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Formulation and Evaluation of *In Vitro* Blood-Brain Barrier Penetration of Emtricitabine Niosomes Using Immobilized Artificial Membrane Phosphatidyl Choline Column Chromatography

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ABSTRACT

The main objective of the study is to formulate emtricitabine, a nucleoside reverse transcriptase inhibitor used in the treatment of Human Immuno deficiency Virus (HIV) infections as a niosomal formulation to improve the Central Nervous System (CNS) penetration of the drug and evaluate its CNS penetration using *in vitro* blood-brain barrier penetration study using immobilized artificial membrane phosphatidylcholine column chromatography. Emtricitabine encapsulated niosomes were prepared by thin layer evaporation (TLE)-paddle stirring method with Span 60 as main surfactant. Cholesterol (CHL), Solulan C24 (SOL) and N-palmitoyl glucosamine (NPG) were also included in the niosomal formulations. The ratio of Span 60:CHL:SOL:NPG was 50:40:10:10 with total concentration of components as 38 mM. The hydration temperature was maintained at 65 ^{0}C. Sonication method was employed for size reduction of the niosomes. The formulation was evaluated for CNS penetration by *in vitro* blood-brain barrier penetration study using immobilized artificial membrane phosphatidylcholine column chromatography. The Scanning electron microscopic images showed good formation of the niosomal vesicles. The mean particle size and encapsulation efficiency were found to be 154±4 nm and 64.45±1.14% respectively. *In vitro* blood-brain barrier (BBB) penetration of emtricitabine from drug loaded NPG niosomes using immobilized artificial membrane phosphatidylcholine column chromatography showed an improved CNS penetration of the drug with (k_{IAM}/MW^4) X 10^{10} values of 2.79±0.05 at pH 5.5 and 8.48±0.18 at pH 7.0. The results showed an increased CNS penetration when the drug was encapsulated in niosomes and may be considered as a potential alternative to improve brain targeting of emtricitabine and thus minimize HIV Associated Neurocognitive Disorders (HAND).

Keywords: Niosomes, Emtricitabine, CNS penetration, Brain targeted drug delivery.

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INTRODUCTION

The novel approaches for drug targeting to a particular organ or site or tissue has evolved as a great field of interest as it offers the advantage of targeting the drug to a particular organ or site or tissue, thereby localizing the drug at the required site and thus reducing the systemic exposure of the drug to other sites.

Brain targeted drug delivery has been explored vastly as it poses a great challenge for targeting the drugs to the CNS due to its highly protective barrier, the blood brain barrier (BBB). The tightly packed BBB acts as a rate limiting barrier in the penetration of many drug molecules to the CNS. Thus, exploration of the various approaches in efficient targeting of such drugs to the brain where the effective penetration of the drugs through the BBB plays a vital role as in the treatment of tumours of brain, diseases of brain and neurocognitive disorders offers a great scope in pharmaceutical research.

The various approaches explored like the molecular approaches (lipidization, prodrugs, transport processes), BBB opening (physical approaches like ultrasound and microbubbles, convection enhanced delivery, electromagnetic radiation and chemical approaches), alternative route of drug delivery (intraventricular, intracerebral and intrathecal, intranasal), novel drug delivery systems (polymeric nanoparticles, micelles, solid lipid nanoparticles, liposomes, nanogels, dentrimers, miscellaneous) have their own limitations of being invasive, having incidences of haemorrhage, cerebrospinal fluid leak, neurotoxicity and central nervous system infection [1]. Among these approaches targeted drug delivery using niosomes has evolved as an important field of interest.

Niosomes or non-ionic surfactant vesicles or surfactant membrane vesicles are osmotically active and stable unilamellar or multilamellar vesicles wherein an aqueous solution is enclosed in highly ordered bilayer made up of non-ionic surfactant with or without cholesterol and dicetyl phosphate. They are vesicular systems similar to liposomes that can be used as carriers of amphiphilic and lipophilic drugs [2]. Niosomes offer the advantages of having good stability, minimized toxicity, and minimized side effects and are also economically feasible with wider formulation versatility. The surfactants used for forming niosomes are biodegradable, non-immunogenic and biocompatible. These vesicles improve the therapeutic efficacy of drugs by reducing the clearance rate, targeting to the specific site and by protecting the encapsulated drug. Drug targeting reduces the dose which leads to subsequent decrease in side effects [3, 4].

Functionalized niosomes with glucose analogues have been suggested as a tool to deliver drugs to the brain, considering the high level of cerebral glucose uptake. In this regard, the derivatized

surfactant N-palmitoyl glucosamine (NPG) has been successfully explored for targeting of drugs to brain. Although the exact mechanism of action of NPG-bearing niosomes has yet to be clarified, recognition of glucosamine exposed on the surface of the vesicles by the glucose transporter GLUT-1, highly expressed on the BBB cells, has been hypothesized [5].

Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI) used for the treatment of HIV infection in adults and children. The CSF of HIV patients acts as a distinct virologic compartment for HIV-1 virus. In early stages, CSF and peripheral virus are similar but in later stages, CSF virus is phylogenetically distinct from virus in the periphery. Antiretroviral therapeutic concentrations in CSF varied but were always lower than in plasma. Persistent HIV replication with sub-therapeutic concentrations of Anti Retro Viral (ARV) drugs in the nervous system may subject the patients to the risk for HIV Associated Neurocognitive Disorders (HAND). ARV treatment regimens that can control CSF HIV replication may help to prevent HAND. Thus, penetration of drugs to the CNS and thereby increasing their concentration in the CSF plays a vital role in the effective treatment of Anti-Retroviral Therapy (ART) of HIV patients.

Emtricitabine is classified as a drug with intermediate CNS penetration with a CNS Penetration-Effectiveness (CPE) score of 0.5 [6]. The CSF/plasma ratio was about 36% and that concentrations of emtricitabine exceed the wild-type IC_{50} in most individuals and may inhibit viral replication in the nervous system [7].

By considering the potential benefits of functionalized niosomes as carriers to improve the delivery of drugs to brain, the use of Emtricitabine encapsulated glucose-bearing niosomal CNS targeted drug delivery may be a promising novel drug delivery system for increasing its concentrations in the brain to treat patients suffering with HIV Associated Neurocognitive Disorders.

MATERIALS AND METHOD

Materials

Emtricitabine (Abhott Laboratories as gift sample); cholesterol (CHL), sorbitan monopalmitate (Span 40, HLB 9.8), sorbitan stearate (Span 60, HLB 4.7), sodium hydroxide and potassium dihydrogen ortho phosphate (SD Fine chemicals); Solulan C24 (Poly-24-oxyethylene cholesteryl ether, SOL), palmitic acid N-hydroxysuccinimide, glucosamine and Dulbecco's phosphate-buffered saline (DPBS) (Sigma-Aldrich) containing 2.7 mM potassium chloride, 1.5 mM potassium dihydrogen ortho phosphate, 137 mM sodium chloride and 8.1 mM disodium hydrogen orthophosphate. All other reagents used were of analytical grade.

Synthesis of glucose-derivatized surfactant N-palmitoyl glucosamine (NPG)

N-palmitoylglucosamine (NPG) was synthesized by slightly modified method of Dufes *et al* [8]. Glucosamine (1.27 g) was added to a mixture of 1.5 mL of triethanolamine and 220 ml of Dimethyl sulfoxide (DMSO). The mixture was stirred for 30 min at room temperature. Palmitic acid N-hydroxysuccinimide (2.5 g) dissolved in chloroform was then added to the mixture, left stirring for 72 h at room temperature, protected from the light. This was immersed in an ice bath and 100 ml of cold water was added. The precipitated product was collected by filtration, washed with water, DMSO and ethanol. Then it was dried at 40 °C for 48 h and stored at 4 °C protected from the light [5].

Method of preparation

Emtricitabine loaded NPG niosomes were prepared by thin layer evaporation (TLE)-paddle stirring method. The surfactant or surfactant-lipid mixture was dissolved in chloroform and introduced in a round-bottom flask. The solvent was evaporated under vacuum to form a thin layer on the flask wall and was hydrated with 25 ml of phosphate buffer saline. A paddle was then introduced in the flask and the suspension was stirred for 30 min at 65 °C. The aqueous phase consisted of 20 ml emtricitabine solution (2 mg/mL) [5].

Size Reduction of Niosomes

The formulated niosomal suspensions were centrifuged for 15 min at 4000 rpm. The supernatant was collected and sonicated for 5 min with the instrument set at 80 % of its maximum power and the size reduced samples were stored at 4° C protected from the light [5].

Standard calibration of emtricitabine in distilled water at 281 nm

Accurately 100 mg of the drug emtricitabine was weighed and dissolved in distilled water in a 100 ml standard volumetric flask. The volume was made up to the mark with distilled water. From this stock solution containing 1 mg/ml, solutions containing 5, 10, 15, 20 and 25 µg/ml concentrations of drug were prepared and the absorbencies determined spectrophotometrically at 281 nm using UV-Visible Spectrophotometer. The values are tabulated in Table 3. A graph was plot by taking concentration of drug on X-axis and absorbance on Y-axis and is represented in Figure 1 [10].

Characterization of niosomal suspensions

Particle size of vesicles

Particle size analysis was carried out using optical microscopy with calibrated eyepiece micrometer. About 200 niosomes were measured individually and the mean diameter was calculated [11]. The results are tabulated in Table 1.

Formation of vesicles

The effective formation of the vesicles was investigated by scanning electron microscopy (SEM). The SEM image is shown in Figure 2.

Drug encapsulation efficiency

Encapsulation efficiency of the niosomal dispersion was indirectly determined by dialysis method. From the emtricitabine-NPG-niosomal dispersion, 3 ml of the dispersion was dropped into a cellulose acetate dialysis bag (Spectra/Por®, MW cut-off 12000), immersed in 150 ml of distilled water and magnetically stirred at 30 rpm. The unencapsulated drug was separated by replacing the receiving medium every 30 min, until the level of dialysed drug was undetectable. The collected samples after suitable dilutions were analyzed spectrophotometrically at 281 nm using UV-visible spectrophotometer from which the amount of drug dialysed were determined. The total amount of drug initially present in each niosomal suspension was determined by disrupting the vesicles by addition of TritonX-100 followed by ultra-centrifugation (as described above) of 1 ml of non-dialyzed sample (to disrupt the vesicles) followed by emtricitabine assay in the supernatant by UV-visible spectophotometric analysis at 281 nm. The percent of encapsulation efficiency (EE%) was determined by the following equation:

$$EE\% = [\text{Total drug}] - [\text{Diffused drug}] / [\text{Total drug}] \times 100$$

Each result is the mean of three separate experiments [5]. The results are tabulated in Table 1.

***In vitro* blood-brain barrier penetration of emtricitabine using immobilized artificial membrane phosphatidylcholine column chromatography [12]**

High-performance liquid chromatography conditions

Shimadzu high-performance liquid chromatography system (Tokyo, Japan) consisting of a model SPD-10A UV-Vis detector, SCL-10A system controller, LC-10AT pump, SIL-10A auto sampler, and CTO-10A column oven was used in the assay. The analytical column was an IAM. PC. DD column (4.6 mm i.d.×10 cm length, particle size 5 µm, pore size 300 Å) purchased from Regis Technologies (Morton Grove, IL). The mobile phase was a mixture of acetonitrile and DPBS (20:80 v/v) with pH adjusted to 5.5 and 7.0 using hydrochloric acid. The flow rate of the mobile phase was maintained at 0.5 ml/min at 37°C, and the ultraviolet absorption wavelength set at 281 nm.

Determination of IAM partition coefficients

The CNS penetration of the formulation was evaluated by the method as described by Yoon *et al.* Emtricitabine niosomal preparation was dissolved in a mixture of acetonitrile:distilled water (50:50, v/v) at a concentration of 100 µg/ml, and a portion (5 µl) was injected to the chromatograph (n = 3 each). The IAM capacity factor (k_{IAM}) was calculated as

$$k_{IAM} = t_r - t_{o/}\ t_o \quad (1)$$

Where,

t_r = the retention time of the drug and

t_0 = the holdup time of the column.

The membrane permeability (P_m) of a drug following passive diffusion may be expressed based on correction by the molecular size:

$$P_m \propto \frac{k_{IAM}}{MW^n} \quad \ldots\ldots\ldots\ldots\ldots\ (2)$$

Prediction of CNS penetration potential based on IAM partition coefficients

The k_{IAM} values were determined at the mobile phase pH of 5.5 and 7.0. The differentiation between CNS+ and CNS- drugs was made based on k_{IAM} corrected by the molecular size with the power function set at n = 4 as at this power function of the capacity factors (k_{IAM}/MW^n), the values separated into two ranges.

The values for human CNS penetration and physicochemical parameters ($pk_{a,}$ Clog P, and PSA) of emtricitabine obtained from literature are tabulated in Table 2 [13, 14, 15, 16].

The classification of blood-brain barrier (BBB) penetration of drugs based on immobilized artificial membrane (IAM) capacity factors (k_{IAM}/MW^4 x 10^{10}) using IAM chromatography are tabulated in Table 3 [12].

In vitro blood-brain barrier penetration values of emtricitabine from the niosomal formulation using immobilized artificial membrane phosphatidylcholine column chromatography are tabulated in Table 4.

RESULTS AND DISCUSSION

Synthesis of glucose-derivatized surfactant N-palmitoyl glucosamine (NPG)

N-palmitoyl glucosamine was synthesized by the method described by Dufes *et al* and was obtained as white powder.

Method of preparation

Emtricitabine niosomes were prepared by thin layer evaporation-paddle method using Span 60 as surfactant with cholesterol and Solulan C24. Cholesterol (CHL), Solulan C24 (SOL) and N-palmitoyl glucosamine (NPG) were also included in the niosomal formulations. The ratio of Span 60:CHL:SOL:NPG was 50:40:10:10 with total concentration of components as 38 mM. The total concentration of components was fixed to 38 mM, since higher lipid amounts produces more

viscous samples. The concentration of SOL was kept at 10 mol%, since high levels of SOL are potentially haemolytic [16]. The hydration temperature was maintained at 65 ^{0}C since it is above the phase transition temperature of Span 60, which is the component of the lipid mixture with the highest value of transition temperature (50 °C) [9].

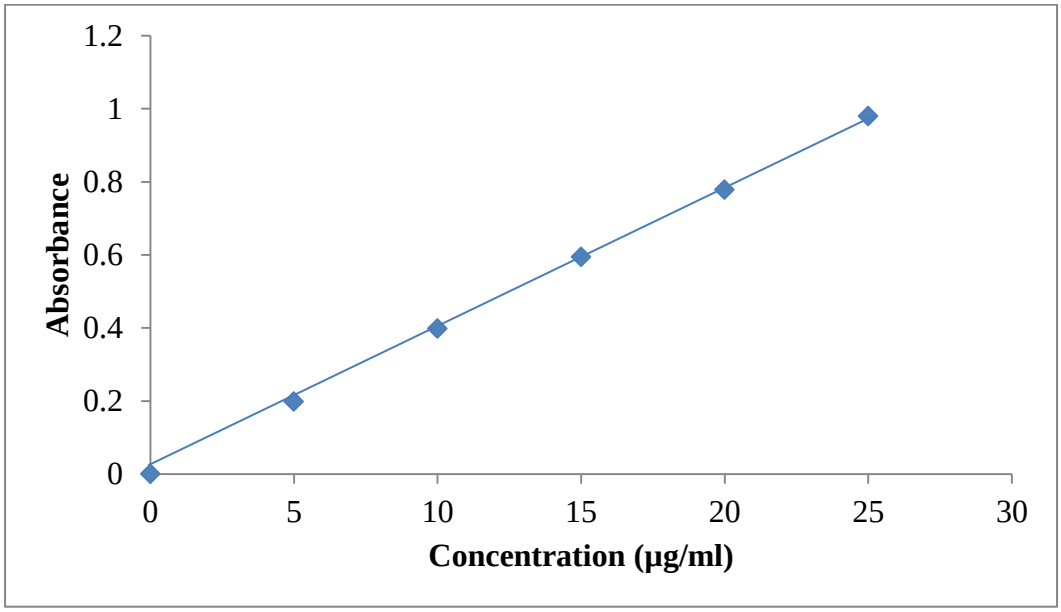

Figure 1: Standard calibration graph of Emtricitabine (FTC) in distilled water at 281nm

Size Reduction of Niosomes

Sonication method was employed for size reduction of the niosomes as it was found to be more efficient in reducing the particle size than size extrusion method [5].

Characterization of niosomal suspensions

Particle size of vesicles

The mean particle size was found to be 154±4 nm (Table 1).

Table 2: Values for the human CNS penetration and physicochemical parameters (pK_a, Clog P, and PSA) of emtricitabine obtained from literature [13, 14, 15, 16]

Drug	MW (g/mol)	CNS penetration	pK_a	Clog P	PSA (Å^2)
Emtricitabine	247.248	Intermediate	2.65	-0.43	88.15

PSA: Polar surface area

Formation of vesicles

Scanning electron microscopic images showed good formation of vesicles with spherical shape (Figure2).

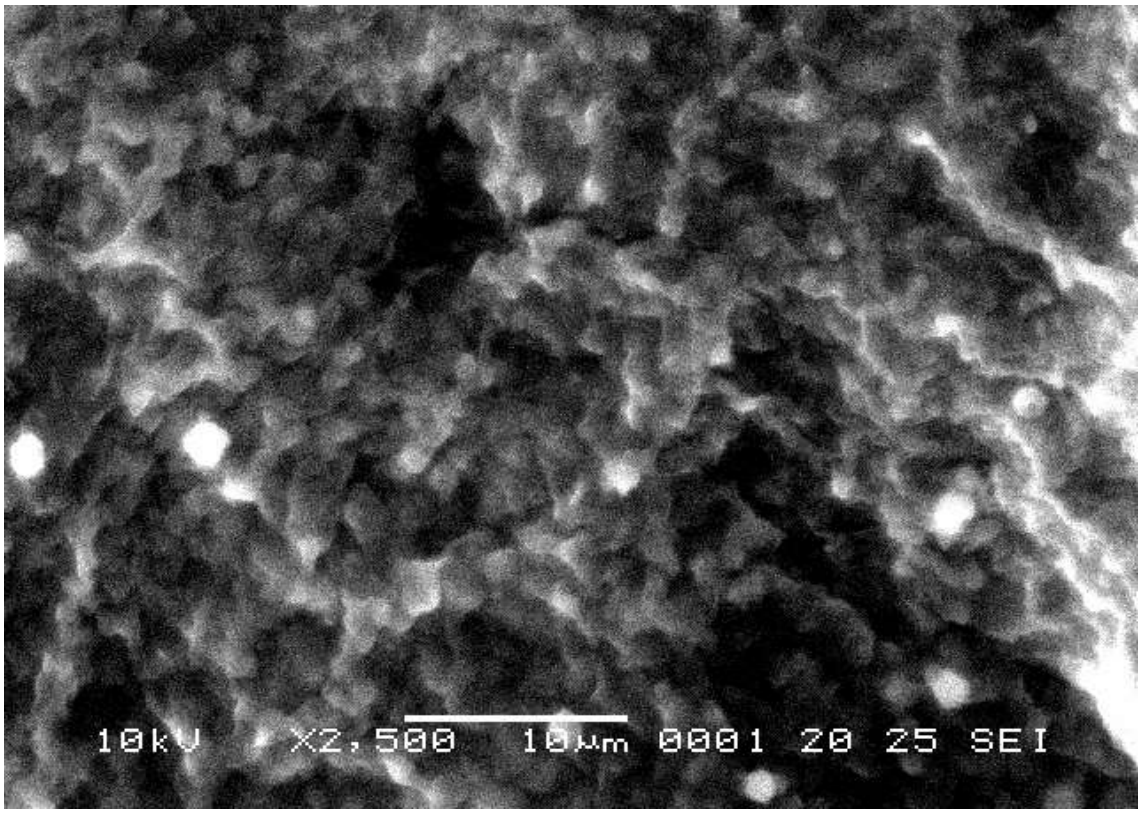

Figure 2: Scanning electron microscopic images of formulations prepared by Thin Layer Evaporation - paddle method with Span 60

Drug encapsulation efficiency

The encapsulation efficiency of the vesicles by dialysis method was found to be 64.45±1.14% (Table 1).

Table 1: Particle size of FTC-NPG-niosomes

Mean particle size ±S.D (nm)	Encapsulation efficiency ±SD (%)
154±4	64.45±1.14

***In vitro* blood-brain barrier penetration of emtricitabine using immobilized artificial membrane phosphatidylcholine column chromatography**

The Prediction of CNS penetration potential based on IAM partition coefficients by *in vitro* blood-brain barrier penetration of emtricitabine from drug loaded NPG niosomes using immobilized artificial membrane phosphatidylcholine column chromatography showed an improved CNS penetration of the drug with (k_{IAM}/MW^4) X 10^{10} values of 2.79±0.05 at pH 5.5 and 8.48±0.18 at pH 7.0 (Table 1) based on the values shown in Table 3 [11]. This might be attributed to the increased penetration efficacy of the surfactants Span 60 and Solulan included in the formulation [5, 17, 18].

Table 3: Classification of blood-brain barrier (BBB) penetration of drugs based on immobilized artificial membrane (IAM) capacity factors (k_{IAM}/MW^4 X 10^{10}) using IAM chromatography [12]

BBB penetration	IAM capacity factor (k_{IAM}/MW^4 X 10^{10})	
	pH 5.5	pH 7.0
High	>1.01	>0.85
Uncertain	0.65-1.00	--
Low	<0.64	<0.84

Table 4: *In vitro* blood-brain barrier penetration of emtricitabine from the formulated emtricitabine niosomes using immobilized artificial membrane phosphatidylcholine column chromatography

20% Acetonitile, (k_{IAM}/MW^4) X 10^{10}			
pH 5.5		pH 7.0	
$\bar{X} \pm SD$	CNS Penetration	$\bar{X} \pm SD$	CNS Penetration
2.79 ± 0.05	CNS+	8.48 ± 0.18	CNS+

SUMMARY AND CONCLUSION

Emtricitabine is a nucleoside reverse transcriptor inhibitor used in the treatment of HIV infections. It is a drug with intermediate CNS penetration efficiency. In the present study emtricitabine niosomes were prepared and characterized for formation of vesicles, mean particle size and encapsulation efficiency.

The formulated niosomal preparation was evaluated for CNS penetration of the drug from the niosomal formulation by *in vitro* blood-brain barrier penetration using immobilized artificial membrane phosphatidylcholine column chromatography. The results showed an improved CNS penetration of the drug when encapsulated in niosomal formulation.

To conclude, by considering the potential benefits of the drug formulated as niosomal dispersion, emtricitabine loaded NPG niosomal formulation with improved CNS penetration may be considered as a potential alternative to improve brain targeting of emtricitabine and thus minimize HIV Associated Neurocognitive Disorders. Thus, this formulation may be considered for further *in vivo* studies.

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