RAMIPRIL LOADED NON-IONIC SURFACTANT VESICLES: CHARACTERIZATION AND *IN-VITRO* PERMEATION STUDIES

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ABSTRACT

The purpose of present study was to design non ionic surfactant vesicles containing ramipril with different spans. The vesicles known as niosomes with a small particle size and good sphericity were prepared by thin film hydration method. Ramipril niosomes were analyzed for their morphological aspects, vesicle size and drug entrapment efficiency. Transdermal permeation of ramipril through rat abdominal skin was determined by Franz diffusion cell. The *in-vitro* permeation profile of optimized formulation was compared with that of ramipril solution. Superior flux [18.53±0.83] was observed in span 40[1:1] niosomes. Further stability studies were carried out at 4ºC for a period of 12 weeks. The results of physicochemical characterization and *in-vitro* permeation studies of the prepared vesicles were promising to formulate transdermal drug delivery system.

Keywords: **Ramipril, Transdermal, Niosomes, Non-ionic surfactants, vesicle delivery, permeation.**

INTRODUCTION

Niosomes have become vehicle of choice as drug delivery carriers, nowadays. The vesicles formed from non-ionic surfactants are known as niosomes or non-ionic surfactant vesicles. These vesicles can be prepared from phospholipids, non-ionic surfactants and mixed anionic and cationic surfactants. They are known as analogous of liposomes and have been widely investigated as drug carriers by several research groups. They have been considered of particular interest because they offer several advantages over liposomes with respect to lower cost, high purity, content uniformity, availability of large numbers of non-ionic surfactants and greater stability [1]. The amount of drug encapsulated can vary upto 90 % and both hydrophilic and lipophillic drugs can be entrapped successfully either in aqueous layer or in vesicular membrane. Drug toxicity and side effects can be reduced using these vesicles because of their non-ionic nature. They also offer the possibility of controlled drug release and drug targeting as reported in literature.

Transdermal drug delivery systems are ideally suited for diseases that demand chronic

treatment. In recent years, owing to advantages offered by transdermal administration a number of antihypertensive agents have been studied by various authors for continuous permeation and transdermal drug delivery. Antihypertensive agent ramipril which is angiotensin converting enzyme inhibitor is selected as drug candidate. Chemical name of ramipril is [2S,3aS,6aS]-1-[[2S]-2-{[[2S]-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino}propanoyl]-octahydrocyclopenta[b]pyrrole-2-carboxylic acid. It is marketed as tablets and capsules. It has absorption and bioavailability of 60% and 28% respectively. It is freely soluble in methanol and polar organic solvents and buffered aqueous solution. It is sparingly soluble in water having melting point 105-110^{0}C and partition coefficient 3.32 [2].

In literature, nanoemulsions and multiunit particles were reported for ramipril [2, 3]. There are no reports on vesicular delivery system for ramipril. However, some other ACE inhibitors have been explored for transdermal delivery. Captopril has been investigated as lyphollised aqueous based polymer matrices and proniosomes for transdermal delivery [4, 5]. Mucoadhesive films of enalpril maleate for buccal delivery were developed using HPMC, PVP and sodium CMC by [6]. Matrix type transdermal system of trandolapril was prepared and evaluated in-vitro and ex-vivo by [7].

Aim of present study was to investigate the feasibility of formulation of niosomes for transdermal administration of ramipril. Drug loaded non-ionic surfactant vesicles were prepared by thin film hydration method and formulations composed of different percentages of cholesterol and non-ionic surfactants were studied for their effect on the size of vesicles. This method was widely reported for niosomal preparation. Non-ionic surfactants are reported as biodegradable, biocompatible and non-immunogenic. Among these spans are more commonly used for niosomal preparation and hence selected for present study. The formulated vesicles were further analyzed for particle size, encapsulation efficiency and in-vitro permeation studies. Finally stability studies were also carried out.

MATERIALS AND METHODS
Materials
Span 40 and 60 were purchased from Shah Scientific, Mumbai [India]. Cholesterol was purchased from Central Drug House Pvt. Ltd, New Delhi [India]. Dialysis membrane [MW cut off 8000-10,000] was purchased from Himedia Laboratories Pvt. Ltd, Mumbai. All other chemicals and solvents were of analytical grade. Ramipril was supplied as a gift sample by Ranbaxy, Ponta Sahib, H.P [India]

Methods
Preparation of rat abdominal skin membrane
The in-vitro experiments using rat skin were carried out after obtaining approval of the Institutional Animal Ethics Committee of M.M. University, Mullana, India and their guidelines were strictly followed. Male Wistar rats were killed by cervical dislocation and the full length shaved abdominal skin with adhering connective tissue was excised with care. Prior to use, the skin was stained with haematoxylene and eosin and was inspected for any damage using microscopy.

Preparation of non-ionic vesicles
Unilamellar vesicles were prepared according to thin film hydration method reported in literature [8]. Table 1 lists the composition of the formulations tested in present study. Accurately weighed quantities of the drug [ramipril], non-ionic surfactant and cholesterol [ratios reported in Table1] were dissolved in 10 ml chloroform into a long necked quick fit round bottom flask. The organic solvent was slowly evaporated at 60°C under reduced pressure, using rotary evaporator [Perfit India] at 150 rpm such that a thin dry film of the components was formed on the inner wall of the rotating flask. After removal of last traces of organic solvents, the film was hydrated by 10 ml of phosphate buffered saline [PBS, pH 7.4] at 60°C for 1hr [9]. The niosomal suspension was left to mature overnight at 4°C.

Method reproducibility
To assess the reproducibility of film hydration method formulations were prepared several times and % entrapment efficiency of each batch was measured.

Vesicular Characterization
Optical microscopy
Small amounts of the niosomes formed were spread on a glass slide and examined for the vesicle structure and the presence of insoluble drug crystals using light microscopy [Carl Zeiss, Berlin and Germany]. Photomicrographs were taken at a magnification of 40X using digital camera fitted with microscope [Nikon coolpix S220, Japan] [10].

Vesicle size determination
Mean size and polydispersivity index of niosomal formulations were measured at 25^oC by photon correlation spectroscopy, using a Delsa Nano-C [Beckman Coulter Instruments]. Light scattering was monitored at 25°C at a scattering angle of 90°. The polydispersivity index of niosomes was performed as a measurement of the size distribution of system.

Entrapment efficiency [%] [EE]
Percentage entrapment efficiency was studied by centrifugal method. A niosomal suspension was centrifuged at 18000 rpm for 40 min at 4^0C. The supernatant was taken and diluted with PBS [pH 7.4]. The drug concentration in the resulting solution was assayed by UV method at 210 nm [11]. The percentage of drug encapsulation was calculated by the following equation:

$$EE [\%] = [[C_t - C_f]/ C_t] \times 100$$

Where C_t is the concentration of total drug and C_f is the concentration of unentrapped drug.

Transmission electron microscopy
The morphology of hydrated niosomal suspension was determined using transmission electron microscope. A drop of noisomal dispersion was applied to a carbon-coated 300 mesh copper grid and left for 1 min. to allow some of the niosomes to adhere to the carbon substrate. The remaining dispersion was removed by absorbing the drop with the corner of a piece of filter paper. A drop of 2% aqueous solution of uranyl acetate was applied for 35 sec. The remaining solution was removed by absorbing the liquid with the tip of a piece of filter paper and the sample was air dried. The sample was observed in a Hitachi 7500 transmission electron microscope at 90kV [12].

In-vitro permeation studies
In-vitro skin permeation studies of ramipril niosomes were performed using modified vertical Franz- diffusion cell with an effective area of 0.785 cm^2. The control solution was prepared by dissolving drug [2mg/ml] in PBS [pH 7.4]. Full thickness albino rat skin was sandwiched securely between donor and receptor compartment with the epidermis site facing the donor compartment. The receptor compartment was filled with 5ml PBS [pH 7.4] solution which was continuously stirred and thermostated at 37^oC $\pm1^oC$ throughout the experiment. After 1 hr equilibrium, 1ml of either ramipril entrapped vesicle in aqueous suspension or ramipril solution were placed on to the skin surface. Before starting the experiment the donor cell was sealed with parafilm and covered with aluminum foil to prevent exposure to light. At predetermined time interval for 24 hr [1hr, 2hr, 4hr, 6hr, 8hr, 10hr, 12hr, and 24hr], 1ml of aliquots were withdrawn and replaced with an equal volume of fresh PBS [pH 7.4] to ensure sink conditions.

Stability of ramipril niosomes
The samples were stored at 4^oC for 12 weeks and encapsulation efficiency of all these samples was determined after 12 weeks [13].

Statistical analysis
Statistical analysis of entrapment efficiency [%]and *in-vitro* release studies among niosomal formulations was performed by using one – way analysis of variance [1- way ANOVA] and paired t-test respectively[Graph Pad, version 3.0, San Diego, CF]. The level of significance was taken at p value < 0.05.

RESULTS AND DISCUSSION
Optical microscopy
The morphology of all the niosomal vesicle formulations were determined by optical microscope equipped with digital camera. The photomicrographs of N2 and N4, niosomal formulations are shown in Figure 1 and 2. These photomicrographs confirmed the formation of vesicular structures. The microscopic

appearance of all formulations showed spherical vesicles. Unfortunately, information concerning microstructure of niosomes could not be visualized by the low-magnification power of optical microscope; therefore transmission electron microscope was employed to elucidate morphology of niosomal vesicles.

Transmission electron microscopy

The microstructure of niosomes can be visualized with high magnification power of electron microscope. Formulations N2 and N4 were selected here for transmission electron microscopy as these possessed high entrapment efficiency values and maximum vesicle size as compared to other formulations. Transmission electron photomicrographs of ramipril niosomes N2 and N4 [at 80 KV with magnification 40000X and 30000X respectively] are illustrated in Figure 3 and Figure 4. These figures showed spherical vesicles which were unilamellar with no aggregation or agglomeration.

Table 1. Compositions of various niosomal formulations

Niosomes code	Drug (mg)	Span 60: Cholesterol (molar ratio)(mg)	Span 40: Cholesterol (molar ratio)(mg)
N1	20	1:0.5	-
N2	20	1:1	-
N3	20	1:1.5	-
N4	20	-	1:0.5
N5	20	-	1:1
N6	20	-	1:1.5

Table 2. Vesicle size of different niosomal formulations

| Sr. No.	Formulations	Particle size (nm)	PI
1	N1	915.2	0.284
2	N2	1066.8	0.393
3	N3	1196.0	0.038
4	N4	792.4	0.487
5	N5	849.8	0.218
6	N6	986.4	0.364

Table 3. Entrapment efficiency (%) for different niosomal formulations

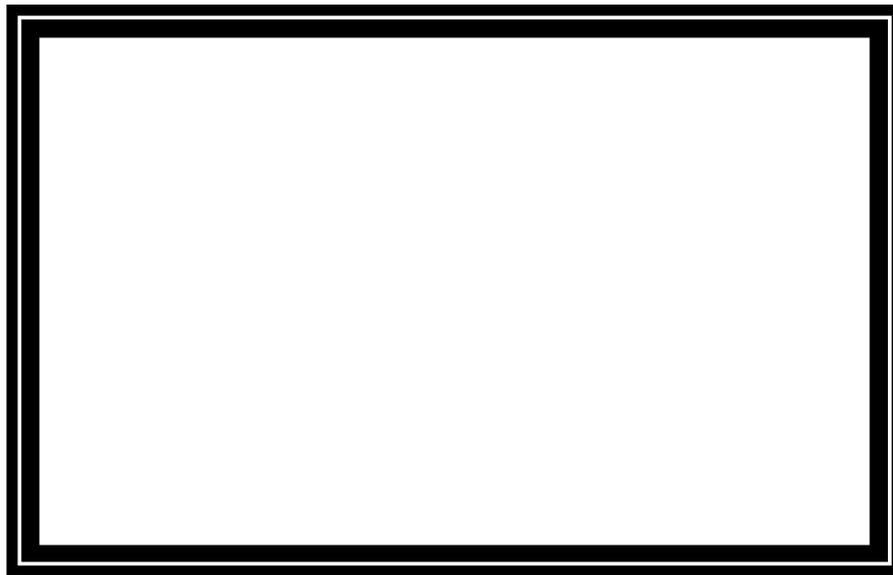| Formulation code	Entrapment efficiency (%)			Mean ± S.D
	1	2	3	
N1	20.95	23.5	25.26	23.23± 2.16
N2	62.4	68.87	58.87	63.38 ± 5.07
N3	41.34	46.87	47.56	45.25 ± 3.4
N4	22.37	20.18	19.2	20.58 ± 1.62
N5	40.31	38.87	44.25	41.14 ± 2.78
N6	27.37	31.5	24.81	27.89 ± 3.37

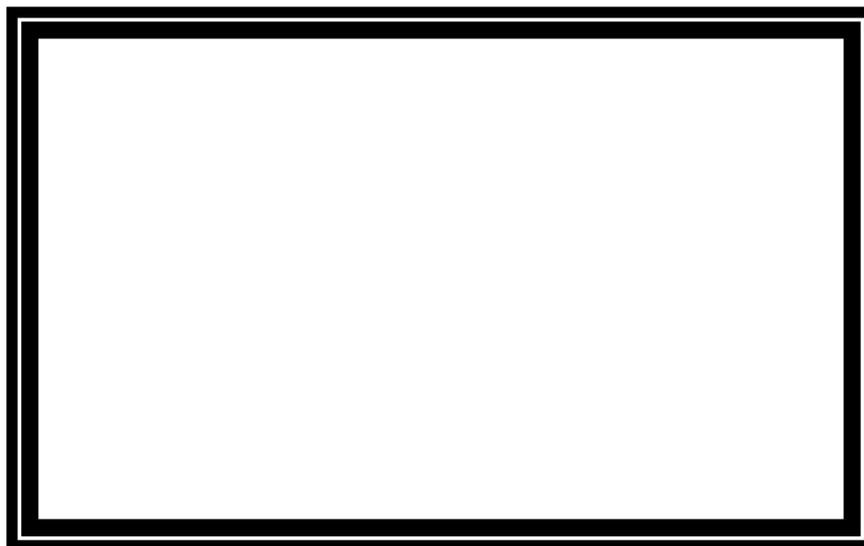*All values are average of three determinations (n=3)

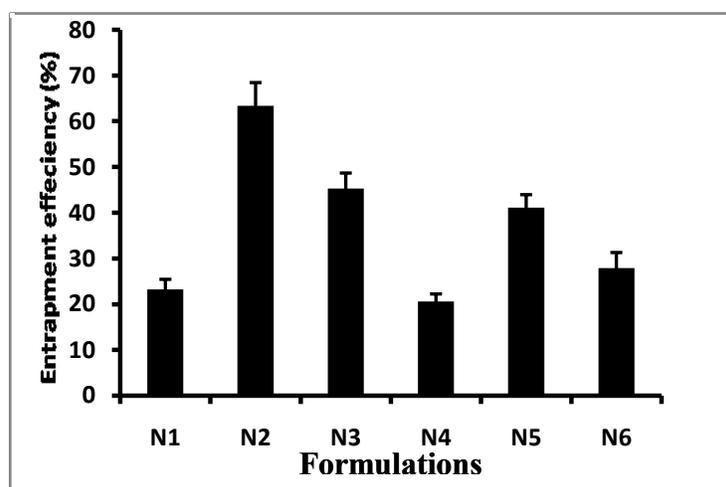**Figure 1.** Photomicrograph of formulation N2
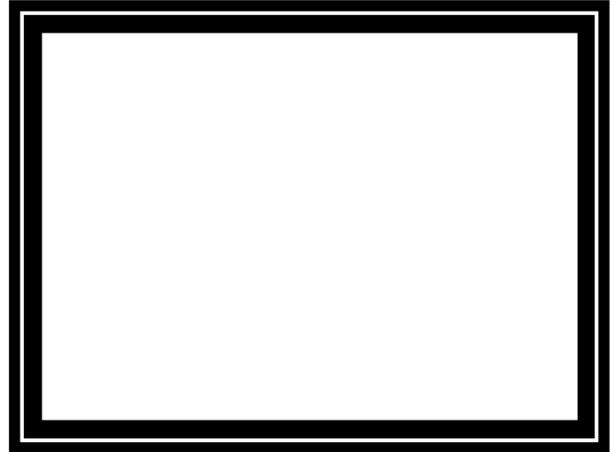
Figure 2. Photomicrograph of formulation N4

Figure 3. TEM of formulation N2 [at 80kv with magnification 40000X]

Figure 4. TEM of formulation N4 [at 80kv with magnification 30000X]

Figure 5. Comparison of entrapment efficiency [%] of different formulations

Vesicle size determination

The average size of vesicles was measured using Delsa Nano-C [Beckman CoulterInstruments, CA, USA]. The vesicle size was found in range from 792 nm to 1196 nm as shown in Table 2.

The vesicle size of ramipril niosomal formulations was found to be in decreasing order as N3>N2>N6>N1>N5>N4. Among these formations, N3 was found to have maximum vesicle size as compared to other formulations. HLB value of Span 60 and Span 40 is 4.7 and 6.7 respectively. More HLB value means more hydrophobic character, therefore, due to more hydrophobicity, Span 40 [N4, N5, and N6] resulted in smaller vesicular size than Span 60 [N1, N2, and N3] as shown in Table 2. Further it was observed; increase in cholesterol content resulted in increased vesicle size.

Encapsulation efficiency [EE] [%]

Percentage encapsulation efficiency was measured by "centrifuge method", in which unentrapped drug was calculated

spectrophotometrically and entrapped drug was determined using formula:

$$EE\ [\%] = [[C_t - C_f]/\ C_t] \times 100$$

Where C_t is the concentration of total drug and C_f is the concentration of unentrapped drug.

The entrapment efficiency of all formulations was found to be in decreasing order as N2>N3>N5>N6>N1>N4. EE [%] of different formulations are represented in Table 3. And comparison of EE [%] of different formulations has shown in Figure 5. Entrapment efficiency of N2 and N4 formulations was found to be approximately 63.50% and 20.50% respectively. The entrapment efficiency is the most important parameter from pharmaceutical view point in niosomal formulations. A high percentage of entrapment would mean less time and effort involved in removal of unentrapped material. To study the effect of increased cholesterol ratio on the amount of drug entrapment in niosomes, a series of formulations were prepared with increasing cholesterol molar ratio [0.5, 1 and 1.5] at a fixed amount of ramipril [20 mg]. The effect of cholesterol on ramipril entrapment was varied according to the nonionic surfactant used. Cholesterol was found to have significant effect on the ramipril entrapment into sorbitan ester niosomes. In niosomal formulations prepared using sorbitan monoesters, Span 60 showed the maximum entrapment efficiency at 1:1 cholesterol molar ratio, as it has the longest saturated alkyl chain, followed by Span 40 [Figure 5]. By increasing cholesterol content

from 0.5 to 1 molar ratio lead to a significant increase [$P > 0.05$] in the entrapment efficiency of sorbitan ester niosomes from 23.23 ± 2.16% to 63.38 ± 5.07% and 20.58 ± 1.62% to 41.14 ± 2.78% for Span 60 and Span 40 niosomes, respectively [Figure 6]. The improvements in drug entrapment with increased cholesterol content [0.5–1] and the major reduction in drug entrapment when cholesterol content was further increased [1–1.5] may be due to two conflicting factors: [1] with increased cholesterol, the bilayer hydrophobicity and stability increased [14] and permeability decreased [15], which may lead to efficient trapping the hydrophobic drug into bilayers as vesicles formed. [2] In contrast, higher amounts of cholesterol may compete with the drug for packing space within the bilayer, hence excluding the drug as the amphiphiles assemble into the vesicles. This explains why sorbitan monoester niosomes at 1:1 cholesterol molar ratio showed higher entrapment efficiencies than those showed at high cholesterol molar ratio at 1.5. According to literature reports, the nature of the hydrophobic alkyl chain affects the encapsulation efficiency of CarboxyFluorescein [16] and Doxorubicin [17] in unsonicated Span containing niosomes. Span 60 [C18] and Span 40 [C16] gave the greatest encapsulation efficiency for CF niosomes due to the fact that these Span surfactants had the highest phase transition temperature [16]. This accounts for the higher entrapment observed

for both Span 40 and Span 60 than Span 20 niosomes at 1:1 molar ratio.

In-vitro permeation studies

Niosomes are composed of non-ionic surfactants which are biocompatible and relatively non toxic and themselves serve as excellent permeation enhancer [13]. In this study in order to assess the influence of the drug carrier on the diffusion of drug through skin, *in-vitro* permeation studies [Figure 7] using albino rat skin and vertical Franz diffusion cell was carried out.

In-vitro permeation flux 18.35±0.83 for span 40 [1:1] and 12.57±1.46 for span 60 [1:1] respectively [Figure 8]. Superior ramipril skin permeation was obtained with smaller niosomes of Span 40 [849 nm] than with large Span 60 niosomes [1066.8 nm]. This could be due to low phase transition temperature of these surfactants and smaller size of these niosomes [16].

Less permeation of drug was observed from Span 60 niosomes. It was suggested that the niosomes prepared from low phase transition temperature surfactant mixture [more fluid membrane] showed higher permeation than the one having higher phase transition temperature [13, 17].

There are several mechanisms which could explain the ability of niosomes to modulate transfer across skin. One of the mechanism by which niosomes may contribute to transdermal drug delivery may be described to the fusion of vesicles on the surface of the skin which might lead to the establishment of large concentration gradients of the intercalated drug across the skin and hence enhanced skin permeation [17, 18]. Moreover, it has been proven that niosomes enhances penetration and retention of topically applied drugs [19]

Stability studies

Stability studies were performed on Span 60 [1:1] and Span 40 [1:1] for a period of 12 weeks by subjecting them to aging at $4^{\circ}C$ [Figure 9]. A direct relationship between the percentage leaching of the drug out of the vesicles and aging was observed i.e. as the storage period increases, the degree of leaching increased. It was observed that Span 40 niosomes showed higher stability over Span 60 niosome this could be due to low electrostatic repulsion. Such trends were also observed by Balakrishnan*et al* [13].
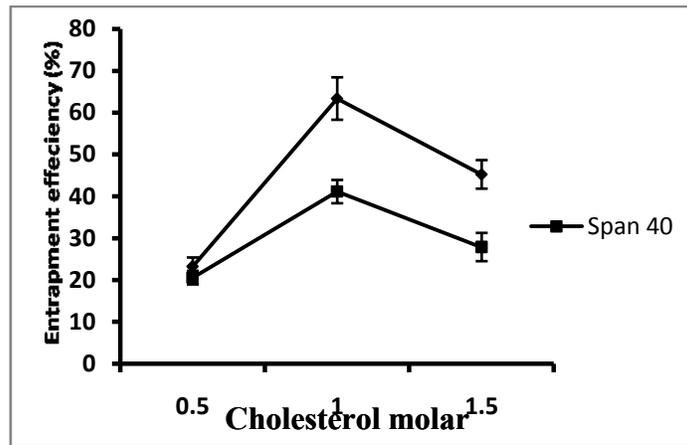
Figure 6. Effect of cholesterol on entrappment effeciency [%]
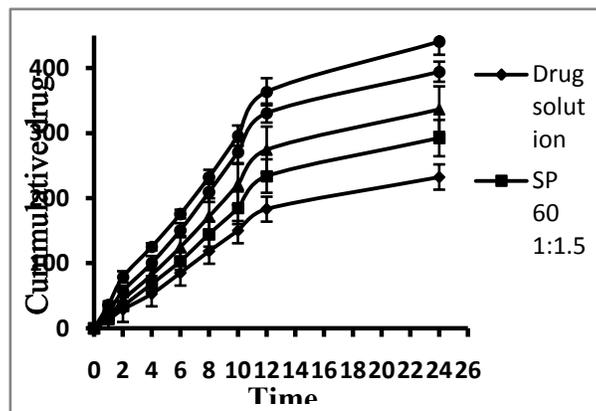

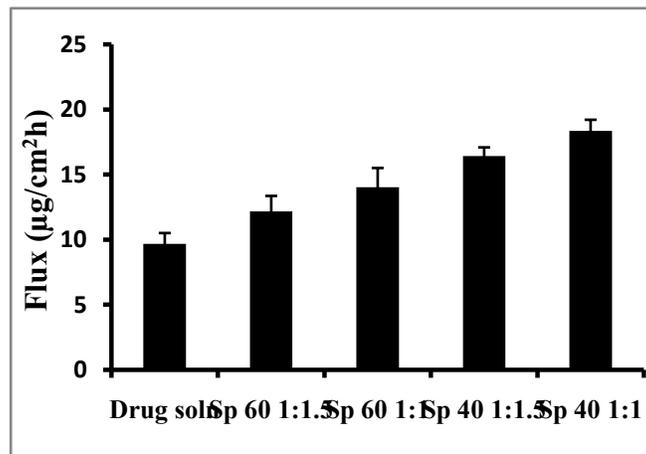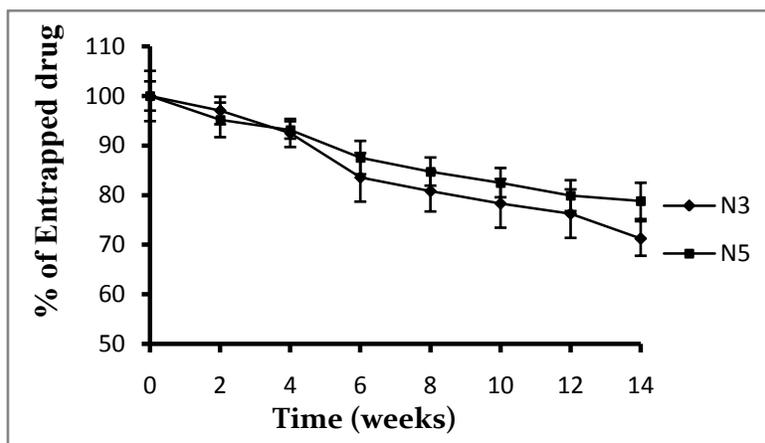**Figure 7.** Cumulative drug release for different ramipril niosomal formulations

Figure 8. Comparison of flux for different niosomal formulations

Figure 9. Comparison of EE [%] of different niosomal formulations stored at 4˚C

CONCLUSION

Thin film hydration method used for the preparation of ramipril niosomes was found to be a good technique to encapsulate hydrophobic drug in non ionic surfactants. The non ionic surfactant prepared showed reasonable drug entrapment, suitable size and good permeation of the drug. The prepared span 40 [1:1] niosomes were found stable at 4ºc for 12 weeks and could potentially be used for transdermal delivery of ACE inhibitor.

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DECLARATION OF INTEREST

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