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PREPARATION, OPTIMIZATION AND *IN VITRO* MICROBIOLOGICAL EFFICACY OF ANTIFUNGAL MICROEMULSION

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ABSTRACT

Recently much attention has been paid to the application of microemulsion as drug delivery system. Part of this interest appears as a consequence of their transparency, ease of preparation and long-term stability. These properties as well as their ability for incorporating drugs of different lipophilicity are some of the reasons why microemulsions have been thoroughly considered for pharmaceutical purpose. The aim of the present study is to improve the solubility of Terbinafine Hydrochloride, a slightly water-soluble antifungal drug by formulating a microemulsion made up of oil, surfactant and solubilizing agent/cosolvent. Tween 80, ajowan oil and peppermint oil were selected for preparing a microemulsion. Propylene glycol (PG) was used as a solubilizing agent. The effect of formulation variables on droplet size distribution was investigated. Droplet size was measured with Malvern Zetasizer instrument based on Photon Correlation Spectroscopy principle. The mean droplet diameter of microemulsions containing 1% w/w of terbinafine hydrochloride was below 100 nm and for the optimized formulation it was 8.1 nm. The optimized microemulsion was found to be stable and showed no physical changes when exposed to freeze-thaw cycles for 72 hours. Also it showed promising results in terms of achieving *in vitro* drug concentration above the MIC.

INTRODUCTION: Over the years, microemulsions have attracted more interest as potential drug delivery systems. Part of this interest appears as a consequence of their transparency, ease of preparation, and long-term stability. These properties as well as their ability for incorporating drugs of different lipophilicity are some of the reasons why microemulsion has been thoroughly considered for pharmaceutical purpose [1].

Microemulsions are optically transparent, low viscous and thermodynamically stable dispersions of oil and water stabilized by an interfacial film of a surfactant, usually in combination with a cosurfactant. In pharmaceutics, microemulsions are used as vehicles to deliver many kinds of drugs because of their thermodynamic stability, ease of preparation, and good appearance. In general, microemulsions can be separated into 3 types: water-in-oil (water/oil), bicontinuous, and oil-in-water (oil/water) [2, 3].

A microemulsion is a good candidate for oral delivery of poorly water-soluble drugs because of its ability to improve drug solubilization [4]. Absorption rate of a drug increases as its thermodynamic activity in the vehicle increases.

The thermodynamic activity can be expressed approximately in terms of relative solubility (the ratio of the current concentration of the drug to the concentration in saturated vehicle).

Hence, absorption rate should be very high from supersaturated vehicles. However, because of the physical instability of supersaturated systems, there are no common dosage forms available. This problem might be overcome by application of systems that become supersaturated *in situ*. Such systems are represented by microemulsions [5].

Fungal infections are common in human beings, which are either topical or severe systemic infections. Invasive fungal infections are being identified with an ever-increasing frequency in premature infants, immuno-compromised hosts, and patients receiving immunosuppressive agents and those with acquired immuno- deficiency syndrome (AIDS). The prevention of fungal infections has been improved by the antifungal agent such as Terbinafine [6].

Terbinafine hydrochloride is a synthetic allylamine antifungal compound. It is currently employed as an agent against fungal infections of toes or fingernails caused by the fungus, Tinea unguium. It has shown activity *in vitro* against most strains of other microorganisms including *Candida albicans.* Terbinafine hydrochloride is a slightly water-soluble drug therefore its release rate is slow, resulting in drug concentrations in the body fluids which are well below the Minimum Inhibitory Concentrations.

In the present study, a microemulsion system composed of oil, surfactant and co solvent was designed and developed to enhance the availability and efficacy of antifungal drug Terbinafine HCl against Candida albicans.

MATERIALS AND METHODS: Terbinafine hydrochloride was supplied by Dr. Reddy's Lab, Hyderabad. Ajowan oil, sodium benzoate and propylene glycol were supplied by Panacea Biotec Ltd., New Delhi. Peppermint oil was obtained from Quest International, India. Polysorbate 80 was obtained from Croda chemicals, Europe and Polysorbate 20 was obtained from S.D. Fine Chemicals, Delhi. All other chemicals were of reagent grade and used without further purification. The reference strain of *Candida albicans* used in this study was obtained from NCIM, Pune.

Solubility study of Terbinafine Hydrochloride: Solubility study was performed in about forty different solvents for selection of excipients for micro emulsion formulation. One gram of excipient was taken and terbinafine hydrochloride was added with vortexing till saturation. The mixture was kept at ambient temperature for 48 hours and allowed to attain the equilibrium. A Solubility criterion chosen was visual examination for any undissolved particulate matter. The solubility of Terbinafine hydrochloride in different excipients is given in **Table 1**.

TABLE 1: SOLUBILITY OF TERBINAFINE HCL IN DIFFERENT EXCIPIENTS

Sr. No.	Excipient (1 g)	Solubility	Sr. No.	Excipient (1 g)	Solubility
1	Tween 80	< 50 mg	19	Squalene	N.S.
2	Lecithin	N.S.	20	PEG 4000	N.S.
3	Corn oil	N.S.	21	Poloxamer F127	N.S.
4	Sunflower oil	N.S.	22	Poloxamer F68	N.S.
5	Propylene glycol	50 mg	23	Glycerin	<50 mg
6	Isopropyl myristate	N.S.	24	White soft paraffin	N.S.
7	Squalene	N.S.	25	Liquid paraffin	N.S.
8	PEG 4000	N.S.	26	Stearic acid	N.S.
9	Oleic acid	N.S.	27	Sesame oil	N.S.
10	Triacetin	N.S.	28	Soyabean oil	N.S.
11	Triethyl citrate	N.S.	29	Ethyl oleate	N.S.
12	Tween 20	N.S.	30	Cetostearyl alcohol	25 mg
13	Benzyl benzoate	N.S.	31	S LS	N.S.
14	Span 80	25 mg	32	Cetomacrogol 1000	N.S.
15	Peanut oil	N.S.	33	Ajowan oil	>50 mg
16	Triethanolamine	<25 mg	34	Peppermint oil	200 mg
17	Diethyl phthalate	50 mg	35	Orange oil	N.S.
18	Dibutyl sebacate	N.S.	36	Lemon oil	N.S.

Note: N.S. stands for 'Not soluble as solubility was less than 0.5% w/w

Preparation of Microemulsion: A total of 100 mg of terbinafine hydrochloride was mixed with 100 mg of propylene glycol (Cosolvent) and an oil-surfactant mixture consisting of different ratios of Tween 80 to either ajowan oil or peppermint oil. Different compositions selected are shown in **Table 2**. Premicroemulsion concentrate containing terbinafine hydrochloride was added to double distilled water to make up the weight to 10g (1% w/w). The preparation was vortexed for 1-2 minutes to get a uniform product.

TABLE 2: PREMICROEMULSION CONCENTRATE FORMULA

Batch No.	Oily vehicle		Surfactant (weight in mg)		Cosolvent	Drug
	Ajowan	Peppermint	Tween 80	Tween 20	PG	
A_1	300	-	500	-	100	100
A_2	300	-	-	500	100	100
A_3	200	-	1000	-	100	100
A_4	150	-	1000	-	100	100
A_5	-	150	1000		100	100
A_6	-	200	1000	-	100	100
A_7	-	300	500	-	100	100
A_8	-	300	-	500	100	100

Droplet Size Determination: The droplet size distribution and the average droplet size of microemulsion with drug were measured at 25±0.5ºC by Photon Correlation Spectroscopy. A light scattering spectrophotometer equipped with data processing unit was used for characterizing the droplet size using dynamic light scattering method [7].

Entrapment of drug in Microemulsion: Entrapment of drug in oil droplets of o/w microemulsion was confirmed by diluting the premicroemulsion concentrate with 0.01N NaOH solution instead of distilled water. As NaOH causes precipitation of drug by reacting with its hydrochloride salt, so if drug is present in dispersion medium and not incorporated in oil droplets, it will precipitate out.

Drug Release Study: The release profile of Terbinafine hydrochloride from both microemulsion and gel formulation was generated from the percentage of Terbinafine hydrochloride released into the receptor chamber of modified Franz Diffusion Cell at each sampling point. A test formulation (either microemulsion or gel) at an equivalent amount of Terbinafine HCl (10 mg), was placed on a Durapore membrane (Millipore) in each donor chamber of Franz Diffusion Cell. Distilled water was used as a receptor medium for all tests. The temperature was maintained at $37_{\pm}0.5$ C throughout the experiment and receptor medium was stirred using magnetic stirrer. An aliquot of the sample (0.5 mL) was taken at predetermined time intervals and similar volume was replaced with fresh distilled water. The samples were analyzed spectrophotometrically. The release profiles of Terbinafine HCl from microemulsion and gel formulation were constructed by plotting the percentage of Terbinafine HCl released against time in minutes [8].

Stability of Microemulsion: The stability of microemulsion was evaluated by exposure to freeze-thaw cycles for 72 hours.

MIC Determination of Terbinafine Hydrochloride [9]: MIC was determined by viable counting technique in which viable counts were performed at varying time intervals on the surviving number of a yeast population in contact with the solution of the drug. The culture was standardized to 85% transmittance at 530 nm. Different concentrations of drug were prepared in Dimethyl sulfoxide (DMSO) from 100-400µg/ml. To each concentration (9.9 ml), 0.1 ml of culture was added. DMSO without drug was used as negative control. These dilutions were incubated at 30-35°C and 0.1 ml aliquots from each dilution were plated after 0, 2, 4 and 6 h respectively. Colony count was performed after 18-24 hours.

Determination of Microbiological Efficacy of formulations using Agar Well Method: The test organism was grown in nutrient broth for 18-24 h at 30-35°C. The concentration of cells was adjusted to

85% transmittance at 530 nm using sterilized broth as blank. This yields a cell suspension containing 1-5×10^6 organisms/ml which was confirmed by colony counting. One mL of the suspension was plated with 25 ml of melted and cooled nutrient agar medium. The plates were kept undisturbed for setting of agar.

For efficacy testing, 3-4 wells were dug on the solidified medium plates seeded with test organism using a sterile hole borer (7mm diameter). Weighed amount of each formulation equivalent to 2mg of the drug, including the marketed gel (Daktarin oral gel) were poured into different wells. The plates were then allowed to remain undisturbed for one hour to have even diffusion of drug into agar. The plates were then incubated at 30-35°C without inversion for 18-24 h. At the end of incubation period, zones of inhibition formed around the wells were measured with the help of a divider and scale and expressed in mm.

RESULTS AND DISCUSSION:

Droplet Size Analysis: The average droplet size of microemulsion prepared using Tween 80 or Tween 20 as surfactant; peppermint oil or ajowan oil as oily phase, and a cosolvent was measured using zetasizer analyzer. Among the formulations prepared (A_1-A_8), the most promising droplet size was found with formulations A_4, A_5 and A_6. Also when ajowan oil was replaced by peppermint oil and the two were used in the same oil: surfactant ratio, a decrease in droplet size was observed. The average droplet size is shown in **Figure 1 and Table 3** for the formulations A_4, A_5 and A_6. As droplet size was minimum with formulation A_6, so it was assumed to be the optimum formulation and further studies are done with this optimized formulation.

TABLE 3: AVERAGE DROPLET SIZE AND POLYDISPERSITY INDEX FOR FORMULATIONS

Batch No.	A_4	A_5	A_6
Kcps	128.7	51.5	44.2
ZAve (nm)	13.691	12.434	8.100
Polydispersity Index	0.438	0.206	0.279

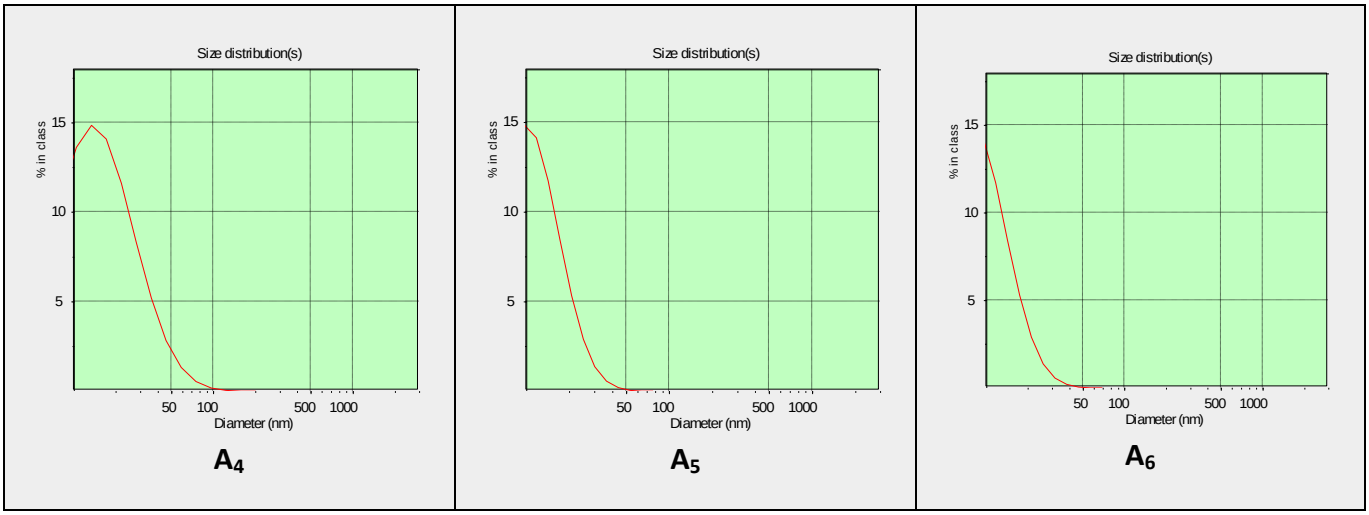

FIG. 1: DROPLET SIZE DISTRIBUTION OF FORMULATIONS

Entrapment of drug in microemulsion: After diluting the premicroemulsion concentrate with 0.01N NaOH solution, no precipitation was seen. This indicates that nearly whole of the amount of drug was entrapped in microemulsion droplets.

Stability study: The stability study of 1% w/w terbinafine hydrochloride- loaded optimized microemulsion was done by exposing it to freeze-thaw cycles for 72 hours. The microemulsion was found to be stable and showed no physical changes.

Drug Release Study: Comparison of Terbinafine HCl release was done for optimized microemulsion formulation A_6 and gel formulation using Franz Diffusion Cell. Results are shown in **Table 4**. Approximately 72% release was achieved with gel formulation whereas micro emulsion enhanced the release to 92% in one hour as shown in Table 4 and **Figure 2**.

TABLE 4: COMPARATIVE DRUG RELEASE PROFILE

Time (minutes)	Percent Release (Mean ± SD) (N=3)	
	Formulation A_6	Gel (1% w/w)
10	89.15 ± 0.29	68.36 ± 0.35
20	91.36 ± 0.29	69.83 ± 0.45
30	91.58 ± 0.26	71.58 ± 0.39
40	91.58 ± 0.26	72.31 ± 0.35
50	91.80 ± 0.40	72.88 ± 0.51
60	92.14 ± 0.60	72.99 ± 0.69
75	92.37± 0.74	73.10 ± 0.45
90	92.65 ± 0.84	73.33 ± 0.48

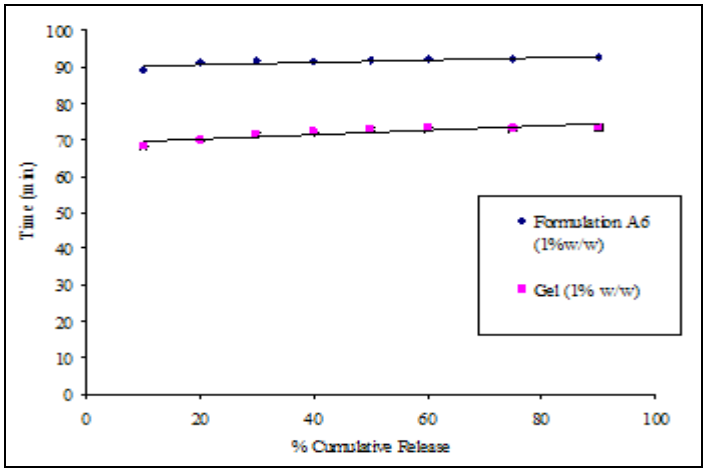

FIG. 2: COMPARATIVE DRUG RELEASE PROFILE

MIC Determination of the Drug: Different drug concentrations were made in DMSO varying from 100-400 µg/ml. This range was selected as reported in literature. Initially there were ~10^6 cfu/ml, these were reduced to negligible in 400 µg/ml concentration after 2 h of incubation only. In all the remaining concentrations, no colony was seen after 4 hours of incubation. So MIC of the drug was found to be < 100µg/ml as depicted in **Table 5**. **Figure 3** gives the pictorial comparison between the optimum microemulsion formulation, marketed gel, plain gel and placebo gel. The measurements of zone of inhibition of different formulations are given in **Table 6**.

TABLE 5: EFFECT OF DRUG CONCENTRATIONS ON CELL COUNT OF *CANDIDA ALBICANS* AT DIFFERENT TIME INTERVALS

Concentration (µg/ml)	0 h	2 h	4 h	6 h
100	1.532×10^6	2.65×10^3	nil	nil
200	1.237×10^6	3.9×10^2	nil	nil
300	1.156×10^6	3.0×10^1	nil	nil
400	1.485×10^6	nil	nil	nil

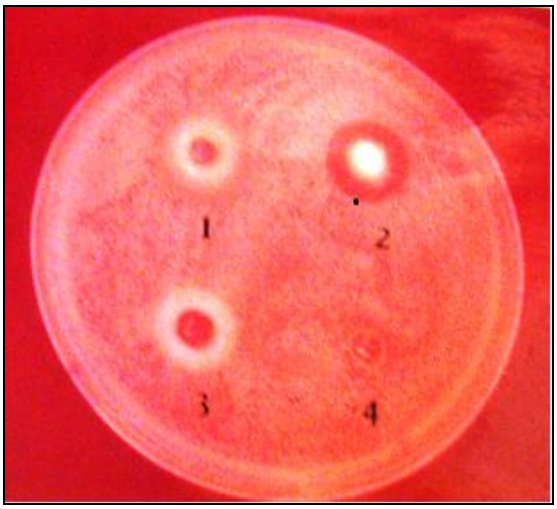

FIGURE 3: ZONE OF INHIBITION 1) MICROEMULSION, 2) MARKETED, 3) GEL (1%W/W), 4) PLACEBO GEL

TABLE 6: ZONE OF INHIBITION OF DIFFERENT FORMULATIONS

Gel type	Zone of inhibition (in mm)
Marketed gel	15
Microemulsion	14
Gel (1% w/w)	No clear zone
Placebo gel	No zone

Zone of inhibition in case of microemulsion was found to be clearer as compared to the gel preparation. Also, zone of inhibition of microemulsion was comparable to the marketed gel.

CONCLUSION: Microemulsion can be formulated to enhance the solubility of slightly soluble compounds and also, to increase the dissolution rate of the drug. Terbinafine hydrochloride as microemulsion showed better efficacy against *Candida albicans* as well. Microemulsion prepared using peppermint oil was the optimized formulation with droplet size less than 10 nm and good stability. It is concluded that new microemulsion formulation has shown promising results in terms of achieving *in vitro* drug concentration above the MIC. These results may be further evaluated under clinical applications in order to have a more effective dosage form for the treatment of oral Candidiasis.

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