Preparation and *in vitro* evaluation of Nystatin micro emulsion based gel

Iram Maqsood[1], Muhammad Irfan Masood[2], Sajid Bashir[3], Hafiz Muhammad Awais Nawaz[2], Aftab Ahmad Anjum[2], Iram Shahzadi[2], Mahmood Ahmad[4] and Imran Masood[4]

[1]Riphah Institute of Pharmaceutical Sciences, Riphah International University, Lahore, Pakistan
[2]University of Veterinary & Animal Sciences, Abdul Qadir Jillani Road, Lahore, Pakistan
[3]Department of Pharmacy, University of Sargodha, Sargodha, Pakistan
[4]Department of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur, Pakistan

Abstract: Nystatin is a polyene antimycotic obtained from *Streptomyces noursei* used in the treatment of topical and transdermal fungal infection. Nystatin is nearly insoluble in water (<0.1) and it is amphoteric in nature. The aim of the present study was to design and develop Nystatin micro emulsion based gel for efficient delivery of drug to the skin by water titration method. The Pseudoternary phase diagrams 1:2, 1:1 and 2:1 were constructed by water titration method. Micro emulsion based gel was prepared by using oleic acid, Tween 20, propylene glycol as an oil phase, surfactant and cosurfactant respectively. Cabopol 940 was used as a gelling agent. *In vitro* evaluation of micro emulsion based gel was done for pH, Viscosity, spreadability and droplet size. Micro emulsion based gel showed greater antifungal activity against *Candida albicans*as compared to control formulations. *In vitro* drug release studies were conducted for micro emulsion based gel and control formulation using Franz diffusion cell. Drug penetration through synthetic skin followed Zero order model as the values for R^2 higher in case of zero order equation. The optimized micro emulsion based gel was found to be stable and showed no physical changes when exposed to different temperatures for a period of 4 week. The results indicated that the micro emulsion based gel system studied would be a promising tool for enhancing the percutaneous delivery of Nystatin.

Keywords: Nystatin, micro emulsion based gel, pseudo ternary phase diagram, antifungal activity.

INTRODUCTION

Hoar and Schulman introduced the micro emulsion concept in 1940s as they titrated the milky emulsion with hexanol and obtained a clear solution (Lawrence and Rees, 2000). The term micro emulsion was subsequently coined by Schulman and coworkers, and then on many occasions it has been defined and indeed redefined. Then, Danielsson and Lindman defined micro emulsion in 1981 as 'a single, transparent and thermodynamically stable system formed by the combination of oil, water and surfactant in conjunction with a co surfactant is known as liquid emulsion' (Lawrence and Rees, 2000).

A newer class of dosage form called gels are formed by entrapment of aqueous or hydroalcoholic liquid in a system of colloidal solid particles. A greater dissolution of drugs in gels takes place due to their higher aqueous component, and when compared with the ointments or creams, gels let easy migration of the drug through a liquid vehicle. Gels are better in terms of patient compliance and patient use (Panwar *et al.*, 2011).

The systemic adverse effects of drugs are reduced or avoided by topical administration. Due to their non invasiveness topical preparations can be self administered and have better patient compliance (Gungor *et al.*, 2013). Fungal infection of the skin is a common dermatological problem now-a-days. The physicians have many choices for treatment from solid dosage forms to semisolid and to liquid formulations (Shah *et al.*, 2009). Stratum corneum is the greatest confront for dermal delivery and new formulation approaches have been investigated to improve permeability across stratum corneum (Gungor *et al.*, 2013).

In 1949, Brown and Hazen discovered Nystatin from a strain of *Streptomyces noursei* in soil. In 1951, it was licensed for use to superficial Candida infections (Khatry *et al.*, 2010). It is a polyene antimycotic act by binding irreversibly to ergosterol (Brennan B, 1997). It causes cell membrane disruption by the release of K+, sugars and other metabolites which is the cause of fungal death (Carrillo-Munoz *et al.*, 2006). It is characterized as very slightly soluble to practically insoluble in water (Abdul-Rasool *et al.*, 2010). Its use is limited to topical use only. Its most common adverse effect is allergic contact dermatitis (Gungor *et al.*, 2013). The present study was conducted to design and evaluate Nystatin micro emulsion based dermal gel which provides fast absorption, increase the residence time of drug on the skin thereby enhance bioavailability, prolonged release and enables reduction in dose.

**Corresponding author:* e-mail: irammaqsood2@yahoo.com

MATERIALS AND METHODS

Materials

Nystatin was kindly received as a gift sample from Lisko Pharmaceuticals Limited Karachi, Pakistan. Oleic acid was obtained from Avonchem Limited, UK. Tween 20 (Polyoxyethylene 20 sorbitan mono laurate), Propylene glycol and Carbopol 940 were obtained from Atlas Chemical Industries Lahore, Pakistan. Double distilled water was used throughout the study. All other chemicals and solvents used in the study were of analytical grade and used as received without further purification.

Methods

Screening of oil and surfactants for nystatin micro emulsion

Solubility study of nystatin

The Nystatin solubility was determined in different oils such as oleic acid, Isopropyl myristate (IPM), Sesame oil, olive oil and Liquid paraffin oil; Surfactants Tween® 20, Tween® 80 and Span® 20 were used as Surfactants and Propylene glycol (PG), Ethanol, Methanol, and n-Butanol were used as Cosurfactants. 5ml of each of the component was taken in 100ml capacity beaker and an excess amount of Nystatin was added to that component. The resultant mixture was then mixed by magnetic stirrer for 72 hours. Then it was centrifuged at 10,000rpm for 15 minutes. After centrifugation the supernatant was separated and filtered through a 0.45µm membrane filter. The filtrate was then diluted with propylene glycol and in diluted filtrate Nystatin concentration was determined by UV spectrophotometer at its maximum wavelength i.e. 307nm. The sufficiently diluted solution of each component in methanol was taken as blank. The component in which Nystatin showed maximum solubility was used for further studies (Elosaily, 2012).

Construction of pseudoternary phase diagrams

Water titration method was used to construct the pseudoternary phase diagrams. The composition of micro emulsions was determined by constructing the pseudoternary phase diagrams. A series of oil, surfactant, and co-surfactant were titrated with water mixtures at room temperature (25°C) to construct phase diagrams. Surfactants and co-surfactants were mixed in different ratios (1: 2, 1:1, 2:1) then each surfactant-cosurfactant mixture was blended with oil. The ratio of oil to the surfactant and co-surfactant mixture was speckled at 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 (w/w). Then drop-by-drop water was added to the mixture under continuous stirring. During titration, clear, crystal liquid phase, and common emulsion phases were noted with turbid to clear or clear to turbid transitions occurring (Chudasama *et al.*, 2011; Patel and Patel, 2012).

Preparation of O/W Micro emulsion Loaded with Drug

After construction of pseudoternary phase diagrams, O/W micro emulsions were prepared. The calculated quantities of oil, surfactant and co-surfactant were weighed accurately. Nystatin 0.4% w/w was dissolved in the mixture of oil, surfactant and co-surfactant. The mixture was stirred with a magnetic bar on magnetic stirrer hot plate at ambient temperature i.e. 25°C. During continuous stirring weighed amount of water was added drop by drop, until a clear, transparent system obtained (Chudasama *et al.*, 2011; Patel and Patel, 2012).

Formulation of micro emulsion based gel of nystatin

Micro emulsion based gel of Nystatin was formulated by using method ofChudasama *et al.*, (2011)with further modification. Nystatin O/W micro emulsions were chosen for further progress to micro emulsion based gels. Different gelling agents e.g. Carbopol 940, Xanthan gum, sodium alginate, hydroxy propyl methylcellulose (HPMC) and Carboxy methyl cellulose (CMC) were tested for their ability to gel Nystatin micro emulsion. Carbopol 940 was selected as Gelling agent and it was soaked overnight in water. Then gel was added to adjust the viscosity of the system. Mixing was done with the help of Overhead stirrer. The creamy, white, smooth micro emulsion based gels of suitable viscosity were obtained (Chudasama *et al.*, 2011).

Characterization of nystatin micro emulsion based gel color, odor, appearance and feel

The formulated micro emulsion based gels were inspected visually for color, presence of any clog and the feel the formulated gels were evaluated by applying gels on skin and feel were practiced (Tyagi *et al.*, 2012).

PH determination

The pH of the Micro emulsion based gel was determined using Digital pH meter (PCSIR Laboratories Complex Karachi), which was previously calibrated using appropriate buffer solutions (Patel and Patel, 2012).

Spreadability determination

Spreadability of micro emulsion based gel was calculated in terms of diameter of circle produced by gel when placed between two glass slides. On the glass slide, a circle of 1 centimeter was drawn by a permanent marker. Then 0.5gm gel was accurately weighed and placed within a circle on a slide, and a second slide was placed carefully over it. The slides allowed remaining in place for 5 minutes. The increase in the diameter of circle was noted due to spreading of the gel (Patel and Patel, 2012).

Viscosity determination

The viscosity of a fluid is a measure of the resistance to its steady deformation by shear stress or tensile stress. Viscosity of the Nystatin gel was determined by Brookfield Digital viscometer (RV DV-II + Pro model using spindle No 6 at 25˚C at different RPM. Viscosity of gel was measured in triplicate at each RPM and then mean was calculated with standard deviation (Patel and Patel, 2012).

Stability Studies

Nystatin microemulsion based gels were prepared and were kept at 4°C, 25°C, 40°C and at 40°C+75% RH (relative humidity) and shelf life of the stored micro emulsion system were evaluated by visual inspection (phase separation), viscosity, conductivity, pH, centrifugation. Stability was evaluated at different intervals (fresh, 24 hrs, 48 hrs, 72 hrs, 1st week, 2nd week, 3rd week and 4th week) (Araya *et al.*, 2005).

Table 1: Solubility of Nystatin in different oils, surfactants and cosurfactant

Component	Solubility (mg/mL)
Oil Phase	
Olive Oil	0
Sesame oil	0
Isopropyl Myristate (IPM)	194.5 ± 0.025
Liquid paraffin oil	920.53 ± 0.125
Oleic Acid	980.8 ± 0.005
Surfactant	
Tween® 20	26.94 ± 0.02
Tween® 80	12.56 ± 0.12
Span® 20	10.45 ± 0.05
Cosurfactant	
Propylene glycol	13.43 ± 0.015
Ethanol	5.10 ± 0.012
Methanol	3.45 ± 0.025
n-Butanol	0.48 ± 0.110

Table 2: Evaluation of organoleptic properties of gel

S. No.	Parameter	Gel
1	Color	White
2	Odor	Pleasant
3	Feel	Smooth
4	Clogging	No

Table 3: pH and spreadability values of gel

Formulation	pH	Spreadability (cm/5 min.)
F1	4.34±0.005	4.4±0.11
F2	4.55±0.017	4.4±0.29
F3	4.20±0.02	4.3±0.25
F4	5.05±0.01	4.1±0.15
F5	4.88±0.005	3.83±0.15
F6	4.72±0.00	3.73±0.05

Table 4: Viscosity of F4Gel

RPM	Viscosity (cps)
	Using Spindle S-4
10	2820±0.577
20	1745±1.000
30	1433±0.577
50	1100±0.577
60	915±1.150
100	753±0.577

Table 5: Zones of inhibition of different formulations after 24 hrs

S. No.	Zone of Inhibition in mm		
	Control	Micro emulsion based Gel (0.2% w/w)	Placebo Gel
1	3	2.5	0.1
2	2.4	2.4	0
3	2.5	2.9	0
4	2.6	2.8	0.2
5	3.1	2.6	0
Mean	2.72	2.64	0.08
Std. Dev.	±0.3114	±0.2702	±0.0837

Microbiological study

Then antifungal efficacy of Nystatin micro emulsion based gel, Placebo Gel and commercial Nystatin suspension (Control) was determined by performing agar cup diffusion test. The efficacy of the prepared Nystatin micro emulsion based gel and Placebo Gel against *Candida albicans*was observed by applying 1gm of each prepared formulation and an equivalent weight of control on the separate sabouraud dextrose agar plates which were previously seeded with *Candida albicans*. The Petri plates were then incubated at 25°C for 24hrs. The effectiveness of the prepared Nystatin micro emulsion based gel 0.2% w/w of Nystatin were compared with plain gel and control that contains 100,000 I.U. of Nystatin/1ml. The growth inhibition zones were measured for all the tested samples. In triplicate each type of the samples was tested. The growth inhibition zones of *Candida albicans* were measured in mm after 24 and then mean inhibition zones were calculated (Marzouk *et al.*, 2012).

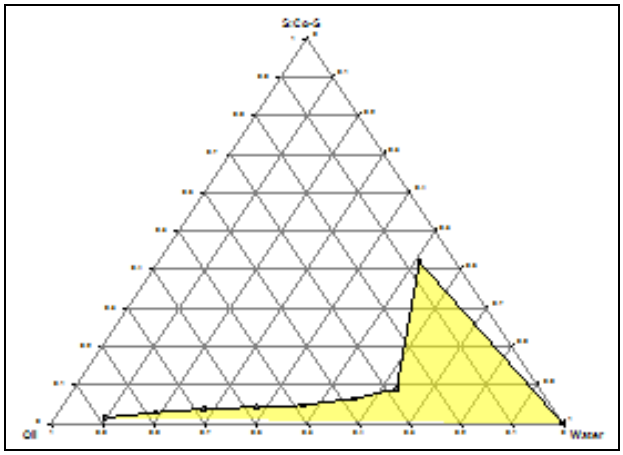

Fig. 1: Construction of Pseudoternary Phase Diagrams with 1:2, S: Co-S.

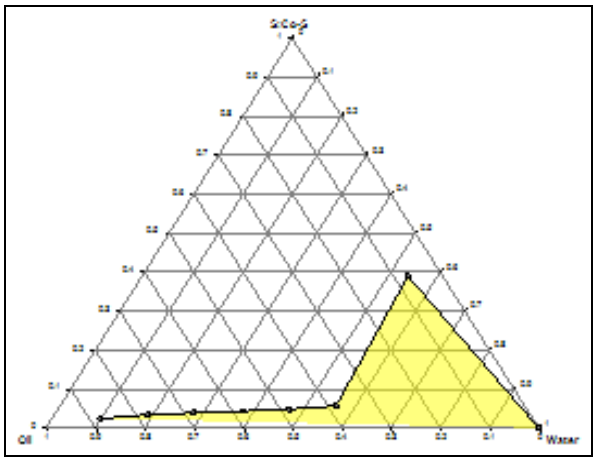

Fig. 2: Construction of Pseudoternary Phase Diagrams with 1:1, S: Co-S

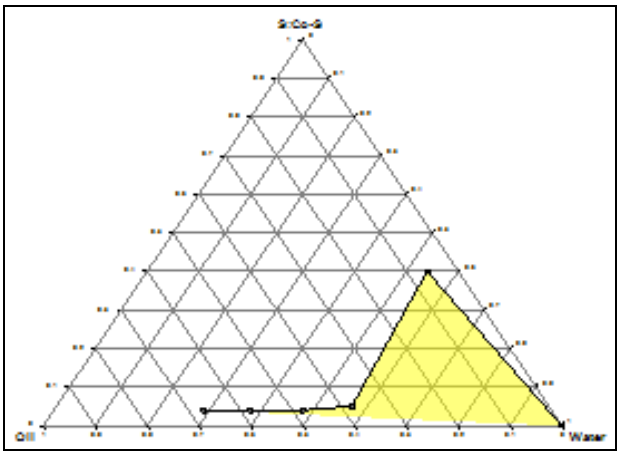

Fig. 3: Construction of Pseudoternary Phase Diagrams with 2:1, S: Co-S

In vitro release of formulations by franz diffusion cell

The experimental set up included a Franz cell, with an open cap, and flat ground glass, with an orifice. It contains receiver volume of about 10ml with an area of about 0.95 cm^2, total of 6 Franz cell system was used for each *in vitro* release testing. The recipient or the receiver temperature was maintained at 30±2°C by setting circulating thermostat bath temperature to 32.5°C. Magnetic stirrer was used for stirring of the receptor medium at 600 rpm. Franz cell was covered with a hydrophobic (nylon) membrane at the top and the cap of Franz cell was fixed over the membrane by using a clamp. All Franz cells should be free of air bubbles. The system was equilibrated for 30 min before the start of experiments. The used receptor media in the cell was composed of 80 ml of Methanol and 20ml of Dimethyl sulphoxide (DMSO). The receptor medium was filtered through a 47-mm, 0.45 membrane filter. Then 2ml of formulations were added via a syringe on top of membrane and test was started. At each sampling time point 1ml sample was collected using a 1ml syringe through the sampling port. The stirring was stopped during sample withdrawal and then resumed after sample was withdrawn. Sample was withdrawn at different time intervals e.g. 0.5, 1, 2, 3, 4, 5, 6 and 12 hours. At each sampling time, volume replaced with fresh medium. The drawn 1ml samples were diluted with 2 ml methanol and DMSO mixture and analyzed by UV-Visible spectrophotometer at 307 wavelength (Harwansh *et al.*, 2010).

STATISTICAL ANALYSIS

All measurements were performed in triplicates for each sample and results were represented as mean ± S.D. Two-way analysis of variance (ANOVA) and LSD test at 5% significance were used to observe the change of each parameter at different time intervals and temperature levels during stability study of Nystatin Micro emulsion based gels; and to differentiate the *in vitro* release rate of two dosage forms i.e. micro emulsion based gel and control. Antifungal activity of the dosage forms was statistically analysed using one-way analysis of variance (ANOVA) and LSD test at 5% significance level to test the difference between the means of zones of inhibitions (Z.O.I).

RESULTS

Solubility study of nystatin

Solubility of Nystatin in various oils, surfactants and cosurfactants was determined as presented in table 1.

Construction of pseudo ternary phase diagrams

The pseudo ternary phase diagrams with different weight ratio of surfactant (Tween 20) to cosurfactant (Propylene glycol) are described in fig. 1, 2 and 3.

Preparation of micro emulsion and micro emulsion based Gel of nystatin

Pseudo ternary phase diagram based upon 1:2 (surfactant/cosurfactant mixture) was used to prepare nystatin micro emulsion. F1-F6 Micro emulsion based gel was prepared by adding preformulated 2% carbopol 940 gel in micro emulsion to get better efficacy.

Characterization of micro emulsion based gel

Evaluation of Organoleptic Parameters

Organoleptic parameters of Micro emulsion based gels were observed as presented in table 2.

pH determination

pH values of the micro emulsion based gel have been determined and presented in table 3.

Spreadability

Spreadability of F1-F5 Micro emulsion based gel was determined which was in the range of 4.4-3.73 (table 3).

Table 6: Model fitting results of formulations using hydrophobic membrane

Formulations	Zero Order		First Order		Higuchi Equation		Hixson Crowell Equation		Korsmeyer-Peppas Plot	
	R^2	k	R^2	k	R^2	K	R^2	k	R^2	n
F4 Gel	0.981	5.321	0.766	0.166	0.95	22.08	0.98	-0.124	0.942	0.664
Control	0.994	4.884	0.819	0.172	0.961	20.24	0.99	-0.106	0.984	0.683

Viscosity determination
Viscosity of F4 gel was measured using Brookfield viscometer with spindle 6 at various rpm at temperature 25±0.5ºC as presented in table 4.

Stability studies of F4 nystatin micro emulsion Gel
Stability of F4 micro emulsion based gel was assessed by observing changes in different physical and chemical parameters subjected at different normal and accelerated conditions for a given period of time.

Anti fungal studies
Antifungal activity of marketed Nystatin Suspension (Control), Nystatin micro emulsion, F4 Nystatin micro emulsion based gel and placebo gel was determined against *Candida albicans* and zone of inhibitions were compared as presented in table 5.

In vitro release study of Nystatin
In vitro drug release studies were conducted for F4 micro emulsion based gel and control formulation using Franz diffusion cell. Franz diffusion cell fitted with nylon membrane was used. Permeation data obtained up to 12 hrs for two dosage forms were 72% and 64.9% release by F4 gel and control respectively (table 6).

DISCUSSION

Solubility study of nystatin
Oleic acid, tween 20 and propylene glycol were selected as oil, surfactant and cosurfactant respectively on the basis of its highest solubility in these components. The solubility data was in agreement with many researchers (Kumar *et al.*, 2010; Mehta and Bhatt, 2011; Patel and Patel, 2012).

Construction of pseudo ternary phase diagrams
The pseudo ternary phase diagrams were constructed to find out quantities of different ingredients of micro emulsion. Three phase diagrams were constructed at surfactant to co surfactant ratios of 1:1, 1:2 and 2:1. Percentage quantities of various ingredients were found for various micro emulsion formulations. Pseudo ternary phase diagram is the commonest optimization approach for preparing micro emulsions reported by many researchers (Mandal and Mandal, 2011; Sharma and Bajpai, 2011).

Preparation of micro emulsion and micro emulsion based Gel of nystatin
Micro emulsion loaded with 0.4% (w/w) of Nystatin was successfully prepared by using oleic acid, Tween 20 in combination with propylene glycol and water as an aqueous phase. This method of micro emulsion formulation was in agreement with some researchers (Shah *et al.*, 2009; Harwansh *et al.*, 2010).

Carbopol 940 was used as gelling agent by the method used by many researchers to prepare micro emulsion based gel (Jadhav *et al*., 2010; Lee *et al*., 2010; Barot *et al*., 2012; Patel and Patel 2012). 2% Carbopol 940 was added in 1:1 ratio with micro emulsion to prepare Miro emulsion based gel. For topical application gel is more suitable (Lapasin *et al.*, 2001; Lakshmi *et al.*, 2011).

Characterization of micro emulsion based gel
Evaluation of Organoleptic Parameters
Micro emulsion based gels showed yellowish white color and smooth feeling and no clogging was observed and the result was in agreement with the researchers (Patel and Patel, 2012; Tyagi *et al.*, 2012).

PH determination
The skin has a pH of 4-6, and topical are designed to be in that pH range. The pH of formulation product can influence not only the solubility of drug in the formulation, but may also affect its potential to cause skin irritation. Changes in pH throughout the shelf life of product may also be indicative of stability problem (Anjali *et al.*, 2010; Harwansh *et al.*, 2010). pH values of the micro emulsion based gel varied from 5.05-4.34 (table 4.25). So, no need for pH adjustment as pH values of all formulations was in the ideal skin pH range (Harwansh *et al.*, 2010; Patel and Patel, 2012; Tyagi *et al.*, 2012).

Spreadability
For topical dosage forms, spreadability is an important criterion to determine the ease of application. The gel which showed larger diameter had good spreadability as stated by some scientists (Soliman *et al.*, 2010). The results showed that spreadability was inversely proportional to apparent viscosity (Patel and Patel, 2012).

Viscosity determination
All the formulations exhibited Newtonian and psudoplastic flow characteristics before and after gelling

respectively. The results were in agreement with the researchers (Soliman *et al.*, 2010).

Stability studies of F4 nystatin micro emulsion Gel

F4 micro emulsion based gel was selected for stability studies. Their stability was determined by keeping them at different storage conditions i.e. 4°C, 25°C, 40°C and 40°C+75% RH for a period of 4 weeks. The gel was characterized for organoleptic parameters, pH and Viscosity at different storage condition at different time intervals e.g. fresh, 24hours, 48hours, 72hours, 1st week, 2nd week, 3rd week and 4th week. The visual inspection showed no change in color, no phase separation, and no significant changes in pH and viscosity for total period 4 weeks (Soliman *et al.*, 2010).

Anti fungal studies

Micro emulsion showed greater zone of inhibition as compared to micro emulsion and control. Micro emulsion based gel showed slightly lesser antifungal activity than micro emulsion. Activity of gel might be increased by incorporating more drugs. Placebo gel showed no fungal activity (Tyagi *et al.*, 2012).

In vitro release study of Nystatin

In vitro drug release studies were conducted using Franz diffusion cell (Sharma and Bajpai, 2011; Patel and Patel, 2012). The ability of Micro emulsion based gel to modulate drug transfer across the skin can be explained through various factors. The most important factor which contributes to transdermal drug delivery was good adhesion to surface of skin and cooling effect due to appropriate viscosity.

Drug release through F4 micro emulsion based gel followed Zero order model and Drug release through control also followed Zero order model which was in agreement with many researchers (Harwansh *et al.*, 2010; Patel and Patel, 2012).

CONCLUSION

Micro emulsion based gel of Nystatin was formulated with good release and consistency. The gel comprised of Oleic acid as an oil phase, Tween 20 as surfactant, Propylene glycol as cosurfactant and Carbopol 940 as gelling agent was deemed promising as a successful topical delivery system of Nystatin for the treatment of fungal infections of skin. Although, the micro emulsion based gel preparation was more effective *in vitro* as an antifungal agent than the conventional skin formulations, more comparative clinical studies are needed to confirm the benefit it provides over the available marketed products.

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