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Formulation and *in vitro* evaluation of ethosomes as vesicular carrier for enhanced topical delivery of isotretinoin

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Abstract

The purpose of the present research was to evaluate the ability of ethosomes for topical delivery of isotretinoin. The ethosomal vesicles were prepared with various concentrations of lecithin and ethanol by using hot method. The ethosomal based isotretinoin gel (GEL-ES) was compared to that of marketed formulations isotretinoin (GEL-MF) by using hydrophobic hydroxyl propyl methyl cellulose as gel base. The physicochemical and stability of ethosomal based isotretinoin and a marketed gel (control) were evaluated for organoleptic properties, drug entrapment, drug content uniformity and *in vitro* drug release and skin permeation studies. F2 ethosomal vesicles containing 2%w/w lecithin and 30%w/w ethanol was found to have shown the best entrapment percentage (99.21%) and also showed suitable physicochemical characteristics for topical administration. Physical stability studies were also conducted for 45 days at 4 C and 25 C. GEL-ES and GEL-MF were applied to rat skin and penetration was assessed by Franz diffusion cells. *In vitro* release studies showed that less than 10% of isotretinoin reached the receptor compartment compared to GEL-MF till 8 h. On comparing F2 and F4 gel formulations, F2 gel has shown better controlled release by *in vitro* drug release and *in vitro* skin permeation profile than F4 gel. However, the *in vitro* skin permeation was increased with the addition of enhancers. From the experimental data, it may be concluded that the ethosomal vesicles and enhancers increased the skin permeation and depot formation of drug in the skin.

Keywords: Ethosomes, topical delivery, isotretinoin, N-methyl-2-pyrrolidone, eugenol.

Introduction

Isotretinoin, a derivative of retinoic acid (13-cis-retinoic acid), has been commonly used for the treatment of severe acne and the other dermatological diseases [1]. However, it has obvious adverse side effects by oral administration. The launched topical preparations such as creams and gels also show systemic absorption and significant skin irritation [2]. So, it is necessary to improve the skin uptake and reduce systemic absorption of isotretinoin using a carrier with an ability of skin targeting.

According to Zouboulis [3], topical retinoids alone or in combinations are regarded as first-line treatment for both comedogenic and mild inflammatory acne [4]. It is believed that topical isotretinoin has similar properties as oral isotretinoin. However, the application of topical isotretinoin is associated with the problems of photolability and low solubility. There is substantial degradation of drug on the skin surface due to photolability [5]. Isotretinoin is highly lipophilic drug, hence, could not penetrates into deep skin layers.

Ethosomes are designed as non-invasive drug carrier system [6]. Touitou (1996) had discovered that ethosomes are lipid vesicular systems embodying ethanol in relatively high concentration [7]. They are soft and malleable vesicles. High concentration of ethanol in ethosomes formulation was suggested to be the main reason for their better skin permeation ability compared to conventional liposomes [8]. Touitou *et al*, reported that ethanol acts as a penetration enhancer in ethosomal system. It penetrates into intercellular lipids, in turn increases the lipid fluidity and decrease the density of lipid multilayer of cell membrane [8]. Increased lipid fluidity results in increased skin permeability. Therefore, ethosomes permeate easily into the deep skin layers, where it is fused with skin lipids, leading to release of drug in deep skin layers [8].

Ethosomes vesicles are preferred as a carrier of isotretinoin due to the absence of ethanol in liposomes. Besides, ethosomes are more permeable to cations as compared to liposomes [6]. Ethosomal vesicles have more benefits in comparison to other transdermal delivery systems [8]. Ethosomal vesicles enhance drug permeation through the skin and may deliver large molecules of compounds [9]. Chemical enhancers which are being used in the research are eugenol or N-methyl-2-pyrrolidone. Generally, chemical enhancers act to enhance permeation of drug through the skin. These enhancers mainly disrupt the structure of stratum corneum lipid, interact with intercellular protein and improve drug partitioning into stratum corneum [10]. The present study was aimed to evaluate the potential of the ethosomes for topical delivery of isotretinoin, the physiochemical properties of the ethosomes vesicles, and the

in vitro drug release and skin permeation behaviour of the ethosomal gel formulation with or without enhancers.

Material and Methods

Materials

Isotretinoin was purchased from Yang Zhou Kai Peng, china. Soy lecithin (Lipamin PC 50) was purchased from Lecico, Hamburg, Germany. 1,2-Propanediol (Empura®) was purchased from Merck, Hohenbrunn, Germany. Pharmasolve was purchased from ISP Technologies, Inc., Alps Road Wayne, NJ, USA. Eugenol was purchased from Spectrum Chemical MFG, Corp., Gardena, New Brunwick, NJ, USA. Sangelose 60L and Sangelose 90L were kindly gifted from Daido, Japan.

Preparation of ethosomal vesicles

Isotretinoin ethosomal formulations were prepared by "Hot Method" [11]. The drug concentration was fixed as 0.05% w/w. The soy lecithin (phospholipid) and ethanol concentration was in the range of 1- 4% w/w and 20-40% w/w respectively. Accurately weighed quantity of drug was dissolved in ethanol and propylene glycol was added to it. The soy lecithin was dissolved in water at 40 C in another beaker. The drug solution was then added slowly to soy lecithin dispersion of water at 40 C with 1,700 rpm in a closed vessel and was stirred for 30 minutes. The final preparation was subjected to ultra-sonication for an hour with a cycle of 10 minutes. The evenly dispersed ethosomal vesicle was formed. The composition of ethosomal vesicles are shown in the Table 1.

Preparation of isotretinoin ethosomal gel

The isotretinoin ethosomal vesicles with the best entrapment efficiency result, F2 and F4 were chosen for gel formulation. A specific amount of Sangelose 60L was added in minimum amount of water and left to swell for an hour. An appropriate amount of F2 or F4 was incorporated to the swollen Sangelose with continuous stirring at 700 rpm and the temperature was maintained at 30 C until homogenous ethosomal gel was formed. pH of the gel was then adjusted by adding small amount of triethanolamine (TEA) and stirred slowly. Final gel formulation was subjected to ultrasonication [12].

Organoleptic properties

The colour, texture, physical appearance and homogeneity of the gels were evaluated by visual observations [13].

Particle size analysis of ethosomes

Particle size was measured using the laser dynamic scattering Mastersizer 3000 (Malvern, England) immediately after diluting the ethosomes with phosphate buffer saline solution. The average particle size of ethosomes was calculated based on the measurements of 5 batches of ethosomes.

Drug content and uniformity

An aliquot of gel (0.1g) was extracted with 10ml of methanol and then centrifuged with 1,000 rpm at 4 C for 20 minutes. The supernatant obtained was analysed by using HPLC [14].

Drug entrapment efficiency

The ability of ethosomes to efficiently entrap drugs was measured by centrifugation technique [14]. The vesicles (2ml) were centrifuged at 4 C with 14,000 rpm for 3 hours. The supernatant and sediment obtained were then analysed for drug content using HPLC. The entrapment efficiency was calculated by the following equation.

Entrapment Efficiency (%) = $(D/D_T) \times 100\%$

Where, D = Amount of drug in sediment

D_T = Total amount of drug in supernatant and sediment

PH measurements

The pH of ethosomal formulations was measured by using the pH meter. The electrode was dipped into the vesicles as long as covered by the vesicles.

Stability study

The stability of ethosomal vesicles were determined by identifying the change in drug entrapment efficiency. The vesicles were stored in two different temperatures, 4 C and 25 C±2 C. Then, the drug content of the vesicles was estimated every 15 days for 45 days.

In vitro drug release study

In vitro drug release study of drug in ethosomes was carried out by using Franz diffusion cell. A specified amount of gel formulation was applied on the cellulose acetate membrane at the donor compartment. The receptor compartment was filled with PBS:THF (50:50) as receptor solution. Sample solution (0.5mL) was withdrawn at 1 hour interval for 8 hours and immediately replaced with the same volume of receptor solution. The samples were then analyzed by using HPLC [15].

In vitro skin permeation study

A section of freshly excised rat skin was immersed in isotonic solution (0.9g sodium chloride dissolved in 100mL of R.O water). Ethosomal vesicles and gel were on rat skin and placed on top of the donor compartment of Franz diffusion cell. The dermal side of the skin must just touch the receptor liquid surface for permeation. All other analysis conditions were similar to the in vitro release study [15]. And skin retention of dug was analyzed by HPLC method [16].

HPLC Assay

Isotretinoin was estimated in skin deposition study by the HPLC method as reported by Tashtoush et al. [17]. Trifluoroacetic acid (0.01%) and acetonitrile (15: 85 v/v%) was used as mobile phase and delivered at 1.0 mL min–1. The injected fluid (20 μL) was eluted in C 18 column at room temperature and isotretinoin was monitored at 341 nm using a UV detector. The calibration curve with in a concentration range from 0.5 to 10.0 μg/mL was used to measure the isotretinoin concentration. The relative standard deviation around the calibration line ranged from 1.0% to 4.5% and the squared correlation coefficient was 0.9972.

Statistical analysis

The results are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to analyze the experimental data collected. Post-hoc Tukey-HSD (Honestly Significant Difference) test was performed if statistically significant difference was obtained. A statistically significant difference was considered at $p < 0.05$.

Results and Discussion

Physicochemical properties of ethosomal gel

The results of physicochemical properties of gel formulations are shown in Table 1 and 2. All the gel formulations, including the marketed formulations, were in yellow colour, transparent in nature, have good homogeneity and smooth texture. The impact of different concentrations of phospholipids as well as ethanol such as 1.0, 2.0, 3.0% and 20, 30, 40% respectively on the entrapment efficiency of ethosomes was evaluated.

Results showed that both the particle size of ethosomes and the entrapment efficiency of isotretinoin ethosomes increased with the increasing concentration of phospholipids, whereas decrease with increasing percentages of ethanol (Table 1). As a vesicle-structured neotype of drug carrier, ethosome has the characteristics of good deformability, high entrapment efficiency, good permeability and good stability, which enable them to effectively carry the drug through the skin, and to penetrate through cuticle to get into deeper layers. It was the good fluidity and deformability given by ethanol that enabled ethosomes to carry drug to penetrate through the eyelet, 1/5 to 1/10 less than its size without much change in shape under the pressure of hydration, so as to penetrate through cell membrane and release drug more effectively than liposomes [18]. Generally, ethosome comprises of high concentration of low molecular weight alcohol (ethanol, propylene glycol, and isopropyl alcohol), phospholipid and water, and sometimes the addition of cholesterol [19].

Table 1: Composition of various ethosomal vesicles.

Batch No	Composition (% w/w) in 100mL					Vesicle Size (μm)	EE (%)
	Drug (%w/w)	Lecithin (%w/w)	Ethanol (%w/w)	Propylene Glycol (%w/w)	Water		
F0	0.05	1	20	20	q.s.	3.46	94.78
F1	0.05	2	20	20	q.s.	3.58	98.06
F2	0.05	3	20	20	q.s.	3.64	99.21
F3	0.05	1	30	20	q.s.	2.46	97.63
F4	0.05	2	30	20	q.s.	2.60	98.59
F5	0.05	3	30	20	q.s.	2.80	98.15
F6	0.05	1	40	20	q.s.	1.96	89.57
F7	0.05	2	40	20	q.s.	2.10	93.43
F8	0.05	3	40	20	q.s.	2.24	92.73

Impact of phospholipid and ethanol concentration on entrapment efficiency

The size distribution of ethosomes ranged between about 200 nm to 360nm and is known to be influenced by the composition of ethosomes. Table 1 shows that the size of the vesicles decreased when the ethanol concentration was increased from 20% to 40%. The largest vesicles of 3.64 ± 18 nm size were present in the preparation containing 20% ethanol, while the smallest vesicles of 1.96 ± 23 nm size were present in the preparation containing 40% ethanol. This significant difference in the size of ethosomal formulations is because of the presence of different concentrations of ethanol. Probably, ethanol causes a modification in net charge of the system and confers it some degree of stearic stabilization that may lead to decrease in mean vesicle size. The composition of

different ethosomal formulations is summarized in Table 1. The entrapment efficiency for isotretinoin in optimized ethosomal formulation was 57.2% ± 2.5% (F2) and 41.4% ± 1.7% (F4), respectively. The higher entrapment efficiency of isotretinoin in ethosomes in comparison to marketed formulation was probably due to the presence of ethanol in vesicle membrane (18). Increasing the concentration of ethanol from 20% to 40% increased the entrapment efficiency owing to increase in fluidity of membranes. However, further increase in the ethanol concentration in the lower phospholipids formulations F6 (89.57%) probably made the vesicle membrane more leaky, thus leading to decrease in entrapment of lamivudine (Table 1). The range of drug entrapment efficiency of the isotretinoin ethosomal formulation was about 78.53% to 99.21%. F2 containing 2%w/w of phospholipid and 30%w/w of ethanol had maximum entrapment percentage of 99.21% among ten ethosomal vesicles (Table 1).

pH meter was used to measure the pH of ethosomal vesicles. The pH of the vesicles was fall within pH 4.0-5.0 (Figure 1). The result of pH revealed that the pH increases as the ethanol concentration increased when the lecithin concentration was constant. However, the pH decreases as the lecithin concentration increase when the ethanol concentration was constant.

Table 2: Evaluation on the physicochemical properties of gel formulations

Batch code	Colour	Physical Appearance	Homogeneity	Texture	Spreadability (gm.mm/min)	Drug Content (%)
Marketed Formulations	Yellow	Transparent	Homogenous	Smooth	505	35.73
F2	Yellow	Transparent	Homogenous	Smooth	482.5	41.21
F4	Yellow	Transparent	Homogenous	Smooth	500	44.26

Stability study

The stability of ethosomal vesicles were identified by measuring the pH and drug entrapment efficiency at 0 C and 25 C from 0 to 45 days. The figures 2 and 3 shows that the ethosomal vesicles had better drug entrapment efficiency by storing at 4 C than at 25 C even at 45th days. Even in the case of pH also ethosomal formulations were more stable at 4 C than at 25 C that data and figures are not presented in this manuscript. Both the drug and concentration of ethanol influence the transition temperature of vesicular lipids. Storage stability of ethosomal systems can be determined by comparing the shape, average size and entrapment capacity of the vesicles over time at different storage conditions.

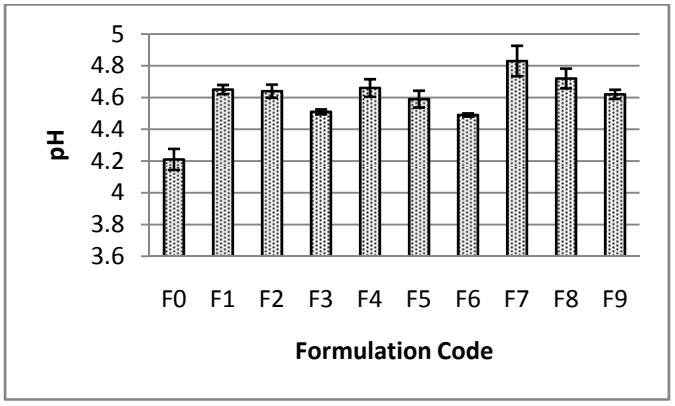

Figure 1: Variation in pH of Isotretinoin ethosomal vesicles

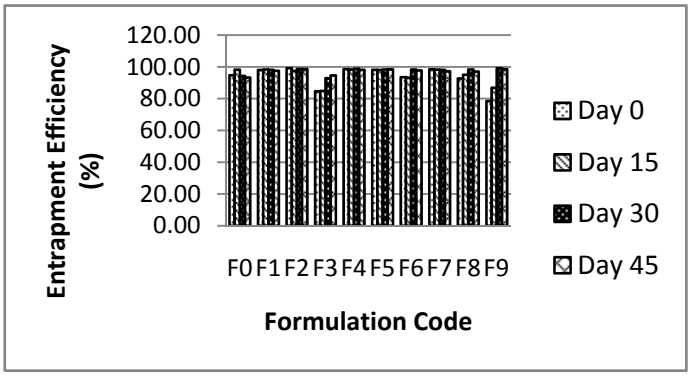

Figure 2: Drug entrapment efficiency of the vesicles from day 0 to day 45 at 4 C.

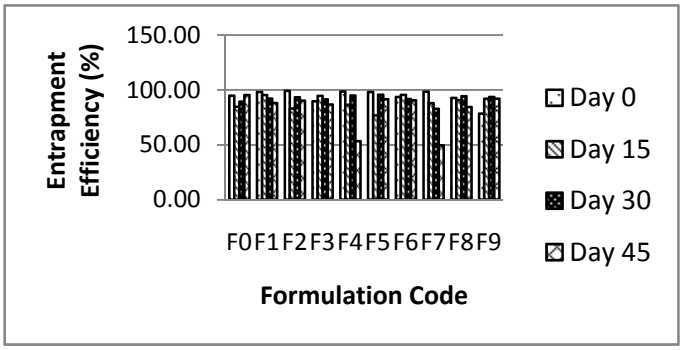

Figure 3: Drug entrapment efficiency of the vesicles from day 0 to day 45 at 25 C.

Based on various stability studies performed, researchers suggest refrigerated condition (4- 8◦C) as the suitable storage condition for ethosomal formulations. Higher temperatures may cause degradation of vesicular lipids loss of structural integrity of vesicles and an accelerated leakage of the entrapped contents. [20-21]. Loss of drug from the vesicles stored at elevated temperatures may be attributed to the effect of temperature on the gel to liquid transition of lipid bilayers together with possible chemical degradation of the phospholipids, leading to defects in membrane packing [22]. Lower percentage of drug leakage at higher temperatures, as observed in storage stability studies clearly showed that ethosomes are more stable in comparison with other vesicular delivery systems. The optimized gel formulation (F2 gel) was used in this study. As shown in figure 4, the drug content was more stable with F2 gel stored at 4 C than at 25 C.

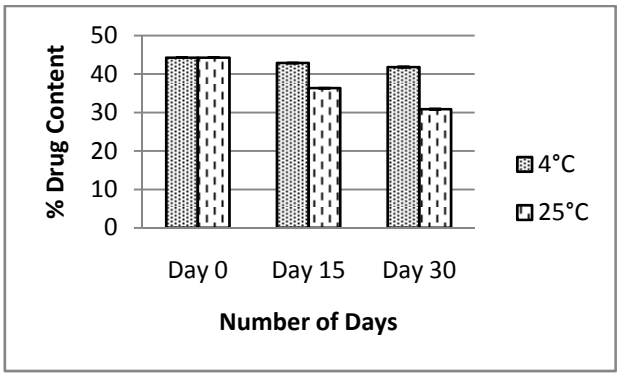

Figure 4: Comparison of Drug Content (%) between 4 C and 25 C.

In vitro drug release study

The drug release profile of marketed product showed significantly ($p<0.05$) better release compared to F4 and F2 gel as shown in Figure 5. Phosphate buffer saline of 7.4 is generally used as the receptor phase in release and skin permeation studies. Isotretinoin solubility in PBS is very low (1.8 ± 0.4 µg/ml) and could not provide the perfect sink condition. In such a condition, an co-solvents or solubilizers becomes essential. To find a suitable receptor liquid, preliminary solubility study with isotretinoin using three receptor phases propylene glycol, Tween and tetrahydrofuran (1:20 PBS 6.8 solutions (01:1) and PBS 7.4 alone. Results showed that the tetrahydrofuran solutions increase the solubility of isotretinoin and provide a perfect sink condition. Solubility of isotretinoin in this receptor phase was found 58.1±4.2 µg/ml.

The percentage cumulative release after 8 h from different formulations of ethosome shown in figure 5. The cumulative release of drug on a percentage basis from F2, F4 and MF was plotted against the square root of time (t½) in Figure 5. From the plots presented it is clear that the release of the drug from the ethosome vesicles followed the diffusion controlled model in which

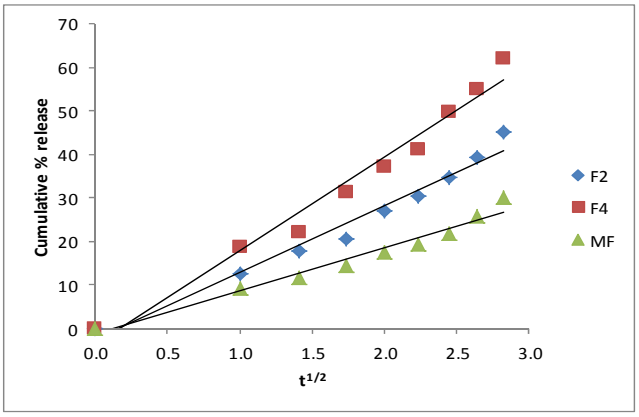

Figure 5: Comparison of in vitro drug release profile of various gel formulations.

the total percentage of drug released is proportional to the square root of time. The drug release profile of marketed product showed significantly ($p<0.05$) higher percent release (30%) compared to F2 and F4 gel. In comparison between F2 and F4 gel, the total release after eight hours from F2 gel (45.11 %) was more than two times the total release after eight hours shown by F4 gel (62%). The main reason is that the higher amount of ethanol concentration of batch F4 (30%), increase the vehicle movement more freely through the membrane. On comparing the release kinetics data (Table 3), F2 and F4 gel were found to release the drug in accordance to Higuchi Kinetics which were indicated by the highest correlation coefficient values.

Table 3: Release Rate Constant (k) and correlation coefficient (R^2) of gel formulations

Gel Formulations	Zero Order Kinetics		First Order Kinetics		Higuchi Kinetics	
	k	R^2	k	R^2	k	R^2
Marketed Formulations	2.568	0.951	1.842	0.951	0.412	0.901
F2 gel	3.759	0.7989	1.983	0.813	0.986	0.973
F4 gel	1.444	0.961	1.995	0.958	0.307	0.985

In vitro skin permeation study

After review of all the factors including physiochemical properties, release pattern, entrapment efficiency before and after storage, the ethosome F2 batch and MF were selected for skin permeation studies. Each selected formulation was evaluated using the rat skin model, and the average of three determinations reported. Generally the in vitro drug release and skin permeation studies showed that the skin is the rate-limiting factor because the in vitro release of the drug was greater from each the formulations compared with the respective in vitro drug permeation rate. To overcome this stratum corneum barrier we have added two

different chemical enhancers in F2 batch such as N-methyl-2-pyrrolidone (NMP) and eugenol (Eu).

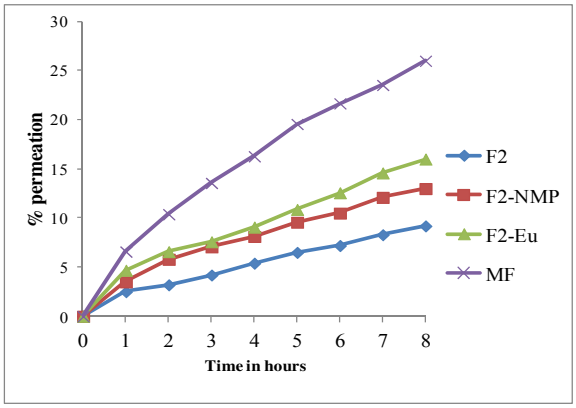

Figure 6: *In vitro* skin permeation profile of F2 gel without or with enhancers.

On comparing the permeation profiles of isotretinoin with and without enhancers gels formulation (F2, F2-NMP and F2-Eu) to marketed product (MF) (Figure 6), the permeation profile of marketed formulation showed significantly amount ($p<0.05$) permeated (26%) through the skin into the receptor compartment. Whereas ethosomal gel (F2 – 9.2%) with chemical enhancers both F2- NMP (13%) and F2-Eu (16%) formulations permeated into the receptor medium respectively up to 8 hours. It may be due to presence of ethanol in ethosomal gels which furnishes the vesicles with a flexibility that allows them to penetrate into deep skin layers more easily [25]. Also phospholipids are hygroscopic in nature and bind easily with water. For this reason we assume that this property increases the skin humidity and that leads to maximal hydration of the horny layer. In addition any residual ethanol would be synergistic with the phospholipds [24]. The cumulative drug permeation as a percentage from different formulations was plotted against the permeation time in hours (t) in Figure 6. In each plot, the rate of drug permeation is fairly constant over time and the permeation profiles exhibit the concentration dependent first-order kinetics. Skin deposition study was carried out with the objective to determine the percent deposition effect of ethosome in the deeper layer of skin. For an effective anti-acne agent, it must deliver the drug to deeper layers of skin. Figure 7 shows that the percent skin deposition of isotretinoin ethosomal gels with and without enhancers and marketed formulation. The amount of drug deposited was 15-folds higher in case of ethosomal gel (F2).

Skin deposition study showed that the F2 batch gel showed better deposition and skin-targeting effect of ethosomal formulation, may be due to ethosomal vesicle is too large to enter into the deeper skin tissue directly; this kind of action could help the isotretinion bypass the cutaneous capillary circulation and hence they may act as depot and sustain the drug release locally. In the case of enhancers containing gels, depot formation is lower due to these enhancers as well as higher amount of ethanol may potentiate the skin lipid hydrolysis and leads to increase drug permeation across the skin and reached into the receptor medium.

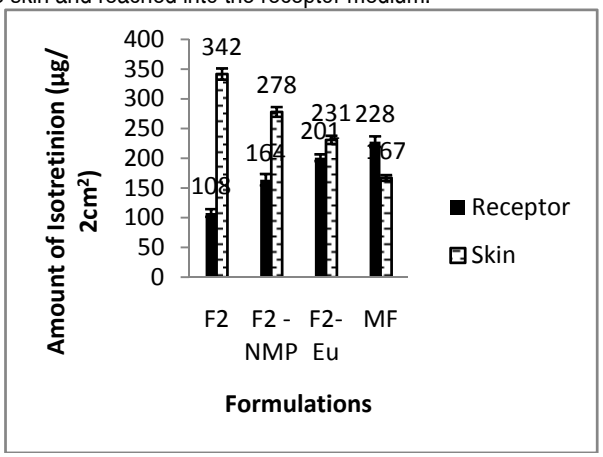

Figure 7: *In vitro* skin deposition profile of different formaulations.

Whereas the marketed formulation does not have the vesicles system unlike ethosome that may be the reason this formulation also reached into the receptor medium without formation of depot drug delivery. This kind of release pattern from F2 –Eu and MF allows the drug intradermal capillary and once it reaches into the dermis region of the skin and then it will reach the systemic circulation [23].

Conclusion

The presence of ethanol in ethosomes plays an important role in permeation of the isotretinoin through the upper layer of the skin. Phospholipids may increase the drug absorption and retain the drug in the skin tissues for longer periods as shown in the experimental findings. The enhancers incorporated in the ethosomal gel also increase the drug permeation through the skin. However, the result was insignificant. Based on the physiologic barrier and limitations in reaching effective component to the lesion areas, this study introduces a new method for the preparation of ethosome with lecithin, ethanol and water, which has advantages of even distribution of particles and its vesicles with high entrapment efficiency. Further experimental studies need to be carried out to find out lecithin distribution behavior in the skin tissues. The clinical efficacy of a topically applied drug depends not only on pharmacological properties but also on the availability of drug at target site. However, F2 and F2-NMP formulation may avoid systemic circulation, photo stability problem and hence reduce the skin irritation and oral formulation relation side effects.

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