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Formulation of Amphotericin B-lipid derivatives and feasibility for nebulization and their stability study

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ABSTRACT

The aim of study was water insoluble amphotericin B (AmB) to be converted into highly water soluble and stable form of solution or micronized suspension form for nebulization. Amphotericin B (AmB) is the drug of choice for most commonly used to treat life-threatening conditions such as cryptococcosis, histoplasmosis, and invasive pulmonary aspergillosis. AmB in lipid drug carriers (SDCS, SC, SDC, KC and KDC) were used to prepare reconstituted dry power formulations by lyophilization process (freeze drying). Among five lipid carriers, KC and KDC were successfully synthesized in a laboratory and characterized by FTIR. AmB-lipid derivatives were reconstituted with distilled water to obtain 5mg/ml of AmB. Their physicochemical parameters (particle size, zeta potential and UV spectroscopy) were monitored. In these formulations all particles were quite stable in size range 17.2 to 73.9 nm during one week after reconstitution. Zeta potential values were found in range between – 29.17 to – 45.53 mV. The nebulizer flow rate was 8 L/min for 2 min and air flow rate in ACI was 28.3 L/ min was operated by vacuum pump suction. The MMADs of all AmB-lipid formulations were obtained between 1.70-2.05 µm with high FPF (70-80%) were characterized by using jet nebulizer (West med) and Andersen Cascade Impactor (stages 0 to 7) (ACI) in vitro study. The results suggest that all AmB-lipid formulations are uniform and stable with in the storage period of six months in the air tight opaque container in a refrigerator condition (2-8ºC). The drug content and assay was carried out by HPLC method. An assay of the AmB showed that the all AmB-lipid formulations contents were closed to 100%. Among different lipid derivatives, sodium deoxycholate sulfate (SDCS) gave the highest particle size and zeta potential value. UV spectra were recorded between 300 to 450 nm, it was confirmed that no markedly visible shifted spectra were observed during first and seven days of reconstituted samples.

Keyword: Nebulizer, Size Characterization, Lipid derivative, Amphotericin B, and MMAD.

INTRODUCTION

Lung fungal infections caused by *Aspergillus* species, which is the prime cause of morbidity and mortality for immunocompromised patients [1]. It is commonly found in the patients who are severely immunocompromised and categorized as nosocomial infection [2]. Poor lung function coupled with immune suppression resulting from lung transplantation can predispose patients to infections caused by *Aspergillus* [3]. Mortality among infected patients is high death rate in excess of 90% has been shown in study [4]. Most of these invasive mould infections are acquired through the respiratory tract [5]. AmB is the drug of choice for the treatment of lung fungal infections caused by *cryptococcosis*, *candidiasis*, *histoplamosis*, and invasive *aspergillosis* [6]. It is a natural polyene macrolide antibiotic that consists of seven conjugated double bonds, an internal ester, a free carboxyl group and a glycoside side chain with a primary amino group [1] and originally synthesized from *Streptomyces nodosus*, yellow colored product [7]. It is insoluble in water, alcohol and soluble in very few organic solvents: dimethylsulphoxide (30-40 mg/ml) and dimethylformamide (4 mg/ml) [6]. Its water solubility at physiological pH (6-7) is less than 1 µg/ml but it increases at pH < 2 or pH >11. However, in these extreme pH conditions AmB is not stable and AmB may form salts with better solubility but its antimycotic activity is less than the basic forms [8]. It is sensitive to light, high temperature

above 8°C and should be protected from them. Oral dosage form of AmB has not been absorbed from gastrointestinal tract and poor bioavailability, because of that it has been administered by intravenously, can cause fever, chills, hemolysis, vomiting and nephrotoxicity. These adverse effects are manifested due to a narrow therapeutic window of AmB [9]. 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, hydrogen ions and eventually cell death [2, 10] . The conventional dosage form Fungizone® consists of mixed micelles of AmB with sodium deoxycholate. The newer formulations are formulated incorporating AmB to lipids which reduce the nephrotoxicity and possible to be given higher doses of AmB [9]. Lipid formulations have been reported to have excellent safety and efficacy but cost of treatment is very expensive as compared to AmB-deoxycholate [11]. At present, two different types of formulations are available in market, first one is a micellar solution of AmBD (Fungizone®), which is still widely used in developing countries due to its low cost though it has been severe nephrotoxicity and hemolysis. The second one is less toxic but high cost products are lipid based formulation such as Ambisome®, Amphocil® and Abelcet® [12]. To reduce the drug toxicity, nebulizer is an alternative and attractive option, as high local drug concentrations are achieved with minimal systemic exposure [13]. Air–jet nebulizers are well established in pulmonary drug delivery [14]. Nebulizer administration delivers drug directly to the lungs to allow concentration of AmB at the site of infection and equally important, reduces systemic exposure. As a result, nebulizer administration of AmB maximizes efficacy and limits toxicities associated with AmB [15]. The physical properties of AmB formulations, one of the most important factors determining deposition into the small airways and alveoli are the particle size or mass median aerodynamic diameter (MMAD). The optimal size for aerosol drug delivery is 1 to 5 µm. Particles that are 1 µm or less are likely to be eliminated during exhalation, whereas particles that are 5 µm or greater are deposited into the oropharynx and swallowed [16]. The main advantages of jet nebulizers are inexpensive, disposable, do not require special equipment and trained personnel as well as can be used for emergency management [17]. These formulations are especially designed for nebulization with improved efficacy and tolerability, inhalation of AmB can be used prophylactically against *Aspergillus* infection following lung transplantation [18]. To achieve best result of aerosolization, small aerosolized particle sizes with MMAD ranging from 0.90 to 2.43µm are required [19]. Unlike the oral route of drug administration, pulmonary inhalation is also not subjected to first pass metabolism. However, solubility and stability are important physicochemical characteristics that affect absorption of the drug and its therapeutic effectiveness. The consequences of poor aqueous solubility can lead to failure in the development of suitable formulations. AmB is amphoteric and can form salts in acidic or basic media, both of which are more water soluble, but have lower antifungal activity than the parent drug. However, sodium deoxycholate forms only a weak micelle and it has poor stability after reconstitution. Therefore the reconstituted preparation has to be used immediately or shortly after formation. Therefore, in the present work, we hypothesized that lipid derivatives could be a better compound to improve the solubility and stability of AmB as a nanoparticulate colloid after reconstitution from a lyophilized dry powder. The physicochemical characteristics of the reconstituted powder such as its pH, particle size and zeta potential were evaluated. The stability of AmB in the reconstituted colloid was determined by a UV absorption spectrometer. The content and assay was analysis by HPLC method. After nebulization, the particle size of both formulations was determined by mass median aerodynamic diameter (MMAD). Our AmB-lipid based formulations were suitable for pulmonary drug delivery system via nebulization, preparing particle size ranging from 1.70- 2.05 µm, drug can be deposited deeply inside the lung for treatment of lung fungal infections.

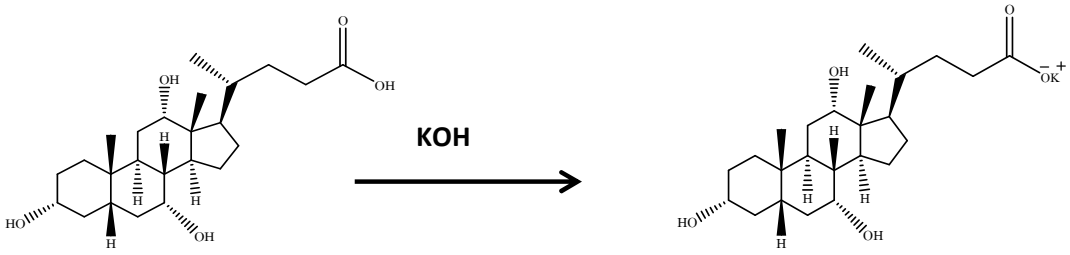
EXPERIMENTAL SECTION

Materials

AmB was obtained from Ambalal Sharabhai Enterprises Pvt. Ltd., Vadodara, India. Deoxycholic acid, cholic acid, sodium cholate and sodium deoxycholate were purchased from Sigma-Aldrich, St. Louis, USA. Sodium deoxycholate sulfate, potassium cholate and potassium deoxycholate were synthesized in a laboratory. Acetonitrile and methanol were purchased from Labscan Asia, Bangkok, Thailand. Dimethylsulfoxide was purchased from Riedel-de Haën, Seelze, Germany. All chemicals were used as received without further purification except tetrahydrofuran (THF). All other reagents and chemicals are analytical grade.

Synthesis of potassium cholate (KC)

The distilled water 20 mL was taken in a beaker (50 mL) and 0.207 g potassium hydroxide and 1.513 g cholic acid (CA) were added to this solution with constant stirring (500 rpm) in a magnetic stirrer Heidolph MR Hei-Mix L (Helodolph Instruent, Schwabach, Germany) for overnight. Cholic acid was insoluble in water but after reaction, potassium cholate was water soluble. After completion of reaction (Fig. 1), the solution was poured into a separating funnel and left overnight for sedimentation and clear solution portion was filtered 0.45 µm size and filled into 10 mL each vial for lyophilization by a freeze dryer (Dura Dry™ MP, FTS Systems Inc., NY, USA). The white amorphous powder was formed.

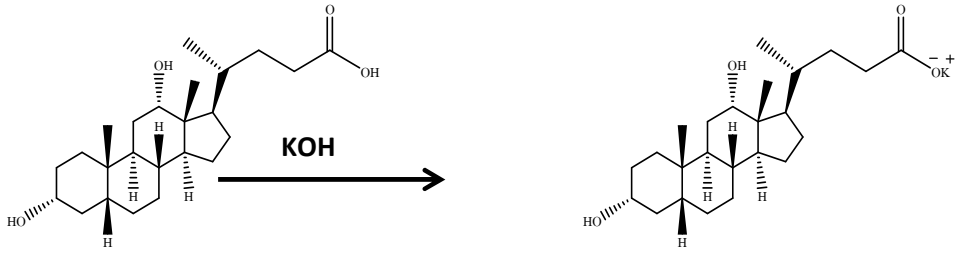


Cholic acid **Potassium cholate**

Figure 1 Synthesis of potassium cholate from cholic acid [20] (Morrison and Boyd, 1987)

Synthesis of potassium deoxycholate (KDC)

The distilled water 20 mL was taken in beaker (50 mL) and 0.197 g potassium hydroxide and 1.379 g deoxycholic acid (DCA) were added to this solution with constant magnetic stirring (500 rpm) in a magnetic stirrer Heidolph MR Hei-Mix L (Helodolph Instruent, Schwabach, Germany) for overnight. Deoxycholic acid was insoluble in water but after reaction, potassium deoxycholate was water soluble. After completion of reaction (Fig. 2), the solution was poured into a separating funnel and left overnight for sedimentation and clear solution portion was filtered 0.45 µm size and filled into 10 mL each vial for lyophilization by a freeze dryer ((Dura Dry^{TM} MP, FTS Systems Inc., NY, USA). The white amorphous powder was formed.



Deoxycholic acid **Potassium deoxycholate**

Figure 2 Synthesis of potassium deoxycholate from deoxycholic acid [20] (Morrison and Boyd, 1987)

Characterization of synthesized lipid derivatives dry powder (KC and KDC) by FTIR.

FT-IR technique was used to determine the interaction and complex formation between drug and lipid derivatives carrier. FT-IR graph provides the useful information about the presence of functional groups in complex. Shifts of wave number or intensity change in the characteristic of substance indicating the complex existence. Each spectrum was recorded in the frequency range of 4000-400 cm^{-1} and 16 scans were obtained at 4 cm^{-1} resolution by using PerkinElmer precisely (PerkinElmer Inc., Hercules, CA, USA). One mg of AmB, lipid derivatives dry powder was mixed with dry KBr (10 mg). The KBr disc was prepared by compressing the KBr-KC and KBr-KDC mixture of powder under hydraulic press of 10 tons. The mixture pellet was taken for FT-IR spectrum instrument.

Preparation of AmB-lipid dry powder formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS)

Sodium deoxycholate (SDCS, 245 mg) was taken in beaker (100 mL) containing 30 mL distilled water and added sodium hydroxide (2.7 mL, 0.2M) to this solution with constant stirring (500 rpm) in a magnetic stirrer Heidolph MR Hei-Mix L (Helodolph Instruent, Schwabach, Germany). After obtained clear solution, then amphotericin B powder was slowly added (AmB, 250 mg) in part wise. When AmB was dissolved completely, then it was formed a clear yellowish color solution at room temperature. Now the pH of the solution was adjusted by adding phosphoric acid (0.2 M) to obtain a pH of 7.4 for an *in situ* phosphate buffer using pH meter (Precisa pH 900, Dietikon, Switzerland). The final volume of the solution was made to 50 mL by adding distilled water. The solution was filled into 10 mL on each vial and lyophilization by a freeze dryer (Dura Dry^{TM} MP, FTS Systems Inc., NY, USA). The yellowish caked powder was formed. A similar methodology was employed to prepare the sodium deoxycholate (AmB-SDC), potassium deoxycholate (AmB-KDC), potassium cholate (AmB-KC) and sodium cholate (AmB-SC) formulation as that of deoxycholic acid as well as cholic acid and AmB.

Determination of particle size and zeta potential after reconstituted of dry powder AmB-lipid derivatives:

Five formulations of lipid derivatives of AmB dry powders were taken in five test tubes and added filtered distilled water at concentration 5 mg/ml to each test tube. Then measured particle size and zeta potential of the solution by Zetasizer, Nano-ZS instruments (Malvern, Worcestershire WR14 1XZ, U. K.) at 25°C. Each experiment was carried out triplicate.

Stability of AmB-lipid derivatives powder formulations with UV spectrum:
In order to determine the predominant aggregation state of AmB, five lipid derivatives of lyophilized dry powers AmB were reconstituted with distilled water. The final drug concentration was made 10 µg/ml of AmB. The UV spectrum was measured using Thermo Genesys 6 UV-Visible Spectrophotometer between 300 to 450 nm.

Aerosol properties of the reconstituted AmB-lipid dry powders
Around 60 mg of AmB-lipid derivatives (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) lyophilized dry powder (i.e., equivalent to 30 mg of AmB) was reconstituted with 6 mL filtered distilled water (5 mg/mL of AmB) for nebulization. The 6 mL solution was poured into a reservoir of a jet-nebulizer (Westmed Inc., Arizona, USA) and connected to a compressed nitrogen gas cylinder and the gas flow was adjusted to 8 L/min. Then the mouthpiece of the jet nebulizer was connected to an eight stage Andersen Cascade Impactor (ACI), (Atlanta, GA, USA). The ACI was operated at a vacuum flow rate of 28.3 L/min. First, the nebulizer was operated for one min and the aerosol generated was directed into a fume hood. Following this nebulization period, the nebulizer was operated for a 2 min period to the ACI. The mass median aerodynamic diameter (MMAD) was calculated during the 1 to 3 min nebulization time interval. The fine particle fraction was calculated from the AmB deposited on each stage from 1 to stage 7. All the nebulization was carried out at room temperature to avoid any temperature effects on the deposition of the particles. Five experiments were conducted on each formulation. The drug deposited on each of the stages (0 to 7 stages) and the metal inlet of the ACI was extracted by rinsing with 25 mL of DMSO and methanol (1:9 ratios v/v) solution. The drug deposited on each stage was determined by high-performance liquid chromatography (HPLC). For the HPLC conditions, acetate buffer (20 mM, pH at 7.2) and acetonitrile (60:40 v/v) at a flow rate of 1 mL/min is used as the mobile phase. The microbondapak C_{18} column (Phenomenex®, USA) (150 x 4.6 mm i.d., 5 µm) was the stationary phase. UV detection was at a wavelength of 405 nm.

Stability Studies on storage of AmB-lipid formulations.
Five reconstituted dry powder AmB-lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) were prepared by (freeze drying) lyophlization process. Theese products are very light, free flowing powder and hygroscopic in nature. So, they should be kept in the airtight amber vials, protection from light and store at 2-8°C in refrigerator. The stability on storage under the following conditions was assessed:

I. 2-8°C for 6 months in refrigerator
II. 30°C±3°C for 6 months in desiccator at normal room temperature

The samples were withdrawn at initial period and 6 months intervals from the condition I storage condition and drug was not stable in room temperature so condition II storage samples test was not conducted. The products were reconstituted in distilled water at a concentration 5mg/mL of AmB and accessed in particle size, zeta potential, and drug content.

Statistical analysis
Data were presented as mean ± standard deviation (SD) from at least three samples unless indicated. The data were compared using Student's *t* test for independent samples and by analysis of variance (ANOVA). All statistical comparisons were calculated using the SPSS software version 16.0 (SPSS Inc., Chicago, IL). A significance of level of p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Synthesis of potassium deoxycholate (KDC) and potassium cholate(KC)
The two synthesized lipid carriers, potassium cholate (KC) and potassium deoxycholate (KDC) were obtained from cholic acid and deoxycholic acid as the starting material in a simple acid base reaction in the presence of base potassium hydroxide, respectively. The chemical structure in Figure 1 and 2 associated with the compounds was invested by using infrared spectral analysis. The final compound (KC) was confirmed by the FTIR, where carboxylic acid group of cholic acid (v_{max} 1715 cm^{-1}) was replaced by carbonyl group in the spectra (v_{max} 1640 cm^{-1}) (Fig. 3). Similarly, The final compound (KDC) was confirmed by the FTIR, where carboxylic acid group of deoxycholic acid (v_{max} 1716 cm^{-1}) was replaced by carbonyl group in the spectra (v_{max} 1641cm^{-1}) (Fig. 4). Similarly third synthesized lipid carrier sodium deoxycholate sulfate (SDCS) was obtained from deoxycholic acid in three steps of reaction process known as esterification, reduction and sulfation, it was reported [21] .

The yields of KC and KDC were obtained 80 and 82% respectively. These compounds were white amorphous powders and highly water soluble. AmB-lipid formulations were prepared from lyophilization process, which were yellowish powders very light and free flowing. These products were highly water soluble and stable in solution form as shown in Figure 5. These are hygroscopic in nature and sensitive to light. Therefore, they should be stored in

airtight amber bottles below 8 °C in a refrigerator. AmB has amphiphilic behavior due to the apolar and polar sides of the lactone ring. The presence of ionizable carboxyl and amine groups as a consequence of its amphiphilic, zwitterionic nature, the asymmetrical distribution of hydrophobic and hydrophilic groups, AmB is poorly soluble in many organic solvents. During the AmB-lipid formulations, AmB and lipid carriers mole ratio were 1:2 and four moles of AmB combined with eight moles of lipid carriers formed complex compound AmB was solubilized by lipid carrier due to the formation of micelle and this micelle stabilized the AmB to prevent the aggregation of AmB in water and existed in monomeric form, which was less toxic than dimeric or tetramer or hexamer forms. In between AmB and lipid carriers such as SDCS, KDC 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 chosen as carrier to formulate AmB micro-particulate powders of reconstituted of nebulization for the treatment of lung fungal infections.

Fig.3 FTIR of potassium cholate (KC)

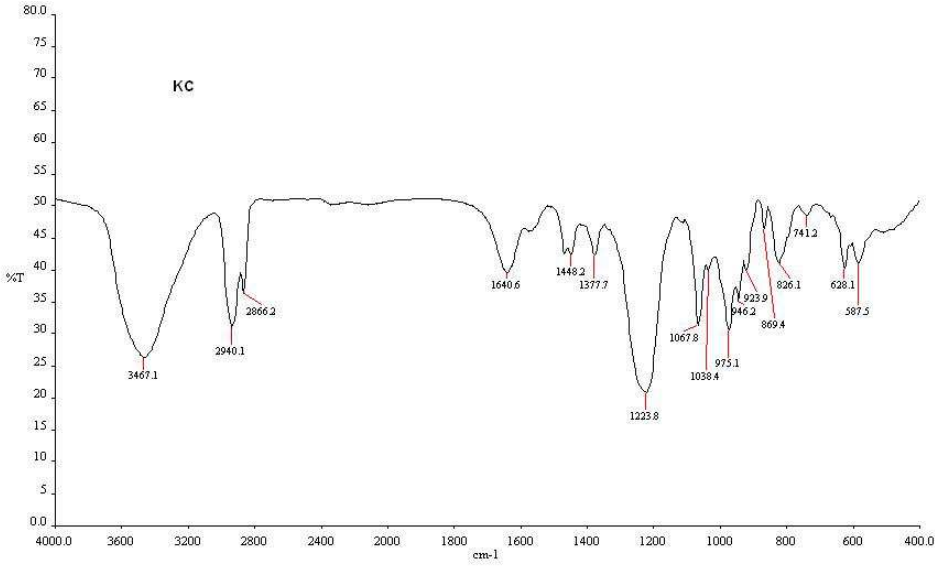
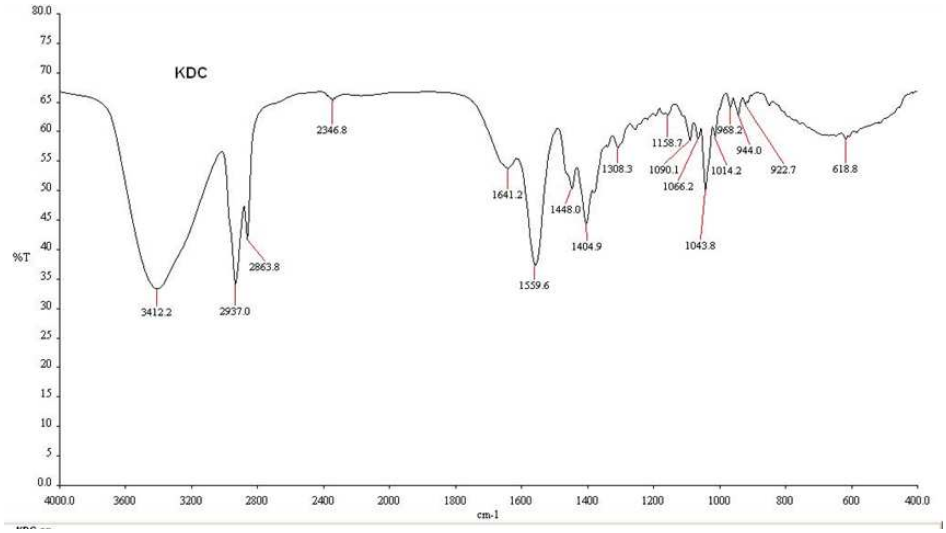


Fig. 4 FTIR spectra of potassium deoxycholate (KDC)



Physical stability

Amphotericin B (AmB) in lipid drug carriers were used to prepare reconstituted dry power formulations by lyophilization process (Freeze drying). AmB-lipid formulations (AmB-KC, AmB-KDC, AmB-SC AmB-SDC and AmB-SDCS) formed solid caked, which was a very light, free flowing, hygroscopic in nature as shown in Fig. 5 (A, B, C). AmB-lipid dry powders are highly water soluble. The amount (around 100 mg) of AmB-lipid dry powder (i.e. equivalent to 50 mg of AmB) was dissolved in 10 mL of distilled water. All reconstituted AmB-lipid dry powders were completely soluble within 1 min as shown in Fig. 5 D. AmB-lipid derivatives were reconstituted with distilled water to obtain 5mg/mL of AmB. Their physicochemical parameters (pH, particle size, zeta potential and UV

spectroscopy) were monitored. In these formulations all particles were quite stable in size range 17.2 to 73.9 nm during one week after reconstitution as shown in Fig. 6 A. Zeta potential values were found in range between – 29.17 to – 45.53 mV as shown in Fig. 6 B and the range of pH was 7.4 to 7.8. Among these five AmB-lipid formulations, sodium deoxycholate sulfate was the highest particle size and zeta potential value. UV spectra were recorded between 300 to 450 nm, it was confirmed that no markedly visible shifted spectra were observed during first and seven days of reconstituted samples as shown in Fig. 7 A and 7 B, respectively.

The particle size and particle distributions are crucial parameters for pulmonary applications to achieve high efficacy and a targeted administration by inhalation [22]. The obtained mean particle size of five formulations was uniform and stable because an electrical double layer developed and they repelled similar charges so there was no agglomeration of particles. Nanoparticles with a high zeta potential (i.e., either positive or negative charges) are one of the important parameters that could play a significant role either for a targeted therapy or the stability of the drug formulations required for its effectiveness as a nanomedicine [23]. If the particle had a low zeta potential value then there would be no force to prevent the particles from flocculating or aggregating due to Van Der Waals inter-particle attractions. In our results, five formulations the AmB-SDCS, AmB-KDC, AmB-KC, AmB-SC and the AmB-SDC nanoparticulate preparations had a high zeta potential (-30 mV) resulting in a highly stable system. The magnitude of the zeta potential provided an indication of the potential stability of the colloidal system [24].The high zeta potential of the AmB-SDCS formulation was not its only satisfactory property but also the particles were distributed homogenously in the solution [25], which was supported by the low polydispersity index (PDI) of the AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC and AmB-SC (0.34 ± 0.06, 0.33 ± 0.06, 0.68 ± 0.06, 0.57 ± 0.03, and 0.26 ± 0.05, n=3, respectively). It was also found that the AmB-SDCS was more stable than that of the AmB-SDC, AmB-KDC, AmB-KC, and AmB-SC. In the aqueous dispersion medium of 59 mM phosphate buffer over the pH range of 7.4-7.8 (i.e., physiological pH).

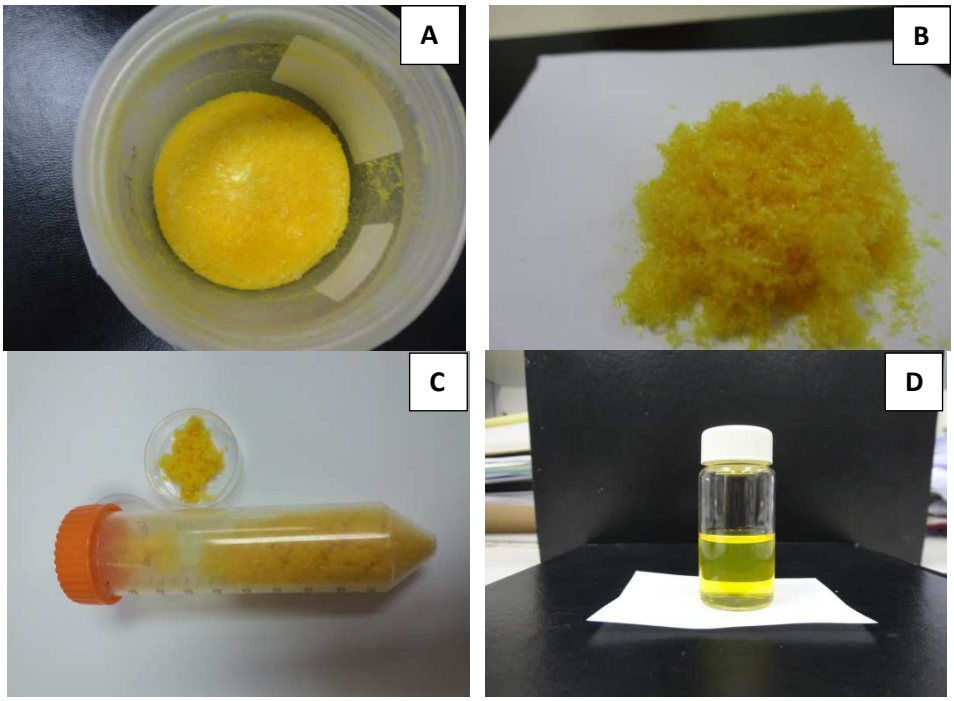


Figure 5. Reconstituted AmB-lipid dry powder caked formed (A), after breaking caked and formed powder (B), stored in the container (C), reconstituted dry powder into distilled water (D)

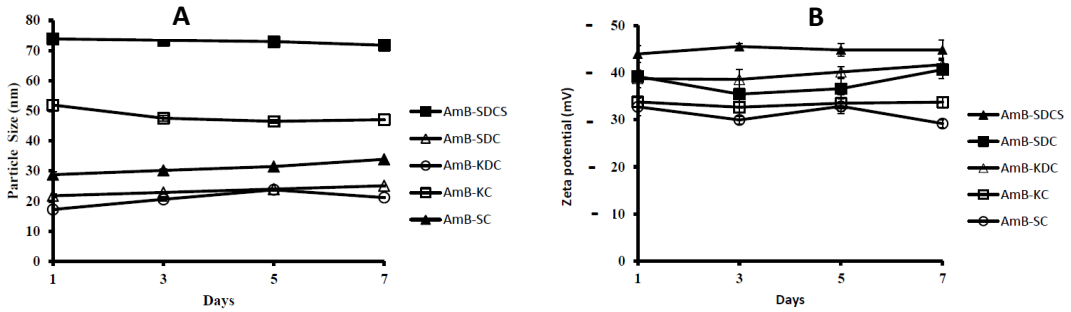


Figure 6. Particle size and the zeta potential of the reconstituted AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC and AmB-SC dry powder in distilled water during 7 day at a conc. 10 mg/mL of amphotericin B

Chemical stability

The aggregation of AmB was dependent on various parameters involved in the stability of AmB such as its concentration, pH, temperature, ionic strength of the aqueous phase and excipients used in the formulations [26]. The molecular state of AmB in an aqueous medium may be the result of self–aggregation. AmB itself is not water soluble and requires organic solvents such as methanol or dimethyl sulfoxide to dissolve [27, 28]. The monomeric-AmB is less toxic than the dimeric-AmB [29]. However the water soluble aggregated state of the dimeric–AmB seems to be the more stable form [30]. The UV absorption spectrum of the AmB-SDCS nanoparticulate solution was similar to that of the AmB-SDC. The state of aggregation of the AmB was directly related to its toxicity. The predominant aggregation state of the AmB formulation can be detected by the size of the particles (Fig.6 A) and UV spectrophotometry as shown in Fig. 7. The UV spectrum of AmB in the aqueous phosphate buffers pH 7.4 exhibited an absorption maximum (Fig. 7). Therefore, AmB in water formed a mixture of water soluble monomers and aggregates. It is important to note that the phosphate buffer was formed *in situ* by reconstitution of the formulations with water. Some AmB molecules were present in the dimeric forms that were characterized by a peak at 328-331 nm. The other smaller intensity bands at 360-363, 383-385 and 408-410 nm were from the monomeric form. Although some aggregation of AmB was observed, the spectra did not change from the 1st to the 7th day. From the UV spectra, there was a decrease in the intensity during the first few days (2nd day and 3rd day) but there was no markedly visible shift of the spectra. The AmB content of reconstituted dry powder formulations of AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC and AmB-SC at initial were 100.2 ± 0.61, 98.0 ± 0.20, 94.3 ± 0.3, 96.9 ± 0.4 and 93.4% ± 0.4% respectively. Whereas on day 7, the content of AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC and AmB-SC decreased significantly (90.2 ± 0.37, 85.9 ± 0.36, 83.3 ± 2.4, 85.7 ± 0.9, and 85.3% ± 1.3%, respectively). This revealed that the longer storage of 7 days caused some degree of AmB degradation. These results may be used to predict the stability of the AmB formulations after reconstitution. It was found that all lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) were unstable without any shift of the spectra. A comparison of the all formulations showed that by day 7, the intensity of the AmB-SDCS had decreased less than that of the AmB-SDC, AmB-KDC, AmB-KC and AmB-SC. Here, spectra were not shifted and slightly decreased intensity. Therefore, all AmB-lipid formulations were unstable after reconstitution and should be immediately used after dilution of solution.

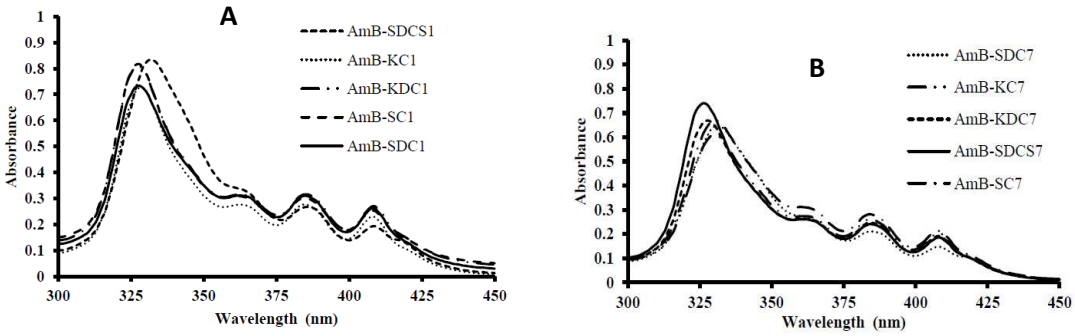


Figure 7. UV measurement of reconstituted AmB-lipid derivatives dry powders after reconstituted into distilled water at conc. 10 µg/mL AmB at day 1 (A) and at day 7 (B).

The reconstituted dry powder AmB-lipid formulations showed no significant change in drug content, particle size and zeta potential at initial period and after 6 months storage at refrigerator conditions as shown in Table 1. There was no apparent difference in physical characteristics such as color of the dry powders remained same as initial period yellowish and free flowing powder in nature. The content uniformity of AmB-lipid formulations was obtained range between 93.4 ± 2.9 to 98.5 ± 3.8%, which was determined by HPLC. The results of accuracy and system precision tests performed on each concentration of AmB is shown in Table 2. The accuracy and precision were presented as % recovery and % RSD, respectively. For accuracy, both intra-day and inter-day run gave the values of % recovery varied between 97-103% which was lined in the acceptable range. In the same way, the system precision was considered to be satisfactory since the % RSD values were less than 2% for both intra-day and inter-day run [31]. The analytical method showed a good correlation coefficient ($r^2 \geq 0.9994$) in the concentration range of between 2.0-10.0 µg/mL of AmB. The percentage recovery of the AmB was more than 97% for all concentration from 2.0-10.0 µg/mL (Table 2). The limit of detection (LOD) and limit of quantification (LOQ) of this method were 0.10 and 0.40 µg/mL, respectively.

At the end of 6 months, this content of AmB was observed by slow rate of degradation of drug on refrigeration. Our result was similar to Darole et al. and Butani et al. reported on (2008) and (2014), respectively [32, 33]. AmB was maintained over 87% for at least six months in all formulations. Among these five formulations, AmB-SDCS was more stable (97.4 ± 4.2%, after 6 months) than other formulations. In addition, the physical properties of reconstituted lipid dry power formulations remained unaltered. Thus, AmB-lipid formulations in dry powders state are likely to be stable. However, these AmB-lipid formulations could be further developed to obtain the good physicochemical properties of dry powder inhaler [34].

Table 1 Results of Stability of freeze dried reconstituted powder AmB-lipid formulations at initial time period and after 6 months at control temperature (2-8 °C) in refrigerator (mean ± SD, n ≥ 3, n=3 for size, zeta potential and n=10 for drug content).

Characteristics	Formulations mole ratio (2:1)									
	I		II		III		IV		V	
	AmB-SDCS		AmB-SDC		AmB-KDC		AmB-KC		AmB-SC	
	Initial 2-8° C	6 months 2-8° C	Initial 2-8° C	6 months 2-8° C	Initial 2-8° C	6 months 2-8° C	Initial 2-8° C	6 months 2-8° C	Initial 2-8° C	6 months 2-8° C
Particle size (nm)	73.0 ± 0.4	73.7 ± 0.2	23.4 ± 0.2	23.7 ± 0.1	21.0 ± 0.5	21.4 ± 0.2	48.2 ± 0.2	47.5 ± 0.6	31.1 ± 0.4	31.6 ± 0.2
Zeta potential (mV)	- 44.9 ±0.6	- 45.6 ±0.4	- 38.0 ± 1.0	- 37.7 ± 0.7	- 39.8 ± 0.5	- 39.5 ± 0.3	- 33.4 ± 0.5	- 32.9 ± 0.4	- 31.2 ± 0.5	- 31.4 ± 0.5
Drug content (%)	98.5 ± 3.8	97.4 ± 4.2	96.8 ± 4.0	89.6 ± 5.4	94.3 ± 3.0	90.0 ± 3.1	96.3 ± 3.0	90.3 ± 3.4	93.4 ± 2.9	87.3 ± 3.2

Table 2 Percent recovery and RSD for accuracy and precision of AmB (n=5).

Conc.	Accuracy (% Recovery ±SD)		Precision(% RSD)	
µg/mL	Inter-day	Intra- day	Inter-day	Intra-day
2	97.1± 1.19	102.9± 0.51	1.24	0.54
4	98.7± 0.20	103.2± 0.71	0.20	0.72
6	98.8± 1.68	99.6± 0.34	1.70	0.35
8	101± 0.88	102.5± 0.65	0.87	0.67
10	98 ± 1.22	100.5± 0.53	1.26	0.54

Aerosol properties of reconstituted AmB-lipid formulations after nebulization

The *in-vitro* deposition of AmB-lipid derivatives in the Andersen Cascade Impactor (ACI) using a reconstituted solution and jet nebulization as described in the materials and method section. The properties of the aerosolized AmB-lipid derivatives (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS,) are presented in Table 3. Part of this research related to the aerosol property of the two formulations, AmB-SDCS and AmB-SDC was previously reported by [21]. The physical properties of the AmB-lipid formulations are the most important factors that determine their deposition in the small airways of the lung for targeting the alveolar macrophages. The FPF was the amount of AmB smaller than 4.7 µm. To achieve the best result of aerosolization for delivery of the drug into a diseased lung, small aerosolized particle sizes with the MMADs that range from 1 to 5 µm are required [19]. MMADs of all the AmB-lipid formulations used in this study were between 1.70 to 2.05 µm (Table 3). The percentages of the FPF of the AmB-lipid formulations were found to be between 70-80%. However, the powders had very poor flow properties, so they were not suitable for use in a dry powder inhalation. For nebulization, the dry powder was reconstituted with distilled water and found to be in a highly soluble and stable form, so this solution was suitable for jet nebulization. It revealed that these formulations were stable without degradation AmB during the nebulization process.

Table 3 Aerosol properties of reconstituted AmB-lipid dry powders with distilled water (Mean ± SD, n=5)

Material	% content	MMAD (µm)	% FPF (< 4.7 µm)
AmB-SDC	100.8 ± 1.8	1.70 ± 0.3	70 ± 3.9
AmB-SDCS	101.9 ± 3.4	1.74 ± 0.4	80 ± 2.3
AmB-KDC	100.4 ± 0.3	1.81 ± 0.3	71 ± 4.9
AmB-KC	100.2 ± 0.5	1.89 ± 0.4	72 ± 4.2
AmB-SC	99.5 ± 0.4	2.05 ± 0.3	74 ± 3.3

CONCLUSION

Five lipid derivatives such as KC, KDC, SC, SDC and SDCS, were chosen as lipid drug carriers. Among them, SDCS, KDC and KC were synthesized successfully in a laboratory and their yields were obtained 78 (data was taken from previously reported [21], 82 and 80%, respectively. SDC and SC were purchased from the market. These carriers were applied to develop as lipid drug carriers system as a reconstituted dry powder AmB-lipid formulations. AmB-lipid formulations were successfully prepared by lyophilization process (freeze drying) in mole ratio 1:2 (AmB:lipid carrier), formed solid caked, which was a very light, free flowing, hygroscopic in nature. These formulations were highly water soluble and stable in solution form with negatively charge developed. 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. The products were stable for six months on storage of refrigerator at 2-8 °C. The contents of AmB were determined during this period. They were obtained above 87% for AmB-SC and rest of four AmB-lipid formulations were 90% over. Among these four formulations, AmB-SDCS was the best stable product found; its content of AmB was 97% over. All formulated dry powders were dissolved completely within 1 min with pH ranges were 7.4-7.8 (data was not shown). Aerosolization characteristics such as MMADs were obtained ranges 1.70- 2.05 µm with high FPF 70-80%. Although, these lipid drug carriers are vital role play to dissolve the poorly soluble AmB into highly soluble and stable solution form. Among five AmB-lipid formulations, it can be concluded that SDCS was the best lipid drug carrier in terms of its physical, chemical properties and aerosol property for the treatment of lung fungal infections by nebulization.

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