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Fabrication of niosomes containing N-acetyl glucosamine: *In-vitro* and *ex-vivo* characterizations

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Abstract: This study was designed to formulate N-acetylglucosamine (a skin lightening agent) containing advanced dosage form, niosomes. Thin film hydration technique was used to produce niosomes. The *in-vitro* stability studies were performed to check different parameters as pH, conductivity and rheology for the period of three months. The particle size, zeta potential, TEM analysis were also conducted. *Ex-vivo* permeation studies were done using Franz cell on albino rat skin. The particle size of optimum formulation (NA 5) was 445.8nm and zeta potential was -25.2. TEM analysis of niosomes suspension showed round smooth vesicles were formed. *In-vitro* stability studies showed that there was no significant (p>0.05) change in pH, conductivity and viscosity throughout the study period. The rheological analysis showed that niosomal gel follow pseudoplastic behavior. *Ex-vivo* permeation studies were showed that percent drug permeated after 24 hours was 42.76±0.02%.The percent drug retained in the skin was 44.95% which was significantly (p<0.05) more as compared to control gel. The targeting efficiency and enhancement ratio were also calculated that were 2.64 and 1.14 respectively. A stable niosomal gel was produced which has improved drug penetration and deposition in the rat skin.

Keywords: N-acetylglucosamine, niosomes, permeation studies, TEM, *in-vitro* studies.

INTRODUCTION

N-acetylglucosamine is a component of hyaluronic acid which has molecular weight of 221.21g/mol and its molecular formula is $C_8H_{15}NO_6$. It is sweet in taste, white in colour and its melting point is 211°C. It is soluble in water and it forms clear and transparent solution at concentration of 1 %. It can be safely used orally and topically. A 2% concentration of N-acetyl glucosamine is used for skin lightening (Draelos, 2007). It is a carbohydrate and water soluble (Pawar *et al*., 2012).

Skin is composed of different layers and protects the body from tactless external environment as sunlight UV radiations or dry environment. The stratum corneum is the upper layer and it regulates the skin water content and texture. N-acetylglcucosamine is a building unit of hyaluronic acid which is essential component for maintaining the skin moisture and skin elasticity. With the passage of time hyaluronic acid content in the skin decreases that results in the wrinkle formation.

N-acetyl glucosamine improves skin condition by increasing the collagen production from the fibroblasts. It increases the moisture content of the skin and improve complexion by reducing skin melanin. It causes the reduction in the genes of epidermal turnover and melanosomes transport in the cytoskeleton thus reduces the melanin production. It also increases the production of keratinocytes, skin fibroblasts and hyaluronic acid in the skin and can be used in wound healing. These effects are confirmed through the animal testing and clinical trials (Chen *et al*., 2010).

The vesicles formed from the non-ionic surfactants are known as niosomes. Niosomes can entrap hydrophilic drug as N-acetylglucosamine (NAG). NAG reduces hyperpigmentation by inhibiting the enzyme tyrosinase in the melanocytes. When NAG incorporated into the niosomes its penetration is improved that was confirmed by testing permeation through rat skin on the Franz diffusion cell.

The niosomes were produced from the combination of Span 60 and Tween 60. The formed niosomes has ability to increase the NAG localization in the skin to treat hyperpigmented skin.

The fabrication of NAG into niosomes is advantageous when compared to conventional drug delivery systems as they act like drug reservoir and drug delivery to the target site. When it is incorporated into niosomes its stability and penetration into the viable epidermis was improved (Shatalebi *et al*., 2010).

Tween 60 and Span 60 formed spherical vesicles when used in combination. These vesicles were stable at 25°C in terms of entrapment efficiency, no sedimentation, phase separation during the period of three months (Tavano *et al*., 2011).

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MATERIALS AND METHODS

Materials

N-acetylglucosamine was purchased from "Sigma-Aldrich", Span 60, Tween 60 from "Daejung", Cholesterol were obtained from "AppliChem", Carbopol 940® and Chloroform by "Merck". The materials and solvents used were of analytical grade.

Preparation of Niosomes

Niosomes were produced using Thin film hydration technique with slight modification. Accurately weighed amount of Span 60, Tween 60 and Cholesterol were dissolved in 10 ml chloroform in 100 ml round bottom flask. Chloroform was evaporated under vaccume using rotary evaporator at 60°C temperature of water bath and 4°C temperature of chiller. A thin film was produced which was then hydrated with 300µm aqueous solution of N-acetylglucosamine. The round bottom flask was again rotated for 45 minutes at 60rpm and 60°C. The niosomes were produced were further sonicated for 5min and homogenized.

Niosomal gel of N-acetylglucosamine was prepared using carbopol 940 using 1% w/w. Carbopol 940 was sprinkled in small amount of distilled water and allowed to soak over night. Niosomes of N-acetylglucosamine were added and mark up weight with quantity sufficient distilled water.

Particle size and zeta potential

The particle size and zeta potential of optimized niosomal formulation were assessed by dynamic light scattering using a Malvern Zetasizer at a temperature of 25°C. Prior to measurements, samples were diluted with deionized water. Zeta potential values were presented as the mean ± standard error of mean (SEM) from triplicate experiments.

Drug entrapment efficiency

The percent drug entrapped was calculated by indirect method. The niosomal dispersion was centrifuged for 30 minutes. The clear supernatant was collected and fresh buffer solution of pH 7.4 was added and centrifuged again for 30 minutes. This process repeated for three times and all collected sample was analyzed under UV-spectrophotometer at wavelength of 210 nm. The percent drug entrapped was calculated by using following formula.

$$\%\ \text{Entrapment efficiency} = \frac{\text{Total amount of kojic acid} - \text{amount of free kojic acid}}{\text{Total amount of kojic acid}} \times 100$$

Transmission electron microscopy (TEM)

Niosomal suspension was analyzed for its morphology by TEM. Sample was placed on copper grid that is coated with carbon and kept it for one minute. A drop 2% solution of phosphotungstic acid was added onto it and a tip of filter paper was used to remove excess of it. The sample was kept to be air dried and analyzed at 60 KV using TEM.

Organoleptic characterization of niosomal and control gel

Organoleptic parameters were analyzed in terms of color, odour, feel and look.

pH measurements

pH meter was used to measure pH values of niosomal gel and control gel for the period of three months. All the measurements were taken in triplicate.

Conductivity measurements

Conductivity of the niosomal gel and control gel were measured to assess the stability using conductivity meter at 25°C. All the measurements were performed in triplicate at time interval.

Viscosity studies

Viscosities of niosomal gel and control gel were measured for analyzing the system stability at 25°C. All the measurements were performed in triplicates on Rheometer. Analyses were done for each sample, and results were obtained as average ± standard deviation.

Ex-vivo permeation studies

Ex-vivo permeation studies were performed on Franz diffusion cell using albino rat skin of abdomen. The skin was washed, fat tissue were removed carefully and cut into pieces. The skin was mounted on the Franz cell that was filled with phosphate buffer of pH 5.5 such that the stratum corneum faced the donor compartment. The temperature of bath was maintained at 37°C. Measured quantity of niosomal gel and control gel was placed on the donor compartment. Samples were taken at different time interval and replaced with phosphate buffer pH 5.5 for the period of 24 hours. The samples were analyzed under UV-spectrometer at 210 nm. Same procedure was repeated by using phosphate buffer of pH 7.4.

Statistical analysis

The IBM SPSS statistic version 20 is used for the statistical analysis of the data. Paired sample t-test is applied to check the difference between two formulations. ANOVA was used to describe eventual changes occurred at different time intervals. The difference was considered significant when value of "*p*" is less than 5% or $p<0.05$.

RESULTS

Particle size, zeta potential and encapsulation efficiency

Table 1 depicted the comparative data regarding particle size, zeta potential and encapsulating efficiency. It is evident from the table that the particle size was in the range of 368.1 nm to 663.5nm.The polydispersity index of formulation was less than 0.5.

The percent drug entrapment of hydrophilic NAG is due to the electrostatic attraction of oppositely charged niosomes and NAG. The EE of Span 60 and Tween 60 in a ratio of 2:1 were greater as compared to 1:1. 61% EE was observed for the optimum formulation of niosomes as indicated by code NA5.

Transmission Electron microscopy

It was summarized from the TEM studies that round spherical shaped vesicles were formed. Fig. 1 revealed the shape.

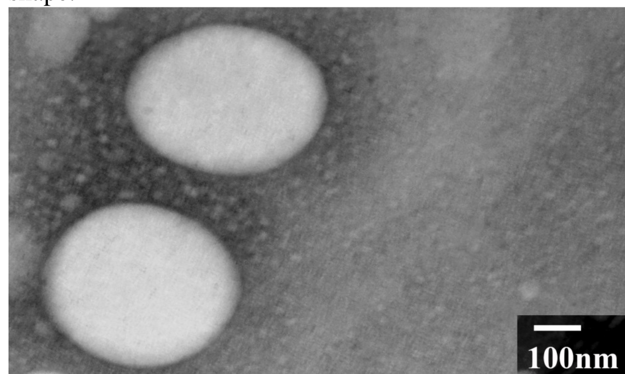

Fig. 1: Transmission electron microscopy (TEM) micrographs of NA5 niosomes.

Physicochemical characterization

There was no change in the color of niosomal gel for the period of three months at temperatures of 8 °C and 25 °C. And it became light yellow at temperatures 40°C and 40 °C ±75% RH at the end of second month and third month. There was no production of odour in the gel. There was no change in the smoothness of niosomal gel for the period of three months at temperatures of 8 °C and 25 °C. And it became slightly thin at temperatures 40°C and 40 °C ±75% RH at the end of second month and third month. The various physical characteristics have been tabulated in table 2.

pH, conductivity and viscosity measurements

The optimized formulation was analyzed for pH, conductivity and viscosity. Statistically it was noted that there was no significant difference (p>0.05) in the pH, conductivity and viscosity of formulation for the period of three months when compared with freshly prepared formulation. The data for pH and rheology and conductivity has been presented in tables 3, 4 and 5 respectively.

Ex-vivo **permeation studies**

Different parameters were evaluated during the *ex-vivo* permeation studies as permeability coefficient, flux, percent N-acetylglucosamine permeated, enhancement ratio and drug targeting efficiency that were represented in table 6. The permeation and percent NAG retained in the skin from niosomal gel is greater than the simple drug solution. Total percent N-acetylglucosamine permeated from niosomal gel at pH 5.5 was significantly (p<0.05) more than the percent permeated from the control gel (N-acetylglucosamine simple gel) which were 42.76±0.02% and 23.71±0.02% respectively as it can be seen from figure 2. Flux of N-acetylglucosamine was also greater from the niosomal gel as compared to control gel. The Enhancement ratio (ER) showed that N-acetylglucosamine permeation was increased by ratio of 1.14. The Targeting efficiency (TE) was also calculated which showed that niosomal gel was more efficient in targeting by ratio of 2.68 as compared to control gel. Total percent N-acetylglucosamine permeated from niosomal gel was significantly (p<0.05) less at pH 7.4 as compared to the percent N-acetylglucosamine permeated from control gel. It indicates that less amount of N-acetylglucosamine was penetrated into systemic circulation which will show side effects and it was deposited more in the skin.

DISCUSSION

Niosomes are a landmark in novel drug delivery systems because of their encapsulating potential for drugs, and other chemotherapeutic agents as well as to overcome the instability, insolubility and rapid degradation of these compounds.

Initial studies (altering surfactant and Cholesterol content while keeping other factors invariant) were carried out to find the effective method of preparation and it was evident that conventional thin film hydration was easy and produced formulation with optimum characteristics.

The polydispersity index of formulation indicated the homogeneous dispersion and narrow size distribution. It

Table 1: Particle size, zeta potential and entrapment efficiency of various niosomes formulations

Sr. No.	Formulation code	S60:T60 (μM)	Surfactant:CHOL (μM)	Size	PDI	Zeta potential	%EE
1.	NA1	1:1	7:3	368.1	0.251	-32.2	54.06
2.	NA2	1:1	6:4	430.7	0.346	-36.5	56.76
3.	NA3	1:1	5:5	647.6	0.383	-38.4	56.44
4.	NA4	2:1	7:3	378.3	0.150	-24.0	55.49
5.	NA5	2:1	6:4	445.8	0.192	-25.2	61.03

was noted that with the increase of hydrophilic surfactant, the particle size reduced as surfactants of high HLB produced small size niosomes (Muzzalupo and Tavano, 2015). Particle size and surface charge of vesicles exert significant influences on stability, release behavior and biodisposition of niosomes. The particle size and EE were in agreement to other researchers who prepared niosomes of various drugs for better stability and bioavailability (Huang *et al*, 2013 and Gelareh *et al*, 2015).

The percent drug entrapment of hydrophilic NAG is due to the electrostatic attraction of oppositely charged niosomes and NAG. The entrapment efficiency of large size niosomes is greater as compared to small size vesicles (Shatalebi *et al*., 2010).

It is reported that the entrapment efficiencies of niosomes were improved when combination of Span 60 and Tween 60 were used as compared to the entrapment efficiencies of niosomes when solely Span 60 or Tween 60 was used. This behavior can be explained on the basis of formation of more number of hydrogen bonds of hydrophilic drug molecule with the hydrophilic head of the Tween 60. Secondly the membrane formed from the Span 60 and Tween 60 have the suitable hydrophobic and hydrophilic characteristics to encapsulate the solubilized hydrophilic drug. The EE depends upon the hydrophobicity of the membrane (Basiri *et al*., 2017; Junyaprasert *et al*., 2012). TEM was performed to analyze vesicle morphology. The observations revealed that the selected niosomes were of uniform size and almost spherical shape.

Table 2: Physical characterization of simple gel (S) and niosomal gel(N) of N-acetylglucosamine at 8°C, 25°C, 40°C and 40°C ± 75%RH kept for the period of three months

Observed parameters	Temp.	Fresh		After 12h		After 24h		After 48h		After 72h		After 7d		After 21d		After 30d		After 60d		After 90d	
		S	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N
Colour	8°C	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	LY	W	LY	W
	25°C	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	LY	W	LY	W
	40°C	W	W	W	W	W	W	W	W	W	W	W	W	LY	W	Y	W	Y	LY	Y	LY
	40°C ± 75%RH	W	W	W	W	W	W	W	W	W	W	W	W	LY	W	Y	W	Y	LY	Y	LY
Odour	8°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40°C ± 75%RH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Feel	8°C	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	sT_h	S_m	sT_h	S_m	sT_h	S_m
	25°C	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	St_K	S_m	St_K	S_m	sT_k	S_m	T_k	S_m
	40°C	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	St_K	S_m	St_K	S_m	T_k	sT_h	T_k	sT_h
	40°C ± 75%RH	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	St_K	S_m	St_K	S_m	T_k	sT_h	T_k	sT_h
Look	8°C	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M
	25°C	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M
	40°C	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M
	40°C ± 75%RH	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M	T	M

W= white, LY= Light yellow, Y= yellow, S_m= smooth, sT_h= slightly thin, T_h= thin, sT_k= slightly thik, T_k= thick, T= transparent, M= milky

Table 3: pH values of niosomes containing N-acetyl glucosamine at different temperatures

Time (days)	8°C	25°C	40°C	40°C±75%
0	6.41	6.41	6.41	6.41
1	6.40	6.40	6.41	6.40
2	6.40	6.40	6.40	6.40
3	6.39	6.39	6.39	6.39
7	6.39	6.38	6.38	6.39
14	6.38	6.39	6.38	6.38
21	6.38	6.38	6.38	6.38
30	6.38	6.38	6.37	6.37
60	6.38	6.38	6.37	6.37
90	6.38	6.38	6.37	6.37

Table 4: Viscosities (cP) of niosomal gel of N-acetyl glucosamine 8°C, 25°C, 40°C and 40°C ±75% at share rate of 60 rpm

Sr. No.	Temperature	Fresh Viscosity	1 day viscosity	14 day Viscosity	30 day Viscosity	2 months Viscosity	3 months Viscosity
1.	8°C	257.88	253.60	249.32	247.05	246.77	246.57
2.	25°C	257.88	251.63	247.35	246.07	245.49	245.29
3.	40°C	257.88	248.05	240.88	240.49	239.91	239.71
4.	40±75°C	257.88	246.72	245.86	245.47	243.89	243.36

Table 5: Conductivity (mS) of niosomal gel containing N-acetyl glucosamine at different temperatures

Time (days)	8°C	25°C	40°C	40°C±75%
0	1.927	1.927	1.927	1.927
1	1.932	1.928	1.928	1.929
2	1.937	1.936	1.938	1.939
3	1.942	1.943	1.944	1.943
7	1.945	1.945	1.948	1.949
14	1.948	1.948	1.950	1.951
21	1.950	1.950	1.951	1.951
30	1.951	1.951	1.951	1.952
60	1.951	1.952	1.952	1.952
90	1.952	1.952	1.952	1.953

Table 6: Permeability coefficient, retention and flux results of niosomes

Formulation	pH 5.5			ER	TE
	Flux (μg/cm².hr)	Papp (cm/hr)	% Drug permeated		
NA niosomal gel	23.12117	4.951×10^{-3}	42.76±0.02%	1.85	2.68
NA control gel	20.22961	1.997×10^{-3}	33.71±0.02%		

Physical characteristics suggested that the optimized formulation was stable in respect of the pH and rheological behaviors.

TEM analysis has demonstrated the presence of individual NA5 niosomes in spherical shape. The image in fig. 1 confirmed the niosome formation. TEM results were also compatible with particle size measurements.

Similar results were seen in another study where the niosomal gel has shown more permeability as compared to control gel (Priprem *et al.*, 2016; Shatalebi *et al.*, 2010). The percent N-acetylglucosamine retained in the skin from niosomal gel was greater as compared to control gel. This difference was observed due to the size of the niosomal N-acetylglucosamine and properties of surfactant involved for the formulation of the niosomes (Budhiraja and Dhingra, 2015). In another study the permeation and percent NAG retained in the skin from niosomal gel is greater than the simple drug solution (Guo *et al.*, 2015). sphere-shaped and nonporous niosomes were formulated form combination of Span 60 and Tween 60 with good percent drug entrapment (Kumbhar *et al.*, 2013). A stable niosomal gel of N-acetylglucosamine was formulated and there was insignificant (p>0.05) difference in pH, conductivity and viscosity were observed. Permeation studies showed that the niosomal gel was good quality can retain drug in the skin and more N-acetylglucosamine was permeated in the skin.

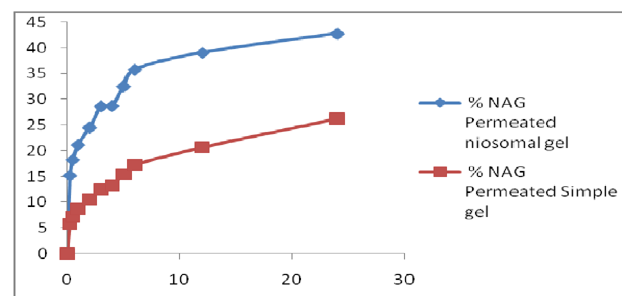

Fig. 2: Permeation of NAG from niosomal gel and control gel

CONCLUSION

This study contributes to the effect of various parameters on niosomal formulation of NAG. The optimized niosomes showed adequate %EE and acceptable stability.

Ex vivo release studies revealed sustained delivery of NAG. This study also indicated that niosomes can be tailored to achieve desired properties using different surfactant, cholesterol ratio. As concluding words, this system was found to be a good drug carrier candidate for NAG delivery.

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