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Investigation of Efficiency of Isopropyl Myristate-Based Oil in Water Microemulsions for Topical Delivery of Fluconazole

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ABSTRACT

This paper investigates the efficiency of isopropyl myristate (IPM)-based oil in water microemulsion formulations for topical delivery of fluconazole (FLZ). IPM was screened as the oil phase of microemulsions due to a good solubilizing capacity of the microemulsion system. The pseudo-ternary phase diagrams for microemulsion regions were constructed using IPM as the oil, Tween-80 (T-80) as the surfactant and propylene glycol (PG) as the co-surfactant. The physicochemical properties of selected microemulsion formulations were examined for pH, viscosity, conductivity, solubility, polarized light microscopy, transparency, drug content and particle size distribution. Efficiency of microemulsion formulations to deliver FLZ through the rat skin was evaluated *in vitro* using Franz diffusion cells fitted with excised rat skins. The *in vitro* permeation data showed that microemulsion increased the permeation rate of FLZ six times over the saturated aqueous solution. Finally the microemulsion containing 2% FLZ was optimized with 14% IPM, 42% T-80/PG (5: 1) and 42% double distilled water (w/w), which showed a highest permeation rate of 67.58 ± 1.26 μg cm^{-2} h^{-1}. The infrared study of optimized formulation showed the absence of any possible interaction between drug and formulation components. *Candida albicans* was used as the model fungus to evaluate the antifungal activity of the best formula achieved, which showed the widest zone of inhibition compared to FLZ reference. The studied microemulsion formulation showed good stability over a period of three months. These results indicate that the studied microemulsion formulation might be a promising vehicle for topical delivery of FLZ.

Keywords: antifungal activity, *Candida albicans*, *in vitro* skin permeation study, rat skin

INTRODUCTION

Fluconazole (FLZ) is chemically 2-(2, 4-difluorophenyl)-1, 3-*bis*-(1H-1, 2, 4-triazole-1-yl)-2-propanol, a synthetic triazole which is one of the most commonly prescribed antifungal drugs. FLZ has emerged as the primary treatment option for virtually all forms of susceptible *Candida* infections in both immunocompetent and immunocompromised hosts (Meis *et al.* 2000). It acts by blocking the synthesis of ergosterol, an essential component of the fungal cell membrane. It has been developed for oral administration and is not practically used as a remedial formulation for external application.

When FLZ-based oral formulation is administered to treat a fungal disease, an excess dose of the formulation is required to obtain the desired curing value (**Table 1**). How-

Table 1 Recommended dose of oral fluconazole for treatment of fungal diseases.

Fungus	Clinical use	Dosage
Candida spp. (except *C. krusei* and *C. glabrata*)	Vaginal candidiasis	150 single dose
	Oropharyngeal candidiasis	100-200 mg/day
	Oesophageal candiasis	200 mg/day
	Candida urinary tract infections and peritonitis	50-200 mg/day
Epidermophyton spp.	Tinea corporis, tinea cruris or tinea pedis	50 mg daily or 150 mg once weekly
Trichophyton spp.		
Microsporum spp.		
Epidermophyton spp.	Onychomycosis	150 to 450 mg weekly
Trichophyton spp.		
Microsporum spp.		
Acremonium spp.		
Aspergillus spp.		
Candida spp.		
Fuserium oxysporum		
Scopulariopsis brevicaulis		
Onychocola canadensis		
Scytalidium dimidiatum		
Cryptococcus neoformans	Cryptococcal meningitis	400 to 800 mg/day
Coccidioides immitis	Coccidioidomycosis	400 to 800 mg/day
Cryptococcus neoformans	Cryptococcosis	400 mg/day
Histoplasma capsulatum	Histoplasmosis	800 mg/day

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Microemulsion formulation components			Drug	*In vitro*/ *In vivo* studies	Membrane/skin /species used	Reference
Surfactant/Cosurfactant	Oil phase	Aqueous phase				
Epikuron 200, benzyl alcohol, sodium taurocholate	Isopropyl myristate, decanol, Ascorbyl palmitate	Water, ascorbic Acid	Apomorphine hydrochloride	*In vivo*	Human	Priano *et al.* 2004
Labrasol, plurol oleique	Isopropyl myristate	Water	Diclofenac	-	-	Djordjevic *et al.* 2004
Cremophor ELP	Labrafil M1944CS	Water	Aceclofenac	*In vitro*	Rat skin	Lee *et al.* 2005
Caprylic/capric Mono-/diglycerides, polysorbate 80	Isopropyl myristate	Water	Celecoxib	*In vitro*	Rat skin	Subramanian *et al.* 2005
AOT	Isopropyl myristate	Water	5-Fluorouracil	*In vitro*	Mouse skin	Gupta *et al.* 2005
Labrasol, transcutol	Labrafil M1944CS, plurol oleique	Water	Dehydoepiandrosterone	*In vitro*	Pig skin	Ceschel *et al.* 2005
Tagat 02, span 80	Isopropyl palmitate	Water	Desmopressin acetate	*In vitro*	Human skin	Getei *et al.* 2005
Brij 58, span 80, isopropyl alcohol, propanol	Soybean oil	Water	Diclofenac sodium	*In vitro*	Rabbit skin	Kantarci *et al.* 2005
Labrasol, transcutol	Lauroglycol, butylated hydroxytoluene	Water	Fluoxetine	*In vitro*	Human skin	Parikh and Ghosh 2005
AOT, tween 85	Isopropyl myristate	Water	Cyclosporin A	*In vitro*	Rat skin	Liu *et al.* 2006
Tween 85, ethanol	Isopropyl myristate	Water	Meloxicam	*In vitro*	Rat skin	Yuan *et al.* 2006
Tween 80, isooctanol	Olive oil	Water	Nimesulide	*In vitro*	Rat skin	Derle *et al.* 2006a
Tween 80, PG	Oleic acid	Water	Valdecoxib	*In vitro*	Human skin	Derle *et al.* 2006b
Lecithin, *n*-propanol	Isopropyl myristate	Water	Tetracaine	*In vitro*	Mouse skin	Changez *et al.* 2006b
Lecithin, *n*-propanol	Isopropyl myristate	Water	Tetracaine	*In vivo*	Rat	Changez *et al.* 2006a
Labrasol, cremophor RH 40	Oleic acid	Water	Theophylline	*In vivo*	Rat	Zhao *et al.* 2006
Brij 30, ethanol	Eucalyptus oil	Water	Cyproterone acetate	*In vitro*	Pig	Biruss *et al.* 2007
Tween 80, ethanol	Isopropyl myristate	Buffer pH 7.4	Diclofenac	*In vitro*	Guinea pig	Kama *et al.* 2007
Brij 30, ethanol	Eucalyptus oil	Water	Progesterone	*In vitro*	Pig	Biruss *et al.* 2007
Tween 80, span 20, ethanol	Isopropyl myristate	Water	Sodium nonivamide acetate	*In vitro*	Rat	Huang *et al.* 2008
Brij 96V, hexanol	Jojoba oil	Water	Diclofenac sodium	*In vivo*	Rat	Shevachman *et al.* 2008
Cremophore EL, ethanol	Oleic acid	Water	Penciclovir	*In vitro*	Mouse skin	Zhu *et al.* 2008
Cremophore El, benzyl alcohol, chlorocresol	Capryol 90	Water	Fluconazole	*In vitro*, *In vivo*	*Candida albicans*, human	Bachhav and Patravel 2009

ever, long-term administration of triazole-based drugs may be a burden for the liver and cause undesired distribution for the drug through thereof over the entire body. The antifungal formulations for external use, especially pharmaceutical compositions containing tolnaftate, clotrimazole, ketoconazole and the like having activity for *Candida* species, are generally available for the remedy of diseases related to the fungi, including fungal infections. However, despite an excellent level of activity relative to a variety of microorganisms these known compositions exhibit poor absorption and/or penetration into the horny layer of the skin resulting in an unsatisfactory treatment (Kim *et al.* 2009).

Accordingly, there is a pressing need to develop a novel remedial formulation for external use containing FLZ as the major active component in order to cure only the fungal infection of skin in a short period of time. The proposed novel microemulsion (ME) formulation is directed to an antifungal composition for external application containing FLZ that substantially obviates the problem discussed above. Thus, a novel microemulsion formulation needs to be designed to provide an antifungal formulation composition for external application containing FLZ which is able to penetrate deep into a horny layer of the skin of a patient to maximize the curing activity of the FLZ and to provide high stability and improved capability of spreading.

Optimal drug delivery vehicles have to exert a high capacity for incorporating both lipophilic and hydrophilic drugs as well as high skin permeability. MEs as colloidal carriers are one of the promising systems that have attracted interest in penetration enhancement because of their localized effect. MEs are liquid dispersions of water and oil that are made homogenous, transparent (or translucent) and thermodynamically stable by the addition of relatively large amounts of a surfactant and a co-surfactant and having a diameter of droplets in the range of 10-100 nm (Danielsson and Lindman 1981). Due to their special features, MEs offer several advantages for pharmaceutical use, such as ease of preparation, long-term stability, high solublization capacity for hydrophilic and lipophilic drugs, and improved drug delivery (Lawrence and Rees 2000).

Penetration enhancement from MEs is mainly due to an increase in drug concentration and thermodynamic activity which provides a large concentration gradient from the vehicle to the skin (Schmalfuss *et al.* 1997). High doses of a drug can be incorporated into this system as a consequence of the supersolvent properties of MEs and the dispersed phase can also act as a reservoir, making it possible to maintain an almost constant concentration gradient over the skin for a long time (Elena *et al.* 2001). Also it has been suggested that the surfactant and the oil from the ME interact with the rigid lipid bilayer structure and acts as a permeation enhancer (Schmalfuss *et al.* 1997). Several studies have reported that ME formulations possess improved dermal and transdermal delivery properties, mostly *in vitro* and *in vivo* (**Table 2**).

Topical drug delivery offers many important advantages. For instance, it is easy and painless, it protects the active compound from gastric enzymes, and it avoids the hepatic first-pass effect. Also, it is simple to terminate the therapy if any adverse or undesired effect occurs. But skin is a natural barrier, and only a few drugs can penetrate the skin easily and in sufficient quantities to be effective. Clinical efficacy of topical antifungal therapy depends on the drug ability to penetrate into the stratum corneum (SC) and the duration of treatment (Piérard *et al.* 1996). The poorly water-soluble nature of FLZ poses problems in designing suitable topical dosage form. Hence, ME formulations appeared to be a viable approach for topical delivery of FLZ. The solubilization of FLZ in MEs would improve its topical availability.

This paper describes formulation and systematically

evaluation of ME formulations containing a lipophilic (isopropyl myristate (IPM)) and two hydrophilic (polysorbate-80 (T-80) and propyleneglycol (PG)) skin penetration enhancers for topical delivery of poorly water-soluble FLZ which is used as model drug. An *in vitro* permeation study was performed using Franz diffusion cells through rat skin to demonstrate the potential of the developed ME system for topical drug delivery of FLZ. The antifungal activity of FLZ using *Candida albicans* as a model fungus has also been evaluated.

EXPERIMENTAL

Materials

Pure powder of FLZ (purity 99%) was procured as a free sample from Alembic Ltd. (Vadodara-India). Isopropyl myristate (IPM), ethyl laurate (EL), ethyl oleate (EO), wheat germ oil (WO), oleic acid (OA), *Arachis* oil (AO), polysorbate 20 (T-20) and polysorbate 20 (T-40) were purchased form National Chemicals (Vadodara, India). T-80 was purchased form Ranbaxy Ltd. (Delhi, India). PG was purchased from Chiti Chem Corp. (Vadodara, India). Span 20 (S-20) and Span 80 (S-80) were purchased from S.D. Fine Chemicals Ltd. (Mumbai, India). A free *Candida albicans* (ATCC 10231) sample was received from the Food and Drug Laboratory (Vadodara, India). Double distilled water was used for the preparation of ME. All other chemicals and solvents were of analytical reagent grade.

Methods

1. Screening of oils and surfactants for microemulsions

The solubility of FLZ was determined in various oils and surfactants. The oils employed were IPM, EL, WO, EO, AO and OA. The surfactants used were T-80, T-40, T-20, S-80 and S-20 having Hydrophilic Lipophilic Balance (HLB) values of 15.0, 15.6, 16.7, 4.3 and 8.6, respectively.

FLZ powder was added in excess (100 mg) to each of the oils and surfactants (5 ml) and then vortexed for mixing. After vortexing the samples were kept for 72 hrs at ambient temperature to attain equilibrium. The equilibrated samples were then centrifuged at 5000 rpm for 30 min to remove the undissolved drug. The aliquots of supernatant were filtered through 0.45 µm membrane filters (Gelman Laboratory, Mumbai, India) and the solubility of FLZ was determined by analyzing the filtrate spectrophotometrically using a double beam Perkin Elmer Lambda 19 (Perkin Elmer, Norwalk, CT) after appropriate dilution (400 µg/ml) with methanol at 258 nm. Appropriately diluted solutions of oils/surfactant in methanol were taken as blank.

2. Construction of pseudoternary phase diagram

In order to find out the concentration range of components for the existing range of MEs, pseudo-ternary phase diagrams were constructed using the water titration method at ambient temperature (Shaji and Reddy 2004). Five phase diagrams were prepared with the 1: 1, 2: 1, 3: 1, 4: 1 and 5: 1 weight ratios of T-80: PG, respectively. For each phase diagram at a specific surfactant: co-surfactant weight ratio, the ratios of oil to the mixture of surfactant and co-surfactant were varied: 0.5: 9.5, 1: 9, 1.5: 8.5, 2: 8, 2.5: 7.5, 3: 7, 3.5: 6.5, 4: 6, 4.5: 5.5, 5: 5, 5.5: 4.5, 6: 4, 6.5: 3.5, 7: 3, 7.5: 2.5, 8: 2, 8.5: 1.5, 9: 1 and 9.5: 0.5. The mixtures of oil, surfactant and co-surfactant at certain weight ratios were diluted with water dropwise, under moderate magnetic stirring (10-100 rpm). After being equilibrated at ambient temperature for 24 hrs, the mixtures were assessed visually and determined as being MEs, crude emulsions or gels. Gels were claimed for those clear and highly viscous mixtures that did not show a change in the meniscus after tilted to an angle of 90°. Stable MEs were also observed under cross-polarized light microscopy (Polarizing Microscope, Carl Zeiss, Jena, Germany) to confirm their isotropic nature (Laithy 2003). No attempt was made to distinguish between oil-in-water, water-in-oil or bicontinuous type MEs.

3. Preparation of FLZ-loaded microemulsions

FLZ was added to the mixtures of oil, surfactant, and co-surfactant with varying component ratio as described in **Table 3**, and then an appropriate amount of distilled water was added to the mixture drop by drop and the MEs containing FLZ was obtained by stirring the mixtures at ambient temperature. All MEs were stored at ambient temperature.

Table 3 Composition of selected microemulsions (%, w/w).

Microemulsion	Fluconazole	IPM	S_{mix}*	Water
ME1	2	14	42	42
ME2	2	14	49	35
ME3	2	14	54	30
ME4	2	7	65	26
ME5	2	7	71	20
ME6	2	7	77	14

IPM = isopropyl myristate; * Smix = surfactant: co-surfactant mixture (5: 1).

Characterization of microemulsions

The formulated MEs were then recognized and characterized on the basis of their physical properties, which not only explains the performance of the system but also helps in modifying their performance attributes.

To measure the solubility of FLZ in the MEs, excess FLZ was added to each ME and then allowed to equilibrate for 72 hrs at ambient temperature (Lianli *et al*. 2002). The equilibrated samples were then centrifuged at 5000 rpm for 30 min to remove the undissolved drug. Aliquots of supernatant were filtered through 0.45 µm membrane filters and the solubility of FLZ was determined by dilution of filtrate with methanol and measurement of absorbance at 258 nm. Appropriately diluted solutions of plain ME in methanol were taken as blank.

ME droplets, being smaller than 1/4 the wavelength of visible light, permit white light to pass through the dispersed system making it transparent or translucent. The ME systems were inspected for optical transparency and homogeneity by visual observation against strong light. The systems were also checked for the presence of undissolved drug or other solid ingredient. This was also confirmed by measuring % transmittance using a colorimeter (Digital Colorimeter, D-801, Photocon) at 570–590 nm.

In order to verify the isotropic nature of MEs, samples were examined using cross-polarized light microscopy (Polarizing Microscope, Carl Zeiss, Jena, Germany). A drop of sample was placed between a cover slip and a glass slide and then observed under cross-polarized light. Isotropic material, such as MEs, in contrast to anisotropic liquid crystals, do not interfere with polarized light (Friberg 1990) and the field of view remains dark because the analyzer absorbs light passing through the polarizer.

The pH values of MEs were determined using a digital pH meter (Orion pH meter 420A, Allometric Ltd., Baton Rouge, LA), standardized using pH 4 and 7 buffers before use.

The viscosity of MEs was measured using a Brookfield Viscometer (Brookfield Engineering LABS, Stoughton, MA) with an LV–III spindle at 100 rpm using a 30-sec interval. All aspects of testing were controlled using optional Rheocalc Software.

The electric conductivity of MEs was measured with a conductivity meter (Equip-Tronics, EQ–664) equipped with an inbuilt magnetic stirrer. This was done by using a conductivity cell (with a cell constant of 1.0) consisting of two platinum plates separated by a desired distance and having liquid between the platinum plate acting as a conductor.

For determination of drug content about 1 g of each ME was weighed in a 10 ml volumetric flask and dissolved in methanol. It was diluted appropriately (400 µg/ml) and analyzed spectrophotometrically at 258 nm. Appropriately diluted solutions of respective plain ME in methanol were taken as blank.

The average droplet size and polydispersity index of ME were measured by photon correlation spectroscopy with an in-built Zetasizer (Nano ZS, Malvern Instruments, UK) at 633 nm. A helium–neon gas laser having a 4 mW intensity served as the light source. Droplet size was calculated using the Stokes–Einstein relationship by Zetasizer Software.

In vitro skin permeation study

The *in vitro* skin permeation study was carried out under the guideline compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Culture, Government of India) and all the study protocols were approved by the Local Institutional Animal Ethics Committee. The abdominal skins obtained from male Wistar rats weighing 230 ± 20 g were used for *in vitro* permeation experiments. After hair was shaved carefully with an electric clipper, the skin was excised from the abdominal region of each sacrificed rat and the subcutaneous fat and other extraneous tissues were removed without damaging the epidermal surface. The excised rat skins were washed and examined for integrity, and then stored at 4°C for 24 hrs in PBS, and then used for the permeation experiments, which were performed using Franz diffusion cells fitted with excised rat skins having the epidermal surface facing outward. The effective diffusion area was 3.14 cm^2 (20 mm diameter orifice), and the receptor compartment was filled with 12 ml of PBS. The diffusion cell was maintained at 37 ± 1°C using a recirculating waterbath and the solution in the receptor chamber was stirred continuously at 600 rpm throughout the experiment. The formulation (1 g) was gently placed in a donor chamber. At 1, 2, 4, 6 and 8 hrs a 2 mL aliquot was withdrawn from the receptor compartment for spectrophotometric determination and replaced immediately with an equal volume of fresh PBS. Average values of three readings of *in vitro* permeation data were calculated and the average cumulative amount of drug permeated per unit surface area of the skin was plotted versus time. The *in vitro* permeation data was subjected to model fitting using PCP Disso 3.0 software, Pune, India.

The permeation parameters of Fick's law equation were calculated from the plot of penetrated amounts vs. time. The slope of the linear portion of the plot was calculated as flux Jss (μg/cm^2/h) (Lianli *et al.* 2002) and the permeability coefficient was calculated using the following formula (Panigrahi *et al.* 2005):

$$Kp = Jss/Cv$$

where Kp is permeability coefficient and Cv is total amount of drug.

Statistical analysis

All the skin permeation studies were repeated three times and data were expressed as the mean value ± standard deviation. Statistical data were analyzed by one-way analysis of variance. A multiple comparison test was used to compare different formulations and $P < 0.05$ was considered to be significant.

Infrared study

The infrared spectra of FLZ, plain ME and optimized drug loaded ME were created using an infrared spectrophotometer (Spectrum GX FT-IR, Perkin Elmer, Norwalk, CT). The plain ME and drug loaded ME were spread as a thin layer on a potassium bromide cell and then scanned between 4000–400 cm^{-1}. The resulting IR spectra of FLZ and plain ME were then compared with drug-loaded ME to detect any possible interaction between the drug and different components used.

In vitro antifungal activity

The antifungal activity of FLZ from the optimized formulation and reference standard (FLZ dissolved in PBS) was determined using *Candida albicans* ATCC 10231 as a representative fungi, adopting cup plate method (El Laithy and El-Shaboury 2002). One gram each of ME and FLZ reference solution containing 2% FLZ were placed in each well with a control (vehicle-free drug). The mean inhibition zone of FLZ released from 5 plates for each formula was calculated and this value was taken as an indicator for the antifungal activity. Statistical analysis using ANOVA test at level of significance of 0.05 was carried out to determine the degree of significance between the test and the reference standard.

Stability studies

Visual inspection: Shelf life as a function of time and storage temperature was evaluated by visual inspection of the ME system at different time period. Stability was monitored at 0-8°C (refrigerator), 25 ±2°C and 50±2°C temperature.

Centrifugation: In order to estimate metastable systems, the selected ME vehicles were centrifuged (Remi Laboratories, Mumbai, India) at 5000 rpm for 30 minute at 0°C.

INVESTIGATIONS, RESULTS AND DISCUSSION

Solubility of FLZ in oils and surfactants

To develop an ME system for topical delivery of FLZ, a suitable oil and surfactant had to be chosen. So the solubility of FLZ was determined in various oils and surfactants (**Table 4**). Among the non-ionic surfactants studied T-80 led to the highest solubility of FLZ (17.78 ± 2.80 mg/ml). Also T-80 is known to be less affected by pH and ionic strength changes and acts as a solublizing agent (Kim *et al.* 2001). On the other hand, there was no significant difference in the solubility of FLZ among the various oils tested except for AO and OA, which exhibited low solubility compared to other oils. However, IPM, a fatty acid ester slightly increased the solubility of FLZ (13.09 ± 2.01 mg/ml) compared with other oils, although it was not statistically significant. IPM is used as a permeation enhancer in topical formulations but its mechanism of action is poorly understood (Goldberg-Cettina *et al.* 1995). Also, with respect to the convenience of formation and use, the IPM oil was a better choice in comparison with other physiologically tolerable oils (Acharya *et al.* 2001). From these solubility results, T-80 and IPM were chosen as a surfactant and oil respectively, for the preparation of ME formulations of FLZ for further studies.

Table 4 Solubility of fluconazole in various oils and surfactants (mean ± SD, $n = 3$).

Vehicle	Solubility (mg/ml)
Oils	
Isopropyl myristate	13.09 ± 2.01
Ethyl laurate	11.48 ± 1.52
Wheat germ oil	10.84 ± 1.30
Ethyl oleate	9.94 ± 1.08
Arachis oil	3.43 ± 0.06 a
Oleic acid	2.32 ± 0.04 a
Surfactants	
Tween-80	17.78 ± 2.80
Tween-40	15.14 ± 1.10
Tween-20	11.55 ± 1.39
Span-80	9.31 ± 1.23 b
Span-20	7.47 ± 1.10 b

SD = standard deviation of the mean; n = number of replicates; a, significantly different from isopropyl myristate ($P < 0.05$); b, significantly different from Tween-80 ($P < 0.05$).

Phase behaviour

Pseudoternary phase diagrams were constructed to obtain appropriate components and their concentration ranges for the MEs (Chen *et al.* 2004). The pseudo-ternary phase diagrams with various weight ratios of T-80 to PG are presented in **Fig. 1**. The transparent ME region is presented in phase diagrams. No distinct conversion from water-in-oil (w/o) to oil-in-water (o/w) ME was observed. The rest of the region on the phase diagram represents the turbid and conventional emulsions based on visual observation. No liquid crystalline structure was observed using a cross polarizer. The area of ME isotropic region changed slightly in size with the increasing ratio of surfactant to co-surfactant.

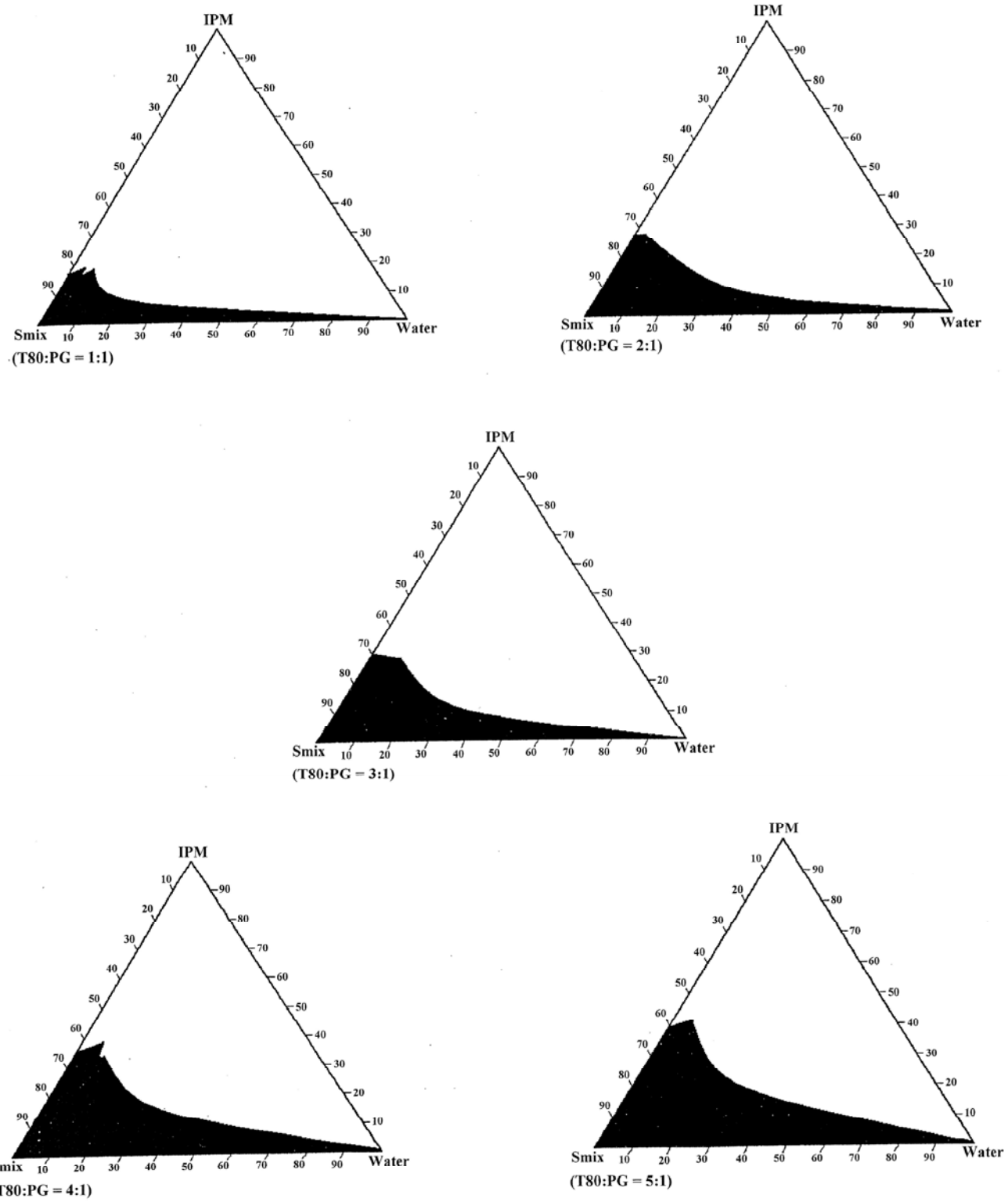

Fig. 1 The pseudo-ternary phase diagrams of the oil-surfactant mixture-water system at the 1: 1, 2: 1, 3: 1, 4: 1 and 5: 1 weight ratio of Tween-80 to Propylene Glycol at ambient temperature, dark area represent microemulsions region.

Preparation and characterization of FLZ loaded microemulsions

Various MEs were selected from the pseudoternaty phase diagram with a 5: 1 weight ratio of T-80 to PG. Here, FLZ was added into the oily phase and the drug-loaded MEs were prepared by mixing the oily phase containing FLZ with water. The physicochemical properties of MEs are reported in **Table 5**.

The samples were examined by ocular inspection in a cross polarizer for sample homogeneity and birefringence. The MEs appeared completely dark when observed under the cross polarizer. The observations indicated that all MEs showed optically isotropic colloidal dispersion.

All the MEs formed were transparent and appeared as a homogenous single-phase liquid, when observed for visual clarity against strong light. No traces of undissolved drug or other solid ingredient were found in all samples. The percentage transmittance of all prepared ME formulations was found in the range of 97 to 99 % T (data not shown).

The drug solubility in the MEs studied was found to increase with increasing surfactant: co-surfactant concentrations (**Table 5**). This could be attributed to higher solubility of FLZ in T-80. However, there was no significant difference in solubility of FLZ in various MEs studied.

The ME formulations had appropriate observed pH

Table 5 Physicochemical parameters of tested microemulsion formulations (mean ± SD, $n = 3$).

Formulations*	Solubility (mg/ml)	pH	Viscosity (mPas)	Conductivity (μS/cm)	Drug content (%, w/w)	Diameter (nm)	Polydispersity index
ME1	27.81 ± 1.23	6.02 ± 0.02	37.54 ± 0.49	155.5 ± 2.0	99.25 ± 1.42	15.8 ± 0.3	0.126 ± 0.021
ME2	29.38 ± 0.64	5.93 ± 0.04	42.41 ± 0.39	104.2 ± 5.0	98.56 ± 2.01	20.9 ± 0.3	0.142 ± 0.016
ME3	30.47 ± 1.07	6.06 ± 0.02	46.55 ± 0.41	70.40 ± 1.0	99.96 ± 1.83	25.6 ± 0.1	0.132 ± 0.027
ME4	33.91 ± 1.60	6.17 ± 0.02	48.92 ± 0.51	54.30 ± 6.0	98.99 ± 1.66	28.4 ± 0.6	0.122 ± 0.010
ME5	35.79 ± 1.37	6.13 ± 0.04	60.42 ± 0.48	43.45 ± 4.0	100.14 ± 1.35	37.5 ± 0.4	0.136 ± 0.014
ME6	37.54 ± 0.77	5.91 ± 0.01	74.01 ± 0.04	35.25 ± 7.0	98.72 ± 1.39	49.8 ± 0.5	0.127 ± 0.012

* See **Table 3** for constituents.
SD = standard deviation of the mean; n = number of replicates.

values varying from 5.9 to 6.2 for topical application (**Table 5**). Incorporation of FLZ did not significantly affect the observed pH value of the ME formulations.

The ME 1 formulation had the lowest viscosity value (37.54 ± 0.49 mPas) among the ME formulations studied (**Table 5**). In general the viscosity of the MEs was found to increase with increasing surfactant: co-surfactant concentrations. There was no significant difference between the viscosities of plain and drug-loaded MEs.

As reported previously, in order to study electrical conduction of non-ionic MEs, a small amount of aqueous electrolyte must be added to provide the charges necessary for the charge transport (Weigert *et al.* 1997). However, the addition of salt can significantly affect the phase behaviors and structural properties of MEs and that even may result in phase separation. For this reason, in this study, the conductivity measurements were performed without deliberate incorporation of an electrolyte. The investigated ME formulations containing non-ionic surfactant mixture, oil and water showed electroconductive behaviour inspite of its non-ionic nature. The ME 1 formulation had relatively high conductivity (155.5 ± 2.0 μS/cm) among the ME formulations studied (**Table 5**). From the electroconductive study it could be concluded that the tested ME formulations were of o/w type. The conductivity results obtained showed that loading FLZ and the addition of the appropriate amount of water phase into the formulation had no negative effects on system stability. When an unstable emulsion system and phase separation occurs, the conductivity values are greatly reduced (Li *et al.* 2003).

The FLZ content of the ME formulations were within the range of 97-99% (**Table 5**).

The ME 1 formulation had the lowest average particle size (15.8 ± 0.3 nm) with a polydispersity index (PI) of 0.126 ± 0.021 (**Table 5**). Average droplet size and PI of all ME formulations ranged from 15 to 50 nm and 0.12 to 0.14, respectively. It was found that 90% of the droplets had a small droplet size, less than 50 nm. PI is a measure of particle homogeneity and it varies from 0.0 to 1.0. The closer to zero the PI value is, the more homogenous are the particles. The PI showed that all ME formulations had a narrow size distribution.

In vitro skin permeation study

Table 6 shows the different ME formulations composed of IPM, T-80, PG and water at different concentrations. The effects of the content of oil and surfactant mixture on the skin permeation of FLZ were evaluated. The skin permeation profiles are presented in **Fig. 2**; the steady state flux (Jss) and permeability coefficient (Kp) values are shown in **Table 6**. Among the ME formulations tested, ME1, which was composed of a 2% FLZ, 14% IPM, 42% Tween-80/1, 2-propylene glycol (5:1, w/w) mixture and 42% water, showed the highest permeation profile. The Jss of FLZ from ME1 was 67.68 ± 1.26 μg/cm^2/h and Kp was 3.3 ± 0.62 cm/h, 6 times higher than those of the FLZ saturated aqueous solution in phosphate buffer solution pH 6.8 (PBS), which were 11.2 ± 0.21 mg/cm^2/h and 0.55 ± 0.11 cm/h, respectively.

Various release kinetic models were applied to elucidate the mechanism of drug release from the ME formulations.

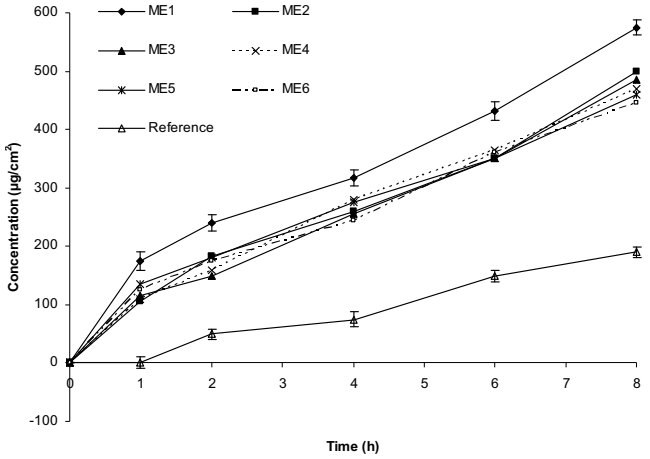

Fig. 2 Permeation profile of fluconazole through rat skins from microemulsion formulations and reference (mean ± S.D. $n = 3$).

Table 6 Permeation parameters of the fluconazole-loaded microemulsions and saturated aqueous solution (mean ± SD, $n = 3$).

Formulation	Jss (μg/cm^2/h)	K_p (cm/h)
ME-1	67.58 ± 1.26 a	3.3 ± 0.62 a
ME-2	45.78 ± 1.53 a	2.3 ± 0.77 a
ME-3	43.42 ± 1.53 a	2.0 ± 0.76 a
ME-4	40.52 ± 2.65 a	2.1 ± 0.13 a
ME-5	36.54 ± 3.79 a	1.8 ± 0.18 a
ME-6	31.90 ± 2.08 a	1.6 ± 0.14 a
Reference (Saturated aqueous solution)	11.2 ± 0.21	0.55 ± 0.11

Jss = steady state flux; Kp = permeation coefficient; SD = standard deviation of the mean; n = number of replicates; a, significantly different from the reference ($P < 0.05$)

Drug release from the optimized formulations ME 1 followed the Peppas models ($R = 0.9872$, $n = 0.43$), respectively, suggesting a diffusion based mechanism of drug release as the diffusion exponent values were less than 0.5 (Costa and Lobo 2001). The permeation rate of FLZ was almost linearly improved as a function of loading dose and the permeation of MEs according to Fick's first diffusion law.

The topical formulations for the treatment of skin infections must provide proper concentrations of the drug in target site for therapeutic activity. In the case of superficial fungal skin infections, in which the main location of the pathogen is in epidermis, the drug must penetrate into the SC in proper concentrations to inhibit the fungus growth (Alberti *et al.* 2001). Interestingly, the percutaneous absorption studies were shown to be superior from the ME formulations. Maximum drug permeation and 6-fold improvement in drug release were achieved in comparison to saturated aqueous solution. These results clearly indicate that FLZ, when used in ME, was more efficiently penetrated compared with saturated aqueous solution.

The higher permeability rate of FLZ from ME formulations is most probably due to the surfactants and the oily phase, which act as penetration enhancers (Lawrence and Rees 2000). The enhancer can increase the transport through skin by modifying the diffusion or partitioning coefficient of the drug (Naik *et al.* 2000). IPM as a permeation enhan-

cer had a strong permeation enhancing effect and could increase the diffusion coefficient in skin, which could result in an increase of the permeation coefficient (Chen *et al.* 2007). It was found that skin permeation of the drug in ME was significantly influenced by the content of IPM. As the content of oil was increased the number of internal phases increased, which further increased the skin permeation rate of the drug (Jang-Hoon *et al.* 2004).

The content of surfactant mixture in ME was also found to affect the skin permeation flux of FLZ significantly, but its mechanism was different from that of the oil. ME containing a lower amount of T-80 and PG provided higher flux. This may be due to an increased thermodynamic activity of the drug in ME at a lower concentration of surfactant and cosurfactant, as FLZ is poorly water soluble and yet solubilized in the surfactant mixture (Rhee *et al.* 2001; Jang-Hoon *et al.* 2004). The thermodynamic activity of a drug in the formulation is a significant driving force for its release and penetration into skin (Walters *et al.* 1998).

Several mechanisms have been proposed to explain the advantages of ME for the topical delivery of drugs. First, a large amount of drugs can be incorporated in the formulation due to its high solubilizing capacity. Second, the steady-state flux of the drug from ME may be increased, since the affinity of a drug to the internal phase in ME can be easily modified to favor partitioning into SC, using a different internal phase, changing its portion in ME or adjusting its property. Furthermore, the surfactant and cosurfactant in the ME may reduce the diffusional barrier of the SC by acting as permeation enhancers (Bianca *et al.* 2000). For efficient percutaneous absorption of drugs, the histological and histochemical structure of the SC must be taken into consideration. Drugs can permeate the SC through two micropathways, one is the intercellular route and the other is the transcellular way. Of these routes, the intercellular route plays a major role in the percutaneous uptake of drugs. It is known that a complex mixture of essentially neutral lipids that are arranged as bilayers with their hydrophobic chains facing each other, form a hydrophobic bimolecular leaflet. Most of the lipophilic drugs pass through this region, and it is called the lipid pathway. Polar head groups of lipids face an aqueous region forming a polar route that hydrophilic drugs generally prefer (El-Laithy and El-Shaboury 2002).

Topically-applied ME is expected to penetrate the SC and to exist intact in the whole horny layer, altering both the lipid and the polar pathways (Thachrodi and Panduranga 1994). The lipophilic domain of the ME can interact with the SC in many ways. A drug dissolved in the lipid domain of the ME can directly partition into the lipids of the SC, or the lipid vesicles themselves can intercalate between the lipid chains of the SC, thereby destabilizing its bilayer structure. In effect, these interactions will lead to increased permeability of the lipid pathway to FLZ. On the other hand, the hydrophilic domain of the ME can hydrate the SC to a greater extent, and plays an important role in the percutaneous uptake of drugs. When the aqueous fluid of the ME enters the polar pathway, it will increase the interlamellar volume of the SC lipid bilayers, resulting in the disruption of its interfacial structure. Since some lipid chains are covalently attached to corneocytes, hydration of these proteins will also lead to the disorder of lipid bilayers. Similarly, swelling of the intercellular proteins may also disturb the lipid bilayers; a lipophilic penetrant like FLZ can then permeate more easily through the lipid pathway of the SC (El-Laithy and El-Shaboury 2002).

Moreover, the particle size of the ME may also affect its efficiency, where its small particle size makes it an excellent carrier for promoting FLZ percutaneous uptake as the number of vesicles that can interact on a fixed area of SC will increase when the particle size decreases (Jang-Hoon *et al.* 2004).

In fact, no stout mechanism could be considered in explaining the superiority of the ME over the other vehicles, but the combined effect of both the lipophilic and hydrophilic domains as well as the particle size of the ME was responsible for its enhancing activity.

Finally, the optimized composition of ME containing 2% FLZ was confirmed as 14% IPM, 42% Tween-80/1, 2-propylene glycol (5:1, w/w) mixture and 42% water which showed the highest permeation profile with appropriate physicochemical characters.

Infra-red study

The infrared spectra of FLZ powder, plain and optimized drug-loaded ME are as shown in the (**Fig. 3**). The spectrum of FLZ shows one absorption band at 3400 cm^{-1} due to hydroxyl stretching (El-Laithy and El-Shaboury 2002). This band was not affected by the drug formulation in ME, which emphasized the absence of any possible interaction between the drug and formulation components used.

In vitro antifungal activity

The values of mean zone of inhibition (the antifungal activity) of the tested ME formulation was larger than that of reference standard (2% FLZ dissolved in PBS) (**Table 7**). The plain formula used in the study showed no antifungal activity. ANOVA showed that there was a significant difference in the tested ME zone of inhibition in comparison to the reference standard at $P<0.05$, where the calculated F was larger than the tabulated F. The enhanced *in vitro* antifungal activity of the tested ME may be attributed to enhanced penetration of oil globules containing FLZ through fungal cell walls to inhibit ergosterol synthesis.

Stability studies

Stability studies of the ME formulations were carried out by subjecting them to visual inspection (without stress) and centrifugation (under stress). The visual inspection test was carried out for 3 months by drawing samples at weekly intervals for the first month and at monthly intervals for the subsequent months. The visual observation conducted by showing no evidence of phase separation or any flocculation or precipitation. These samples also showed no sign of phase separation under stress when subjected to centrifugation at 5000 rpm for 30 min.

CONCLUSION

In the treatment of superficial and localized infection topical application of an antifungal seems to be an appropriate strategy to restrict the therapeutic effect to the affected area and to reduce systemic incrimination. In this paper, the application of ME systems for topical delivery of FLZ was investigated. The results suggested that the ME had a permeation enhancing effect. Compared with saturated aqueous solution, the skin permeation ability of FLZ was significantly increased by ME, which might result from the special characteristics of ME. These studies indicated that FLZ ME formulations could be a viable alternative to the current topical formulations available for the treatment of candidiasis. Nevertheless, significant work still needs to be carried

Table 7 Antimicrobial activity of optimized microemulsion in comparison to reference standard using *Candida albicans* (mean ± SD, $n = 3$).

Formulation	Zone of inhibition (mm)					
	1	**2**	**3**	**4**	**5**	**Mean ± SD**
Microemulsion	20	22	25	23	26	23.2 ± 2.39 a
Reference	11	12	15	13	10	12.2 ± 1.92

SD = standard deviation of the mean; n = number of replicates; a, significantly different from the reference ($P < 0.05$)

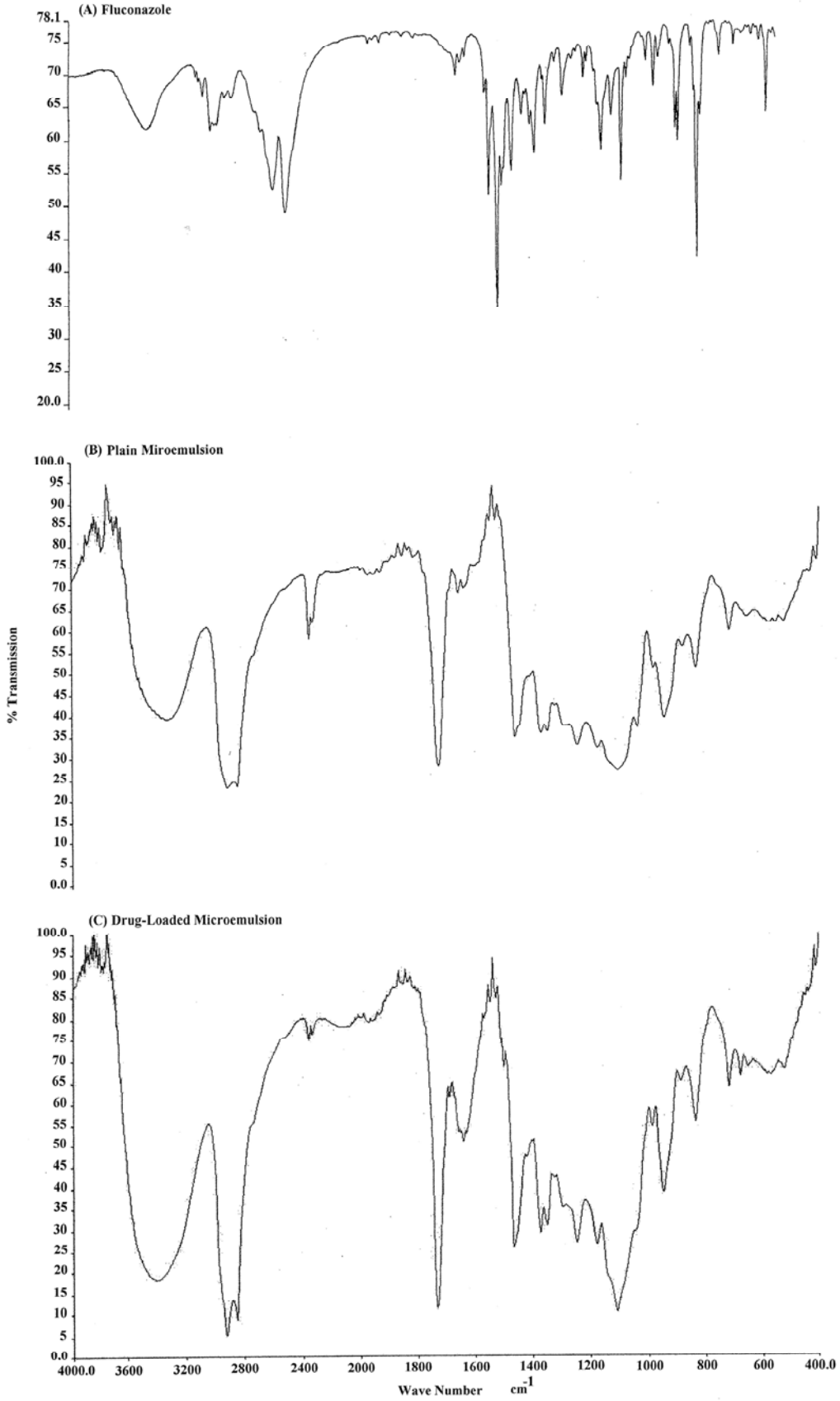

Fig. 3 IR study of (A) fluconazole, (B) plain microemulsion and (C) drug-loaded microemulsion.

out to elucidate the mechanisms of drug delivery into the skin which are dictated by internal structure and the special characteristics of the ME.

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