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Formulation, *In-Vitro* and *In-Vivo* Evaluation of Lamivudine-Loaded Solid Lipid Nanoparticles (SLNs)

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ABSTRACT

Background: The solubility and permeability of drugs could be improved using lipid based drug delivery systems (LBDDS). Drugs are usually classified into four groups according to Biopharmaceutical classification system. Lamivudine is a class 3 drug with high solubility and low permeability. **Objectives:** Lamivudine-loaded solid lipid particles were formulated and evaluated using different lipid matrices consisting of phospholipon 90H® and softisan® 154 in the ratio of 1:2, and *in-vitro* and *in-vivo* characterizations were done on solid-lipid nanoparticles (SLNs). **Materials/Method:** fusion method was used in the preparation of the lipid matrices using mixtures of phospholipon® 90H and softisan® 154 in the ratio of 1:2. They were melted together in a crucible using a water bath and subsequently stirred with a glass stirrer at 70 oC to get a homogenous transparent yellow melt. The homogenous mixture was subsequently stirred at room temperature to get solidification. **Results:** The pH range of the batches formulated with lipid ratio 1:2 was 4.9. After 3 months interval, there was no significant change in pH of the formulated SLNs, which resulted in no significant degradation of the drug and excipients that were used in the formulation. The encapsulation efficiency (EE) of batch F1 was 84 %, while batches F_2 and F_3 were 75 % and 71 % respectively. Loading capacity for the three batches F_1, F_2 and F_3 were 4.2 %, 7.5 % and 14.2 % respectively. The release studies obtained showed that the SLN had a percentage drug release of 63.5 % to 72.8 % after 10 h in simulated intestinal fluid (SIF) while in simulated gastric fluid (SGF), the percentage drug release was 49 % to 60 %. The *in-vitro* result obtained, showed that batch P (Pure standard lamivudine) recorded increased $CD4^+$ count from day 0 to 21, while batch N (control) showed no sign of $CD4^+$ count depletion. Conclusion: The method was used successfully.

Keywords: $CD4^+$ Count, Lamivudine, Nanopartcles, Phospholipon 90H,

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Softisan®, Solid-Lipid Nanoparticle (SLN).

INTRODUCTION

Poor oral bioavailability and inefficient dose proportionality is usually associated with lipophilic drugs when taken orally [1,2]. High stability, ease of incorporation of hydrophobic substances, high drug carrier capacity is usually associated with lipid drug delivery systems (LDDSs). Lipid formulation is one technique that can be used to increase drug solubilization of water insoluble drugs [3]. Some of the advantages associated with SLNs are good biocompatibility, non-toxicity, high stability and biodegradability. Lamivudine is an antiretroviral agent that belongs to a class of nucleoside analogue reverse transcriptase inhibitor [4]. Combination of lamivudine and zidovudine, inhibits the replication of some retroviruses. It also exhibits anti-hepatitis properties which makes it useful in the treatment of chronic hepatitis B [5].

Therapeutic substance can be introduced into the body using a drug delivery system, which helps to control the rate, time and release mechanism of the drug in the body [6]. Lipid based drug delivery systems are classified as solid lipid particulate dosage forms, lipospheres, emulsion-based system, solid lipid tablets, and vesicular systems [5].

Oral bioavailability of poorly water soluble drugs can be improved using SLNs [7]. The size range is between 50-1000 nm. Some of the advantages associated with SLNs include control and targeted drug release, improved stability, increase drug content, high stability, biocompatibility [8], while the disadvantages include poor drug loading capacity, particle growth and unpredictable gelation tendency [9]. There is numerous formulation techniques used in the preparation of SLNs. They include high pressure homogenization, micro emulsion, solvent evaporation and super-critical fluid method [10].

MATERIALS AND METHODS

Phospholipon 90H (Phospholipid GmbH, Koln, Germany), Softisan 154® (Schuppen Chondea, chemei, GmbH, Germany), Sorbic acid (Sigma Chemical company, USA), Tween 80 (Sigma Chemical company, USA), Sorbitol (Wharfedale laboratories, Otley, UK), Monobasic potassium phosphate (Sigma Chemical company, USA), Sodium hydroxide (Sigma Chemical company, USA), distilled water (UNN Water Resources, Nsukka, Nigeria), Lamivudine (Paucocyclo, Kwality Pharmaceuticals, India).

Formulation of Lipid Matrices

Lipid matrices were prepared using the fusion method which comprised of phospholipon 90H and softisan® 154 in the ration of 1:2. For each of the formulations, the lipids were weighed and melted together in a crucible using a water bath and stirred with a glass stirrer at a temperature of 70 °C to obtain a homogenous, transparent yellow melt [11].

Formulation of SLNs

Formulation of solid lipid nanoparticles was carried out using hot melt homogenization technique. A 10 g quantity of the lipid matrix was melted at 80 °C on a water bath and a weighed amount of lamivudine was incorporated into the lipid melt. Sorbitol was incorporated into the formulation which served as a lyoprotectant. This was dissolved in distilled water at a temperature of 80 oC with the lipid melt together with Tween 80 and ascorbic acid in each case. The hot aqueous phase was poured into the lipid melt and immediately subjected to high shear homogenization with ultra-turax at 18000 rpm for 15 minutes. Oil in water emulsion was formed by phase inversion. A control was also used which contained no drug.

Characterization of lipid matrices

Particle size determination

The average particle size was done microscopically using the BET (Brunauer, Emmett and Teller) as outlined in Nthabeleng Molupe (1991), surface area measurement by Transmission Electron Microscope (TEM) [12,13]. Samples were analyzed using a micromeretics TriStar 3000 gas adsorption instrument which calculated a BET surface area. From this, an average particle diameter was calculated assuming nonporous spherical particles and a theoretical density of the individual materials.

Determination of pH of the SLNs

The pH of dispersions of the SLMs from each batch was taken in a time dependent manner for 1 week, 1 month and 3 months using a pH meter (Suntex TS-2, Taiwan). Each determination was done in triplicates and the average taken.

Drug content and encapsulation efficiency analysis

A 10 ml quantity from each batch was centrifuged at 3000 rpm for 30 minutes and the supernatant was collected and transferred into a beaker and made up to volume, stirred and filtered with Whatman no 1 filter paper. The lamivudine

content of appropriate dilutions was determined at 271 nm (Model 450 UV/Vis Pye Unicam spectrophotometer). Duplicate determinations were made and average obtained. The absolute drug content and encapsulation efficiency (EE) were calculated.

Preparation of Beer's calibration plot

The wavelength of maximum absorption was obtained, by scanning pure sample of lamivudine solution at 271 nm. A 100 mg quantity of drug was dissolved in 100 ml of simulated intestinal fluid pH 7.2 in a 100 ml volumetric flask to obtain a concentration of 1 mg/ml. The solution was serially diluted. The absorbance of the different dilutions were noted and a calibration curve was obtained at ten concentration levels of lamivudine standard solution (0.01 to 0.1 mg/100ml). The above procedure was repeated using simulated gastric fluid as the dissolution media.

In vitro drug release

Beer's plot was obtained for lamivudine in simulated intestinal fluid at pH 7.2 and simulated gastric fluid at pH 1.2 at a pre-determined wavelength of 271 nm and 248 nm respectively. The dissolution medium consisted of 250 ml freshly prepared simulated intestinal fluid, pH 7.2 and simulated gastric fluid, pH 1.2, maintained at 37 ± 1 °C. The polycarbonate dialysis membrane (MWCO 6000-8000, Spectrum Labs, Breda, Netherlands) selected was pre-treated by soaking in the dissolution medium for 24 hours prior to use. A 3mls quantity of the SLN from each batch was placed in a polycarbonate dialysis membrane securely tied with a thermo resistant thread and placed in the appropriate chamber of the release apparatus. The beaker was filled with 250 ml of the dissolution medium and maintained at a temperature of 37 ± 1 °C by means of a thermostatically controlled water bath with agitation at 100 rpm. Aliquots of 5 ml was removed and placed by an equal volume of the receptor phase at different time interval up to 10 hours and the sample collected analyzed for lamivudine content using spectrophotometer. The volume of the dissolution medium was kept constant by replacing it with 5ml of fresh medium after each withdrawal to maintain sink condition. The drug content of each time point was calculated by reference to Beer's calibration in each medium.

In vivo pharmacodynamics studies

Healthy rats weighing between (100-180 g) were divided into six groups of three each including both positive and negative controls. All animal experimental protocol was carried out in accordance with guidelines of the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka for animal experiments with approved Number: UNN/004/89745. The rats were first allowed to acclimatize to the new experimental environment for 1 week, weighed, properly labeled for identification and housed separately in cages. They were allowed free access to food and water throughout the study. Cyclophosphamide 12 mg/kg was administered to induce leucopenia and immune suppression. A period of 4 days was allowed to elapse and then the rats were treated with 5 mg/kg lamivudine every day for 21 days.

Blood collection

Blood collection was through the retro orbital plexus (optical puncture) of the rat using a heparinized hematocrit capillary. Blood was collected prior to induction by cyclophosphamide, before treatment with lamivudine, and subsequently every 7 days throughout the experimental period. The blood samples were placed in ethylenediaminetetraacetic acid (EDTA) bottles for storage and collection for experiment.

Determination of $CD4^+$ count

The $CD4^+$ count was performed using the volumetric Single Platform Flow Cytometer. The Cyflow counter used in this study was an automated two parameter flow cytometer. In the volumetric single platform protocol, $CD4^+$ cells are marked with a single monoclonal fluorochrome (phycoerythrin) conjugated antibody in a known volume of blood. The fluorochrome marked $CD4^+$ cells are counted by excitation with a green solid- state laser operating at 532 nm by side scatter analysis. A 50 µl quantity of EDTA blood were placed in a test tube and mixed with 10 µl monoclonal $CD4^+$ antibody (clone EDU-2, DIATEC, Oslo, Norway). After incubation period of 15 mins at room temperature in the dark, 800 µl of no-lyse dilution buffer (phosphate buffered saline) was added. A total sample of 860 µl was then analyzed with a Cyflow counter. The results were indicated on the display.

Data and statistical analysis

Statistical package for the Social Sciences (SPSS) version 22.0 (SPSS Inc. Chicago, IL.USA) was used. The data was analysed by One-Way Analysis of Variance (ANOVA), Excel 2010 and differences between means were assessed by a two-tailed Student's t test.

RESULTS AND DISCUSSION

Results of the particle size obtained showed that lamivudine loaded SLNs prepared with LM 1:2 (F1) containing 0.5% lamivudine showed the lowest mean particle size (41 mm) while SLNs prepared with LM 1:2 containing 1% lamivudine exhibited the largest mean particle size of (45 nm) . The particle size was affected by the ratio of drug to lipid matrix used.

Table 1. Particle size of SLN batches (A)

Batch	Mean Particle size (nm)
F1 (1:2)	41 ± 0.82
F2 (1:2)	45 ± 0.82

F1 contain 0.5 % lamivudine, F2 contain 1 % lamivudine

A1 and B1 contained 0.5% lamivudine, A2 and B2 contained 1% lamivudine

In Table 2, it was observed that lamivudine-loaded SLNs prepared with LM 1:2 (A1 – A2) exhibited the lowest mean particle sizes, while SLNs prepared with LM 1:2 and containing 0.5 % and 1 % lamivudine i.e. batches B1and B2 had the largest mean particle size of 89 nm and 96 nm respectively. The ratio of drug to lipid matrix used and the ratio of the lipid matrix in the formulations affected the particle size of the SLNs. Highest particle size was seen in SLNs loaded with 1 % lamivudine in all formulations. Also the ratios of the two lipids used in the SRMS affected the size of the SLNs. SLNs formulated with LM 1:2 had the smallest particle size, however SLMs prepared with LM 1:2 (Phospholipid : Softisan® 154) exhibited the largest particle sizes of the SLNs. The particle size diameters of the formulations are within the normal range. According to Gugu et al; [14], the solid-lipid microparticles (SLMs) were spherical and smooth in shape. The results showed that particle size was not directly proportional to drug loading, while their particle size increased significantly with encapsulation efficiency (EE) without a significant difference ($p < 0.05$).

Table 2. Particle size of SLN batches (B)

Batch	Mean Particle size(nm)
A1(1:2)	32 ± 0.82
A2(1:2)	37 ± 0.81
B1(1:2)	89 ± 0.82
B2(1:2)	96 ± 0.82

pH study

The results obtained for the different batches of SLNs showed that all the batches formulated with lipid ratio 1:2 had an average pH range of 4.9. There was no significant change ($p < 0.05$) in pH of the SLNs formulated and stored at room temperature after three months.

Table 3. pH of lamivudine SLNs (A)

BATCH	Mean pH (1 week)	Mean pH (1 month)	Mean pH (3 months)
F1 (1:2)	5.00 ± 0.02	5.50 ± 0.03	5.20 ± 0.02
F2 (1:2)	5.50 ± 0.03	5.20 ± 0.02	5.60 ± 0.03
F3 (1:2)	5.30 ± 0.02	5.50 ± 0.03	5.50 ± 0.03
F4 (1:2)	5.40 ± 0.03	5.30 ± 0.02	5.60 ± 0.03

Drug content, encapsulation efficiency, Loading capacity

The encapsulation efficiency showed that SLN batches F1 loaded with 0.5 % lamivudine had the highest EE % of 84 % followed by batch F2 formulated with 1 % lamivudine (Table 4). On the other hand, batch F3 containing 2 % lamivudine showed the least EE of 71 %. The EE % of the lamivudine in the SLNs showed a decrease in the quantity of lamivudine entrapped with increasing drug concentration. The lipid matrices incorporating 0.5 % lamivudine (F1) had the highest percentage entrapped while those containing 2 % lamivudine (F3) had the least percentage entrapped. The EE % defines the total weight of drug added to the dispersion

Table 4. Actual drug content and encapsulation efficiency of SLNs (A)

Formulation code	TDC (%)	ADC (%)	EE (%)
F1(1:2)	0.3 ± 0.12	0.42 ± 0.01	82.6 ± 1.25
F2(1:2)	1.1 ± 0.08	0.75 ± 0.01	75 ± 0.82
F3(1:2)	2.6 ± 0.47	1.42 ± 0.01	71 ± 0.47
F4(1:2)	0.0 ± 0.0	-	-

ADC is actual drug content, TDC is theoretical drug content, EE is encapsulation efficiency

The loading capacity (LC) increased with increase in drug load (Table 5). Batch F3 with 2 % lamivudine exhibited the highest LC of 14.2 mg API/100 mg of lipid, while batch F1 with 0.5 % lamivudine exhibited the lowest LC of 4.2 mg API/100 mg of lipid. Generally, EE% and LC were affected by the total amount of API in the formulation.

Table 5. Drug loading capacity

Formulation code	EE (%)	LC
F1(1:2)	84 ± 1.25	4.2 ± 0.08
F2(1:2)	75 0.82	7.5 ± 0.08
F3(1:2)	71 ± 0.82	14.2 ± 0.05
F4(1:2)	-	-

EE is encapsulation efficiency, LC is loading capacity

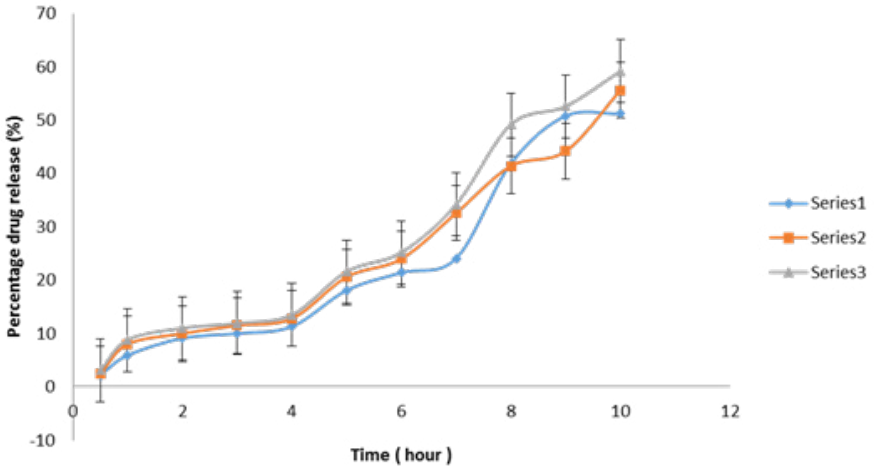

Figure 1. In vitro drug release of lamivudine in pH 1.2 and 7.2

Series 1 =F1, Series 2 =F2, Series 3 =F3

The *in-vitro* release studies were carried out using SGF (pH 1.2) and SIF (pH 7.2) as release media (Figure 1). The release of lamivudine varied with the amounts of the components of the solidified lipid matrices used in the formulations. The SLNs showed a percentage release of 63.5 to 72.8 % at 10 h in SIF and a percentage release of 49 % to 60 % in SGF. The drug concentration was seen to have an influence on the rate of drug release from the SLNs as the percentage drug release was seen to increase with increasing drug concentration amongst the various batches over time. The percentage drug release increased subsequently as the duration of release increased. It was found that more drug was released in SGF than in SIF showing that these lipospheres could release drugs better and faster in the stomach than in the intestine. This difference in drug release was attributed to the pH of the SGF. Generally, the release of the drug from all the batches was diffusion-controlled, as expected of matrix systems [13,15].

***In-vivo* studies**

Batch P (Table 6) which received pure standard lamivudine drug was showed consistently increasing $CD4^+$ count with high $CD4^+$ count values from day 0 throughout the 21 days of study. Batch N which received neither cyclophosphamide nor lamivudine was seen to show no sign of $CD4^+$ count depletion, therefore the $CD4^+$ count for the 21 days of study was stable with values of 527, 602 and 604 cells/mm^3 for the 1st, 2nd and 3rd week respectively.

The highest encapsulation efficiency was recorded in F-1. LM 1:1, gave an increasing $CD4^+$ count throughout the study; however when compared to the standard drug, lesser $CD4^+$ count values was seen throughout the 21 days. This means that with the extension of treatment of the immunocompromised rats with the SLN formulation of lamivudine, there was a high probability of restoring the $CD4^+$ count to normalcy. The rationale of the SLN formulation was to ensure that the CD4 count increased within the shortest period of time.

Table 6. SLN formulation (Batch A) on the $CD4^+$ count of immunocompromised rats

$CD4^+$ count (cells/mm^3)					
Batch	Baseline	0 day	7 days	14 days	21 days
F1(1:2)	501	435	484	521	601
P	347	309	412	582	723
N	498	513	527	602	604

Batch P is positive control, N is negative control, F1 (1:2) contained 0.5% lamivudine.

CONCLUSION

The hot melt homogenization method was used to successfully formulate a sustainable release lamivudine-loaded solid lipid nanoparticles using phospholipon 90 H and softisan 154 in the ratio of 1:2. The *in-vitro* drug release studies obtained showed that more drug was released in SGF than in SIF which indicated that the lipospheres would release drugs better and faster in the stomach compared to the intestine. The *in-vivo* study showed that the CD4 count increased appreciably within the shortest period of time. The advantage of the formulation over the commercial include low cost of ingredients used in formulations, low cost of equipment, enhanced absorption and ease of preparation.

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AUTHORS CONTRIBUTIONS

Salome AC: Conceptualization

Iyanyi CJ: Formal analysis

Ezegbe CA: Formal analysis, investigation

Onunkwo GC: Conceptualization

Okafor NP: Data curation

Ezegbe GA: Data curation

All authors revised the article and approved the final revision.

DATA AVAILABILITY

Data will be available to anyone on request to the corresponding author.

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