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Characterization and Stability Study of Reduced L-glutathione-loaded Niosomes

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RESEARCH ARTICLE

Abstract

Background: : Glutathione is a major antioxidant in the body that serves as a substrate for conjugation reactions and regulates cell proliferation. Low levels of glutathione have been linked to cancer, liver problems and other chronic diseases. Studies have shown that oral supplementation is not effective in increasing the glutathione level in the body.
Objectives: The purpose of the study was to prepare a niosomal formulation of glutathione and to characterize the niosomal formulation. Furthermore, the study compared the effect of the charge inducer in the formulation.
Methodology: The method was divided to the preparation, characterization and stability study of the niosomal formulation. The niosomal formulation was produced by thin film hydration with varying Span 60 (Sorbitan monostearate) and cholesterol ratios. Niosomal formulation with highest entrapment efficiency was further characterized for mean particle size, surface morphology, and in vitro drug release.
Results and Discussion: Formulation A entrapped 98.21% of the glutathione. Addition of charge inducer increased its entrapment efficiency to 98.91%. Furthermore, both niosomal formulations released glutathione at pH 7.4 in 1.0M phosphate buffer saline (PBS). The mean vesicular size obtained was 1,242.97 + 40.52nm. Differential Scanning Calorimetry revealed compatibility between glutathione and its excipients. Both formulations do not cause cytotoxicity in human dermal fibroblast. The stability study also revealed that it was stable at 5°C and 40°C for 3 months.
Conclusion: Results of this study suggested the potential use of niosomes in the targeted delivery of glutathione. This is the first report on the use of niosomal preparations through thin film hydration technique in the delivery of reduced L-glutathione.

Keywords: glutathione, niosome, drug delivery, formulation, bioavailability, cytotoxicity and stability

Introduction

Chronic diseases are characterized by their occurrence for more than three months and can neither be prevented by the use of vaccines nor cured by medications[1]. Depleted level of glutathione in blood is associated to some chronic illnesses like cataracts, liver problems, diabetes mellitus, lung diseases and others. Low levels of blood glutathione have been a risk factor for developing chronic diseases [2]. Moreover, diseases associated to glutathione deficiencies are hemolytic anemia, metabolic acidosis, mental retardation, neuropsychiatric syndrome, Parkinson's disease, human immunodeficiency virus (HIV)[3], cystic fibrosis [4], Crohn's disease, circulation disorders, metal storage (Wilson's disease), pancreatitis, and neurodegeneration [5]. Glutathione is a tripeptide composed of glutamic acid, cysteine, and glycine that when polymerized forms gamma-glutamylcysteinylglycine (GSH) [5]. It is also an endogenous antioxidant that prevents oxidative damages to cells. Reduced glutathione (GSH) in the body acts as an electron donor preventing free radical or oxidative damages to cells. Oxidized glutathione (GSSG) is formed via disulfide bond from the sulfhydryl group of the cysteine moiety of two glutathione molecules. The oxidized glutathione also reacts to another electron donating molecule or an antioxidant (NADPH or vitamin C) for it to be reused as an antioxidant. However, oral supplementation of glutathione is not effective in increasing its levels in the blood [6]. It was also reported

that glutathione is easily degraded by chemical or enzymatic hydrolysis.

Upon oral administration, glutathione is acted upon by proteolytic enzymes in the intestine, resulting to small amounts of glutathione reaching the portal or hepatic circulation. Moreover, pH gradient and low epithelial permeability prevent the delivery of proteins and peptides via the oral route [6]. In this regard, protection and stabilization of glutathione via drug delivery system can increase its bioavailability following the enteral route (pH 6.8) [7,8].

Vesicular system is one of the novel ways to deliver drugs in a controlled manner. This achieves better bioavailability and prolongs the duration of action of drugs [9]. Vesicles are concentric bilayer colloidal particles of amphiphilic molecules serving to entrap molecules in aqueous solutions. This vesicular drug delivery system can be classified as either lipoidal or non-lipoidal in composition. Lipoidal vesicles include liposomes, pharmacosomes, transferosomes, and emulsomes while non-lipoidal vesicles include niosomes and bilosomes [10].

The vesicles formed from phospholipids are called liposomes whereas those formed from non-ionic surfactants and cholesterol are called niosomes [7]. Liposomes encapsulate and effectively deliver the drugs to the target site; however, it imposes high cost and short shelf life due to oxidative degradation, while niosomes give a great advantage of low cost and better shelf life compared to liposomes. Niosomes can act as drug reservoir that releases the drug in a controlled manner [10]. Some examples of drugs that are entrapped within a niosomal vesicle include aceclofenac [11], diclofenac [12], Rofecoxib [13], ketoprofen [14], acetazolamide [15], cefuroxime [16], gliclazide [17], and griseofulvin [18].

This paper presents the formulation and characterization of a niosomal reduced L-glutathione in terms of its release time, surface morphology, particle size, entrapment efficiency, drug excipient compatibility, cytotoxicity and stability.

Methodology

Materials

Reduced L-glutathione, USP grade was obtained from Health Sources Nutrition Co., LTD in China. Other chemicals, analytical grade reagents and materials used in the study were acquired from Sigma Aldrich and Laboratory Equipment and Supplies Office (LESO) of University of Santo Tomas, Manila, Philippines.

Preparation of Niosomes

Glutahione-loaded and empty niosomes were prepared by thin film hydration method as described by Pando et al. (2015) [19]. Formulations consisting of Span 60 and cholesterol were prepared at different molar ratios: Formulation A (1:1), Formulation B (3:2), and Formulation C (7:3). Both Span 60 and cholesterol were dissolved in a 10 mL mixture of dichloromethane and methanol (1:1). Having the highest entrapment efficiency, Formulation A was then added with a charge inducer, dicetylphosphate, composed of 49.5:49.5:5 of Span 60, cholesterol and dicetylphosphate, respectively to come up with Formulation D.

Different niosomal formulations (100 μM) were prepared by dissolving Span 60 and cholesterol in dichloromethane-methanol mixture. The resultant solution was concentrated in vacuo at 65°C at 100 rpm to form a thin film in the inner walls of the flask. A 10mL pre-warmed (65°C) glutathione in 1.0M phosphate buffered saline (PBS) pH 7.4 (1 mg/mL) was added to the thin film and allowed to stand for 15 min at 65°C while stirring at 25 rpm. The niosomes formed were stored at 5 + 2°C for 24 h to allow complete hydration and vesicle formation. The formation of the pellets are due to the self-assembly of the surfactant molecules.

The niosomes were subjected to ultracentrifugation at 20 000 x g at 4°C for 90 min [26]. The supernatant containing free glutathione was decanted and stored for future use, while the niosomal pellets were washed with 10mL PBS, and re-centrifuged for 90 min. The resulting supernatant liquid was also decanted and mixed with the previously collected supernatant. Finally, the niosomal pellets were re-suspended in 10mL PBS solution. In Formulation D, dicetylphosphate was added and was prepared in a similar manner as that of the previous formulation.

In vitro Characterization

Entrapment Efficiency

Entrapment efficiency was determined by glutathione assay (Glutathione Assay Kit CS0260 SIGMA) [27]. The amount of glutathione detected was the non-entrapped/free glutathione in the supernatant liquid using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) to form a yellow colored chromophore, which is 2-nitro-5-thiobenzoate (TNB) and then read at 412nm. The Entrapment efficiency

was computed using the formula: E.E. (%) = [(TC – FC) 100]/TC where E.E. (%) = Percent Entrapment Efficiency, TC = Total Concentration of glutathione used (µg/µL) and FC = Free glutathione detected in the supernatant liquid (µg/µL).

Particle Size Determination

The average size of the niosomes was determined using laser diffraction particle size analyzer (Nanoplus 1 – Micromeritics Particulate System). Phosphate buffered saline (PBS) has a refractive index of 1.33, viscosity of 0.8882 cP at 25°C and a dielectric constant of 79. Particle size determination was done at the Department of Metallurgical, Mining and Material Engineering, College of Engineering, University of the Philippines, Diliman, Philippines.

Surface Morphology

Niosomal formulations (with glutathione and blank) were lyophilized prior to Scanning Electron Microscopy (SEM, JEOL, JSM-5310). Each lyophilized niosomes were mounted onto screw shaped stubs using a double-sided carbon adhesive tape. The niosomes were coated with gold in an argon atmosphere under vacuum conditions in an ion sputtering chamber. The morphology of the niosomes was determined at 20,000X – 35,000X magnification and 25,000V accelerating voltage [28]. The surface morphology study was done at the Physics Surface Laboratory at De La Salle University, Taft Avenue, Manila, Philippines.

In vitro drug release

In vitro drug release study was performed at different simulating environments such as the stomach (pH 2.0), small intestines (pH 6.8) and blood (pH 7.4) using an open-end glass tube with a dialysis membrane covering the other end[28]. A 10mL niosomal glutathione suspension in PBS pH 7.4 was poured into the open-end tube (lined with dialysis membrane, 12 kDa molecular weight cut-off). The dialysis membrane was submerged into a release medium of 200 mL solution of 0.1M HCl (pH 2.0). The set-up was stirred at 75 rpm for 3 h at 37 + 0.5°C. An aliquot was collected from the receptor compartment (release media) every hour followe\ volume of aliquot taken. The dialysates were used to determine the concentration of glutathione. Two separate simulations were also done at pH 6.8 and at pH 7.4.

Differential Scanning Calorimetry

Melting point determination of pure glutathione, Span 60, cholesterol, dicetylphosphate, and binary mixtures were performed at zero (0) time and after storing under 40°C and 75% Relative Humidity using a Differential Thermal Analysis, Thermo-Gravimetric Analysis instrument (DTG-60H) at the Physics Laboratory of De La Salle University, Taft Avenue, Manila, Philippines. The samples (10-20 mg) were heated in an aluminum crucible using nitrogen (50mL/min) as effluent gas. Analysis was carried out at temperatures ranging from 50 to 250°C at a rate of 10°C/min.

Cytotoxicity Assay

Cytotoxicity assay was done using the MTT assay. Human Dermal Fibroblast, neotanal (HDFn – ATCC CRL-2522) was used as the cell line to determine the toxicity of the samples. The cells were seeded (5 x 105 cells/mL) onto a 96-well plate and were incubated at 37ºC in a humidified incubator with 5% CO2 for 24 hours. The cells were then treated with 100µL of 200, 100, 50, 25 and 12.5µg/mL test samples. Doxorubicin-HCl at 20, 10, 5, 2.5, and 1.25µg/mL was used as the positive control. Treated cells were incubated for 24 h at 37oC in a humidified incubator with 5% Co2.

The culture medium was removed after incubation and 20µL of 5 mg/mL of 3-(4,5-dimethylethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate buffer saline, pH 7.2, was added to the treated and untreated cells. The cells were incubated for another 4 h at 37ºC in a humidified incubator with 5% CO2. A 200 µL of dimethyl sulfoxide was added to each well and the absorbance was read 570nm in a microtiter plate reader (Corona®). After the absorbance was read, the formula used to compute for percent inhibition was: (%) Percent Inhibition = 100-{[(At-Ab)/(Ac-Ab)]*100} where At is the absorbance value of the treated cells while Ab is the absorbance value of the blank, and Ac is the absorbance value of the untreated cells. The median inhibitory concentration was computed using Graphpad prism version 6 [29].

Stability Study

The stability of the niosomal glutathione stored in colorless glass vials with push on cap sealed with paraffin film was determined for three months at 5°C and 40°C, respectively as prescribed by the International Conference on Harmonization (ICH) [30]. The samples were stored at 5°C in an Electrolux refrigerator. Likewise, samples at 40°C were placed in a stability oven, Binder-APT Line Series BD/ED/FD (E2). Every month, a sample of the niosomal suspension was pulled out and subjected to glutathione assay to determine the amount of glutathione retained

from the niosomes. The order of reaction kinetics and shelf life were determined.

Statistical Analysis

Data, presented as mean + standard error of the mean (SEM), was treated with the statistical analysis using One-way Analysis of Variance (ANOVA). To further determine the differences among group, post hoc analysis using Tukey honesty significant difference (HSD) was applied. Statistical analysis was carried out at 95% confidence interval.

Results and Discussion

Physical Characteristics

The suspensions of prepared niosomes were found to be white, colloidal, and odorless.

In vitro characterization

Entrapment efficiency

Statistical analysis one-way ANOVA found that three formulations differ significantly at $p < 0.001$. Tukey HSD Post-Hoc analysis revealed that the mean entrapment efficiency for all three formulations has a decreasing relationship and are ranked as follows: Formulation A (98.21 + 0.4%) > C (97.92 + 0.01) > B (97.80 + 0.07). This indicates that Formulation A was the most suitable for further characterization. Investigating further, a charge inducer was added to Formulation A which increased the entrapment efficiently by 0.71% (Table 1).

Factors affecting the characteristics of the formulation are the following, the choice of surfactant, hydrophilic lipophilic balance (HLB), cholesterol level and hydration medium. Non-ionic surfactants are composed of both polar head (hydrophilic segment) and non-polar (hydrophobic) tail. They form vesicles which are lamellar in structure and nanoscopic due to their self-assembly upon hydration. Surfactants are classified based on their HLB values. HLB values of 14-17 did not form any niosomes while HLB value of 8.6 demonstrated the highest entrapment efficiency. For this study, span 60 (sorbitan fatty ester) was used due to its high phase transition temperature (Tc) but having an HLB value of 4.7.

Table 1. *Entrapment efficiency of formulations A, B, C and D based on the dialysate assay for glutathione content*

Formulation	Glutathione Content (%)
I	98.21 + 0.4
II	97.80 + 0.07
III	97.92 + 0.01
IV	98.91 + 0.001

Glutathione content was determined in the supernatant obtained using a glutathione assay kit. *n* = 9. Data were expressed as Percent glutathione content + S.E.M.

However, as the HLB value decrease from 8.6 to 1.7, it resulted to lower entrapment efficiency due to the difficulty in forming the niosome vesicles [7,9]. To facilitate the formation of niosome and enhancing the entrapment efficiency of the vesicles with span 60, cholesterol must be added at a concentration of 30-50%. At 50% concentration of cholesterol, it would produce the best entrapment efficiency as shown in the comparison of the three formulations (A, B, and C). Formulation exhibited the best entrapment efficiency due to a 1:1 molar ratio resulting to a more compact and well organized membrane [24]. Cholesterol level in the formulation greatly affects the leakage of the drugs in the vesicles as cholesterol fills the gaps between the surfactant molecules. As cholesterol level (at most of 50%) imparts rigidity and prevents drug leakage [17]. The study has not optimized the best pH and temperature of the hydration medium due to a previous study in the formulation of niosomes [7].

The use of phosphate buffer saline pH 7.4 had demonstrated the best medium for hydrating the niosome vesicles. The method of thin film hydration used the temperature of 65°C due to the transition temperature of Span 60 (50-55°C) which should be exceeded to facilitate the self-assembly of the vesicle. The structure of the drug also affects the entrapment efficiency into the niosomal vesicles.

The drug, glutathione which is composed of glutamic acid, cysteine, and glycine [25], is considered as an amphiphilic molecule due to the hydrophobic and nonpolar character of glycine. Due to this amphiphilic property, the drug molecule can be incorporated between the bilayer of the vesicles. Another factor to consider with the compound is the presence of an electronegative atom (oxygen and nitrogen) which permits the drug to interact with the cholesterol and span 60 through hydrogen bond[26].

Table 2. *Particle size distribution of formulation A and D*

F	T	Ave Dia (nm)	PI	D(50%)
I	1	1280.5	0.256	1460.3
	2	1200	0.224	1330.1
	3	1248.38	0.348	1490.3
	Average	1243 + 40.52277	0.276 + 0.064374	1426.9 + 85.16267
IV	1	1315.7	0.236	1490.3
	2	1618.7	0.306	1863.7
	3	1867.3	0.299	2254.2
	Average	1600.6 + 276.2467	0.28 + 0.038553	1869.4 + 381.9819

Legend: F – Formulation code; T – Trial number; Ave Dia – average diameter in nanometers; PI – polydispersibility index; D(50%) – median diameter

Particle size was determined by a laser diffraction particle size analyzer. Samples were suspended in PBS (refractive index = 1.33, viscosity = 0.8882 centipoise at 25 °C and dielectric constant = 79).

The polydispersibility index indicates the homogeneity of the particles in the samples. A value of less than 1 indicates a good homogeneity of the sample.

Table 3. *Differential scanning calorimetry results showing the melting point of the pure components (in oC)*

C	Melting Point		DT	Significant
	F	S	Max: 5%	Yes/No
GSH	204.3	200.6	1.811062	No
S60	56.23	56.73	0.889205	No
CHO	154.5	150.2	2.783172	No
DCP	79.8	79.8	0	No

Legend: C – Component; F – Fresh Sample; S – after stress test; DT – difference in the melting point; GSH – Glutathione; S60 – Span 60; CHO- Cholesterol; DCP- Dicetylphosphate

Data are presented as average melting points of the samples. n = 3. Samples were subjected to DSC analysis from 50°C to 250°C. Stress Test was carried out at 40°C for 30 days.

The difference in the melting point of the sample indicates possible incompatibility. The significant difference was noted when there is a DT > 5%. Incompatibility results from the thermal method suggest only possible incompatibility thus further testing using non-thermal method is recommended.

Addition of dicetylphosphate, which is a negatively charged surfactant, imparted difference in the characteristics of the formulation A. The negatively charged phosphate head of the dicetylphosphate interact the positively ionized amino group of the glutathione (pKa =9.67). This electrostatic interaction resulted to an increase in the entrapment efficiency of Formulation D as compared to Formulation A [24,27].

Surface Morphology and Particle Size Determination

Niosomes are found to be spherical but Formulation A niosomes appeared to have more edges as compared to the smooth edge particles of Formulation D loaded niosomes.

Performing dynamic light scattering technique, the mean particle size of Formulation A niosomes (1,242.97nm + 40.52nm) is not statistically significant with Formulation D niosomes (1,600.57nm + 276.25nm) with a p value of 0.091. The polydispersity index (P.I.) of the two formulations was reported.

As discussed above, addition of dicetylphosphate imparts charge to the particle and thus leads to the repulsion of the structure which results to an increase in the particle size and smoother edges. As the particle size increases, the capacity in entrapping the drug also increases[6]. The polydispersity index is the measure of the particle size distribution. The value of 1 indicates heterogeneity in the particle size of the *suspension whiles values of 0.5 and less are considered to be homogenous. Both formulations have PI values less than 0.5 thus considered to be homogenous. The greater value of particle size of both formulation A and D might be due to the* shorter time of sonication of the niosomes. The median diameter (D(50)) (in nm) of formulation A was determined to be 1426.9nm + 49.17nm while formulation D was determined to be 1869.4nm + 220.54nm. Median diameter for formulation A indicates that half of the particles possess less than 1426.9nm and that the other half of the particle possesses greater than 1426.9nm. Same as to be applied to formulation D, half of the particles possess less than 1869.4 nm diameter and the other half possess greater than 1869.4 nm [28].

In vitro Drug Release

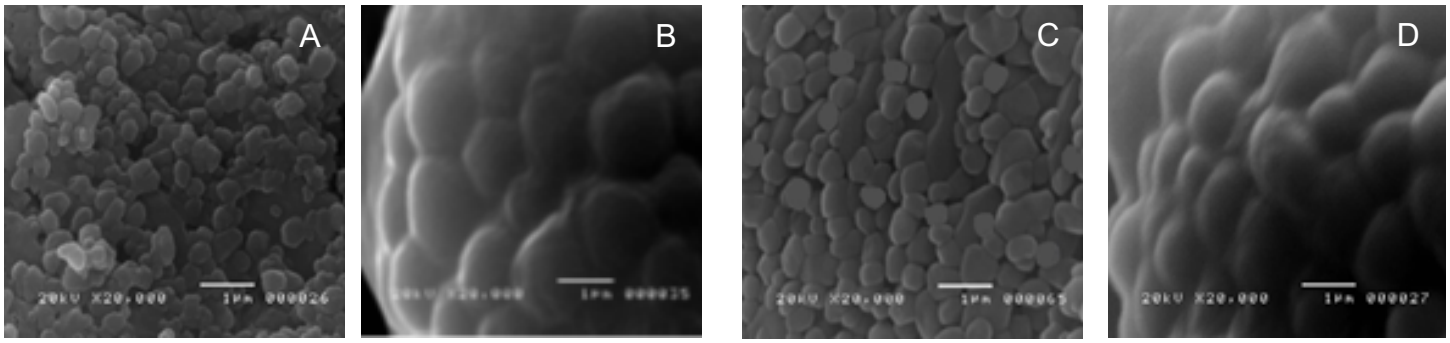
L-glutathione in Formulation A was not released at acid (pH 2.0) and neutral (pH 6.8) environments but then released 1.12mg + 0.09mg (11.21%) at the third hour into the neutral pH 7.4 (Fig. 2a). In contrast, incremental release of drug was observed for Formulation D during the first hour with 0.22mg + 0.11mg (2.20%), 0.35mg + 0.19mg (3.50%), and 0.54mg + 0.67mg (5.40%) (Fig. 2b).

The mechanism of drug release in the niosomal vesicle is either bursting of the niosomal vesicles, resulting to the release of its content or the efflux of the drug from the aqueous core through the lipid bilayer [27]. Results suggest that the release of glutathione in the three media: 0.1 N HCl, PBS pH 6.8 and PBS pH 7.4 had become a pH selective release in which the vesicles released its content only in PBS pH 7.4. The possible mechanism of pH selective release is due to the hydrogen ion (H+) ion concentration that is different in the three media used in the simulation. Since the niosomal glutathione was prepared using the PBS pH 7.4, the pH of the aqueous core and the donor compartment differ in the release media which can cause water movement due to osmosis. Both 0.1 N HCl (pH 2) and PBS pH 6.8 had greater H+ ion concentration than PBS pH 7.4 (aqueous core). Due to this H+ ion gradient, water diffuses from the aqueous core and the donor compartment to the release media which prevents the diffusion of glutathione through the semi-permeable niosomal vesicle.

The efflux mechanism H+ ion gradient was not present in PBS pH 7.4 release medium because the aqueous core and the donor compartment also contained PBS pH 7.4. This allowed the movement of glutathione outside of the semi-permeable niosomal bilayer vesicle. Another mechanism of drug release is the movement of water from the release media to the donor compartment to the aqueous core of the niosome leading to swelling and bursting of the niosome resulting to the release of drug content.

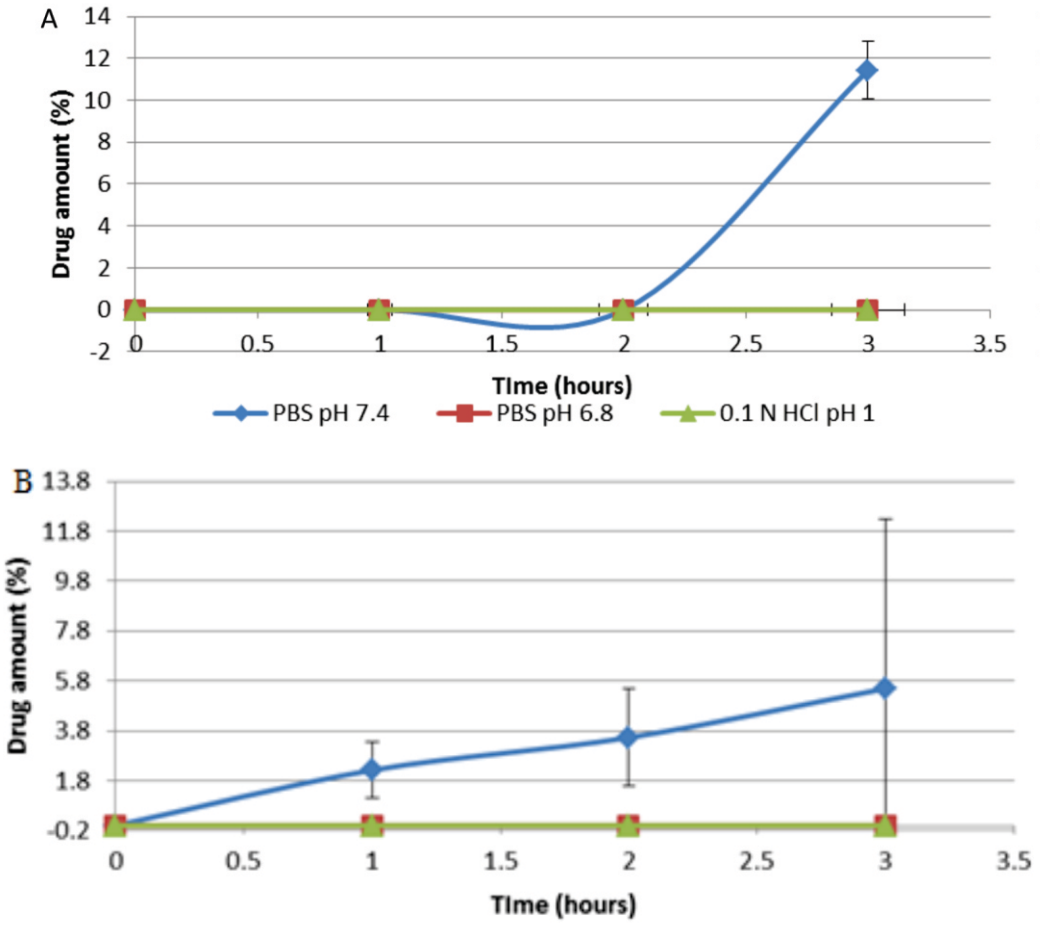
As discussed above, cholesterol imparts rigidity and orientational order and thus, it markedly reduces drug efflux due to the ability of cholesterol to fill the pores in the bilayer vesicles and thus abolishes the gel-liquid phase of the surfactant in the niosome resulting to a less leaky bilayer system. Therefore, cholesterol would aid to sustain the drug release by acting as a membrane stabilizing agent. Release pattern of the drug was affected by the incorporation of the charge inducer, dicetylphosphate. Presence of dicetylphosphate in the niosomal preparation resulted to an increase in the drug release and also extended the release time of the drug [29].

Inclusion of dicetylphosphate produces electrostatic repulsion of the bilayer and thus enhances drug release from the vesicle. Changing the molar ratio of cholesterol



Formulation I containing no charge inducer appears to have nearly spherical morphology compared to Formulation IV containing the charge inducer, dicetylphosphate. The difference in the surface morphology is accounted to the surface charge of the particle resulting to a spherical morphology.

Figure 1. *Micrograph of (A) Formulation I (blank), (B) Formulation II (blank), (C) Formulation I (glutathione loaded), (D) Formulation IV (glutathione loaded) scanned at 20,000X. n = 3*



Legend: Release media: - 1M Phosphate buffer saline pH 7.4; - 1M Phosphate buffer saline pH 6.8; -0.1N Hydrochloric Acid pH 2

Both formulations were noted to release their respective contents at 1M PBS pH 7.4 simulating the blood pH. The difference in the hydrogen ion concentration of the release media and of the niosomal formulation prevented the release of the glutathione.

Figure 2. *Mean Glutathione Release of (A) Formulation I, (B) Formulation IV at 3 h simulation. n = 3. Data Presented as mean + standard deviation. Release media used were 0.1N HCl (pH 2), 1M PBS pH 6.8 and 1M PBS pH 7.4*

Table 4. . *Differential scanning calorimetry results showing the melting points of the binary mixtures (1:1) (in oC)*

Mixture	C	Melting Point		DT	Significant
		F	S	Max: 5%	Yes/No
1	GSH	194.6	190.8	1.9527235	No
	S60	56.59	57.3	1.2546386	No
2	GSH	206.5	203.1	1.6464891	No
	CHO	156.1	150.9	3.331198	No
3	GSH	200.2	200.6	0.1998002	No
	DCP	79.73	79.93	0.2508466	No

Legend: C – Component; F – Fresh Sample; S – after stress test; DT – difference in the melting point; GSH – Glutathione; S60 – Span 60; CHO- Cholesterol; DCP- Dicetylphosphate

Data are presented as average melting points of the samples. n = 3. Samples were subjected to DSC analysis from 50°C to 250°C. Stress Test was carried out at 40°C for 30 days.

The difference in the melting point of the sample indicates possible incompatibility. The significant difference was noted when there is a DT > 5%. Incompatibility results from the thermal method suggest only possible incompatibility thus further testing using non-thermal method is recommended.

and surfactants may also change the release pattern of the drug[27]. In comparison with the previous study of Kamboj *et al.*, (2013), niosomes can serve both as drug depot and as controlled release drug delivery system[10].

Differential Scanning Calorimetry

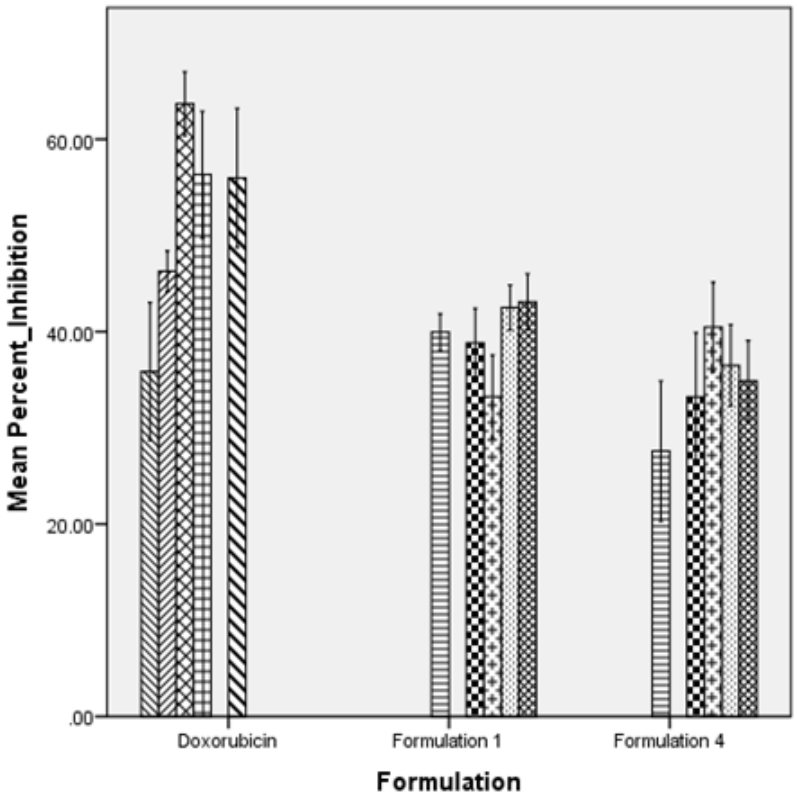
Identification of the presence of drug excipient interaction is a crucial step in preformulation and formulation studies. Presence of incompatibilities resulted to poor stability of drug product. Interactions may alter the physical, chemical and as well as therapeutic property of the active ingredient. Accelerating the interaction of the components is achieved by placing the components and the binary mixtures at 40 °C. Determination of the compatibility testing includes thermal method and non-thermal method. In this study, differential scanning calorimetry was employed to determine drug excipient incompatibility[30].

Drug-excipient compatibility before and after one month storage under accelerated temperature condition (40C) and 75% RH was observed for any change in appearance. All the pure compounds and binary mixtures remained comparable to the initial color (white) and physical state (powder). Likewise, melting point of fresh sample was determined and compared with one-month old samples through DSC (Table 3a and 3b).

No liquefaction after stress test was observed on powdered samples indicating that the mixtures are physically compatible. Change in the melting point however, was evaluated according to ICH criteria[30]. Since % changes from initial value was less than 5%, such changes were deemed insignificant (Table 4).

Cytotoxicity Assay

Cytotoxicity assay was done to assess the potential toxicity of the formulations to normal cells. In this study, Human dermal fibroblast was used. Based on figure 4, both formulations A and D exhibited a non-dose dependent activity wherein formulation A at 200 µg/mL showed 43.09 % inhibition, 42.50 % at 100 µg/mL, 33.24 % at 50 µg/mL, 38.83 % at 25 µg/mL and 39.95 % at 12.5 µg/mL while formulation D at 50 µg/mL concentration showed the highest inhibition which is 40.46 % inhibition, 34.88 % inhibition has been noted at 200 µg/mL, 36.50 % at 100 µg/mL, 33.20 % at 25 µg/mL and 27.60% at 12.50 µg/mL. Formulation A and D exhibited a median inhibitory concentration (IC50) of 41,240 µg/mL and 219 µg/mL, respectively. These IC50 of both formulations



Legends: - 1.25 µg/mL; - 2.50 µg/mL; - 5.0 µg/mL; - 10.0 µg/mL; - 12.50 µg/mL; - 20.0 µg/mL; - 25 µg/mL; - 50 µg/mL; - 100 µg/mL; - 200 µg/mL

Cytotoxicity assay using Human dermal fibroblast was used to assess the in vitro toxicity of the formulations. The positive control, doxorubicin, has an IC50 value of 2.18 µg/ml compared to the IC50 values of formulations A and D which are 41,240 µg/ml and 219 µg/ml, respectively.

Figure 3. *Mean percent inhibition of formulations A and D against human dermal fibroblast proliferation, Doxorubicin was used as positive control. n = 3. Data presented as mean percent inhibition + S.E.M.*

Table 5. *. Stability of the niosomal glutathione under 5°C and 40°C*

Temp (°C)	Pull-Out (d)	Ave GSH (mg)
5	0	9.89
	30	9.78
	60	9.73
	90	9.64
40	0	9.89
	30	9.73
	60	9.69
	90	9.61

Legend: Temp – Stability Temperature; Pull-Out – Sampling day; Ave GSH – average glutathione content

The data are presented as average glutathione content. n = 3. Samples were placed at 5 ° and 40 ° in an oven and were pulled-out after 30, 60 and 90 days, respectively.

A difference in the glutathione content of less than 5% left after every pull-out suggests stability in a particular temperature condition.

indicate that they are nontoxic to the normal human dermal fibroblast as compared to the IC_{50} of Doxorubicin which is 2.18 µg/mL. IC_{50} which are more than 20 µg/mL are considered to be nontoxic[29].

Stability study

Stability is the ability of the drug substance or product to retain its physical, chemical, microbiological and biological properties[30]. Samples remained as white vesicles having no visible aggregation of the niosomes at 30, 60 and 90 d pull out both at 5°C and 40°C. It was also revealed that surface morphologies of the niosomes retained its nearly spherical shape.

Table 5 presents a comparison of glutathione content for samples stored at 5°C and 40°C. Both samples were stable after three months, exhibiting degradation of not exceeding

5% from initial. Using the ICH criterion, the shelf life is estimated at this point to be twice that of apparent stability period i.e. six months. The calculated first order kinetics, 2.8448 x 10-4 mg/d and 3.1911 x 10-4 mg/d for 5°C and 40°C respectively, are yet to be validated through a long term actual stability testing. The stability study conferred with the result of the compatibility testing [30].

Conclusion

Niosomes yield a high entrapment rate for glutathione using Span 60 and cholesterol at a molar ratio of 1:1. Furthermore, addition of 5 µM charge inducer (dicetylphosphate) to the formulation increased its entrapment by 0.71%. Dicetylphosphate increased the particle size by 357.7 nm. Particles of niosomes containing dicetylphosphate tend to have fewer edges as compared to the particles devoid of dicetylphosphate. The formulations of niosome displayed a pH selective release at 1 M PBS pH 7.4. This is due to the difference in the hydrogen ion concentration of PBS pH 7.4 as compared to 0.1 M HCl and 1 M PBS pH 6.8. Glutathione is compatible with its excipients using DSC analysis. Both formulations A was stable at 5°C and 40°C when packed in a colourless glass vial with push on cap sealed with paraffin film following the ICH guidelines for stability testing. Niosomes, made from nonionic surfactant and cholesterol, have the potential for improving the delivery and bioavailability of certain drugs.

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