RESEARCH ARTICLE

Component Screening of Miconazole Nitrate Nanoemulsion

Shinde P. B.*

JSIPH, Hadapsar, Pune

ABSTRACT

The aim of the present study was to investigate the potential of a nanoemulsion formulation for vaginal delivery of Miconazole Nitrate(MCZ). Various oil-in-water nanoemulsions were prepared by the spontaneous emulsification method. The nanoemulsion area was identified by constructing pseudoternary phase diagrams. The prepared nanoemulsions were subjected to different thermodynamic stability tests. The nanoemulsion formulations that passed thermodynamic stability tests were characterized for pH, refractive index, viscosity, droplet size, drug content, transmission electron microscopy, stability study and in vitro drug release through modified dissolution apparatus. A significant increase in drug release, and good in vitro and in vivo antifungal efficacy were observed in optimized NE formulation F4 which consist of 1%wt/wt MCZ, 20%wt/wt Captex200EP, 40% wt/wt Twen80:CapmulMCM(2:1) and 40% wt/wt distilled water. *In vitro* antifungal efficacy of formulation F4 showed a significant increase in percent inhibition when compared with MCZ cream on candida albicans induced vaginal infection in mice. These results suggested that NEs are potential vehicles for improved vaginal delivery of MCZ.

Keywords:- Miconazole Nitrate, nanoemulsion, screening, irritation study.

1. INTRODUCTION

Miconazole nitrate (MCZ) is a broad-spectrum antifungal agent of the imidazole group (1). It acts by means of a combination of two mechanisms: ergosterol biosynthesis inhibition, which causes lysis of fungal cell membranes because of the changes in both membrane integrity and fluidity, and direct membrane damage of the fungal cells. The drug is primarily used as a topical treatment for cutaneous mycoses (2); poor dissolution and lack of absorption make it a poor candidate for oral administration. However,MCZ can be used as a systemic antifungal agent when amphotericin B or ketoconazole is either ineffective or contraindicated. MCZ's poor skin-penetration capability presents a problem in the treatment of cutaneous diseases by topical application. For effective treatment, the drug must be delivered in sufficient concentration to the site of infection (3). Various approaches have been used to enhance the access of such poorly skin-partitioned drug molecules. For example, the use of complexation with cyclodextrins has been reported to improve oral and topical delivery of MCZ (4,5). Other approaches have used submicron emulsions of MCZ for improved topical delivery (6,7) and chewing gum containing MCZ for buccal delivery (8,9).

To design of effective formulations for MCZ has long been a major challenge, because efficacy can be severely limited by instability or poor solubility in the vehicle. One of the most promising technologies is the nanoemulsion drug delivery system, which is being applied to enhance the solubility and bioavailability of lipophilic drugs. The nanosized droplets leading to an enormous increase in interfacial areas associated with NE would influence the transport properties of the drug[10,11]. nanoemulsions are isotropic, thermodynamically stable transparent (or translucent) systems of oil, water, and surfactants with a droplet size usually in the range of 10–100nm. Their long-term stability, ease of preparation (spontaneous emulsification), and high solubilization of drug molecules make them promising as a drug delivery tool. They have found wide applications in oral drug delivery to enhance the solubility and bioavailability of the lipophilic drugs viz. MCZ. Recently, there has been a surge in the exploration of nanoemulsions for vaginal drug delivery. They are also being investigated ardently for potential applications in ocular, pulmonary, nasal, transdermal, and parenteral drug delivery. These systems often require high surfactant concentration, and this may lead to toxicity and irritancy

*Corresponding author: Shinde P. B. | JSIPH, Hadapsar, Pune | Email: pbshinde_2011@yahoo.com

problems. Therefore, judicious selection of surfactants along with their optimum concentration is required & determination of the influence of the surfactant-to-cosurfactant mass ratio (Smix) on the nanoemulsion formation region also formed an important aspect of the study. Optimum selection would aid in better formulation with desirable attributes[12].

The main objective of this study was to provide an efficient screening approach for the proper selection of oils, surfactants, and cosurfactants for the nanoemulsion formulation and vaginal irritation study.

2. MATERIALS AND METHODS

2.1. Materials:-

Miconazole was a gift sample from Camlin Fine Chemicals Ltd. (Bombay, India). Gift samples of Propylene Glycol Dicaprylocaprate (Captex200EP), Glycerol monocaprylocaprate (CapmulMCM) from USabitec (US) and Propylene glycol monocaprylate (Capryol 90), caprylocaproyl macrogol-8-glyceride (Labrasol) (Gattefosse, Gennevilliers, France) from Colorcon Asia (Mumbai, India) and polyoxy-35-castor oil (Cremophor EL) purchased from Sigma Aldrich (St.Louis, MO). Isopropyl myristate, castor oil, methanol, and ammonium acetate were purchased from E-Merck (Mumbai, India). Polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monostearate (Tween 60), polyoxyethylene sorbitan monooleate (Tween 80), ethanol, isopropyl alcohol, PEG 400, and propylene glycol were procured from S.D Fine Chemicals (Mumbai, India). Water was obtained from Milli Q water purification system (Millipore, MA). All other chemicals and solvents were of analytical grade.

2.2. Methods:-

2.2.1. Screening of components for preparation of nanoemulsion:-

Screening of oil:- The solubility of MCZ in various oils was determined by adding an excess amount of drug in 5 mL of the oils(Ethyl oleate, Oleic acid, Olive oil, Captex200P, Capryol90, Isopropyl myristate) separately in 10-mL-capacity stopper vials, and mixed using a vortex mixer. The mixture vials were then kept at 25±1.0°C in an isothermal shaker (Nirmal International, Delhi, India) for 72 h to reach equilibrium. The equilibrated samples were removed from the shaker and centrifuged at 3,000 rpm for 15 min. The supernatant was taken and filteredmthrough a 0.22-μm membrane filter. The concentration of MCZ was determined in oils using a UV spectroscopy at 272nm.

1.2 Screening of Surfactant:-

Five types of surfactants were screened for nanoemulsion formulation, which included Labrasol, Cremophor EL, Tween 20 and Tween 80. In water, 2.5 mL of 15 wt.% surfactant solution was prepared, and 4 μL of oil was added with vigorous vortexing. If a one-phase clear

solution was obtained, the addition of the oil was repeated until the solution became cloudy.

Screening of Cosurfactant:-

Selected surfactant was combined with three types of solubilizers as cosurfactants, namely CampulMCM, PEG 400 and propylene glycol. At a fixed Smix.(Surfactant and cosurfactant mixture) ratio of 1:1, the pseudoternary phase diagrams were constructed. Nine different combinations in different weight ratios of oil and Smix, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9(w/w) were taken so that maximum ratios were covered to delineate the boundaries of phases precisely formed in the phase diagrams.

2.2.2. Effect of surfactant & cosurfactant mass ratio on nanoemulsion region in pseudoternary phase diagram:-

Surfactant was blended with cosurfactant in the weight ratios of 3:1, 2:1, 1:1, 1:2, and 1:3. These Smix ratios were chosen in decreasing concentration of surfactant with respect to cosurfactant and increasing concentration of cosurfactant with respect to surfactant for detailed study of the phase diagrams. Aqueous titration method was used for the construction of the pseudoternary phase diagrams, which involves stepwise addition of water to each weight ratio of oil and surfactants, and then mixing the components with the help of vortex mixer at 25°C [13]. The nanoemulsion phase was identified as the region in the phase diagram where clear, easily flowable, and transparent formulations were obtained based on the visual observation. Nine different combinations in different weight ratios of oil and Smix, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9(w/w), were taken. One axis of the pseudothree-component phase diagram represented the aqueous phase, the other represented the oil phase, and the third represented Smix at a fixed weight ratio.

2.2.3. Selection of nanoemulsion Formulations:-

From each phase diagram constructed, different formulas were selected from the nanoemulsion region so that the drug could be incorporated into the oil phase[14]. Exactly 1% wt/wt of MCZ, which was kept constant in all the selected formulations, was dissolved in the oil phase of the nanoemulsion formulation. Selected formulations were subjected to different thermodynamic stability tests.

Formulation Code	FM 1	FM 2	FM 3	FM 4	FM 5	FM 6	FM 7	FM 8	FM 9
S:Cos ratio	1:1			2:1			3:1		
S:Cos Conc.	40	50	60	40	50	60	40	50	60
Water	40	30	20	40	30	20	40	30	20

*Oil conc.of 20%wt/wt was constant for all nanoemulsion formulation
Table 1 Composition of selected nanoemulsion formulations.

2.2.4. Thermodynamic stability:-

To overcome the problem of metastable formulation, thermodynamic stability tests were performed[15].

Selected formulations were taken for the heating and cooling cycle. Six cycles between refrigerator temperature 4°C and 45°C with storage at each temperature of not less than 48 hours were done. The formulations, which were stable at these temperatures, centrifuged at 3500rpm for 30 minutes. The formulations that did not show any phase separations were were subjected to a freeze-thaw cycle test. Three freeze-thaw cycles were done for the formulation between −21°C and +25°C. The formulations that survived thermodynamic stability tests were selected for further study.

2.2.5. Characterisation of nanoemulsion:-
All formulations were evaluated for pH (Digital pH meter), viscosity (Brookfield DV III ultra V6.0 RV), refractive index (Abbe-type refractometer) and drug content (potentiometric titration-USP2004).

2.2.6. Transmission Electron Microscopy:-
Morphology and structure studied using transmission electron microscopy (TEM), with Topcon 002B operating at 200 kV (Topcon, Paramus, NJ) and capable of point-to-point resolution. To perform the TEM observations, a drop of the nanoemulsion was directly deposited on the holey film grid and observed after drying[16].

2.2.7. Droplet Size:-
Droplet size distribution determined by photon correlation spectroscopy that analyzes the fluctuations in light scattering due to Brownian motion of the particles, using a Zetasizer 1000 HS (Malvern Instruments, Worchestershire, UK). Light scattering was monitored at 25ºC and at a 90ºangle [17].

2.2.8. In-vitro drug release:-
The drug release kinetics studied using a modified method. A glass cup with a cross-sectional area of 1.5 cm^2 was filled with 0.2ml of the nanoemulsion, covered with a cellophane membrane, sealed with a rubber band and adhesive tape, and inverted under the surface of 30 ml of simulated Vaginal fluid of pH 4.2 at 37°C ± 0.5°C in USP XXIII Type I Dissolution Test Apparatus with a speed of 30 rpm. 1ml of aliquots were withdrawn at specified time intervals and immediately replaced with fresh dissolution medium. The drug content in the withdrawn samples was determined spectrophotometrically at 272.5nm, keeping simulated vaginal solution as a blank. The drug content in sample was determined by software PCP disso version 3.08. simulated Vaginal fluid composition and preparation was similar to the one developed by Owen and Katz [18].

2.2.9. In-vitro antifungal efficacy:-
Ditch plate technique was used. It is a technique used for evaluation of bacteriostatic or fungistatic activity of a compound. Previously prepared Chloramphenicol yeast glucose agar medium (25ml) sterilized plates were used. 1ml NE formulations are placed in a ditch cut in the plate. Freshly prepared culture loops of *candida albicans* are streaked across the agar at a right angle from the ditch to the edge of the plate.

After incubation for 18 to 24 hours at 25°C, the fungal growth was observed and the percentage inhibition was measured as follows(19):

% inhibition = L2 / L1 × 100

Where L1 = total length of the streaked culture, and
L2 =length of inhibition.

2.2.10. Stability study:
Stability studies performed as per ICH guidelines. The samples were kept at four different conditions of temperature and relative humidity (%RH) as 40 ∘C/75% RH, 30∘C/65% RH, 25∘C/60% RH and refrigeration condition. The stability was observed over a period of 3 months. The samples were evaluated for particle size, viscosity and drug content[20,21].

2.2.11. Animal study:
All animal studies were performed on swiss albino female mice weighing 25-30gm. The pairs of mice were caged on wire in temperature and humidity controlled rooms and permitted food and water ad libitum. Animal study were approved and conducted in National toxicological centre, India and all animal care procedure were conducted according to committee for purpose of control and supervision of experiments on animals (CPSCEA, India) guidelines.

2.2.11. Vaginal irritation study:
Each group of 5 swiss albino female mice was administered intravaginally with 0.2ml of formulation twice daily for 10 days. The toxic manifestation if any on the vaginal region were then accessed by observing the vaginal mucosa at pre-selected time intervals between &after treatment for 10days. The findings were recorded in terms of the numerical scores for each animal as follows;

    0 = no visible irritation
    1 = mild irritation
    2 = moderate irritation
    3 = intense irritation.

The scores for the treated and the control groups were then compared.

2.2.12. Statistical analysis:
All *in vivo* irritation experiments results, as score data, were expressed as the mean value±S.D. The statistical significance of the treated group mean with that of control group was analysed using the one way analysis of variance, followed by Dunett's multiple comparison test using INSTAT3 software. The difference was considered statistically significant for P less than 0.05.

3. RESULTS AND DISCUSSION:-

3.1. Screening of oil:-
Lipophilic drugs are preferably solubilized in o/w nanoemulsions, whereas w/o systems seem to be a better choice for hydrophilic drugs. Drug loading per formulation

is a very critical design factor in the development of nanoemulsion systems for poorly soluble drugs, which is dependent on the drug solubility in various formulation components. The volume of the formulation should be minimized as much as possible to deliver the therapeutic dose of the drug in an encapsulated form. Solubility of the drug in the oil phase is an important criterion for the selection of oils. This is particularly important as the ability of nanoemulsion to maintain the drug in solubilized form is greatly influenced by the solubility of the drug in the oil phase. If the surfactant or cosurfactant is contributing to drug solubilization, there could be a risk of precipitation, as dilution of nanoemulsion will lead to lowering of the solvent capacity of the surfactant or cosurfactant[22,23]. Thus, an understanding of factors influencing drug loading capacity while maintaining the capability of the system to undergo monophasic dilution with water and minimizing the tendency for drug precipitation or crystallization in diluted systems is essential to the design of stable and appropriately low-volume nanoemulsion systems for drug delivery applications. As of late, novel semisynthetic medium chain derivatives, which can be defined as amphiphilic compounds with surfactant properties, are being preferred. Fig. 1 indicates the order of solubility of Miconazole nitrate is Captex200EP>Oleic acid>Capryol90>Ethyl oleate. This may be attributed to the non-polar nature of the poorly water soluble drugs that favors their solubilization in oils with small/medium molecular volume (containing medium chain triglycerides or mono- or diglycerides[24,25]. Among the various oils tested, Captex200EP made the most suitable oil phase viz. a synthetic ester (Propylene Glycol Dicaprylocaprate) with good stability, ability to dissolve large amounts of lipophilic drugs hence low volume required for solubilisation of drug and emulsification can be achieved with smaller quantities of surfactants with no risk of toxicity due to large concentration.
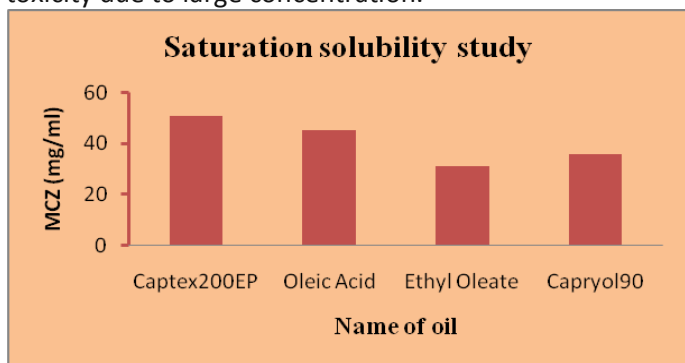

Fig. 1 Saturation solubility of Miconazole nitrate in different oils.

3.2. Screening of Surfactant:-

The most critical problem related to the nanoemulsion based systems is the toxicity of the components. Large amounts of surfactants may cause skin irritation when administered topically. Therefore, the proper selection of surfactants becomes necessary. It is, therefore, important to determine the surfactant concentration properly and use the minimum concentration in the formulation. Nonionic surfactants are relatively less toxic than their ionic counterparts and typically have lower CMCs. Also, o/w NE dosage forms for topical use based on nonionic surfactants are likely to offer in vivo stability[26]. Therefore, proper selection of surfactants becomes a crucial factor. Another important criterion is the selection of surfactant with proper HLB value. Hydrophilic surfactant and cosurfactant are considered to prefer the interface and to lower the necessary energy to form the nanoemulsions, consequently improving the stability. For example, the required HLB value to form o/w nanoemulsion is greater than 10[27]. The right blend of low and high HLB surfactants leads to the formation of a stable nanoemulsion upon dilution with water. After selection of captex200M as the oil phase, the goal was to identify the surfactant that has the highest solubiliza-tion capacity for the oil. In the present study, five nonionic surfactants, namely, Labrasol, Cremophor EL, Tween 20, and Tween 80, were chosen for screening.

Nonionic surfactants were selected since they are known to be less affected by pH and changes in ionic strength, are generally regarded as safe, and are biocompatible. Ionic surfactants were excluded from the study due to toxicological concerns. Although some authors had selected surfactants on the basis of drug solubility, we suggest that solubilization of oil with the surfactant is also an important factor. It is not necessary that the same surfactant that has good solubilizing power for drugs would have equally good affinity for the oil phase. Here, we have selected the surfactant giving the maximum nanoemulsion area alone, i.e., without the addition of the cosurfactant. The greater the nanoemulsion area is, the greater the nanoemulsification capacity of the surfactant is. As Tween 80 solubilized the maximum amount of Captrex200EP, i.e., 2.84 wt.%, it was chosen as the surfactant for the nanoemulsion development. Surfactant–oil miscibility can thus give an initial indication on the possibility of nanoemulsion formation with this system.

3.3. Screening of Cosurfactant

A single surfactant may not give a stable nanoemulsion when used at low concentration[28]. Hence, it is necessary to add a cosurfactant to nanoemulsion. The presence of cosurfactant decreases the bending stress of interface & imparts sufficient flexibility to the interfacial film to take up different curvatures required to form nanoemulsion over a wide range of composition. Thus the cosurfactants viz. PG, PEG400 & CapmulMCM were selected. These cosurfactants increase the mobility of the hydrocarbon tail & allows greater penetration of the oil into this region &

may also increase the miscibility of aqueous & oily phases due to its partitioning between these phases. Ternary phase diagrams of water, oil & surfactant-cosurfactant were plotted & nanoemulsion zone was identified. Based on the area of this zone CapmulMCM was selected as cosurfactant. The pseudo-ternary phase diagram with different cosurfactants viz. PEG400, PG & CapmulMCM are described in Fig.2 A, B& C respectively. The shaded portion in phase diagram indicate transparent nanoemulsion region.
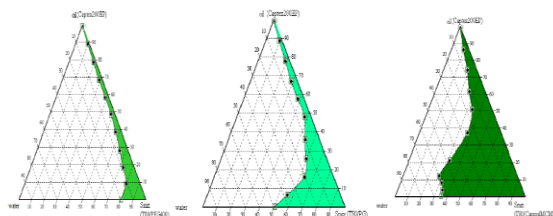

| **A** | **B** | **C** |

Fig.2 Pseudoternary phase diagram of with different S/CoS(1:1) viz. PEG400(A), PG(B) & CapmulMCM(C)

3.4. Effect of surfactant & cosurfactant mass ratio on nanoemulsion **region in pseudoternary phase diagram:-**

nanoemulsion formation is a function of composition of the system. The existence of nanoemulsion formation zone can be illustrated with the help of the pseudoternary phase diagram. The order of mixing of various components is not expected to influence the formation of nanoemulsion if the system is indeed thermodynamically stable (path-independent). Phase diagrams were constructed using Captex200P as the oil phase and Tween 80 and CapmulMCM as the surfactant and cosurfactant, respectively. No distinct conversion from w/o to o/w nanoemulsions was observed. The rest of the region on the phase diagram represents the turbid and conventional emulsions. Formulations were carefully observed so that the metastable systems were not selected, although the free energy required to form a nanoemulsion is very low and the formation is thermodynamically spontaneous. Fig. 3 shows phase diagrams of S:CoS weight ratios 1:1, 2:1, 3:1, 1:2 & 1:3 and gives suggested regions of formation of transparent nanoemulsions The outside regions on the phase diagram represented poorly formed nanoemulsions or conventional emulsions.[29]
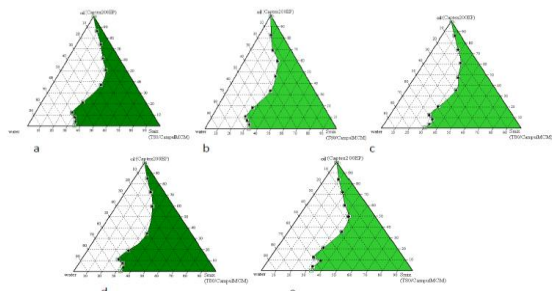

Fig. 3 Pseudoternary phase diagrams using Captex200EP as the oil phase and Tween 80/ CapmulMCM as the S/CoS rept 1:1(a), 2:1(b), 3:1(c), 1:2(d) & 1:3(e)

At 1:1 ratio of S:CoS, a nanoemulsion region was observed, perhaps because of the further reduction of the interfacial tension and increased fluidity of the interface. At this ratio 30%w/w of Smix was required for solubilization of 20%w/w oil and when this ratio increased upto 2:1 (Fig.2 b), a broader nanoemulsion region was observed (Fig.2 b). The maximum Smix. required for solubilization of 20%w/w oil was 28%w/w. When the S:CoS ratio was increased to 3:1 (Fig 2 c), a smaller nanoemulsion region was observed (Fig 2 c). Hence, it is observed that with increase in proportion of surfactant in Smix upto 2:1 there was increase in the nanoemulsion region which further, on increase in the proportion of surfactant, could not enhance the zone. Hence, the areas of one phase nanoemulsion zones are dependent on amount of surfactant in Smix. [30]. On the other hand the zone area was found to be independent of cosurfactant concentration. Thus the nature of cosurfctant & amount of surfactant in Smix. are the key factors influencing the nanoemulsion region. The effect of varying proportions of surfactant in Smix was further studied from pseudoternary phase diagrams.

The usual preference is to select formulations with the lowest surfactant concentration for topical application. However, for topical delivery, where enhanced skin permeation is the aim, it is not purposeful to select the lowest surfactant concentration. The surfactant concentration should be chosen so that it gives the maximum flux, which is an important criterion. This is usually not obtained with formulations that contain the highest amount of surfactant since high surfactant concentration decreases the thermodynamic activity of the drug in the vehicle, and the affinity of the drug to the vehicle becomes greater. Therefore, formulations should be optimized judiciously. As it could be seen from the phase diagrams, the surfactant or Smix that is able to increase the dispersion entropy, reduce the interfacial tension and increase the interfacial area, and thus, lower the free energy of the nanoemulsion system to a very low value with the minimum concentration, and that is thermodynamically stable, is a prospective candidate for efficient drug delivery[31].

3.5 Characterisation of nanoemulsion:-

All nanoemulsion appeared clear & transparent with pH in the range 5.3 to 5.5.

The mean values of the refractive index of drug-loaded formulations and placebo formulations are given in Table 2. When the refractive index values for formulations were compared with those of the placebo, it was found that there were no significant differences between the values. Therefore, it can be concluded that the nanoemulsion formulations were not only thermodynamically stable but also chemically stable and remained isotropic; thus, there

were no interactions between nanoemulsion excipients and drug.

Sr. No	Formulation Code	Placebo nanoemulsion	MCZ nanoemulsion
1	FM1	1.405 ± 0.007	1.401 ± 0.007
2	FM2	1.407 ± 0.006	1.403 ± 0.008
3	FM3	1.408 ± 0.008	1.404 ± 0.009
4	FM4	1.411 ± 0.015	1.409 ± 0.014
5	FM5	1.410 ± 0.014	1.407 ± 0.013
6	FM6	1.414 ± 0.013	1.411 ± 0.015
7	FM7	1.408 ± 0.005	1.404 ± 0.007
8	FM8	1.409 ± 0.008	1.405 ± 0.005
9	FM9	1.408 ± 0.009	1.406 ± 0.003

Table No. 2 Refractive Index of all Nanoemulsion formulation.

Drug content was found to be within pharmacopoeial limit NLT 98% & NMT 102%(United state Pharmacopoeia, 2004).

The viscosity of the selected formulations was determined (Table 3). The viscosity of formulation F4 (134.3 ± 1.41 cP) was lower than that of any other formulation, and this difference was significant (P<.05). The viscosity of formulation F9 was highest (148.3 ± 2.75 cP) attributed to increase in number of droplets(dispersed phase) due to increase in concentration of Smix & S:CoS ratio leading to dense packing within the nanoemulsion, but it was observed that the viscosity of the nanoemulsion formulations generally was very low. This was expected, because one of the characteristics of nanoemulsion formulations is lower viscosity.

The droplet size decreased with the increase in concentration and ratio of S:Cos in the formulations (Table 3). The droplet size of formulation F4, containing 2:1 ratio of S:Cos, was lowest (35.20 ± 1.24 nm). The droplet size of formulation F1 was highest (68.3 ± 5.26 nm). All the formulations had droplets in the nano range, which is very well evident from the low polydispersity values. Polydispersity is the ratio of standard deviation to mean droplet size, so it indicates the uniformity of droplet size within the formulation. The higher the polydispersity, the lower the uniformity of the droplet size in the formulation. Although the polydispersity values of all formulations were very low, indicating uniformity of droplet size within each formulation, the polydispersity of formulation F4 was lowest (0.035).
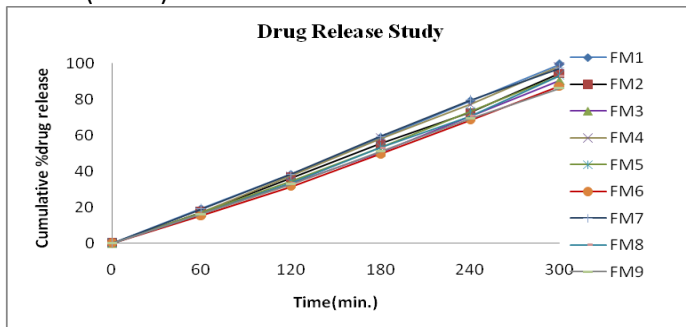

Fig.4. Cumulative % drug release of NEs.

Sr. No	Formulation Code	Conductance (mhos)	Droplet size (nm)	Polydispersity index	Viscosity at 100rpm (cps)	%Drug release (Q$_{300}$)
1	FM1	22.7	68.3±5.26	0.087	135.4±1.41	99.363
2	FM2	18.1	65.3±4.23	0.055	139.2±2.32	94.400
3	FM3	17.4	63.3±6.67	0.065	140.4±2.54	90.319
4	FM4	18.8	35.2±1.24	0.035	134.3 ± 1.41	98.337
5	FM5	15.3	35.1±2.40	0.038	145.5±4.32	93.488
6	FM6	15.0	35.0±2.32	0.045	147.6±3.42	87.392
7	FM7	17.8	36.6±3.33	0.051	140.3±2.56	97.894
8	FM8	14.9	35.9±5.42	0.065	146.5±2.44	93.133
9	FM9	14.3	35.6±4.26	0.058	148.3 ± 2.75	86.490

Table No. 3 Characterization of Nanoemulsion Formulations.

The increase in the concentration of Smix caused linear decrease in release of drug. This is because, Miconazole nitrate partitioned preferentially into internal phase of nanoemulsion due to its lipophilicity . With increase in Smix concentration, partitioning effect increased and hence, caused slow release of the drug. Moreover, with the addition of increasing concentration of Smix, the number of droplets in internal phase of nanoemulsion increased leading, increase in viscosity of internal phase and hence, decreased the release of drug from 99%-86%.

3.6. Thermodynamic Stability:-

In order to exclude the possibility of metastable formulations, stress testing is required. All the nine nanoemulsion formulations were subjected to the thermodynamic stability tests such as heating cooling cycles & centrifugation tests. No phase separation, turbidity, creaming/cracking was observed. All the nine nanoemulsion formulations were found to be stable after heating cooling cycles & centrifugation tests. Thermodynamic stability confers long shelf life to the nanoemulsion as compared to ordinary emulsions. It differentiates them from emulsions that have kinetic stability and will eventually phase-separate.

3.7. Transmission Electron Microscopy:- The droplet size decreased with increase in the concentration of the S:Cos in the formulations. However, the droplet size of all the formulations was in the nano range. The low polydispersibility values observed for all the formulations indicated uniformity of droplet size within each formulation.The droplets in the nanoemulsion appear dark, and the surroundings are bright; a "positive" image was seen using TEM (Fig.8). Some droplet sizes were measured using TEM, as it is capable of point-to-point resolution.
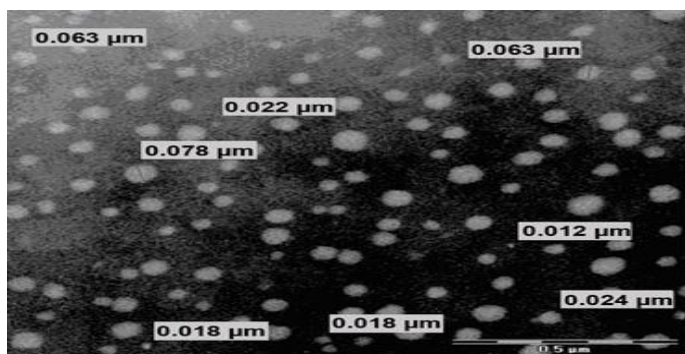
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Fig 5. Transmission electron micrograph of nanoemulsion showing the size of some oil droplets.

3.8. *In vitro* antifungal efficacy:-

The *in vitro* efficacy of the nanoemulsion formulations was compared with commercial cream formulations. The average zone of inhibition of the nanoemulsion formulations (1%w/w MCZ) against *Candida albicans* was ranging from 30$\pm$0.5mm to 33.21$\pm$0.5mm as compared to 33.40$\pm$0.6mm of commercial Miconazole cream(2%w/w MCZ) , indicating significantly efficacy of gel. From the results of *in vitro* antimicrobial activity it is clear that the developed nanoemulsion was as effective as commercial cream. The *in vitro* antifungal activity of Miconazole nanoemulsion may be attributed to enhanced penetration of oil globules containing Miconazole through fungal cell walls to inhibit ergosterol synthesis.

3.9. Vaginal irritation studies:

Vaginal irritation studies in mice were carried out for 10 consecutive days, wherein the irritancy symptoms were assigned numerical scores based on their intensity. Table No.4 lists irritation score of nanoemulsion with the positive and negative control for epithelial ulceration, edema and leukocyte infiltration.

No mortality was recorded in any of the groups during 10 days of vaginal application. Clinical examination of mice prior to application revealed no signs of vulval or vaginal irritation; discharge/bleeding from vagina for all mice of placebo group and nanoemulsion group. Unlike formalin treated mice which induce marked ulceration, severe epithelial loss and leukocyte infiltration (mean score 3 out of 4). None of the treatment group revealed any sign of irritation viz. epithelial erythema or edema or leukocyte infiltration (mean score 0 out of 4).

Group Name	Severity of irritation Symptoms		
	Epithelial Ulceration	Leukocyte infiltration	Edema
I NE Placebo	0$\pm$0	0$\pm$0	0$\pm$0
II NE with Drug	0$\pm$0	0$\pm$0	0$\pm$0
III Standard irritating solution	2$\pm$0.3	3$\pm$0.4	3$\pm$0.4

Table No. 4 Numerical score for in-vivo vaginal irritation study.

4. SUMMARY & CONCLUSION:-

 Proper selection of components is critical to an efficient NE formulation. Low-molar-volume oils viz. Captex200M for Miconazole nitrate are preferable instead of high-molar-volume oils, as they usually show better solubilization of the drug. As of late, novel semisynthetic medium chain derivatives, which can be defined as amphiphilic compounds viz tween80 and capmulMCM with surfactant properties, are being preferred. On the basis of optimum drug release, lowest droplet size, lowest polydispersity, lowest viscosity, and minimum surfactant and cosurfactant concentration, we selected formulation FM4 of MCZ, which contains Captex200EP(20% wt/wt), Tween 80:CapmulMCM(40% wt/wt), and distilled water (40% wt/wt), for use in vaginal irritation study. From in vitro antifungal efficacy and vaginal irritation data it can be concluded that the developed nanoemulsionshave great potential for vaginal drug delivery. Attention should be paid with regard to the tolerability of the constituting excipients. Recent efforts have, therefore, been focused on how to decrease or eliminate the toxicity or irritation of the nanoemulsion formulations. The study clearly illustrated the impact of the surfactant/cosurfactant weight ratio in the formulation of nanoemulsion systems. It is possible to achieve desirable properties by appropriately varying the level of oil, surfactants, and secondary surfactants.

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