

IN VITRO PERMEATION STUDIES ON CLONAZEPAM FROM PRNIOSOMES BASED NIOSOMES FOR TRANSDERMAL DELIVERY

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ABSTRACT

Proniosomal gel of clonazepam (CNZ) for transdermal delivery to provide controlled drug release was prepared by phase coacervation method using non ionic surfactants, cholesterol and egg lecithin. The formulations were investigated for surface morphology by scanning electron microscopy (SEM), particle size, entrapment efficiency, *in-vitro* drug release and *in-vitro* drug permeation. SEM enabled visualization of proniosomes of clonazepam and the vesicle size was found to be in the range of 1492.32 - 9865.68 nm with entrapment efficiency as high as 72.9%. Amongst the ten formulations designed, F2 and F6 were selected as the optimized formulations and incorporation of egg lecithin increased the entrapment efficiency of the selected formulations to more than 90%. The fabricated matrix transdermal patch exhibited more than 70% drug release in 24 hours.

Key words: *Clonazepam; proniosomes; transdermal delivery; in vitro permeation.*

INTRODUCTION

Liposomes or niosomes in dispersions can carry hydrophilic drugs by encapsulation or hydrophobic drugs by partition of these drugs into hydrophobic domains. However, there remains significant problem in the general application of liposomes for drug delivery. In a dispersed aqueous system, liposomes have problems associated with degradation by hydrolysis or oxidation and sedimentation, aggregation, or fusion of liposomes during storage.¹ Other problems include difficulties in sterilization and large scale production. One alternative involves formation of liposomes like vesicles from hydrated mixtures of cholesterol and nonionic surfactant such as monoalkyl or dialkyl polyoxethylene.^{2,3} These niosomes can entrap solutes, are quite stable and require no special conditions, such as low temperature for production and storage. Preliminary studies indicate that niosomes behave *in-vivo* like liposomes, prolonging the circulation of entrapped drug to alter its organ distribution and metabolic stability and to prolong the contact time of drug with the applied tissue in topical application.⁴ Proniosomes exhibit superior stability and are free from other limitations of niosomes and liposomes like fusion and drug leakage. Proniosomes seem to convert into niosomes *in situ* by absorbing water from the skin.⁵ Proniosomes form niosomes following topical application under occlusive conditions, due to hydration by water from skin itself.⁶ The aim of this study was to investigate the feasibility of using proniosomes as a transdermal drug delivery system for clonazepam, an anticonvulsant benzodiazepine widely used in the treatment of epilepsy, particularly in children.⁷ The biological properties of clonazepam, such as high first

pass metabolism, wide blood level fluctuations, side effects, low dose size, need for long-term treatment⁸ and repetitive dosing, make this drug an interesting candidate for transdermal administration.

EXPERIMENTAL

Materials and Methods

Clonazepam was procured from Esteem Pharmaceuticals, Agra, India as gift sample. Span 60, 20 and Tween 80, 20 were purchased from Qualigens fine chemicals, Mumbai, India. Egg lecithin and Himedia membrane 150 (MW cut off) were procured from (Himedia Laboratories Pvt. Ltd., Mumbai, India. Carbopol 934 was procured from Central Drug House, New Delhi.

Preparation of proniosomes

Proniosomes were prepared using various nonionic surfactants span 60, span 20, tween 20 and tween 80, cholesterol and egg lecithin. The composition of formulations is given in Table 1. Cholesterol, surfactant and drug were taken in dry wide glass tube, to this 95% ethanol (2ml) was added. Open end of the glass tube was covered with a lid to prevent loss of solvent and heated at 60 °C on water bath, till clear solution was formed. To this clear solution aqueous phase 0.5 ml of 0.1%v/v glycerol was added and warmed on water bath till clear solution is formed which on cooling converts into proniosomal gel. Formulations F11 and F12 were prepared in a similar manner mentioned above with incorporation of egg lecithin (2% by wt.), for enhancement of entrapment efficiency. Clonazepam dispersed in carbopol 934 gel (2% w/w pH 7.4) was used as control.

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Table 1. Composition of proniosomes of clonazepam

Formulation	INGREDIENTS (% by wt.)						
	Group 1		Group 2				
	Span 60	Tween 80	Span 20	Tween 20	Cholesterol (mg)	CNZ (mg)	Egg lecithin
F1	100	00	-	-	100	2	-
F2	75	25	-	-	100	2	-
F3	50	50	-	-	100	2	-
F4	25	75	-	-	100	2	-
F5	00	100	-	-	100	2	-
F6	-	-	00	100	100	2	-
F7	-	-	25	75	100	2	-
F8	-	-	50	50	100	2	-
F9	-	-	75	25	100	2	-
F10	-	-	100	00	100	2	-
F11	75	25	-	-	100	2	2
F12	-	-	00	100	100	2	2

Fabrication of transdermal patch

The circular aluminum foil of diameter 3.0 cm was used as backing membrane. On this backing membrane a plastic sheet of same size with 1.0mm thickness was stuck with adhesive. Additional plastic sheet was centrally cut (diameter 2 cm, corresponding to 2 cm² area) and stuck with adhesive. The proniosomal gel was evenly spread over this area and covered with the fine nylon mesh.

Polarized microscopy

A thin layer of proniosomal gel was spread in a cavity slide and after placing the cover slip observed under microscope with polarized light. A drop of water was added through the side of the cover slip while under microscope and again observed. Photomicrographs were taken at suitable magnifications before and after addition of water using Nikon HFX-DX, Labophot Microscope, (Germany).

Vesicle size determination

Vesicle size was determined by coulter submicron particle size analyzer, Malvern Mastersizer (Model N4MD, Coulter, UK). The proniosomal gel in a glass test tube was diluted with 10 ml water to determine vesicle size. 3 ml of diluted suspension was taken into disposable sizing glass cuvette at 25°C for a period of 20 seconds.

Scanning electron microscopy (SEM)

The surface morphology of selected formulations were examined by scanning electron microscope (Joel, JSM-840A, Tokyo, Japan). The samples were previously sputter coated with Au/Pd under an argon atmosphere at 180 mA for 1 minute. Electron micrographs were obtained using a field-emission SEM operating at 1 or 2 kV.

Entrapment efficiency⁹

The fraction of drug associated with the niosomes was measured using centrifugation assay. The niosomes were prepared using proniosomes gel by addition of hot buffer IPBS pH 7.4 (PEG 400 25% v/v) and the suspension was centrifuged in Remi Centrifuge (Jindal Scientific Industries, India) at 12000 rpm using polypropylene micro centrifuge tubes for 15 minutes at room temperature to produce soft pellet. The supernatant for each niosomes preparation was further

diluted with buffer and centrifuged again to ensure complete removal of niosomes. Drug concentration was measured in the supernatant and was used directly as aqueous phase concentration. Control samples containing same amount of drug as used for niosomal preparation was suspended in carbopol gel (2%w/w pH 7.4), diluted with IPBS (PEG 400 25% v/v) and subjected to centrifugation at 12000 rpm and analyzed. Control samples without niosomes provided the total drug concentration and the difference (control-aqueous) gives the amount entrapped into niosomes. The supernatants were analyzed for drug concentration at 261 nm.

In vitro diffusion and permeation studies

In vitro drug diffusion and permeation studies were carried out in Franz diffusion cell having surface area of 2 cm² and receptor cell volume of 22 ml of IPBS (PEG 400, 25% v/v) as medium at 37 °C for a period of 24 hours. Samples equivalent to 1mg of CNZ were used in each study and were placed over Himedia membrane 150 in the donor compartment, and rat skin was used instead of membrane in case of permeation studies of optimized formulations. A 3 ml of aliquot from receptor compartment was withdrawn at different time intervals, and replaced with 3 ml of fresh medium. The samples were suitably diluted if necessary and assayed for CNZ by measuring absorbance at 261 nm. The diffusion studies were conducted in triplicate.

Evaluation of Transdermal Patch

In vitro permeation studies

In vitro permeation study of matrix type transdermal patch was done using rat skin as barrier membrane in Franz diffusion cell at 37 °C for 24 hours using 22 ml of IPBS (PEG 400 25% v/v).

Skin irritation studies

Skin irritation studies were done on rabbits. Desired patch of 2 cm² was applied on the clean hairless dorsal side of rabbit (n=3) and observed for any sign of irritation (erythema and edema) after 2,12,24,48 and 72 hours.

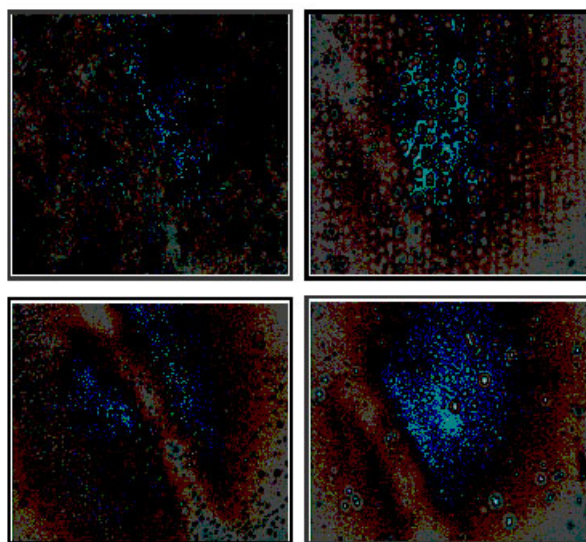
RESULTS AND DISCUSSION

Surface morphology and physical analysis

The mean vesicle size of niosomes formed from clonazepam proniosomes formulation is presented in Table 2. The differences in vesicle size among the niosomes prepared with different ratios of span and tween were significantly different (P<0.05). Niosomes prepared with 100% span60, tween80, tween 20 and span 20 resulted in F1, F5, F6 and F10 respectively that were smaller in size than those prepared with mixture of span and tween in different ratio. Maximum vesicle size was observed when span tween ratio was 1:1 (F8 and F3). Incorporating lecithin formed vesicles of smaller size, because of increase in the hydrophobicity that results in reduction of vesicle size.⁹

Table 2. Physicochemical characterization of proniosomes based niosomes of clonazepam

Proniosome Code	Vesicle Size (nm)	Polydispersity Index	Entrapment Efficiency (%)
F1	1568.58	0.11	72.2
F2	1569.96	0.29	75.2
F3	9865.68	0.15	72.9
F4	3656.98	0.31	71.4
F5	1576.78	0.18	71.5
F6	1791.59	0.17	75.3
F7	6342.90	0.13	70.6
F8	8293.55	0.21	60.2
F9	6254.67	0.18	60.2
F10	1492.32	0.14	59.2
F11	1091.52	0.12	92.4
F12	1049.31	0.10	95.7

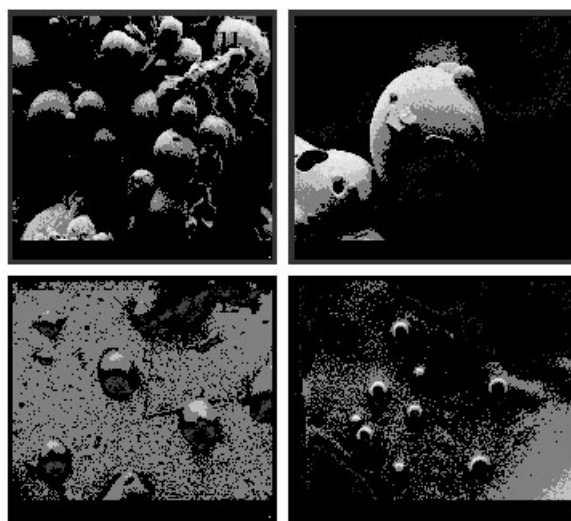
**Fig. 1:** Photomicrograph of proniosomes (F1) under cross polarized microscope at 40x, a: nonhydrated, b: Hydrated at zero time, c: upon hydration after 5 min, d: upon hydration for 10 min**Polarized Microscopy**

Niosomes formed from proniosomes were spherical in shape (Fig.1).The transformation of lamellar liquid crystalline proniosomes to niosomes may be explained by different degree of hydration of span and phospholipids molecules and simultaneously by change in shape of the hydrated molecules characterized by their packing parameter. Addition of water leads to swelling of bilayers as well as vesicles due to interaction of water with polar groups of the surfactant.¹⁰ Above a limiting concentration of solvent, the bilayers tend to form spherical structures. When shaken with water i.e. with excess aqueous phase, complete hydration takes place leading to the formation of niosomes. Formation of niosomes from proniosomes was observed in polarized microscopy which is because of the transformation of lamellar liquid crystalline proniosomes to niosomes. Addition of water leads to swelling of bilayers as well as vesicles due to interaction of water with polar groups of the surfactant. Above a limiting concentration of solvent, the bilayers

tend to form spherical structures. When shaken with water i.e. with excess aqueous phase, complete hydration takes place leading to the formation of niosomes.¹¹

Enhancement in entrapment efficiency was observed due to formation of well organized and compact membrane which prevents leakage of drug and makes the system more stable¹² and highly lipophilic portion of drug is expected to be housed almost completely within the lipid bilayers of niosomes.

The scanning electron microscopy of niosomes prepared from selected proniosomal (F11 and F12) formulation is shown in Fig. 2 Most of the vesicles were spherical and discrete with sharp boundaries and hollow in nature.

**Fig. 2:** Scanning electron micrograph images of F11, F12**Entrapment efficiency**

Proniosomes made with span and tween exhibited 60 – 72% entrapment efficiency as shown in Table 1. Highly lipophilic portion of drug is expected to be housed almost completely within the lipid bilayers of niosomes. Freely soluble nonionic surfactants like tween can form micelles on hydration in presence of cholesterol, so as to entrap the drug easily. Incorporation of egg lecithin to the formulations F2 and F6 resulted in an increase in entrapment efficiency to 95.7 %.

In-vitro drug release study of proniosomal gel

The amount of drug permeated through Himedia membrane 150 at different time intervals are reported in Fig. 3. Maximum drug release was observed in case of formulation F6. This could be due to smaller vesicle size which gives an increased surface area for diffusion. The increase in flux, permeation across skin was observed due to smaller vesicle size which gives an increased surface area for diffusion. Higher drug release could also be due to emulsification effect of surfactant after hydration of the proniosomes, by the

dissolution medium and formation of elution channels within the gel structure due to loss of lipid bilayers. Surfactants with higher hydrophilicity like tween decrease stability of niosomes and increase leakage of free drug thus improving transdermal delivery of lipophilic drugs. Lag time in permeation of drug was observed in formulations F2, F3 and F4. This could be due to difference in the ratio of spans and tweens in the formulation which make the bilayers more compact resulting in reduction of free drug, thus preventing leakage of drug.¹³ All the formulations showed higher release than the control formulated with carbopol 934 as the base. All the formulations displayed a drug release that followed Higuchi matrix model suggesting that the drug is uniformly dispersed in a swellable matrix.

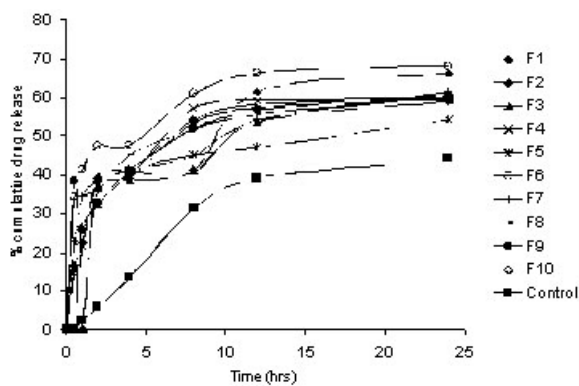


Fig. 3. Comparative cumulative % drug release profile of proniosomal formulations of clonazepam through Himedia membrane 150 using Franz diffusion cell

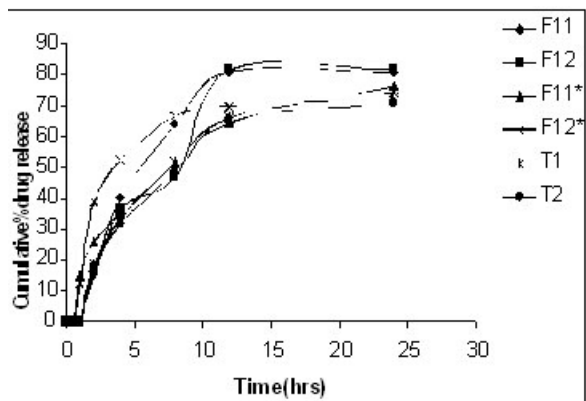


Fig. 4. Comparative cumulative % drug release profile of optimized formulations of clonazepam and transdermal patch Himedia membrane and rat skin(*) using Franz diffusion cell

Selection of the optimized formulation(s)

The formulations F2 (group 1) and F6 (group 2), that showed higher entrapment efficiency and maximum drug release, were selected for incorporation of egg lecithin to obtain F11 and F12. The effect of incorporation was evaluated on entrapment efficiency, *in vitro* drug release and permeation studies through rat skin in order

to assess the suitability for formulation as transdermal patch (T1 and T2) (Fig. 4).

The release of clonazepam across the cellulose membrane is higher than its flux (Table 3) across skin indicating barrier properties of skin for drug. Although interaction between skin and proniosomes may be an important factor for improved transdermal drug delivery. Drug release from both matrix transdermal patch (T1 and T2) was not significantly different. The drug release was 74.26 % and 70.77 % respectively. No significant difference was observed in skin permeation of formulation F11 and F12. The release profile of transdermal patch was similar to F11 and F12 gels with *f*₂ values 61.32 and 66.11 using F11 as reference for T1 and F12 for T2 respectively. It could be due to the reason that there is no rate controlling membrane in T1 and T2 thus allowing free flow of drug through patch. Insignificant decrease in drug permeation across the rat skin could be due to barrier properties of skin. The reasons for better permeability of drug across rat skin may be - a) adsorption and fusion of niosomes onto the surface of skin to facilitate drug permeation, b) the vesicle acts as penetration enhancers to reduce barrier properties of the stratum corneum and c) the lipid bilayers of niosomes acts as a rate limiting membrane for drugs which leads to controlled drug release.

Table 3. Flux of Clonazepam from proniosomes and transdermal patches

Formulation Code	Barrier membrane	Flux ($\mu\text{g}/\text{cm}^2\text{h}^{-1}$)
F11	Himedia membrane 150	18.34 \pm 1.9
F12		14.11 \pm 2.3
F11*	Rat skin	09.31 \pm 3.0
F12*		10.01 \pm 2.7
T1	Rat skin	11.21 \pm 1.2
T2		09.98 \pm 4.1

Skin irritation studies

Skin irritation studies displayed zero score on Draize scale¹⁴. There was no sign of irritation (erythema and edema) on the applied area. This revealed that the matrix transdermal patch is suitable for transdermal use.

Finally it was concluded that proniosomes may be promising carrier for transdermal drug delivery of clonazepam.

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