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Formulation and Evaluation of Niosome Entrapped Pentoxifylline Using *In Vivo* Bronchodilatory Activity in Guinea Pigs

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The objective of this study is to formulate niosomes of pentoxifylline, characterize niosomes in terms of entrapment efficiency, particle size distribution, *in vitro* release and stability and investigate the bronchodilatory activity of plain and niosomal pentoxifylline *in vivo* in guinea pigs. Pentoxifylline was entrapped in niosomes by lipid layer hydration method using Span 60, cholesterol and dicetyl phosphate. The entrapment efficiency of niosomes of pentoxifylline was determined by separating the entrapped drug from the free drug by centrifugation. The *in vitro* release profile of the drug from niosomes was carried out in phosphate buffer saline (pH 7.4). The stability of niosomes was assessed by storage at 4±1°, 25±1°, 37± 1° and 45±1° for one month. The plain (20, 40 and 80 mg/kg) and niosomal pentoxifylline (5, 10, 20 and 40 mg/kg) was injected intraperitoneally to guinea pigs for evaluating bronchodilatory activity. The entrapment efficiency of niosomes of pentoxifylline was found to be 9.26±1.93% giving a sustained release of drug over a period of 24 h and better stability over the period of storage. The plain and niosomal pentoxifylline produced significant bronchodilatory effect in guinea pigs on histamine-induced bronchoconstriction. The study indicates that pentoxifylline may be an effective bronchodilator.

Management of asthma poses a unique challenge to the practicing physician. Part of this challenge is that despite apparently good therapy, the incidence and death rate from asthma are continuously rising[1]. Theophylline has been used in the treatment of asthma for more than 50 years, but has some adverse reactions such as tachycardia and CNS stimulation[2]. Pentoxifylline (PTX), an homologue of theophylline has been reported to be used in US without demonstrated utility as a bronchodilator[3]. PTX has been shown to relax histamine contracted guinea pig tracheal muscle *in vitro*[4]. The tracheal muscle relaxant and atrial beat stimulating effects of PTX were almost similar as those of theophylline. Thus, when PTX and theophylline are used for bronchodilation in asthma, cardiac adverse reactions such as arrthymia may occur[5].

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The half-life of PTX is 0.4-0.8 h with two active metabolites, both with a half-life of 1.0-1.6 h[6]. Due to lack of specificity of delivery and short half-life, the encapsulation of PTX into a proper delivery system such as liposomes or niosomes may be advantageous. The chemical instability, high cost, variable purity of lipids used in liposomes makes niosomes preferable over liposomes. Niosomes, the non-ionic surfactant vesicles, are spherical lipid bilayers which can entrap hydrophilic solutes within the non aqueous domain and hydrophobic solutes within the lipid bilayers. Niosomes are biodegradable, biocompatible, non-immunogenic in nature and exhibit flexibility in their structural characterization[7]. Large multilamellar niosomes are reported to be taken up by the lungs[8,9] which may probably serve our purpose of achieving a selective localization of PTX for the study of bronchodilatory activity. Niosomal formulation of PTX was expected to have a sustained release of the drug,

reduce the adverse effects and increase the specificity of the drug.

In this study, attempts have been made to prepare niosomes of PTX and improve the entrapment and release characteristics of the niosomal drug and study the *in vivo* bronchodilatory activity of plain and niosomal PTX.

MATERIALS AND METHODS

Pentoxifylline was a gift sample obtained from Sun Pharmaceuticals Ltd., Mumbai. Salbutamol was a gift sample obtained from Cipla, Mumbai. Cholesterol was purchased from Loba Chemie, Mumbai, Span 60 was purchased from Koch Laboratories, Mumbai. Dicetyl phosphate, histamine diphosphate were purchased from Sigma, USA. Dialysis sacks, chloroform were purchased from Hi Media, Mumbai.

Preparation of niosomes by lipid layer hydration method:

The lipid layer hydration method was used for preparing PTX niosomes. In brief, different proportions of span 60 (S), cholesterol (CHOL) and dicetyl phosphate (DCP) as mentioned in Table 1 were dissolved in chloroform and the solvent was evaporated using rotary flash evaporator under reduced pressure at 40-50°. The lipid film was hydrated by PTX solution (10 mg/ml) in phosphate buffered saline (PBS) at 40-50°, followed by gentle hand shaking with intermittent vortexing for 20 min to obtain multilamellar vesicles (MLVs) in the case of formulation 1, 2, 3a, 3b and 4 and the flask was shaken on a horizontal shaker bath for 2 h[10] in the case of formulation 3c.

Determination of PTX entrapment in niosomes:

The unentrapped drug was separated from the entrapped drug by centrifugation at 3000 rpm for 1 h. The entrapment was assayed from the free drug in the supernatant on a spectrophotometer (Shimadzu 160A UV) at 274 nm as compared to the initial total amount of drug used. The pellet of niosomes was resuspended in PBS and vortexed. Formulation prepared with sonication (3c) and that without sonication (3a) were used for *in vitro* release studies. Sonicated resuspended niosomes were used for the *in vivo* bronchodilatory study in guinea pigs[8]. Vesicle size of sonicated and non sonicated niosomes was determined using a Malvern mastersizer M-S3.

In vitro release of PTX from niosomes into PBS:

In vitro release profile of formulation 3a and 3c (fig. 1) was determined. The niosomal suspension containing known amount of PTX was transferred to a dialysis tube and subjected to dialysis with the dialysis tube immersed in a receptor medium (50 ml PBS). 5 ml sample was withdrawn from the receptor compartment at different time intervals and same volume was replaced with PBS. The drug content was determined spectrophotometrically at 274 nm and cumulative amount of drug released over a period of 24 h was determined[10].

Stability studies:

Niosome formulation was stored at 4 different temperatures; viz., 4±1°, 25±1°, 37±1° and 45±1° for one month to estimate leakage of PTX from niosomes. At 7 day intervals, these formulations were evaluated for their drug content and mean vesicular diameter on Malvern Autocounter[10].

In vivo studies in Guinea pig:

Male English guinea pigs, (300 - 500 g) obtained form

TABLE 1: ENTRAPMENT EFFICIENCIES AND MEAN GEOMETRIC DIAMETER OF NIOSOMES OF PTX:

Formulation No.	Composition of niosomal formulation (mg:mg:mg)	Per cent entrapment (±S.E.M.)	Size (μm)
1.	S:CHOL (10:10:0)	7.68% (1.85)	4.55
2.	S:CHOL (23.75:23.75:0)	6.12% (1.49)	8.76
3.	S:CHOL:DCP (10:10:2.5)	12.21% (0.73)	7.44
4.	S:CHOL:DCP (23.75:23.75:5)	10.10% (2.19)	11.26

S : Span 60, CHOL : Cholesterol, DCP : Dicetyl phosphate. Entrapment efficiencies and mean geometric diameter of niosomes of PTX of different formulations prepared by hand shaking for 20 min with intermittent vortexing without sonication. Percent entrapment values represents the mean±SEM (n=3)

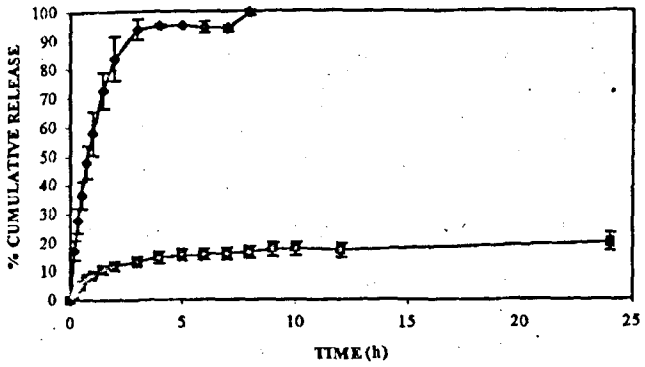

Fig. 1: *In vitro* release profile of PTX
***In vitro* release of PTX from niosomal formulations 3a hydrated for 20 min (-◆-) and 3c hydrated for 2 h and sonicated for 2 min (-□-). Drug release profile from niosomes was assessed in PBS (pH 7.4). Each data point represents mean±S.D. of three individual measurements**

Hindustan Lever Ltd., Mumbai, were used in this study. The histamine aerosol method was adopted[11]. A finely atomized mist of 0.5% solution of histamine diphosphate was blown into the chamber containing guinea pig under 6 psi Hg pressure through the nebuliser for 2 s. The animals were divided into 10 groups (6 animals per group). Two and a half hours after control histamine exposure the animals were injected intraperitoneally with a dose of PTX (20, 40 and 80 mg/kg) or salbutamol (0.25 mg/kg), or empty niosomes, or niosomal PTX (5, 10, 20 and 40 mg/kg). After 30 min of drug administration they were again exposed to histamine and the exposition time was noted. Percent protection afforded by the drug was calculated.

RESULTS

The niosomes of PTX were prepared by the reported lipid layer hydration method[10]. Two composition of S:CHOL with or without DCP were tried. Increasing the concentration of S:CHOL did not show any increase in the percent entrapment of drug, but there was an increase in the particle size. Inclusion of DCP in the formulation showed an increase in the mean vesicle diameter (Table 1).

Hydration of niosomes for 20 min by hand shaking with intermittent vortexing formed niosomes which was a mixture of spherical as well as irregular rod shapes as observed in formulation 3a. Shaking the formulation for 2 h without sonication resulted into uniform spherical MLVs with higher entrapment with no significant change in particle size (formulation 3b). While sonication of formulation for 2 min reduced the mean diameter and entrapment efficiency MLVs as seen in formulation 3c (Table 2).

The *in vitro* release profile for the formulation 3a with 20 min of hand shaking without sonication showed 99.45% release of drug in 8 h while the formulation 3c shaken for 2 h with sonication showed 19.79% release in 24 h (fig. 1). Stability studies were carried out with the potential formulation 3c. Niosomes were observed under microscope and some aggregates of niosomes were seen. Niosomes stored at different temperatures hardly showed any change in the percent entrapment and higher drug retention at all sampling point (Table 3).

In vivo Bronchodilatory study in Guinea Pigs:

The empty niosomes did not give any protection against histamine-induced bronchospasm. The percent protection offered by niosomal PTX is compared with that of the plain PTX and salbutamol (Table 4). It was observed that plain PTX (20 and 40 mg/kg) did not show any significant protection but plain PTX (80 mg/kg) did show significant protection against histamine-induced

TABLE 2: ENTRAPMENT EFFICIENCY AND MEAN GEOMETRIC DIAMETER OF NIOSOMES OF PTX

Formulation No. 3	Hydration time	Per cent entrapment (±S.E.M.)	Size (μm)
a.	20 min	12.21% (0.73)	7.44
b.	2 h	13.14% (1.68)	6.53
c.	2 h with 2 min sonication	9.26% (1.93)	4.96

Effect of different hydration time on the entrapment efficiencies and geometric mean diameter of niosomes of PTX of formulation 3 (Span 60:Cholesterol:Dicetyl phosphate; 10 mg:10 mg:2.5 mg) Per cent entrapment values represents the mean±SEM (n=3)

TABLE 3: EFFECT OF STORAGE CONDITIONS ON STABILITY OF NIOSOMES OF PTX

Temperature	Day 0		Day 7		Day 14		Day 21		Day 28	
	% E	MVD (μm)	% E	MVD (μm)	% E	MVD (μm)	% E	MVD (μm)	% E	MVD (μm)
4±1°	8	4.5	7.65	4.5	7.58	6.0	7.52	6.0	7.51	6.0
25±1°	8	4.5	7.6	6.0	7.5	6.0	7.46	6.0	7.44	6.0
37±1°	8	4.5	7.55	6.0	7.47	6.0	7.46	6.0	7.15	6.0
45±1°	8	4.5	7.51	6.0	7.42	6.0	7.41	6.0	7.02	6.0

Percent entrapment, MVD : Mean Vesicular Diameter. Effect of different storage temperatures on the percent entrapment (%E) and mean geometric diameter of niosomes of formulation 3c at different intervals of stability period

bronchospasm. Niosomal PTX (5 mg/kg) did not show significant protection but niosomal PTX (10, 20 and 40 mg/kg) gave significant protection. The percent protection offered by plain PTX (80 mg/kg) and niosomal PTX (20 and 40 mg/kg) were comparable to the standard drug used salbutamol. The maximum effect for niosomes was reached at 20 mg/kg with no further increase in the percent protection offered (fig. 2).

TABLE 4: EFFECT OF PLAIN AND NIOSOMAL PTX (FORMULATION 3C) ON HISTAMINE - INDUCED BRONCHOCONSTRICTION IN GUINEA PIGS

Drug	Dose (mg/kg, i.p.)	Percent Protection
Plain PTX	20	2.79 (±4.43)
Plain PTX	40	50 (±22.63)*
Plain PTX	80	76.53 (±22.27)*
Empty niosomes	—	Nil
Niosomal PTX	5	29.61 (±9.88)
Niosomal PTX	10	60.94 (±22.09)*
Niosomal PTX	20	79.38 (±16.07)*
Niosomal PTX	40	79.71 (±13.83)*
Salbutamol	0.25	85.0 (±19.92)*

Values represent the mean±SEM. (n=6) of percent protection offered by different treatments on histamine-induced bronchospasm. * indicates significant difference between pretreatment and post treatment time using Student's t-test for paired data ≤0.05.

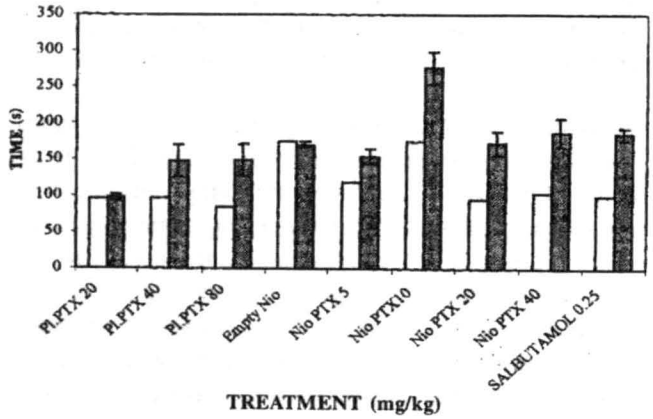

Fig. 2: Effect of various doses of plain and niosomal PTX (formulation 3c) on the pretreatment exposition time of histamine-induced bronchospasm. Each values represent the mean±SEM (n=6). *P<0.05 significant difference between pretreatment and post treatment time by Student's t-test for paired data. (□) pretreatment exposition time (■) post treatment exposition time

DISCUSSION

The lower drug entrapment may be due to the hydrophilic nature of drug. With increase in quantity of lipid, more number of niosomes per ml of the niosomal dispersion are reported to be formed, resulting in an increased per cent entrapment. Further increase in the S:CHOL concentration in formulation 2 than in formulation 1 did not have a proportionate increase in the per cent entrapment which may be because of the system saturation[12]. Large MLVs are taken up by the lungs[8,9]. DCP imparts negative charge on the membrane of the niosomes

thereby increasing their size[7,14]. This was observed in formulation 3. Formulation 3a was prepared by hand shaking for 20 min with intermittent vortexing. The niosomes formed were not uniform in shape. The *in vitro* release profile showed complete release of drug within 8 h probably due to the ununiform hydration of film by hand shaking for a shorter period of time. Increasing the hydration time for formulation 3b by shaking the formulation on a shaker for 2 h gave uniform, spherical, MLVs with increase in vesicle diameter.

Since pulmonary drug delivery requires a niosomal size below 5 μm, sonication seem to be a crucial parameter especially in pulmonary drug delivery. Hence, the formulation 3c was sonicated for 2 min. Sonication causes breakage of larger niosomes to smaller ones and in doing, so, leakage of small quantities of drug takes place leading to a decreased percent entrapment[12]. Also smaller niosomes do not remain in the lungs and pass into the systemic circulation and so further size reduction of niosomes was not carried out.

The *in vitro* release profile showed that formulation 3c gave a sustained release of PTX from the niosomes may be due to a firm film formation. Therefore formulation 3c was used for *in vivo* studies. Niosomes stored at different temperatures hardly showed any change in the percent entrapment. The loss of entrapped drug during storage may be due to leakage of the drug during the fusion of the niosomes and diffusion of the drug across the bilayer due to residual hydrating medium. At all sampling points in the stability studies higher drug retention was observed. This enhanced stability can be attributed to the stabilizing effect of cholesterol on the niosomal membrane reducing the permeability and improving the retention of solute[12]. The mean particle diameter did not increase significantly. This suggests that presence of sufficient cholesterol in the membrane reduces the elastic stress[12].

This study was aimed at preparation of niosomes of PTX and evaluating the formulation *in vivo* in the guinea pigs for the bronchodilatory activity. Histamine when inhaled, has been shown to induce bronchoconstriction by direct H_1 receptor activation and also by a neurally mediated bronchoconstrictor effect via vagal reflexes. Histamine has shown to activate action potentials in the intrapulmonary vagal afferents. PTX was found to significantly inhibit the histamine induced bronchospasm in a dose dependent manner, it could mean that the drug could have an H_1 blocking effect or a direct bronchodilator effect[11]. *In vitro* studies with PTX have shown to produce guinea pig tracheal muscle relaxation upon histamine contracted tissue and increased in cAMP of smooth muscle[4]. The precise mechanism underlying xanthine-induced relaxation of smooth muscle, notably bronchial muscle is not clearly understood. Various studies have been performed to explain the proposed mechanisms for the bronchodilatory activity such as inhibition of cyclic phosphodiesterase isoenzymes resulting in increased accumulation of cAMP. However, inhibition of phosphodiesterase is no longer viewed as the main molecular mechanism to explain the bronchodilatory activity of xanthines. Other proposed mechanisms include prostaglandin antagonism, alterations in intracellular calcium concentrations, effects on IgE-mediated immediate reactions, the late-phase response, release of catecholamine from the adrenal medulla and antagonism of endogenous adenosine[13]. Pulmonary effects of some xanthines, exhibiting a range of potencies as cyclic nucleotide phosphodiesterase inhibitors and adenosine antagonists, on investigation showed that the bronchodilatory effect may occur through multiple molecular mechanisms of actions including one or more unknown mechanism[5].

Studies have also shown that PTX has a lower bronchoselectivity[2]. In this study niosomal PTX (20 and 40 mg/kg) gave a per cent protection against histamine-induced bronchoconstriction which was comparable to plain PTX (80 mg/kg) and standard drug (salbutamol). This may occur due to the targeting of niosomal drug into the lungs leading to bronchoselectivity thereby reducing the dose of the drug required to produce the same effect. The effect produced by niosomal PTX 20 mg/kg was similar to niosomal PTX (40 mg/kg) which may indicate that the ceiling effect was achieved at a niosomal dose of 20 mg/kg.

In conclusion, the study indicates that niosomal entrapment of PTX can markedly prolong the release of PTX under *in vitro* conditions and showed improved *in vivo* bronchodilatory results. Therefore, this approach could be used as a means to control the duration of drug action and the release rate of a promising candidate.

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