S_{mix}, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, were made so that maximum ratios were covered for the study to delineate the boundaries of phases precisely formed in the phase diagrams. Pseudo ternary phase diagrams of oil, S_{mix} and aqueous phase were developed using the aqueous titration method³⁴. Slow titration with aqueous phase was performed for each mass ratio of oil and S_{mix} and visual observations were made for transparent and easily flowable o/w nanoemulsions. The physical state of the nanoemulsion was marked on a pseudo-three-component phase diagram with one axis representing the aqueous phase, the second one representing oil and the third representing a mixture of surfactant and cosurfactant at a fixed mass ratio.

Selection and preparation of nanoemulsion formulation:

From phase diagram constructed, different formulas were selected from the nanoemulsion region so that drug could be incorporated into oil phase. Exactly 5 % w/w of tamoxifen citrate, which was kept constant in all the selected formulations were subjected to different thermodynamic study.

Thermodynamic Stability Studies:

To overcome the problem of metastable formulation, thermodynamic stability test were performed. Selected formulations were centrifuged at 3500 rpm for 30 minutes. Those formulations that did not show any phase separations were taken for heating and cooling cycle. Six cycles between refrigerator temperature of 4°C and 45°C for 48 hours were done. The formulations that were stable at these temperature were subjected to the freeze-thaw cycle test. Three freeze-thaw cycles were done for the formulations between -21°C and +25 °C. The formulations that survived dispersion stability tests were selected for further studies and the compositions of these formulations are given in Table 1.

Table 1. The composition of selected formulations.

Formulation code	S _{mix} ratio	Oil: S _{mix} ratio	% Wt/Wt of Components in Nanoemulsion formulation			Drug %w/w
			Oil	S _{mix}	Water	
A1	1:1	1:9	4.12	37.15	58.73	5
A2	1:1	2:8	7.56	30.33	62.11	5
B1	2:1	1:9	3.74	35.30	60.96	5
B2	2:1	2:8	6.92	28.60	64.48	5

Characterization of nanoemulsions:

The prepared nanoemulsions were characterized by the following techniques.

Nanoemulsion Droplet Size Analysis:

Droplet size distribution of optimized nanoemulsion was determined by photon correlation spectroscopy, using a Delsa Nano-C (Beckman Coulter Instruments). Light scattering was monitored at 25°C at a scattering angle of 90°. The sample of optimized nanoemulsion was suitably diluted with distilled water and filtered through 0.22 µm membrane filter to eliminate multi scattering phenomena. The diluted sample was then placed in quartz cuvette and subjected to droplet size analysis.

Transmission Electron Microscopy (TEM):

Morphology and structure of the true nanoemulsion were studied using transmission electron microscopy (TEM) (Philips CM-10, USA) operating at 200 kV and capable of point-to-point resolution. To perform the TEM experiments, a drop (50 µL) of the true nanoemulsion was suitably diluted with distilled water (1:100), filtered through 0.22-µm filter paper and applied on carbon-coated grid with 2% phosphotungstic acid. It was left for 30 sec for drying purpose. The dried coated grid was taken on a slide and covered with a cover slip for TEM observations.

Refractive Index:

The refractive index of placebo formulation and drug loaded formulation was determined using an Abbe-type refractometer.

Viscosity:

The viscosity of nanoemulsion was determined using Brookfield cone and plate viscometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) at 25± 0.5° C.

Drug Content:

The nanoemulsion was suitably diluted with methanol to obtain required drug concentration of 10 µg/ml and absorbance was recorded by using UV spectrophotometer at 272 nm.

In vitro* skin permeation studies:*Preparation of rat abdominal skin:**

The male albino rats were sacrificed by excess chloroform inhalation (institutional Animals Ethics Committee, MES COP-1211/ac/08/CPCSEA, approved the protocol). Hair on abdominal skin was removed with electrical clipper taking extreme precaution not to damage the skin. The shaved skin was excised from the animal subcutaneous tissue. The full thickness skin thus prepared was soaked in distilled water at 60 °C for 60 s, followed by careful removal of epidermis. Skin was dried in desiccator at 25%RH and wrapped in aluminum foil and stored at 4 °C³⁵.

Procedure:

In vitro skin permeation studies were performed on a modified Keshary Chien-diffusion cell with an effective diffusional area of 1.76 cm² and 35 mL of receiver chamber capacity, using rat skin. The skin was brought to room temperature and mounted between the donor and receiver compartments of the Keshary-Chien diffusion cell where the stratum corneum side was facing the donor compartment and the dermal side was facing the receiver compartment. Initially, the donor compartment was empty and the receiver chamber was filled with phosphate buffer saline (PBS) pH 7.4. The receiver fluid was stirred with a magnetic rotor at a speed of 100 rpm and temperature was maintained at 37 ± 1 °C. The whole PBS was replaced with fresh one after every 30 min to stabilize the skin. It was found that the receiver fluid showed a negligible peak area after 2.5 h and beyond indicating complete stabilization of the skin. After complete stabilization of the skin, 1 mL nanoemulsion formulation was placed into the donor compartment and sealed with paraffin film to provide occlusive conditions. Samples were withdrawn at regular half intervals, filtered through 0.45-mm membrane filter and analyzed for drug content by UV Spectrophotometer at 272 nm.

FT-IR analysis:

FT-IR spectra of all sample of tamoxifen citrate, blank sample without drug and all formulations were recorded on Nicolet Magna 550 (USA) FTIR spectrometer using AgCl plates, in the frequency range 4000-400 cm⁻¹.

Differential scanning calorimetry (DSC):

DSC measurement was performed with (Dupont, USA 990). Sample of tamoxifen citrate and blank sample without drug, formulations were accurately weighed, encapsulated and hermetically sealed in flat bottomed aluminum pan with crimped on lid. The pans were positioned on sample pan holder. The samples were heated in an atmosphere of nitrogen over a temperature range from 5° C to 155° C with a constant heating rate of 10 °C/min.

Skin irritation test:

The authors followed the “Guideline of the institutional Animals Ethics Committee” for this experiment. The hair on the dorsal side of wistar albino rats was removed by clipping one day before the start of the experiment³⁶. The rats were divided into three group (n=6). Group I was the control (i.e., without formulation), Group II received A1 formulation, and group III received a 0.8% v/v aqueous solution of formalin as a standard irritant³⁷. New formulation or new formalin were applied daily for seven days. Finally, the application site was graded according to a visual scoring scale, always by the same investigator.

RESULTS AND DISCUSSION:

Lipophilic drugs are preferably incorporate in o/w nanoemulsions through the surface area of skin, the efficiency of the dosage form applied on the skin depends on the flux of the drug across the skin. Flux of the drug, that a formulator can alter, depends on the formulation components. Oil phase of nanoemulsion, in which the lipophilic drug is solubilized, is an

important criteria in the selection of formulation components. The physicochemical properties of tamoxifen citrate suggest that it has good potential for topical drug delivery³⁸. Tamoxifen citrate has sufficient partition coefficient and it suggests that tamoxifen citrate has sufficient lipophilicity to be formulated in to transdermal systems. The solubilization of tamoxifen citrate was found to be highest in arachis oil (4.26 ± 0.208 mg/ml) as compared to other oils as shown in fig 1. Hence, arachis oil was selected as the oil phase for the development of optimal formulation. The proper selection of surfactant and cosurfactant combination (S_{mix}) will contribute to the formulation of nanoemulsion and improving the stability. Although, the concentration of S_{mix} in the final formulation is less, the solubility of drug in S_{mix} will be additional contribution to the drug loading in nanoemulsion formulation. S_{mix} (Cremophore EL: Ethanol) selected in the study had the comparatively highest solubility of drug in respective components. Fig 1 shows that solubility of tamoxifen citrate was highest in cremophore EL (10.6 ± 0.529 mg/ml) and ethanol ($5.09 \pm .083$ mg/ml) component to other surfactant and cosurfactant respectively, studied.

Pseudo-ternary phase diagram:

The existence of stable nanoemulsion formation zone can be illustrated with the help of the pseudoternary phase diagram. Pseudoternary phase diagrams were constructed separately for each S_{mix} ratio so that o/w nanoemulsion region could be identified and nanoemulsion formulation could be optimized as shown in fig 2.

Fig 1. Graphical representation of solubility of tamoxifen citrate in selected oily phases (Jojoba oil, Coconut oil, Sesame oil, Castor oil, Arachis oil), surfactant (Cremophore EL, Labrafil M 1944CS , Tween 80) and cosurfactant (Ethanol, Butanol, Ethanol: Butanol, Propanol). Mean \pm SD, $n = 3$.

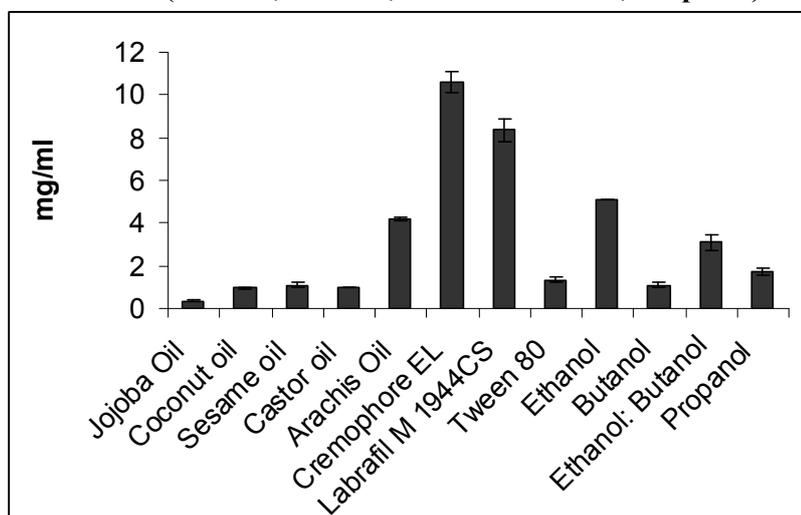
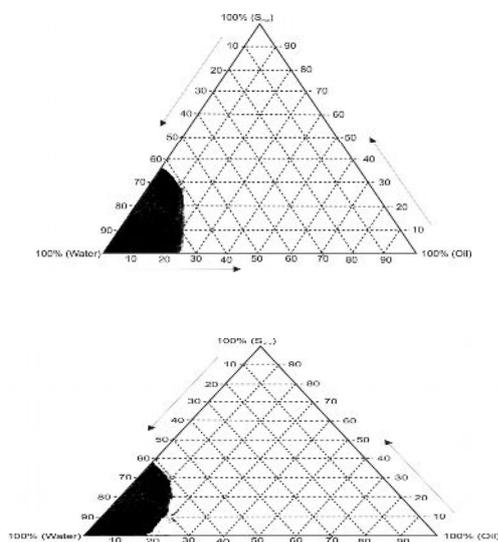


Fig 2. Pseudo-ternary phase diagrams showing the o/w nanoemulsion regions of Arachis oil (oil), Cremophore EL (surfactant), Ethanol (cosurfactant) at different S_{mix} ratios: a) S (surfactant: cosurfactant_{mix}) 1:1, 2:1.



Selection of nanoemulsion formulations:

From Pseudoternary phase diagrams, the formulations in which the amount of oil phase completely solubilized the drug and visual observations were made for transparent and easily flowable o/w nanoemulsion were selected for the further thermodynamic stability study.

Thermodynamic stability studies:

Nanoemulsion are thermodynamically and physically stable systems and are formed at a particular concentration of oil, surfactant and water, making them stable to phase separation, creaming or cracking¹³⁻¹⁴. Thus, the formulations were tested for their physical stability by using centrifugation, heating-cooling cycle and freez-thaw cycle. Only those formulations which survive thermodynamic stability tests were selected for further study as shown in table 1.

Droplet Size analysis:

Table 2, shows that mean droplet size of A1 (68.0 nm) was lower compared to other formulations studied also shown in fig 3. Among the formulations containing $S_{mix}(1:1)$, the mean droplet size increased as the

concentration of oil was increased, and was also increased relatively to same extent as the ratio between surfactant and cosurfactant were varying (i.e 2:1). All the formulation had droplets in the nano range, which is very clear from the low polydispersity values shown in table 2. Polydispersity is the ratio of standard deviation to mean droplet size, so it indicates the uniformity of dispersity, lower the uniformity of the droplet size seen in all formulation. The polydispersity of formulation A1 was lower (0.125) as compared to other formulations.

Transmission Electron Microscopy:

The TEM analysis revealed that nanoemulsions droplet of all formulation were spherical in shape, discrete with size in nanometer range (<100nm). Droplet size of optimized A1 formulation were measured and spherical in shape as shown in fig 4. This observation was consistent with that obtained in the globule size analysis using photon correlation spectroscopy.

Drug content:

Drug content of all the formulations (table 2) was $\geq 97.42 \pm 0.87$ % and less than 99.52 ± 0.52

Table 2. Droplet size, polydispersity and viscosity, drug content of nanoemulsion formulations

Code	Droplet Size(nm)	Polydispersity	Viscosity(cP) ^a	%Drug Content ^a	Flux Jss ^a ($\mu\text{g}/\text{cm}^2/\text{h}$)
A1	68.0	0.125	201 \pm 1	98.30 \pm 0.54	95.98 \pm 7.499
A2	86.2	0.245	288.66 \pm 2.15	99.52 \pm 0.52	61.82 \pm 2.955
B1	72.2	0.120	225.58 \pm 3.45	97.42 \pm 0.87	88.20 \pm 2.290
B2	118	0.257	300.25 \pm 2.40	98.45 \pm 0.25	52.56 \pm 3.037

^a Mean \pm SD, *n* = 3.

Fig 3. Photon correlation spectroscopy particle size analysis of formulation A1 showing average size distribution of the particle and statistical graph measurement by model distribution

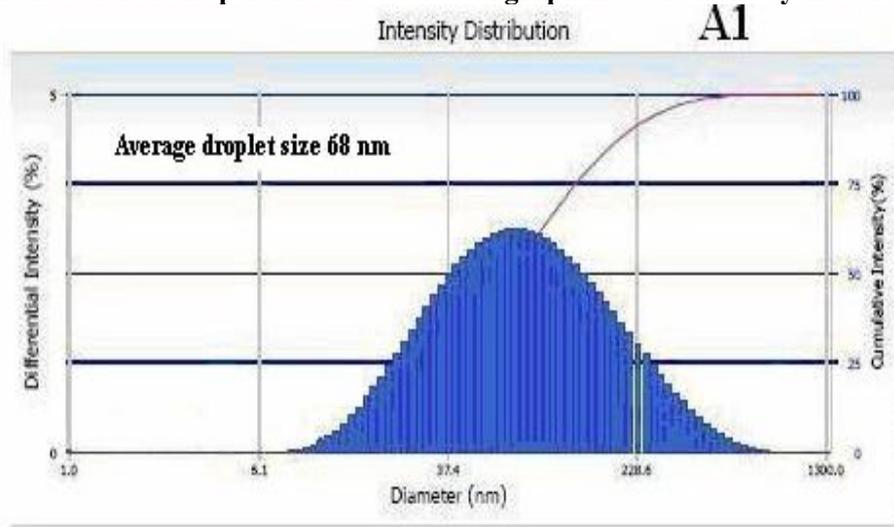


Fig 4. TEM photograph of A1 formulation showing measurement of droplet size less than 100nm.

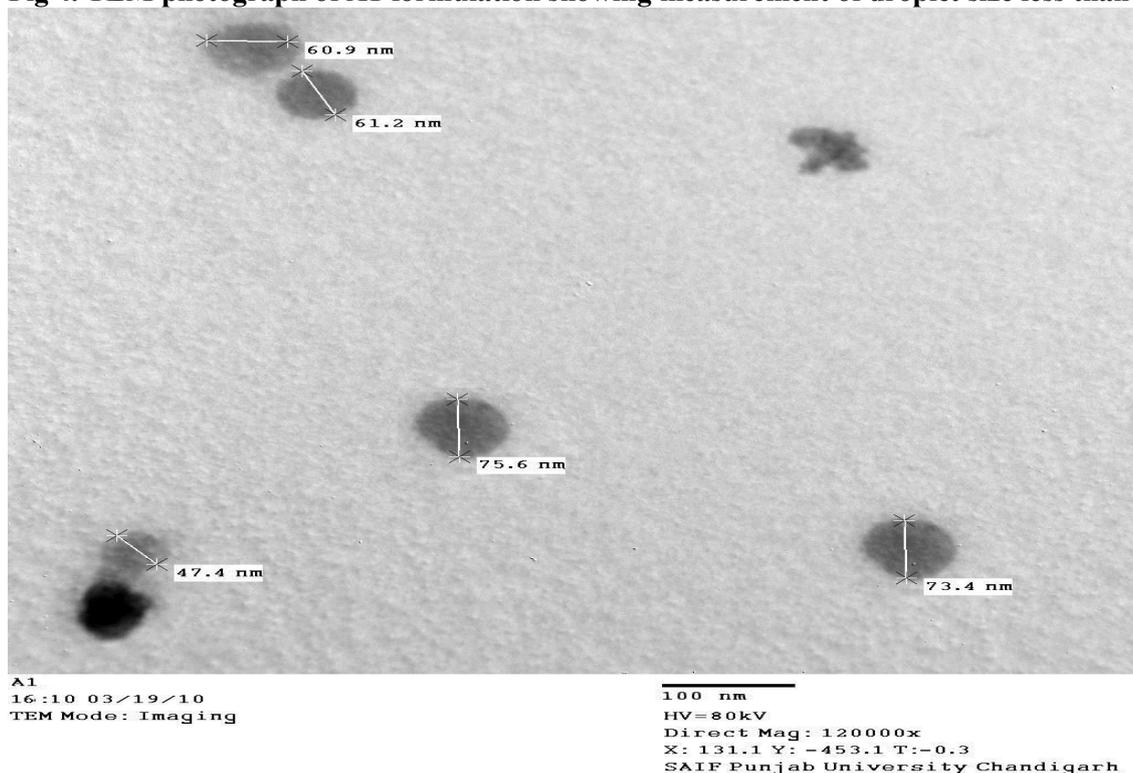


Table 3. Refractive index of nanoemulsion formulations

Code	Refractive Index ^a	
	Formulation	Placebo Formulation
A1	1.390 ± 0.0054	1.391
A2	1.392 ± 0.0065	1.393
B1	1.391 ± 0.0051	1.392
B2	1.391 ± 0.0055	1.392

^a Mean ± SD, *n* = 3.

Refractive Index:

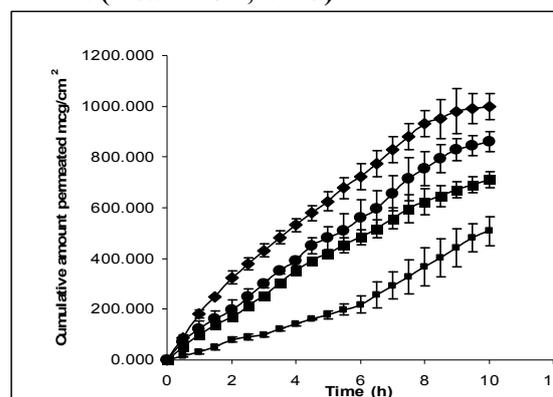
The mean values of the refractive index of drug-loaded formulations and placebo formulations are given in table 3. When the refractive index values for formulation were compared with those of the placebo, it was found that there were no significant difference between the values. Therefore, it can be concluded that the nanoemulsion formulations were not only thermodynamically stable but also chemically stable and remained isotropic. The result revealed that there were no interactions between nanoemulsion components and drug.

In vitro skin permeation studies:

In vitro skin permeation studies were performed in which skin permeation was highest in formulation A1 and lowest in B2 formulation (Fig. 5) Skin permeation profile of A1 was significantly different when compared with other formulation ($p < 0.05$). The maximum release in A1 could be due to having the lowest droplet size and lowest viscosity of all the nanoemulsions. Steady state flux (J_{ss}) of A1 formulation was $(95.98 \pm 7.499 \mu\text{g}/\text{cm}^2/\text{h})$ which is significant different ($p < 0.05$) when compared with other formulation as shown in table 2. To explain the probable mechanism by which nanoemulsions enhance the skin permeation of drugs, the histological and

Fourier Transfer Infra Red Spectroscopy Study:

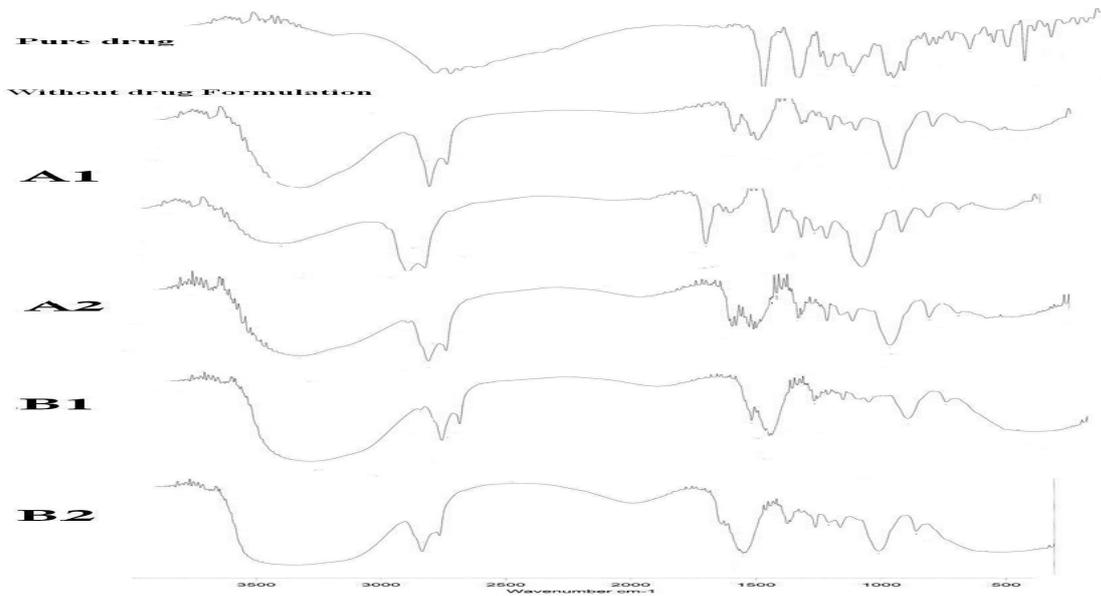
Drug excipient interaction study is one of the most important parameter, which depicts much information regarding the stability of formulation, drug release from them. The IR spectral analysis of tamoxifen citrate alone showed that the principal peaks were observed at wave numbers 3405.50, 1378.13, 1050.83, 1507, 1477, 3180, confirming the purity of the drug. The spectrum fig 6 shows the IR spectra of pure drug and the IR spectra of all nanoemulsion formulation. The major peaks of tamoxifen citrate were observed

Fig.5. In vitro skin permeation profile of tamoxifen citrate containing formulation A1-♦-, B1-●-, A2-■-, B2 -■-. (mean ± SD, *n* = 3)

histochemical structure of stratum corneum must be taken into consideration. Drugs permeate stratum corneum through two micro pathways, *i.e.*, intercellular and transcellular pathways. Of these, the intercellular pathway plays a major role in percutaneous uptake of drugs. It is well known that a complex mixture of essentially neutral lipids, which are arranged as a bilayer with their hydrophobic chains facing each others, forms a lipophilic bimolecular leaflet. Most of the lipophilic drugs pass through this region, and it is called a lipid pathway. The polar head group of lipids faces an aqueous region, forming a polar route that hydrophilic drugs generally prefer. A dermally applied nanoemulsion is expected to penetrate the stratum corneum and to exist intact in the whole horny layer, alter both lipid and polar pathways³⁹. A lipophilic drug like tamoxifen citrate can then permeate more easily through the lipid pathway of stratum corneum. Moreover, droplet size and viscosity of the nanoemulsion may also affect its efficiency, where the small droplet size and low viscosity of the nanoemulsion make it an excellent carrier for enhancing percutaneous uptake of tamoxifen citrate, since the number of vesicles that can interact on a fixed area of stratum corneum will increase when droplet size and viscosity decrease.

such as aliphatic alcohol O-H stretch (3405.50 cm^{-1}), phenolic C-O stretch (1378.13 cm^{-1}), and C-O stretch of ether (1050.83 cm^{-1}), and 1507 cm^{-1} and 1477 cm^{-1} (C=C ring stretching) and 3180 cm^{-1} (NH_2) in all nanoemulsions formulation. These result suggested that there was absence of drug degradation or drug excipient molecular interaction in all formulation. The comparison of IR spectra of blank nanoemulsion formulation with drug loaded nanoemulsion formulation.

Fig 6. FTIR study of Pure drug, Blank formulation without drug, A1, A2, B1, B2 formulations



DSC:

The DSC scan for tamoxifen citrate, blank formulation and optimized formulation are shown in fig 7. Tamoxifen citrate has well defined endothermic peak, with a melting point of 142-146°C and a heat of fusion of 88.95 J/g. The incorporation of tamoxifen citrate change the melting behavior of the excipient studied, producing a wider DSC curve. From fig 7 it is clear

that the formulation of tamoxifen citrate indicates the presence of small endothermic peak of tamoxifen citrate with reduction of intensity and shifting to lower temperature. Thermogram shows more reduction in size and intensity of the endothermic peak of the drug which may be due to its solubility in the nanoemulsion ingredients.

Fig 7. DSC study of pure drug, blank formulation without drug and optimized formulation

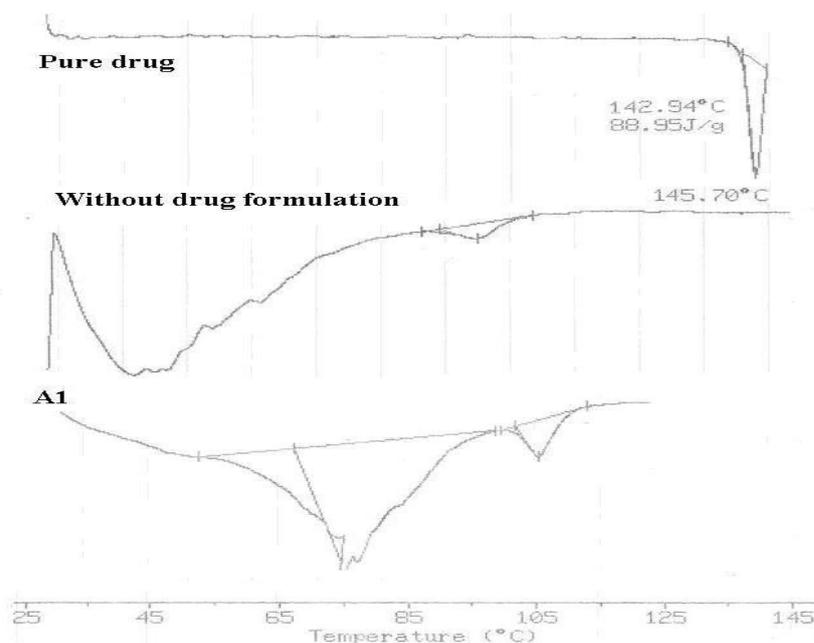


Table4: Skin irritation study of optimized A1 formulation.

Rat No	Control		A1		Formalin (Standard)	
	Erythema	Edema	Erythema	Edema	Erythema	Edema
1	0	0	0	1	1	1
2	0	0	0	1	2	3
3	0	0	1	1	3	2
4	0	0	1	0	2	3
5	0	0	2	1	3	2
6	0	0	0	1	3	2
Average			0.66±0.81	0.83±0.40	2.33±0.81	2.16±0.75
*Erythema scale:0 is none,1 is slight,2 is well defined,3is moderate,4 is scar formation						
**Edema scale:0 is none,1 is slight,2 is well defined,3 is moderate and 4 is severe						

Skin irritation test:

The skin irritation test of formulation A1 resulted in a score of less than 2 (erythema and edema) as shown in table 4. According to Draize et al, compounds producing scores of 2 or less are considered negative (no skin irritation)⁴⁰. From this it was concluded that the optimized nanoemulsion formulation was safe to be used for transdermal drug delivery.

CONCLUSION:

Suitable choice of the components is essential to minimize the irritancy effect and to determine an improvement of the percutaneous permeation of the drug through the stratum corneum. On the basis of highest drug permeation, lowest droplet size, lowest viscosity, we selected formulation of tamoxifen citrate containing 5 % of tamoxifen citrate, 4.12% of oil

phase (Arachis oil), 37.15% of surfactant mixture (Cremophore EL and ethanol) and 58.73% of distilled water as optimized formulation. The obtained results suggest a new opportunity for tamoxifen citrate to be incorporated in a novel transdermal formulation. The in vivo characteristics of the selected formulation are presently investigated.

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