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In Vitro hemolysis study of human erythrocytes at different time interval period with amphotericin b and amphotericin b in lipid formulations

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ABSTRACT

The main purpose of this research work was to observe the hemolysis of human RBC in different interval of time period at a lower to higher concentrations (1 µg/ml to 8 µg/ml) with pure drug Amphotericin B (AmB) and AmB-lipid formulations. Reconstituted amphotericin B in lipid drug carriers was prepared in the laboratory by the freeze drying process. We have taken five lipid carriers (such as SDC, SDCS, KC, KDC, SC) and prepared five different AmB lipid reconstituted dry powders with salts of Na^+ and K^+ which are highly water soluble and feasible for nebulization. Freeze dried product of AmB in lipid formulations were very light, free flowing and yellowish powder caked formed. This was highly water soluble and dissolved within 1 min with yellowish stable solution formation. The aerosols properties were characterized by the determination of particle sizes in the form of MMAD and FPF. Their toxicity was studied on RBC lysis at 0.5 h, 3 h, 6 h, and 24 h and obtained low toxic to erythrocytes than pure AmB. In hemolysis study, this was shown higher viability of cells with all five AmB in lipid formulations as compared to pure drug (AmB). In five AmB in lipid formations, it can be concluded that SDCS was the best drug carrier on the basis of hemolysis test at different interval of time period.

KEY WORD: Hemolysis**,** RBC, AmB-lipid formulations, lyophilization, lipid carriers.

INTRODUCTION

Amphotericin B (AmB) is a broad spectrum polyene marcrolide antibiotic widely applied in the treatment of severe systemic fungal infection diseases such as Aspergillosis, Cryptococcus, Histoplasmosis and Candidiasis. AmB is a yellow or orange-colored product and originally synthesized from *Streptomyces nodusus* (Lemke, 2005). This drug has been introduced in 1956 to till date because of most effective treatment for severe systemic fungal infections (Adedayo, 1997; Moreno, 2001). This is drug of choice for immuno-compromised patients for fungal infections. It has a unique molecular structure as shown in Figure 1and drug was concentrated on cell membranes. This drug is used more than 50 years for clinical application for treatment of systemic fungal infection, till now there has been reported very few cases of drug resistance in the medical practice (Brajturg, 1990). This is the unique antibiotic as compared to others (antifungal drugs) low chance of drug resistance. Therefore, it is to be considered a gold standard for the treatment of fungal infection. But it has several major clinical limitations due to its severe side effects mainly nephrotoxicity and hematological toxicity. Other minor toxicity such as fever, chills, nausea and vomiting were reported by Darole, 2008. The major problem of this drug is low water solubility and poorly absorbed from gastrointestinal tract, due to that drug was administered by parenterally for the treatment of systemic fungal infection. Its main drawback was side effects due to this therapeutic dose may have to be reduced or some cases treatment may be terminated. AmB has amphiphilic characteristic in nature and due to that it tends to aggregate in aqueous solution because of low solubility in water. AmB exerts its antifungal activity on the cell membrane binding to ergosterol, the most abundant sterol found in the cell membrane of sensitive fungi, creating channels or pores. The consequent increase in cell membrane permeability leads to the leakage of sodium, potassium and hydrogen ions and eventually cell death (Kirchgessner, 2008; Moen, 2009). In mammalian cells, cholesterol is a major membrane sterol, whereas in fungi, amoeba and protozoa of genus lieshmania, it is ergosterol. AmB strongly favors ergosterol over cholesterol (Thornton and Wasan, 2009). The binding affinity of amphotericin B for cholesterol and ergosterol are different due to the minor chemical structure difference of membrane sterols and higher affinity to ergosterol containing membranes (Charbonneau, 2001; Vertut-Croquin, 1983). It was proved by Readio and Bittman, (1982) in egg phosphatidylcholine vesicles. The behavior self-association and aggregation of amphotericin B is very complicated and depends on concentration of drugs as well as its local environment. In aqueous solution, Amphotericin B can exist as the combination of species such as monomers and /or soluble or insoluble aggregates of AmB that causes leakage in human RBC. This aggregated stage of AmB caused toxic effect of drug to cells (Bolard, 1991). This aggregation state of drug can be reduced by adding the surfactants and toxicity can be minimized (Barwicz, 1990). In the literatures survey it was found that, the numbers of researchers have demonstrated that monomeric forms of AmB is nontoxic to mammalian cells than self-aggregated species such as dimers, tetramers, hexamers and octamers forms. For AmB, 1mg/ ml is the critical aggregation concentration which is very low amount in water. When the therapeutic doses of AmB 0.50 to 1.5 mg/kg per day dissolved in water for administration by infused intravenously, drug is highly in aggregated state form, which was highly toxic side effects occurred. Therefore, to minimize this aggregated state, during the last decade, a lot of efforts have been done for development

less toxic drug of novel drug delivery systems by chemical modification (Hoeprich, 1987; Hiemenz and Walsh, 1996). In this system, drug should be given to appropriately targeted therapy that will focus on affected parts of organs (disease site) at appropriate concentration without exposing to other tissues for toxicity. For this purpose, the route of drug administration for the treatment of lung fungal infections is directly delivered into the lungs by nebulization or dry powder inhalation. This delivery system has been successfully done by Gavaldà, 2005 and by Shah and Misra, 2004. In this delivery system, drug concentration is required very low as compared to I.V. infusion, because of directly targeted to the infected area, and reduces the kidney toxicity which was the major clinical problem. Oral inhalation of amphotericin B has been improved the efficacy and tolerability of drug when used prophylactically against aspergillus infection to lung translation patient. Another alternative approaches have been done to reduce drug toxicity by modifying the existing formulation on lipid based formulations of amphotericin B have been developed. After lot of efforts have been done to decrease the drug toxicity (AmB) using liposomes, emulsions and other systems all are based on lipid formulations of Amphotericin B, which are commercially available nowadays in market such as AmBisome®, Amphocil®, Abelcet™. The nephrotoxicity is the major drug toxicity of AmB, can be reduced by using liposomes or by formation of complex with various lipids as drug carriers (Fukui, 2003; Jung, 2009). Due to low toxicity, AmB-lipid formulations can be administered at higher doses and obtain the greater efficacy but these lipid formations have drawbacks are low rate of drug elimination from the body and higher dose administrated may accumulate drug inside the body (Heinemann, 1997; Bekersky, 2000; Fielding, 2001). Besides these, market available lipid formations are highly expensive as compared to conventional AmB (Fugnizone®) and due to high cost, the medical practitioners have limited choice for prescribe the patient for the treatment of systemic fungal infection (Persson, 1992).

To solve these problems (drawbacks), I have tried five lipid drug carriers' sodium deoxycholate, sodium deoxycholate sulfate, sodium cholate, potassium cholate and potassium deoxycholate are taken for amphotericin B carriers for lung fungal infections.

It was hypothesized that these AmB-lipid formulations could reduce drug toxicity (hemolysis). In this manuscript, I have tried to show toxicity of drug on hemolysis of erythrocytes of AmB- lipid formulations and comparing them with that of pure drug (AmB) incubation with RBC at 0.5 h, 3 h, 6 h and 24 h respectively.

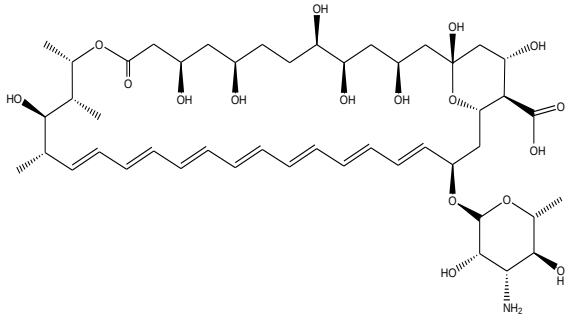

Figure.1. Chemical structure of Amphotericin B

MATERIALS AND METHODS

Materials: The raw material amphotericin B was purchased from Ambalal Sharabhai, Vadodara, India. Sodium deoxycholate sulfate, potassium cholate and potassium deoxycholate were prepared in the laboratory. The remaining two sodium cholate and sodium deoxycholate was purchased from Sigma- Aldrich, Company, USA. All chemical were used in laboratory grade reagents.

Preparation of AmB-lipid dry powder formulations (AmB- SDCS, AmB-SDC, AmB-SC, AmB-KDC and AmB-KC): Sodium deoxycholate sulfate, 245 mg was taken in 100 ml beaker containing 30 mL distilled water then dissolved lipid drug carrier by adding 2.7 ml (0.2 M) of sodium hydroxide to this solution with constant a magnetic stirrer. When solution become clear then added 250 mg of AmB yellowish power slowly by spatula in part wise in the solution. After dissolving the AmB completely it was form a clear yellowish solution. pH of the solution was adjusted by phosphate buffer at 7.4. The final volume was adjusted by adding distilled water to make 50 ml solution and transferred into vial for freeze drying. A yellowish caked powder was formed and similarly other AmB-lipid products were prepared following same procedure.

***In vitro* hemolysis study:** Mehta, (1984) was described for *in vitro* hemolysis study and the experiment was followed the evaluation procedure of human RBC lysis according to him. Human red blood cells (RBC) were isolated from fresh human blood were collected from the Blood Bank, Dept. of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai Thailand. The blood samples were washed three times with phosphate buffer saline solution, PBS. Then the sample was centrifuged with centrifuged machine at 3000 rpm for 5 min at constant temperature 4^0 C. The stock solutions of five AmB-lipid formulations such as AmB-KC, AmB-KDC AmB-SC, AmB-SDC and AmB-SDCS and AmB were added to suspended RBC and the suspension was diluted with PBS to obtain a final

concentration of AmB in the range 1µg/ml, 2 µg/ml, 4µg/ml, 8µg/ml and a final hematocrit of 1 %. The complete solutions containing drug (AmB) and AmB-lipid formulations were incubated in an incubator at temperature 37 ^{0} C for 0.5 h, 3 h, 6 h and 24 h. This solution was transferred into the closed centrifuged tubes and centrifuged to remove unlysed cells from the solution at 3000 rpm for 5 min at constant temperature, 4^{0}C. The hemoglobin in the supernatant was measured by its absorbance was measured using a microplate reader (Biohit BP 800, Helsinki, Finland) at 540 nm. Control sample was prepared without AmB in PBS only and positive sample was prepared by adding 1% TritonX-100 with PBS and cell lysis was 100%. The percent of hemolysis was calculated by equation given below:

% Hemolysis = [Abs- Abs_0/Abs_{100}-Abs_0] ×100

Where: Abs is the absorbance of sample

Abs_0 is the absorbance of negative control

Abs_{100} is the absorbance of positive control

Statistical analysis: In this manuscript, data were mentioned as mean ± SD (standard deviation) from at least three samples were taken in experiment unless indicated. A significance of level of p-value < 0.05 was considered statistically significant value.

RESULTS AND DISCUSSION

All five lipid compounds were white and amorphous powders in nature and highly water soluble. Out of them, three were prepared in the laboratory i.e. sodium deoxycholate sulfate, potassium cholate and potassium deoxycholate. Two others, sodium cholate and sodium deoxycholate were purchased from commercial market. All five AmB-lipid formulation were very light, free flowing and yellowish color in powder as well as hygroscopic in nature prepared by freeze drying process. These products are highly water soluble and stable in solution form and the physical characteristic was shown in Figure 2. Amphotericin B in lipid formulations were prepared in the mole ratio of drug (AmB) and lipid drug carriers were 1:2 and 4 moles of amphotericin B combined with 8 moles of lipid drug carriers by forming a complex compound. Amphotericin B was solubilized by lipid drug carrier by forming micelle. This micelle play vital role for stabilizing the amphotericin B in water or aqueous solution and to prevent the aggregation state and existed in monomeric form, this form was low toxic than other forms such as dimeric, tetramer, or hexamer forms. In between AmB and lipid carriers such as SDCS, KDC, SDC, SC and KC were formed hydrogen bonding interaction different types of cations and anions make into potential with rationale design of lipids. Therefore, these materials are considered as carrier to formulate AmB-lipid reconstituted dry powder formulation as micro-particulate powders for nebulization to treat lung fungal diseases (Gangadhar, 2014).

(A) (B) (C)

Figure.2. A. Reconstituted AmB-lipid dry powder cake formed, B. After breaking the cake powder was formed, C. Reconstituted dry powder into distilled water

***In vitro* hemolysis study:** *In vitro* hemolysis study was carried out on RBC and evaluated by reported method as mentioned in experimental design section. Erythrocytes treated with five AmB-lipid formulations and AmB showed some hemolysis as shown in Fig.3 (A, B, C &D) in different AmB concentration range from 1 µg/ml to 8 µg/ml at the interval of time period was, 0.5 h, 3 h, 6 h and 24 h, respectively.

The similar process was followed for determination of five lipid drug carriers (KC, KDC, SC, SDCS and SDC), toxicity to the erythrocytes were treated with these carriers at concertation range from 1 µg/ml to 8 µg/ml in time period intervals, 0.5 h, 3 h, 6 h, 24 h respectively. It was found nontoxic from lower level to higher level of concentration (8 µg/ml), whose values cell lysis were less than 1% (data was not presented). Therefore, it was confirmed by the test of hemolysis with lipid carriers were found nontoxic at a concentration range from 1µg/ml to 8 µg/ml in different time intervals, 0.5 h, 3 h, 6 h and 24 h. In our study, AmB drug concentration range was selected from 1 µg/ml to 8 µg/ml on the basis of reported by Yu, (1998). The safety level of hemolysis was reported by Yu, (1998), was less than 5%. We have studied 8- fold higher value than normal range, this value was quite safe to know the toxic effect of drug.

In similar way, we studied with increased time of exposure to pure drug (AmB) and AmB-lipid formulations at the interval started from 0.5 h, 3 h, 6 h and 24 h, it was shown that hemolysis was depended on the time of exposures was shown in graph in Fig.3 (A, B, C, and D). we have shown only higher concentration of AmB (8

µg/ml), on this concentration, the hemolysis was obtained 2%, 3.3%, 8% and 17% at 0.5 h, 3 h, 6 h and 24 h respectively. In 24 h, the hemolysis was obtained 17%, which was the highest at AmB (8 µg/ml).

The hemolysis study was done at 0.5 h, AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC, AmB-SC and AmB, each at a concentration of 8 µg/mL AmB, was added to the RBC, then hemolysis data was observed at 0.5 %, 1.1%, 0.6%, 1.1%, 1.2% and 2.0%, respectively. The graphs were shown in Fig. 3A that five AmB-lipid formulations produced less hemolysis than pure drug (AmB). The hemolysis study was done at 3 h, AmB-SDCS, AmB-S DC, AmB-KDC, AmB-KC, AmB-SC and AmB, each at a concentration of 8 µg/mL AmB, was added to the RBC, then hemolysis data was observed at 0.7 %, 1.3%, 1.0%, 1.8%, 1.2% and 3.3%, respectively. The graphs were shown in Fig. 3B that five AmB-lipid formulations produced less hemolysis than pure drug (AmB). The hemolysis study was done at 6 h, AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC, AmB-SC and AmB, each at a concentration of 8 µg/mL AmB, was added to the RBC, then hemolysis data was observed at 2.8 %, 3.8%, 4.5%, 3.7%, 3.6% and 8.0%, respectively. The graphs were shown in Fig. 3C that five AmB-lipid formulations produced less hemolysis than pure drug (AmB). The hemolysis study was done at 24 h, AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC, AmB-SC and AmB, each at a concentration of 8 µg/mL AmB, was added to the RBC, then hemolysis data was observed at 9%, 14%, 14%, 14%, 13% and 17%, respectively. The graphs were shown in Fig. 3D that five AmB-lipid formulations produced less hemolysis than pure drug (AmB). The different time period interval graphs were shown in Fig. 3 (A, B, C and D) and it was observed that the longer duration of time to exposure of RBC produced higher rate of hemolysis of cells than shorter time period of exposure of RBC though the concentration of AmB was same. This proved that time play important role for hemolysis of cell. The appropriate time for exposure to RBC for hemolysis study was 24h, which was applicable for clinical uses. Therefore, we considered, Fig. 3 D was therapeutically effective time period and we had chosen the possibility of maximum to observe toxicity test. Among these five formulations, AmB-SDCS was least hemolysis than others.

In comparison among the five AmB-lipid formulations, AmB-SDCS was observed less hemolysis than other four AmB-lipid formulations. This was due to the sulfate ions interact of lipid drug carrier with amphotericin B drug molecule which formed the complex compound in between drug and carrier molecules and stabilize this by micelle formation in aqueous solution. Because of that, slow release of free drug molecule AmB and free form AmB is less available to formation of dimeric form, which causes for hemolysis. The ratio of the AmB-associated micelle to the monomeric free AmB of the AmB-SDCS in isotonic dextrose solution was shown to be 14:1. In contrast, the ratio of the self-associated AmB to the monomeric free AmB of the pure AmB in isotonic dextrose solution was about 5:1 reported by Adhikari, 2015. Therefore, the AmB-SDCS resulted in a lower toxicity as a similar observation in the AmB nanomicelle formulation reported by Diaz, 2015. Similar report was observed on lecithin-stabilized emulsions studied by Forster, 1988. Similar study was done by Chuealee, 2011 on various cholesterol derivatives were used for preparation of amphotericin B dry powder formulations and which were used on hemolysis and found that AmB was stabilized in a similar manner and hemolysis was prevented.

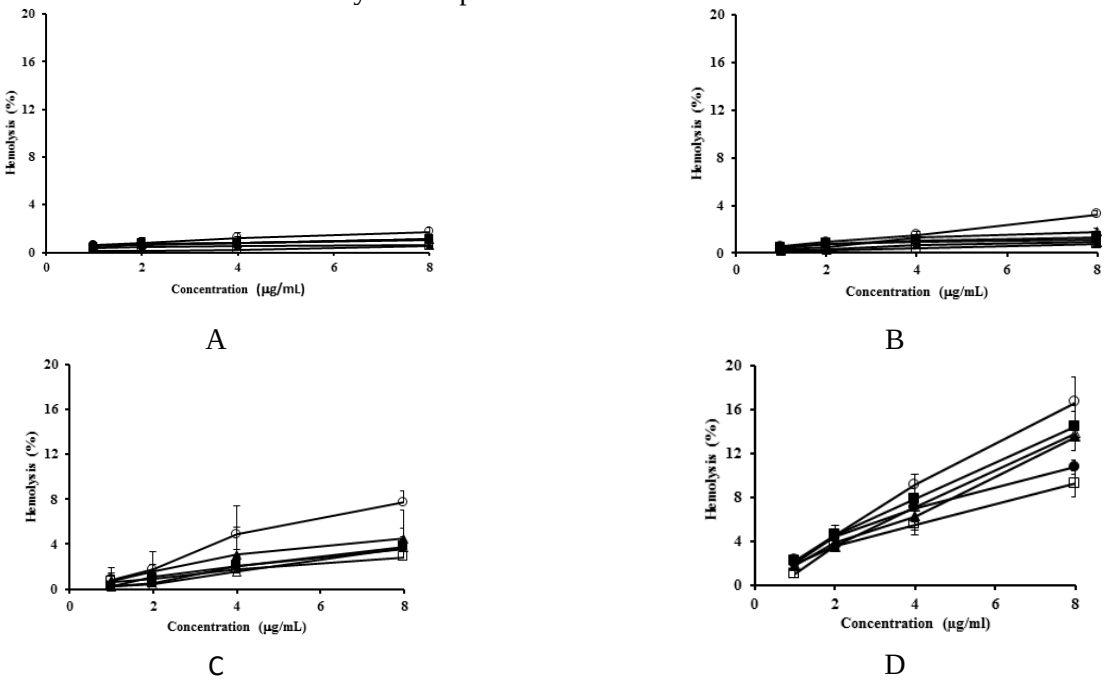

Figure.3.*In vitro* hemolysis after incubation with, AmB-SDC (■), AmB-KC (Δ), AmB-KDC (▲), AmB-SDCS (□), AmB-SC (●) and AmB (O) at mole ratio 1:2 at 0.5 h (A), 3 h (B), 6 h (C) and 24 h (D), (mean ± SD, n=3)

CONCLUSION

KC, KDC, SC, SDC and SDCS were taken as five lipid drug carriers, which were applied to develop as lipid drug carriers system as a reconstituted dry powder AmB-lipid formulations. Lipid drug carriers were playing vital role for dissolving poorly water soluble drug (amphotericin B) into highly soluble and stable solution form. This was due to the forming of micelle with negatively charge developed in solution. The particle sizes were found between 17 to 74 nm and zeta potential was above -30 mV for all formulations and among these, AmB-SDCS was the highest -45 mV was reported by Gangadhar, 2014. The study of hemolysis on RBC was postulated that, the viability of RBC was higher in all five amphotericin B in lipid formulations as compared to pure drug (AmB). Lipid carrier SDCS was less toxic to RBC than other four lipid carriers such as SDC, SC, KC and KDC. All AmB-lipid formulations were low toxic than pure AmB. Therefore, lipid drug carrier was shown as an alternative process to development of reconstituted dry powder AmB-lipid formulations to treat pulmonary aspergillosis by nebulization.

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