Development of Amphotericin B Incorporated In Lipid Derivatives as a Reconstituted Powder for Nebulization

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Thesis Title	Development of amphotericin B incorporated in lipid derivatives as a reconstituted powder for nebulization
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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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ABSTRACT

Amphotericin B (AmB) is the drug of choice for most systemic fungal infections. It is considered to be the gold standard therapy and most commonly used to treat life-threatening conditions such as cryptococcosis, histoplasmosis, and invasive pulmonary aspergillosis. AmB in lipid drug carriers were used to prepare reconstituted dry power formulations by lyophilization process (freeze drying). Five lipid derivatives such as sodium deoxycholate (SDC), sodium deoxycholate sulfate (SDCS), potassium deoxycholate (KDC), potassium cholate (KC) and sodium cholate (SC) were chosen as lipid drug carriers effect in different salts (Na^+ and K^+) in reconstituted dry powder formulations for nebulization. SDCS was successfully synthesized in a laboratory and characterized by FTIR, ^{1}H NMR, mass spectra, its R_f and melting point. KDC and KC were prepared from their corresponding acid such deoxycholic and cholic acid with potassium hydroxide in equal mole ratios. Thus, these compounds provide a desirable feature as a lipid drug carrier for reconstituted dry powder formulation, which was suitable for nebulization. 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. All AmB-lipids formulations were highly water soluble and dissolved within 1 min. These formulations were evaluated for content uniformity and delivery efficiency by Andersen cascade impactor (ACI). The aerosols properties were determined as their mass median aerodynamic diameter (MMAD), and fine particle fraction (FPF). 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). An assay of the AmB showed that the all AmB-lipid formulations contents were closed to 100%. The MMADs of all AmB-lipid formulations were

obtained between 1.70-2.05 µm with high FPF (70-80%). All AmB-lipid formulations had equivalent potency to that of AmB (100%). The MICs and MFCs against *C. neoformans and C. albicans* exhibited a lower value than the pure AmB of all AmB-lipid formulations. All AmB-lipid formulations were less toxic to human red blood cells, respiratory associated cell lines (alveolar cell lines, human bronchial epithelial cells and alveolar macrophage cell lines) and kidney cells than pure AmB. Furthermore, all AmB-lipid formulations did not produce nitric oxide at a toxic level as compared to lipopolysaccharide. The AmB-lipid formulation was successfully targeted alveolar macrophages. Permeation across lipid bilayers was found to be 2-5 fold higher than pure AmB. Though, the amount of AmB had been transferred from left to right chamber was very low but it was enough to kill the fungi. This indicated that AmB in lipid derivatives carriers as a reconstituted dry power formulations are potential candidates to be used for the treatment of lung fungal infections as an alternative option via nebulization to target the AM.

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Rationale

Invasive pulmonary aspergillosis (IPA) is a prime cause of morbidity and mortality in immunocompromised individuals, who is undergoing for lung transplantation (Segal, 2009; Mihara et al., 2014). Mortality among infected patients is high death rate in excess of 90% has been shown in studied by Richardson, 2005. Most of these invasive mould infections are acquired through the respiratory tract. An increased incidence of invasive fungal infection has created major challenges for medical practitioners. In the last decades, the incidence of pulmonary fungal infections that has been increased due to the growing number of immunocompromised patients related to human immunodeficiency virus (HIV), organ transplantations, hematologic disorders and cancer (Limper et al., 2011). Fungal infections of the lung are less common than bacterial or viral infections but it is more difficult for diagnosis in early stage, because the clinical manifestations of invasive fungal and bacterial infections are similar, leading to that treatment will be delayed and more complicated. The organisms causing systemic fungal infections include *Candida albicans* (80%), *Aspergillus fumigatus* (15%), *Candida tropicalis* and *Cryptococcus neoformans* (Brykier, 2005). Amphotericin B (AmB) is the drug of choice for most systemic fungal infections. AmB is considered to be the gold standard therapy and most commonly used to treat life-threatening conditions such as cryptococcosis, histoplasmosis, and IPA.

AmB is originally synthesized from *Streptomyces nodosus*, the soil actinomycetes (Kaminski et al., 2014) and is a polyene macrolide broad spectrum antimycotic (Hamill, 2013; Ruiz, et al., 2014). The chemical structure includes a large lactone ring of 37 carbon atoms in which one side of the ring is a hydrophobic conjugated heptaene chain and the other side is hydrophilic due to the presence of seven hydroxyl groups. The macrolide ring also contains a six-membered ketalic ring to which the aminosugar, mycosamine, is bonded through an α-linkage. The dual hydrophobic-hydrophilic or amphipathic nature of AmB promotes the complexation

with other moieties that can result in altered pharmacokinetics and pharmacodynamics. AmB is a yellow to orange powder and practically insoluble in water as well as most of the organic solvents. It is soluble in very few solvents such as dimethylsulphoxide (DMSO), slightly soluble in dimethylformamide (DMF), very slightly soluble in methanol and insoluble in alcohol. Its water solubility at physiological pH (6-7) is less than 1 µg/mL but it increases at pH <2 or >11. However, in these extreme pH conditions AmB is not stable (Bennett, 2011). It is light sensitive product, should be stored in a well closed airtight, opaque container at 2-8 ºC.

AmB exerts its antifungal activity by binding to ergosterol on cell membrane of 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 (Moen et al., 2009; Gray et al., 2012; Yang et al., 2013). 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 (Gray et al., 2012). Drug toxicity, the nephrotoxicity of AmB is the major clinical problem, which could lead to treatment discontinuation (Labiado-Laborin and Carbrales-Vargas, 2009; Malone et al., 2013, Ruiz et al., 2014). It is inevitable there is a demand to develop less toxic product using new delivery system.

The new advanced drug delivery techniques are applied to successfully development of lipid-based formulations of AmB. On the basis of this technique commercially available products Ambisome®, Amphocil® and Abelcet® are less toxic than micellar formulation amphotericin B deoxycholate. The toxicity of AmB can be reduced by incorporation of liposomes or by complexation with various lipids. All these formulations enhanced therapeutic window of AmB by decreasing its toxicity (Hiemenz and Walsh, 1998; Wade et al., 2013). However, several severe side effects, albeit infrequent, have been reported in patients, including allergic reactions and cardiopulmonary toxicity (Hamill, 2013). The comparable efficacy of lipid-based AmB can be achieved only when they are administered at a higher dose than conventional formulation (Fungizone®). Furthermore, lipid complex formulations are much more expensive than conventional formulation. Although, lipid formulations have been reported to have excellent safety and efficacy but it has two disadvantages,

first, it needs higher dose and secondly, product is very expensive as compared to AmB-deoxycholate (Herbrecht et al., 2003; Butani et al., 2014). The high cost is one of the major obstacles, due to that the clinician cannot freely prescribe lipid based formulations to the patients, who cannot afford it though available in the market. Therefore, low cost production is essential for the third world peoples.

Newer AmB delivery systems have greatly reduced infusion-related reactions and nephrotoxicity. Because of the high toxicity rate of AmB, AmB colloidal dispersion (ABCD) was formulated to reduce nephrotoxicity. In a study comparing these two agents the incidence of nephrotoxicity was 15% for ABCD versus 49% for AmB (Bowden and Chandersekar, 2002). Further toxicity has occurred since the introduction of lipid formulations of AmB, AmB lipid complex (ABLC) and liposomal amphotericin B (L-AmB) (Moen et al., 2009). In one study, L-AmB infusion–related reactions of rigors and chills were significantly less prevalent than with ABLC (23.5% and 79.5%, respectively; $P<0.001$). Nephrotoxicity, defined as an increase of three times baseline serum creatinine, occurred approximately 20% less often with L-AmB versus ABLC ($P<0.001$) (Wingard et al., 2000). Lipid based formulation of AmB shared reduced nephrotoxicity by a mechanism that is not yet clearly known. Liposomal AmB formulations can be pegylated to reduce toxicity and prolong the serum half-time of liposomal AmB formulations. Pegylations induced a hydrodynamic layer on the particle surface which shielded the liposomes and consequently, the particles circulated longer in the blood and were uptaken much less by the phagocytes (Torrado et al., 2008). These lipid formulations have a low rate of elimination and the increased doses of administered AmB may accumulate in the body. Therefore, the lipid-based AmB formulations that were developed in an attempt to reduce the side effects associated with the conventional AmB formulation largely achieved the goal. While the pharmacokinetic properties of the lipid-based formulations are highly variable it is accepted that lipid-based formulations reduce nephrotoxicity. The current paradigm is that lipids provide a protective effect based on the altered affinity of AmB for human cell membranes while preserving the affinity of AmB for fungal membranes (Sawaya et al., 1995). Still, lipid-based formulations have some drawback such as less stable, low potent and required 6-10

fold higher doses than conventional AmB (Fungizone®) to achieve a similar therapeutic effect (Bennett, 2001).

There is continued interest in developing a new AmB formulation that maintains the potency with fewer side effects and has low cost. A promising approach is to deliver therapeutic agent directly to the respiratory tract. Aerosol inhalers are more commonly used for this purpose. Local delivery of medication to the lung is highly desirable, especially in the patient with specific pulmonary disease like cystic fibrosis, asthma, chronic pulmonary infections or lung cancer. The principal advantages of local delivery include reduced systemic side effects and higher dose levels at the site of action (Bennett et al., 2002; Fauvel et al., 2012).

The most prevalent and important fungal infections lung-transplanted patients are caused by *Aspergillus* spp., which give rise to considerable morbidity and mortality. Nebulized AmB has been used successfully in the rat model of invasive aspergillosis and in human (Monforte et al., 2001). Aerosolized AmB prophylaxis may be efficient and safe in preventing Aspergillus infection in lung-transplanted and AIDS patients (Ruijgroka et al., 2001; Monforte et al., 2013). Sorensen and coworkers (1993) used aerosolization of liposomal (Ambisome®) and non-liposomal (Fungizone®) AmB for pulmonary fungal infection treatment. Ambisome® was shown to be more amiable for nebulization than Fungizone®. The small multilamellar AmB liposomes were successfully prepared by a reverse phase evaporation technique and the stabilized by lyophilization. The resulting liposomal dry power inhaler formulation had a shelf life over 1 year with refrigerated store (Shah and Misra, 2004). However, liposomal AmB dry powder inhaler gave a fine particle fraction of only 25%, this was quite low and would be expected to result in low deposition in the lung and high deposition in the upper respiratory tract with the expected consequence of reduced efficacy and increased side effects. Fungizone® often causes serious side effects whereas lipid-based AmB formulations require complex manufacturing method.

Cell culture refers to a culture derived from dispersed cells taken from original tissue, from a primary culture, or from a cell line or cell strain by enzymatic, mechanical or chemical disaggregation. The applications of human cell cultures are done to study drug toxicity of the different formulations product of AmB-lipid

derivatives dry powders and lipid derivatives to evaluate its toxic effects in human organs (especially lung and kidney). Theses cell lines were selected because of well stabilized toxic data are available and easy to correlate such toxicity especially for lung and kidney. The efficacy was monitored by measuring the bioactivity of AmB, AmB-lipid formulations in comparison with pure AmB drug. The safety of AmB with lipid derivatives of reconstituted powder as a nebulization reaching to airways was determined to alveolar macrophages. The toxicity study was carried out to respiratory associated cells such as human lung adenocarcinoma cells (A549), human bronchial epithelial cells (Calu-3), alveolar macrophage cell lines (NR8383), kidney cells (293T/17) and red blood cells.

Potassium cholate (KC), potassium deoxycholate (KDC), sodium deoxycholate (SDC), sodium deoxycholate sulfate (SDCS) and sodium cholate (SC) are lipid derivatives compounds having steroid nucleus with polar functional two or three hydroxyl group. SDC was taken as a control. Carboxyl group of SDC has been replaced by anionic charge group of sulfate in SDCS. It was highly ionizable and soluble in water. Incorporation of active ingredients into the solid lipid matrix offers protection against chemical degradation of the active compound (Jenning and Gohla, 2001). These compounds are highly stable for oxidation and hydrolysis reaction as compared to liposomes. This was the main advantage of AmB-lipid formulations. Liposomes are less stable and undergo hydrolysis and oxidation reaction as well as drug clearance from the body is taken longer time, which is accumulated inside the body and shown in toxicity after repeating the doses. AmB is water insoluble but the AmB-lipid formulations are highly water soluble and stable in solution form. The products were stable as the dry powder form which was stored at 2-8 ^{o}C in a refrigerator in airtight amber bottles for 6 months.

In this study lipid derivatives carriers such as sodium deoxycholate (SDC), sodium deoxycholate sulfate (SDCS), potassium deoxycholate (KDC), sodium cholate (SC) and potassium cholate (KC) were used to prepare AmB-lipid dry powers formulations. It was hypothesized that lipid derivatives would improve stability, enhance the solubility, improve efficacy and reduce toxicity of AmB. The AmB permeability was expected to enhance through lipid bilayer membrane and was able to target the AM.

1.2 The objective of the studies

1. Synthesize and characterize physicochemical properties of sodium deoxycholate sulfate
2. Preparation of amphotericin B with lipid derivatives drug carriers as reconstituted dry powers formulations for nebulization by lyophilization process
3. Study the physiochemical properties and interactions of amphotericin B with lipid drug carriers
4. Evaluate *in vitro* efficacy of AmB-lipid formulations reconstituted dry powders against *Saccharomyces cerevisiae*, *Candida albicans* and *Cryptococcus neoformans*
5. Examine the toxicity of AmB-lipid formulations reconstituted dry powders in respiratory-associated cell lines, kidney cells and red blood cells
6. Determine release of nitric oxide level by alveolar macrophages cell lines after exposures with AmB-lipid formulations
7. Observe phagocytosis of AmB-lipid formulations
8. Determine the permeation of AmB across the lipid bilayer

CHAPTER 2
LITERATURE REVIEW

2.1 Lung fungal infections

Mostly fungal infections are occurred from inhalation. The target organ is the lungs, which is most frequently encountered these pathogenic microorganisms and most often demonstrate the pathogenic changes of fungal disease. Generalized dissemination of the fungus may cause spread of the infection from the lungs to the rest of the body as part of process that has been called primary infection and post-primary disseminations. In this process, the immune system is involved in a completed series of reactions reflecting innate factors as well as humoral and cell mediated immunity. In general, the resistance of the body to infections by fungi, characterized as saprophytic is excellent, in fact, these fungi rarely cause significant infection in health humans. A group of more aggressive fungi regularly caused primary infection in healthy human subjects, but this infection is limited and not associated with significant disease, even when the post-primary dissemination occurs (Baun and Hodes, 1998). There are different types of fungal infections such as histoplasmosis (*Histoplasma capsulatum*), coccidioidomycosis (*Coccidioides* spp.), cryptococcosis (*Cryptococcus* spp.), aspergillosis (*Aspergillus* spp.) and candidiasis (*Candida* spp.) (Limper et al., 2011; Ruiz et al., 2014). Among the fungal diseases, invasive fungal infections are increasing incidence due to *Candida*, *Aspergillus* and *Cryptococcus* (Ghannoum and Perfect, 2010). The incidence of invasive fungal infections that has increased in the last decades partially due to the growing number of immune-compromised patients related to HIV, cancer, hematologic disorders, and organ transplantations (Limper et al., 2011).The organisms causing systemic fungal infections include *Candida albicans* (80%), *Aspergillus fumigatus* (15%), *Candiada tropicalis* and *Cryptococuous neoformans* (Brykier, 2005).

2.1.1 Aspergillosis

Aspergillosis is a disease caused by a fungus of the genus *Aspergillus* which consists of many species. The predominant species is *Aspergillus fumigatus*.

Aspergillus can cause illness three ways, an allergic reaction in asthmatics; a colonization in scarred lung tissue; and an invasive infection with pneumonia. *Aspergillus* is a ubiquitous hyalohyphomycete found throughout the world and prevalence of disease as shown in Fig. 2.1. Over 200 species, only a few have been reported as pathogenic to humans, such as *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*. Among them, *A. fumigatus* is the most common species to cause invasive disease and it is involved in 80% to 90% of human aspergillosis. Conidia are easily aerosolized because of its micronized size 2-3 μm, which are inhaled and deposited inside the deeper part of lungs, where they colonize and a variety of clinical syndrome of diseases may develop for weak immunity patients but in healthy person immune response destroy it and prevent from the disease (Ghannoum and Perfect, 2010). Invasive aspergillosis is prime cause of mortality in immunocompromised patients. *A. fumigatus* is an opportunist pathogen of humans and the commonest aetiological agent of pulmonary aspergillosis, being responsible for 80-90% of cases. Treatment consists of AmB but the mortality rate is extremely high even though appropriate use of AmB.

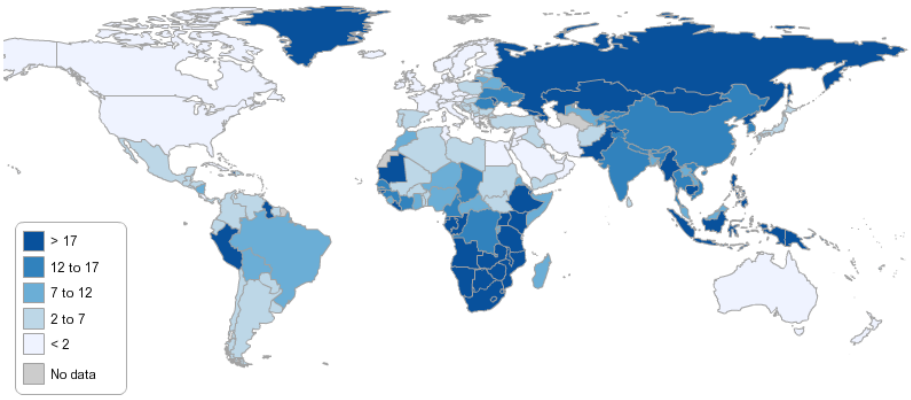


Figure 2.1 Chronic pulmonary aspergillosis prevalence map (numbers represent rates per 100,000)
http://www.gaffi.org/why/burden-of-disease-maps/cpa-prevalence/

2.1.2 Histoplasmosis

Hisptoplasma capsulatum is a dimorphic fungus that is cause of histoplasmosis. It is the most prevalent fungal pulmonary infection in USA and is

common among the AIDS patients. The mortality rate among infants exposed to a large dose of *H. capsulatum* may be 40-50%. After inhalation of *H. capsulatum*, pulmonary macrophages engulf the yeast cells and provide a protected environment for the yeast to multiply and disseminate from the lungs to other tissues. Normal individuals who inhale a large number of *H. capsulatum* spores can develop a non-specific flue like syndrome associated with fever, chills, headache and chest pains after a three week incubation period that resolves without treatment (Hugo and Russell, 2004). Treatment of pulmonary histoplasmosis also depends on the particular type of infections. In acute histoplasmosis generally requires no therapy and is self-limited illness. Disseminated histoplasmosis requires treatment with two antifungal agents, AmB or ketoconazole, depending on the pace of the illness and immunologic status of host.

2.1.3 Coccidioidomycosis

Coccidioidomycosis also affects normal hosts and may have its clinical consequences altered in special categories of patients, especially those with impairment of host defense mechanism. The causative organism, *Coccidioides immitis*, it is also dimorphic fungus. It needs a similar treatment to that of histoplasmosis.

2.1.4 Other fungi

The remaining fungi are less frequent causes of respiratory infections. *Blastomycosis* is seen in normal hosts and is associated with pulmonary disease, as well as with potential problems with other organs systems.

Candida albicans, although an extraordinarily common contaminant of sputum, is an uncommon cause of pneumonia, even in immunosuppressed patients. *Cryptococcus neoformans* is found primarily in immunosuppressed patients, in whom it causes lung disease as well as meningitis. Lastly, *Mucor* is an opportunistic fungus that may cause pulmonary infection in the immunocompromised host.

2.2 Treatment of lung fungal infections

2.2.1 Structure of fungal cells

Fungi are eukaryotic organisms, and there are many similarities between the biochemistry of fungal cell and human cells. Fungal cell membrane consists of lipid particle which is known as sterol. The fungal sterol is different from the mammalian cell membrane. The ergosterol is the main constituent of sterol in fungal membrane; where as in mammalian cell membrane is cholesterol. The chemical structure of ergosterol is shown in Fig.2.2 (A) and cholesterol as shown in Fig. 2.2 (B).

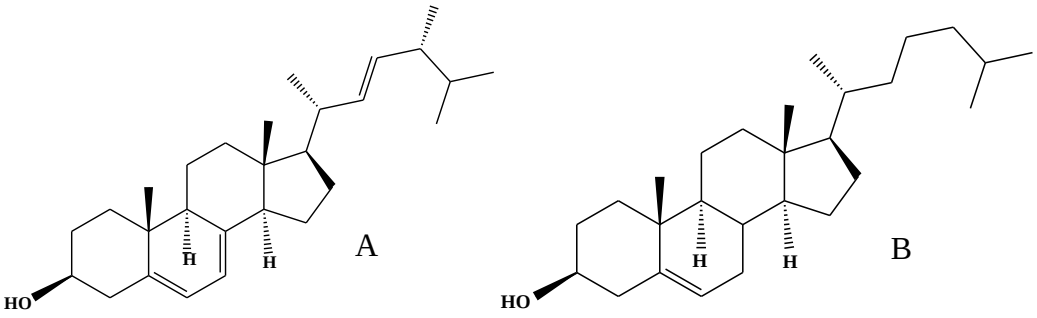


Figure 2.2 The chemical structures of ergosterol (A) and cholesterol (B).

2.2.2 Amphotericin B (AmB)

In seriously ill and immunocompromised patients, the mortality and morbidity rate is significantly high in fungal infections. The rate of frequency is rising because of increasing the number of AIDS and immunocompromised patients. Treatment of fungal infection is a very complicated and often prolonged. Because the infections are difficult to diagnose at early stage due to the similarity of sign and symptoms of bacterial and fungal infections. Cultures frequently remain negative or only become positive in the advanced stages of infection (Ghannoum and Perfect, 2010; Hamill, 2013). Therefore, early detection and treatment of suspected fungal infections, especially in febrile neutropenic patients, is an important goal in current antifungal therapy. There are four different kind of drugs are available for the treatment of invasive fungal infections. These include polyenes (AmB), neucleoside analogs (flucytosine), azoles (fluconazole, itraconazole and voriconazole), and echinocandins (capsofungin) (Wingard and Leather, 2004). Over 40 years, AmB

remains the drug of choice for treatment of life threatening conditions (Matsumori et al, 2004; Chudzik et al, 2013). It is a broad spectrum and least resistance to the systemic fungal infection (Butani et al., 2013; Ruiz et al., 2014). It is produced by a fermentation by-product of *Streptomycetes nodosus*, which was obtained from soil *actinomycetes* (Chudzik et al, 2013; Kaminski et al, 2014).

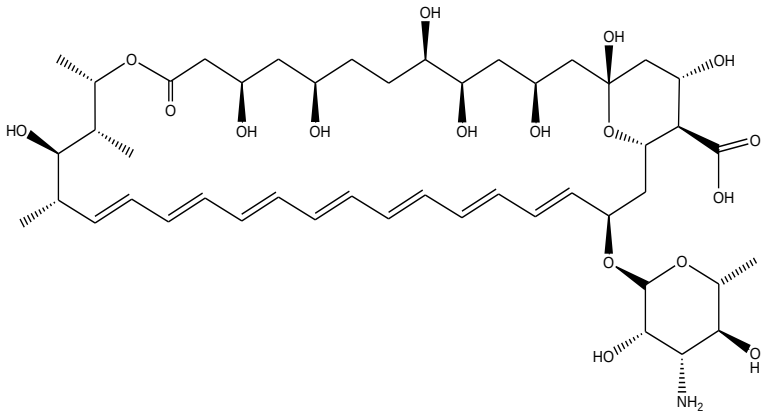


Figure 2.3 The chemical structure of Amphotericin B (AmB)

The chemical structure of AmB (Fig. 2.3) shows a large lactone ring of 37 carbon atoms in which one side of the ring is a hydrophobic conjugated heptaene chain and the other side is hydrophilic due to the presence of seven hydroxyl groups. The macrolide ring also contains a six-membered ketalic ring to which the aminosugar, mycosamine, is bonded through an α-linkage. The dual hydrophobic-hydrophilic or amphipathic nature of AmB promotes the complexation with other moieties that can result in altered pharmacokinetics and pharmacodynamics.

AmB is a yellow or orange powder and practically insoluble in water as well as most of the organic solvents. But it is soluble in very few solvents such as dimethylsulphoxide (DMSO), slightly soluble in dimethylformamide (DMF), very slightly soluble in methanol and insoluble in alcohol. Its water solubility at physiological pH (6-7) is less than 1 µg/mL but it increases at pH <2 or >11. However, in these extreme pH conditions AmB is not stable (Bennett, 2011). It is light and temperature sensitive product. Therefore, it should be stored in a well closed airtight amber bottle, to protect from light and temperature at 2-8 ºC.

2.2.3 Mechanism of action of AmB

The AmB molecular mechanism of action has still not been understood fully to make rational design of new derivatives. It has been known that AmB interacts with the components of cell membrane and forms ion channel (Baginski et al, 2002). These ion channels disrupt membrane functions and cause uncontrolled cation transport through an increase in cell membrane permeability leading to the leakage of sodium, potassium and hydrogen ion, where it associates with membrane sterols to form pores which damage the cell ion, and eventual cell death (Tzu-Sen et al., 2013). AmB has 10-fold higher affinity to ergosterol than cholesterol (Matsumori et al., 2004). The chemical structures of ergosterol and cholesterol are as shown in Fig. 2.2. The creation of a barrel pore is shown in Fig. 2.4 (Brajtburg and Bolard, 1996).

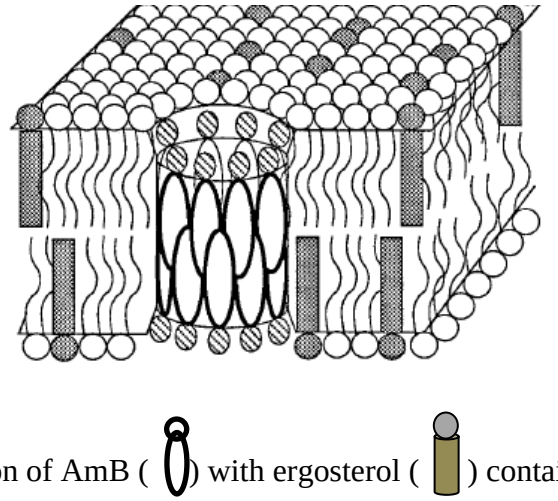


Figure 2.4 Model illustrating the possibilities for the formation of channels by AmB interacting with ergosterol containing membrane (adapted from Brajtburg and Bolard, 1996)

The binding of AmB with fungal cell membrane involves in the hydrogen bonds between the hydroxyl groups of the sterol and the carboxyl group at C18 of the AmB molecule. The strong intermolecular AmB-sterol interactions within the channel are responsible for AmB stability (Cotero et a.l, 1998). This binding is strengthening by participation of the amino group of the amino sugar as shown in Fig. 2.5.

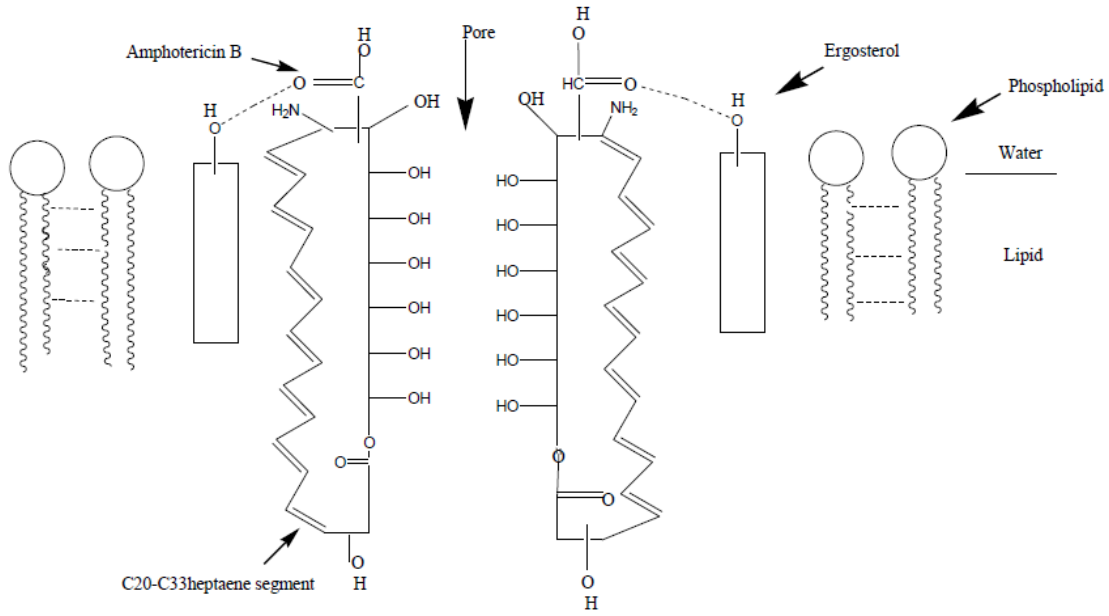


Figure 2.5 Schematic representation of hydrogen bond formation (adapted from Brajtburg et al., 1990)

2.3 Targeting of Drugs to the Respiratory Tract

The pulmonary system has an important function to exchange of gases between CO_2 and O_2 in the lungs with environmental atmosphere. The average capacity of normal healthy human lungs can hold about 6 L of air. The respiratory tract can be divided as an upper airway, a lower airway and the lungs. The upper airway includes the nose and nasal passages paranasal sinuses as well as the pharynx as shown in Fig. 2.6. The lower airway includes the larynx, trachea, bronchi and bronchioles. The lungs include the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. The alveoli are tiny little air sacs in the lungs where gas exchange takes place. In a normal healthy person there are estimated to be as many as 400 million alveolar sacs and the total surface area of lungs 75 m^2 (Taylor, 2002). The alveoli are rich with capillaries and the red blood cells (RBC) absorb oxygen from the air in the form of oxyhaemoglobin, to keep alive the cells. The RBC also carries CO_2 away from the cells in the form of carboxyhaemoglobin and releases it into the alveoli through the alveolar capillaries. Lungs and airways are a site for gas exchange and contact with the exterior, being exposed to organic, inorganic and biological components that can cause disease. In WHO reports, it is mentioned that infections of

the lower respiratory tract are among the top three major causes of morbidity worldwide and first in low income countries, being responsible for approximately 3.5 million deaths annually (WHO, 2008). Respiratory infections are a major burden problem to the health systems in the worldwide mainly due to the intra-hospital infections, which are more easily affected to the immune-compromised patients (Andrade et al., 2013).

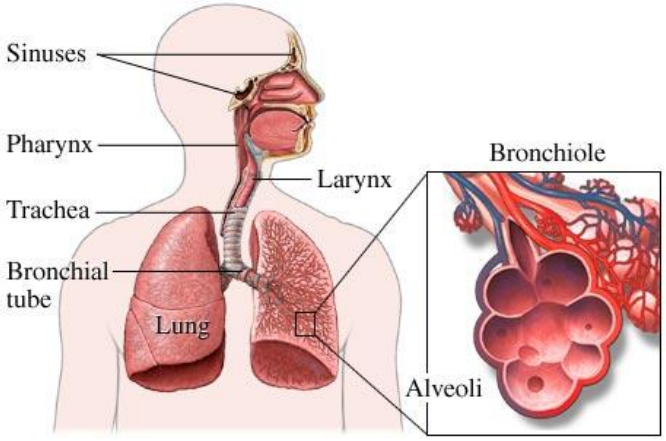
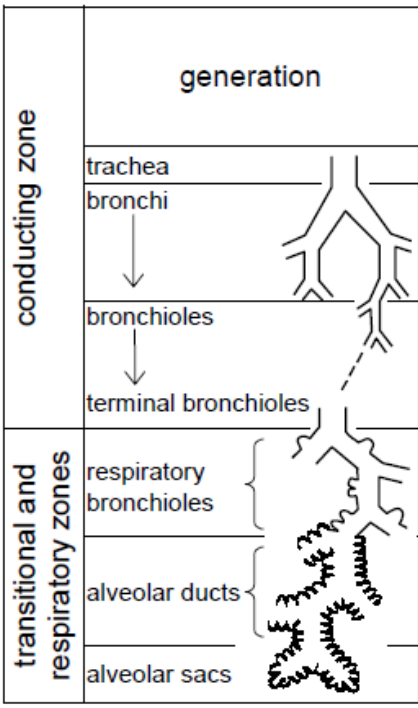


Figure 2.6 Anatomy of respiratory tract

In Fig. 2.7, physiological of the airway duct shows reduction in diameter from conducting area to respiratory zone, related with reducing from the largest internal diameter that is about 1.8 cm in trachea to smallest diameter about 40 µm in alveolar sacs. Larger than 2 µm of the respired particles will retain at the upper respiratory tract and only smaller than 2 µm particles can penetrate into the deeper part of lung.



	generation		diameter (cm)	length (cm)	number	total cross sectional area (cm^2)	Powder deposition by particle diameter
conducting zone	trachea	0	1.80	12.0	1	2.54	7 - 10 µm
	bronchi	1	1.22	4.8	2	2.33	2 - 10 µm
		2	0.83	1.9	4	2.13	
		3	0.56	0.8	8	2.00	
	bronchioles	4	0.45	1.3	16	2.48	
	terminal bronchioles	5 ↓ 16	0.35 ↓ 0.06	1.07 ↓ 0.17	32 ↓ $6 \cdot 10^4$	3.11 ↓ 180.0	
transitional and respiratory zones	respiratory bronchioles	17	↓	↓	↓	↓	0.5 - 2 µm and < 0.25 µm
		18					
		19	0.05	0.10	$5 \cdot 10^5$	10^3	
	alveolar ducts	20	↓	↓	↓	↓	
		21					
		22					
	alveolar sacs	23	0.04	0.05	$8 \cdot 10^6$	10^4	

Figure 2.7 A schematic representation of airway branching in the human lung (adapted from Koning, 2001).

Targeting of drug to the respiratory tract refers to a noninvasive route of administration for delivering drugs to the body via the lungs for local and systemic effects by inhalation. It can be used as an alternative to the oral drug delivery. Inhalation provides most direct access to drug target. Most of the water insoluble drug products are soluble in lipid and deliver to lungs. The lungs are able to absorb water and lipid soluble drug product into the tissues. Carriers such as micro-particles, nanoparticles, and liposomes can be used in lung targeting. The human lungs have capacity to actively exchange various materials between the external environment and interior of the body. This ability of lungs makes them a very convenient and safe route for administration of a number of drugs that are considered unsuitable for administration via other routes such as oral and parenteral routes. Recent advances in drug delivery systems have made it possible to formulate and deliver almost any drug via the lungs (Patton et al., 2004). Targeting of drug to the respiratory tract can be utilized for the delivery of the variety of molecules into the body. The major class of molecules being investigated includes systemically active peptides and proteins such

as insulin, interferones, growth hormone-releasing peptides and more recently, hepatitis B vaccine (Gonda, 2006; Thomas et al., 2008). The targeting approach has been utilized mainly for the treatment of various lung diseases such a non-small-cell lung cancer, cystic fibrosis and pulmonary arterial hypertension (Gettinger, 2008; Haj et al., 2006; Ramalingam et al., 2007). Targeting of drug to the respiratory tract offers various advantages, such as first; the most important is the possibility of needle-free treatment or painless treatment. Secondly, the ease of administration without help of health professionals, thirdly bypasses metabolism and degradation by the enzymes in the gut and liver, thus delivering the drugs to the body very efficiently without generating toxic metabolites. Lastly, the pulmonary drug delivery is a very convenient and safe route for administration of a number of drugs that are considered unsuitable for administration via other routes, such as oral and parenteral routes.

Nowadays, the advance development of technology in the scientific field, inhalation drug is not constrained to respiratory tract only, inhalation has been clinically evaluated and used to treat both, local and systemic diseases (Nasr et al., 2012) such as asthma (McAllen, et al., 1974), TB (Muttil et al., 2009), and other bacterial infections (Geller et al., 2011), influenza virus infection (Bernstein et al.,1988), fungal infections (Mohammad and Klein, 2006), cystic fibrosis (Aitken et al., 1992), chronic obstructive pulmonary disease (Jones et al., 2011), diabetes (de Galan et al., 2006), or cancer (Huland et al., 2003; Videira et al., 2012). Beside this, inhalation has been also tested as non-invasive vaccination platform. In this context, a new door has been opened in the field of inhalation therapy and new advances could be expected in the near future (Hokey and Misra, 2011; Lu et al., 2010). Currently there are a few inhaled formulations (insulin, tobramycin, ipratropium, albuterol) are available in the market for the treatment of systemic and local diseases; an ever-increasing number of inhalable drugs for treating systemic diseases are in the pipeline. Recent advances in drug delivery systems have made it possible to formulate and deliver almost any drug via the lungs (Patton et al, 2004). As per a report published by the Kalorama Foundation, the market for pulmonary drug delivery was expected to grow 34 billion by 2010 with a 10% increase in annual growth, whereas in 2002, 25.5 billion was (Kalorama Research Foundation, 2007).

2.4 Aerosol formulation

Aerosol therapy refers to the delivery of a drug to the body via the airways by delivering it in an aerosolized form. Whereas the aerosolized drug may be intended for systemic use utilizing the vast surface area for absorption provided by the respiratory tract, but majority of the aerosols products are only for topical application. The last few years have seen a major evolution in aerosol delivery to the human kinds. Modern technology along with increasing understanding of human pulmonary physiology has aided the development of improved systems of aerosol delivery. This form of therapy has revolutionized the management of patients with various pulmonary diseases. Generally bronchodilators, anti-inflammatory agents (steroids), antibiotics and antifungal agents are available for pulmonary drug delivery system (Khilani and Banga, 2004). Inhalation drug delivery have been used for many years for the delivery of pharmacologically active agents to treat respiratory disease, asthma therapy with bronchodilators, steroid, mast cell stabilizer and anticholinergic drug has primarily used in pressurized meter-dose inhaler (pMDI). However, this delivery system is now under increasing threat from the environmental concern regarding chlorofluorocarbon (CFC) propellants. A range of alternative devices, such as dry powder inhalers, which do not contain propellants, are being evaluated and developed. Similarly, nebulizers are non-propellants based, in which distilled water or IV fluids (normal saline or 5% dextrose solution) are used for dilution or dissolving the drug in solution or suspension forms are being evaluated and developed.

2.4.1 Aerosol Devices

In pulmonary route, aerosol generating devices paly important role as an equivalent to that of formulation aspect. It is almost impossible to be administered a formulation through pulmonary route without suitable devices.

The aerosol generating devices can be classified as follows.

I Pressurized Metered-Dose Inhaler (pMDI)

II. Dry Powder Inhaler (DPI)

III. Nebulizer

a) Jet nebulizer

b) Ultrasonic nebulizer

2.4.2 Nebulizer

Two types of nebulizers are available for use as aerosol generators in clinical practices: jet nebulizer and ultrasonic nebulizer. These work on different principles but have many features in common. These are non-propellant based, do not require patient coordination and can be used to deliver high doses of a particular drug over a short time, such as during acute exacerbations of obstructive airway diseases in emergency settings. Nebulizers generate an inhalable drug aerosol from a solution or suspension. They are useful for treatment of respiratory diseases as asthma, COPD and cystic fibrosis (CF) (Knoch et al., 2005). The most common nebulizer type is the jet nebulizer, which generates aerosols from the liquid medicament using a source of compressed gas as shown in Fig. 2.8. Although relatively inexpensive, treatment with jet nebulizers has long treatment time, the air compressors are bulky and noisy, and expensive medications are wasted in considerable residual volumes (Fisher, et al., 2009). Many of the pitfalls are addressed by more recent vibrating-mesh nebulizers which have much greater portability and operate silently. The low velocity plume and minimal residual volume greatly enhance drug delivery to the lungs (Daniels, et al., 2013; Rubin, 2011). However, these new devices may have a much higher upfront cost and there is a lack of open literature demonstrating equivalent dosing of existing products to the more commonly used jet nebulizers. Other types of liquid aerosol generation systems include the Respimat® SoftMist™ Inhaler and AERx® systems, which generate liquid aerosol by mechanically forcing a drug solution through a nozzle array (Dalby et al., 2004; Cipolla et al., 2010). Effective nebulizer therapy requires a device that repeatedly and quickly delivers sufficient drug to the site of action, with minimal wastage, at a low cost (O`Callghan and Barry, 1997). With many nebulizers only 10% of the prescribed dose may reach the lung (Rubin, 2011).

Optimization of this existing nebulizer technology has focused on maximizing aerosol lung deposition with each breath. Nebulizers typically generate aerosols throughout the entire respiratory cycle of the patients, leading to a significant loss during expiration. Thus mechanical regulation of aerosol generation has been implemented in breath-enhanced (Pari LC® Star) or breath-actuated (e.g. Trudell AeroEclipse® II) jet nebulizers. These limit aerosol loss by mechanically restricting the majority of aerosolization to the inspiratory phase (Knoch et al., 2005; Watts et

al., 2008; Arunthari et al., 2012) and have been shown to shorten treatment time whilst minimizing wastage of expensive medications (Leung, et al., 2004; Rau et al., 2004). However, these are relatively crude compared to the more recent integration of digital control systems with nebulizers, which provide much more precise regulation of aerosol delivery. These electronic systems further reduce treatment time by personalizing aerosolization to an individual patient's breathing pattern, thereby maximizing lung drug deposition.

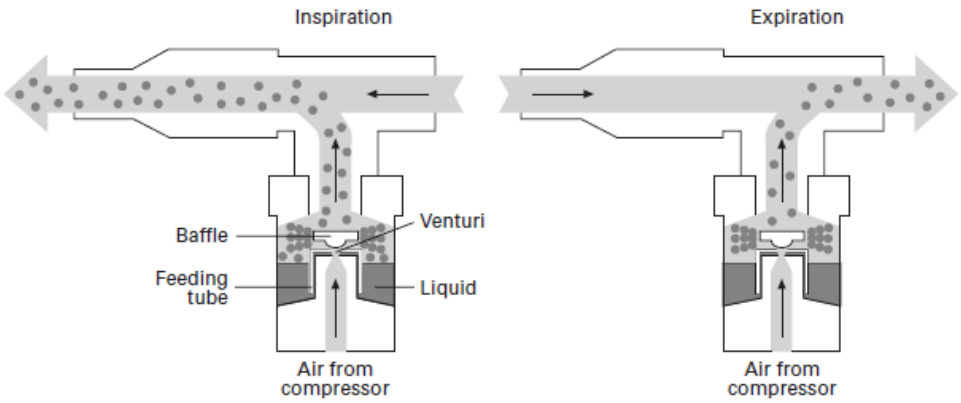


Figure 2.8 Conventional nebulizer design (adapted from O`Callaghan and Barry, 1997).

2.4.3 Development of AmB aerosols

The most prevalent and important organism causing fungal infections in lung-transplanted patients are *Aspergillus* spp. That remains the leading cause of morbidity and mortality after lung transplantation. Current treatment involves administration of AmB but serious adverse complications related to dose-dependent acute and chronic toxicity are seen. The rational approach to the problems requires that the drug should be target to the macrophages in such a way that the interaction of the free drug with non-targeted tissue could be minimized. The maximum tolerated dose of AmB is low in mice. The LD_{50} is 1.2 mg/kg, and doses higher than 1.6 mg/kg causes acute toxic reactions following by cardiac-respiratory arrest (Lopez-Berstein et al., 1983). Treatment of disseminated fungal infections by liposomal AmB results in a lower toxicity and significantly increased survival time (Brajtburg et al., 1990). It is probably that increased concentrations of drug in macrophages through passive

liposomal uptake may improve its therapeutic index (Janknegt et al., 1992, De Marie et al., 1994). Thus it is expected that ligand-mediated active targeting to the macrophages would significantly increase the rate and extent of macrophage accumulation of drug. This may reduce the required doses of liposomal AmB in pulmonary aspergillosis.

Nebulized AmB has been used successfully in the rat model of invasive aspergillosis and in human, nebulized AmB prophylaxis may be efficient and safe in preventing Aspergillus infection in lung transplanted patients (Monforte et al., 2001). Aerosolized AmB has been used in the treatment of pulmonary fungal infections as well as intravenous AmB. In particular, prophylactic use of AmB aerosol is effective in the prevention of pulmonary aspergillosis in an experimental animal model (Conneally et al., 1990; Beyer et al., 1993). Sorensen and coworkers (1993) have used aerosolization of liposomal (AmBisome®) and non-liposomal (Fungizone®) AmB for pulmonary fungal infection. Liposomal AmB (AmBisome®) was able to nebulize much better than non-liposomal (Fungizone®) and to highly effective as a prophylactic treatment in an immune compromised murine model of pulmonary aspergillosis. In addition, the pharmacokinetics of nebulized AmB was studied by Koizumi et al., (1998). The results showed the peak AmB concentration in bronchial-wash fluid was observed at 30 min after that, AmB was slowly eliminated over 24 h. AmB is still the most effective agent currently available for the treatment and prevention of serious fungal infection. However, it must be more clinical and experimental studies to determine the best tolerated and effective regimen of AmB.

The small multilamellar AmB liposomes were successfully prepared by reverse phase evaporation technique (Shah and Misra, 2004). Liposomal dry powder inhaler was chosen to stabilize the liposomal system. Drug lipid ratio was 1:10 with membrane composition of hydrogenated soy phophatidylcholine, cholesterol and either saturated soy phosphatidylglycerol (7:3:0.5) or stearylamine (1:1:0.1) were used to prepare negatively and positively charge liposomes, respectively. The shelf life of the powder was over 1 year at refrigerated condition (2-8 ºC).

Effectively chemotherapy through dug targeting to alveolar macrophages can be practically implemented particularly in pulmonary aspergillosis

using ligand-anchored liposomes, aerosolized liposomal AmB has also been reported for treatment of pulmonary fungal infections (Gilbert et al., 1992; Allen et al., 1994; Ruijgroka et al., 2001). The encapsulation of AmB into the liposome with modification of liposomal surface by anchoring lung macrophages-specific ligands will certainly improve efficacy against pulmonary aspergillosis (Vyas et al., 2005).

2.4.4 Formulations of AmB for lung infection

2.4.4.1 Conventional AmB formulation

The conventional formulation of AmB, Fungizone® (Bristol-Myers Squibbs, USA) was used in invasive fungal infection for more than 40 years. Fungizone® was prepared from AmB complex formation with the sodium deoxycholate, as solubilizing agent. Each vial of Fungizone® containing 50 mg of AmB with 41 mg of sodium deoxycholate and 20.2 mg of sodium phosphate. In clinical application, conventional AmB has been manifested a variety of adverse reactions such as nephrotoxicity, fever, chills, electrolyte imbalance, cardiac toxicity and phlebitis at the site of injection. Nephrotoxicity is the prime cause of drug discontinuation during long term treatment as well as dose limits (Deray, 2002). AmB is a very low solubility to water and usually precipitated during the dilution of AmB with 5% dextrose solution, which may cause the acute renal toxicity (Yu et al., 1998).

2.4.4.2 Lipid based AmB formulation

Lipid based AmB formulations have been successful prepared due to the recent advance development of drug delivery technology in field of nanotechnology. On basis of this technology, AmbiSome®, Amphocil®, Abelcet® formulations have been developed. AmBisome®, the first to be licensed, was a unilamellar liposome of about 80 nm in diameter. This formulation employed saturated phospholipid with cholesterol to stabilize the liposomal membrane. Charged phospholipids (phosphatidylglycerol) were also included to stabilize the AmB liposomes through ionic interactions. Further stabilization could result from the direct interaction of AmB with the cholesterol via its sterol binding region. Finally, it was anticipated that the small size of these liposomes (<100 nm) would provide

stabilization in vivo and prolong the circulation of drug-containing liposomes in the plasma.

Pharmaceutical characteristics of AmB formulations are shown in Table 2.1. Those formulations differ widely in their composition and physical properties: one has a mixed micelle structure, and the others are AmB-lipid formulations (liposomal bilayer, disc-like and ribbon-like structure).

Table 2.1 Industrial manufacturer produced intravenous formulations of AmB (Storm and van Etten, 1997).

Formulations	Manufacturer	Carrier	Shape
Fungizone®	Bristol Myers- Squibb	Sodium deoxycholate	Micelle
AmBisome®	NeXstar Pharmaceuticals	HSPC/DSPG/cholesterol	Unilamellar
Amphocil® (ABCD)	Sequus Pharmaceuticals	Cholesterol sulfate	Disc complex
Abelcet™ (ABLC)	The Liposome Company	DMPC/DMPG	Ribbon

ABCD: AmB colloidal dispersion, ABLC: AmB lipid complex, DMPC: dimyristoylphosphatidylcholine, DMPG: dimyristoylphophatidylglycerol, HSPC: Hydrogenated soy phophatidylcholine, DSPG: distearoylphosphatidyl glycerol

2.5 Lipids

Lipids are a group of naturally occurring molecules that include fats, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides, triglycerides, phospholipids and others. The main biological functions of lipids include storing energy, signaling and acting as structural components of cell membranes. It may be broadly defined as hydrophobic or amphiphilic small molecules, the amphiphilic nature of some lipids allow them to form structures such as vesicles, liposomes or membranes in an aqueous environment. Lipids have applications in the cosmetic and food industries as well as nanotechnology.

2.5.1 Lipid derivatives as drug carriers

Lipids, one of the major natural products, usually have high biocompatibility and low toxicity. Due to their proper physicochemical properties, they are the most commonly used materials for building modern drug delivery systems, especially nanocarriers. However, the lipids can be modified or synthesized to meet special to impart new functions. Using lipids or lipid derivatives, various lipid-based drug or gene delivery systems have been developed and show potential in pre-clinical and clinical applications (Ying and Lin, 2014).

Lipid based drug delivery systems (LDDS) consist of diverse group of formulations, each consisting of varying functional and structural properties that are amenable to modifications achieved by varying the composition of lipid excipients and other additives. LDDS has evolved from micro to nano-scale enhancing the efficacy and therapeutic application of these systems. Generally, most lipid drug delivery systems used as drug carriers have high stability, high carrier capacity, feasibility of incorporating both hydrophilic and hydrophobic substances and feasibility of variable routes of administration, including oral, topical, parenteral and pulmonary routes (Chime and Onyishi, 2013). Lipid-based excipients such as glycerides, fatty acids, ionic and non-ionic surfactants are known permeability enhancers (Kuentz, 2012) which may be due to increased membrane fluidity.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

AmB was obtained from Ambalal Sharabhai Enterprises Pvt. Ltd., Vadodara, India. Deoxycholic acid, cholic acid, sodium cholate, sodium deoxycholate and ergosterol were purchased from Sigma-Aldrich, St. Louis, USA. Sodium deoxycholate sulfate, potassium cholate and potassium deoxycholate were synthesized in a laboratory. Sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate dihydrate were purchased from Ajax Finechem Pty Ltd, NSW, Australia. Acetonitrile and methanol were purchased from Labscan Asia, Bangkok, Thailand. Dimethylsulfoxide was purchased from Riedel-de Haën, Seelze, Germany. Polyamide membranes with a pore size of 0.22 µm and 0.45 µm were obtained from Sartorius, Gottingen, Germany. All chemicals were used as received without further purification except tetrahydrofuran (THF). All other reagents and chemicals are analytical grade.

3.2 Synthesis of sodium deoxycholate sulfate

3.2.1 Synthesis of methyl deoxycholate

Methyl deoxycholate ester compound was prepared from deoxycholic acid (3.0 g,) using conc. sulfuric acid (0.6 mL) in methanol (50 mL) under reflux for 4 h by esterification reaction (Fig. 3.1) (Huang et al., 2011). The progress of the reactions was monitored by analytical TLC. After completion of this esterification reaction, the mixture was washed five times with distilled water a neutral pH was obtained and confirmed by pH meter (Precisa pH 900, Dietikon, Switzerland). The product was then extracted with ethyl acetate in a separating funnel. The ethyl acetate layer was collected in a dry conical flask and dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure using a rotary evaporator (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The crude mixture was purified by silica gel column chromatography by elution with hexane and ethyl acetate (40:60, v/v) to obtain pure methyl deoxycholate in a yield of 98% (3.0 g).

3.2.2 Reduction of methyl deoxycholate ester to the corresponding alcohol

Sodium borohydride (1.2 g, 0.0316 mol) was added to dry the THF (10 mL) in the round bottom flask and stirred (IKA C-MAG HS-7, Staufen, Germany) for 5 min at room temperature. Methyl deoxycholate (2.5 g, 0.0061 mol,) in dry THF (30 mL) was added slowly dropwise for 10 min and the resulting mixture was left for 30 min at a reflux temperature of 65 °C in a nitrogen atmosphere. Then methanol (30 mL) was added dropwise at room temperature and the reaction was continued at a reflux temperature for 8 h. After completion of the reaction the solution was quenched with 2N HCl (5 mL) and stirred (IKA C-MAG HS-7, Staufen, Germany) at room temperature for 30 min (Fig. 3.1) (Saeed and Ashraf, 2006). The progress of the reactions was monitored by analytical TLC. The mixture was then extracted by ethyl acetate and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure using a rotary evaporator (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) to obtain a crude mixture. The crude mixture was purified using silica gel column chromatography by elution with hexane/ethyl acetate (20:80, v/v) to obtain the deoxycholic alcohol compound. The alcohol was obtained as a single pure product using chromatography on a silica gel column and eluting with hexane/ethyl acetate (20:80, v/v). In the IR spectrum, the appearance of a broad absorption peak at 3350 cm^{-1} and the complete absence of a carbonyl ester stretching peak at 1721 cm^{-1} showed that the 24-carbonyl group in methyl deoxycholate compound had been converted to a deoxycholic alcohol compound.

3.2.3 Synthesis of sodium 3α,12α-dihydroxy-5β cholan-24-ol sulfate

Deoxycholic alcohol compound (0.68 g, 0.0018 mol) in a mixture of DMF (4 mL) and DCM (2 mL) were stirred in a magnetic stirrer (IKA C-MAG HS-7, Staufen, Germany) at 0 °C in ice-bath for 10 min and sulfur trioxide pyridine complex (0.420 g, 0.0027 mol) was added slowly in small portions at 0 °C for 1 h. After completion of the reaction, saturated sodium bicarbonate (15 mL) solution was added to the reaction mixture and the reaction was continued at the reflux temperature for 2 h (Fig. 3.1) (Lazer et al,. 2004; Sawada et al., 2005). The progress of the reactions was monitored by analytical TLC. Then the volatile solvents were removed under reduced pressure using a rotary evaporator (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo,

Japan) to obtain a crude mixture. The crude mixture was dissolved in methanol and stirred at room temperature for 30 min. The methanol soluble fraction was dried under reduced pressure and purified by silica gel column chromatography by elution with ethyl acetate/methanol (60:40, v/v) to obtain the pure compound sodium deoxycholate sulfate (Burns et al., 2011).

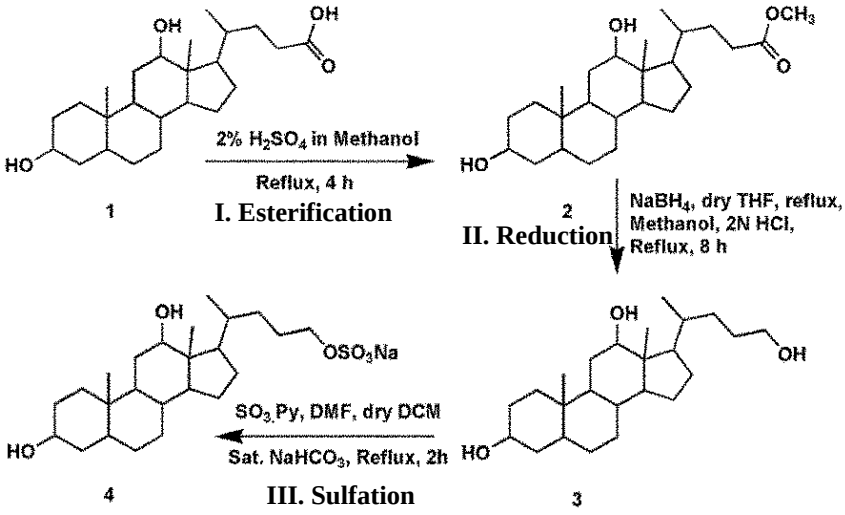
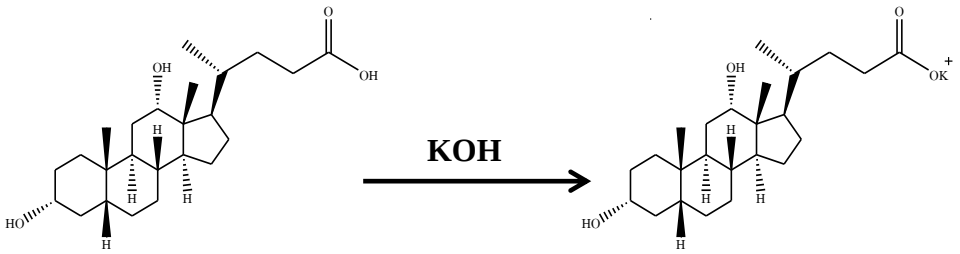


Figure 3.1 Synthesis route of sodium deoxycholate sulfate in 3 steps reaction (**Scheme 1**) (Huang et al., 2011; Saeed and Ashraf, 2006; Lazer et al., 2004; Sawada et al., 2005; Burns et al., 2011). The number (1), (2), (3) and (4) denoted the compund as deoxycholic acid (DCA), methyl deoxycholate ester, deoxycholic alcohol (DAL) and sodium deoxycholate sulfate (SDCS), resepectively.

3.3 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. 3.2), 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.

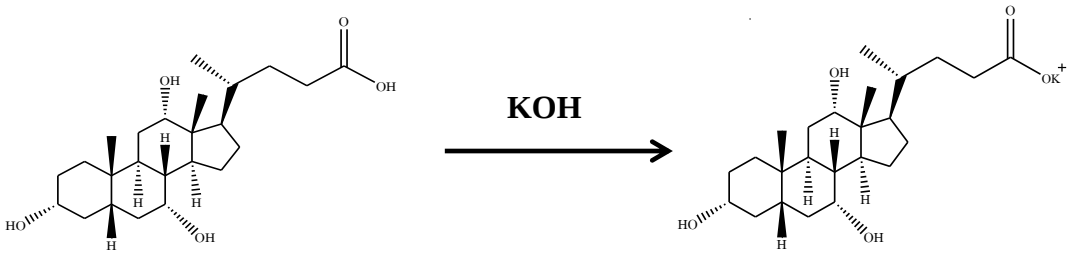


Deoxycholic acid Potassium deoxycholate

Figure 3.2 Synthesis of potassium deoxycholate from deoxycholic acid (Morrison and Boyd, 1987)

3.4 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. 3.3), 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.



Cholic acid Potassium cholate

Figure 3.3 Synthesis of potassium cholate from cholic acid (Morrison and Boyd, 1987).

3.5 Charactrization of sodium deoxycholate sulfate (SDCS), potassium cholate (KC) and potassium deoxycholate (KDC)

A) Fourier Transform Infrared Spectroscopy (FTIR)

The functional group of carbonyl esters were recorded by using PerkinElmer precisely (PerkinElmer Inc., Hercules, CA, USA) in the frequency range 4000- 450 cm^{-}.[1] A small amount of sample was sealed into KBr pellets by a hydraulic press prior to measurement at ambient temperature.

B) Nuclear Magnetic Resonace Spectorcopy (NMR)

The The ^{1}H-NMR and ^{13}C-NMR spectra are performed on Varian Unity Inova 500 spectrometer at 500 MHz (Varian, Germany). All the NMR spectra are recorded at 298 K using deuterochloroform ($CDCl_3$) as solvent. ^{1}H-NMR chemical shifts are referenced to internal standard TMS, for ^{13}C-NMR spectra resonance line of $CDCl_3$ at 77.00 ppm was taken as reference line.

C) Mass Spectrometry (MS)

The electrospray ionization (negative mode) mass spectra were obtained from micro mass LCT mass spectrometer (Bruker, Breman, Germany).

3.6 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.

The stability test was carried out reported method by Darole et al. 2007. The formulated product sample was packed into the air tight amber bottle and stored at below 8 °C in a refrigerator for 6 months to study stability test. For the initial period of time, the stability test was performed immediately after formulation and stored at below 8 °C in a refrigerator until test was not carried out. The stability test was determined into particle size, zeta potential and drug content as follows. The product was reconstituted in distilled water to obtain an AmB concentration of 10 mg/mL. The physicochemical properties such as particle size and zeta potential were determined by Zetasizer (Malvern Instrument Ltd., Worcestershire, UK) as well as stability was evaluated by spectrophotometer (Thermo Genesys 6 UV-visible Spectrophotometer) between 300 to 450 nm. Analyses were made in triplicate. The drug contents assay was carried out in HPLC method (Waters, Singapore). 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.

3.7 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.

3.8 *In vitro* hemolysis assay

The human red blood cells lysis was evaluated as described by Mehta et al. (1984). Briefly, erythrocytes (Blood Bank, Department of Pathology, Faculty of Medicine, Songklanagarind Hospital, PSU, Thailand) were isolated from fresh human blood, washed three times with phosphate buffer saline solution (PBS) and centrifuged at 3000 rpm for 5 min. Stock solutions of AmB, AmB-KC, AmB-KDC AmB-SC, AmB-SDC and AmB-SDCS were added to the suspended erythrocytes and the suspension was diluted with PBS to give a final AmB concentration in the range 1-8 µg/mL and a final hematocrit of 1%. The solutions were incubated at 37 °C in an incubator for 24 h. The unlysed cells were removed by centrifugation at 3000 rpm for 5 min and the hemoglobin in the supernatant was determined by its absorbance at 540 nm. PBS without AmB was used as a negative control and 1% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) was used as a positive control for 100% lysis. According to the equation 1, % hemolysis can be calculated.

$$\% \text{ Hemolysis} = [Abs - Abs_0/Abs_{100} - Abs_0] \times 100 \quad \text{..... Equation.....1}$$

Where: Abs is the absorbance of sample

:Abs_0 is the absorbance of negative control

:Abs_{100} is the absorbance of positive control

3.9 The cytotoxicity assay in respiratory cell lines and kidney cells

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

The viability of human lung adenocarcinoma cell line (A549), human bronchial epithelial cells (Calu-3), alveolar macrophage cell line (NR 8383) (ATCC CRL-2192), human embryonic kidney cells (293T/17) were determined by using the MTT assay to detect functioning mitochondria. Live mitochondria transform 3-(4,5 dimethylthiazole-2yl)-2 diphenyltetrazolium bromide or MTT (Sigma Chemicals, St. Louis, MO, USA) to formazan, which was measured with a spectrophotometer. To determine the MTT assay, the respiratory associated cell lines (A549, Calu-3, NR 8383) and kidney cells (293T/17) were cultivated in a cell culture flask using a different complete media for different cells as mentioned here. First, the complete media were prepared as follows. A549 culture media was prepared from Kaighn's F12K supplemented with 4 mM L-glutamine, 10% fetal bovine serum (FBS), and 50 units/mL penicillin and 50 µg/mL streptomycin. Similarly, Calu-3 complete media was prepared from Minimum Essential Medium Eagle Supplemented (MEMES) with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 µg/mL streptomycin. Alveolar macrophage (AM) complete media was prepared from F12K supplemented with 15% heated–inactivate fetal bovine serum (FBS), 50 units/mL penicillin and 50 µg/mL streptomycin. Kidney cells complete media was prepared from Dulbecco`s Modified Eagle's Medium (DMEM) supplemented with 2mM L-glutamine and 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 µg/mL streptomycin. To prevent cell contamination, each experiment was done at once time and after completing culture process then all materials were cleared from the hood and cleaned

properly before to start next cell culture process. After preparing four different complete media, 5 mL each media of cell line A549, calu-3, NR 8383 and kidney cells were taken in four different cultures flasks inside hood as an aseptic condition. Then four different cell lines were taken out from the liquid nitrogen gas cylinder and thaw on incubator until liquefied. After that individual cell line was transferred into the each culture flask inside the laminar flow hood and was mixed properly and closed the cap tightly. All these cells were taken out from the hood and incubated in an atmosphere of 5% CO_2 and 95% humidity incubator at 37°C. After successfully cultivated four different cells (A549, Calu-3, NR8383 and 293T/17) in four different culture flasks, then cells were seeded into the 96 well plates for the MTT assay test as follows.

The MTT (3-(4,5 dimethylthiazole-2yl)-2 diphenyltetrazolium bromide) colorimetric assay, as developed by Mosmann (1983) and modified by Edmondson et al. (1988), was employed to assess the cytotoxicity of AmB and AmB-lipid formulations on the A549, Calu-3, AM NR 8383 and kidney (293T/17) cells. A549, Calu-3 and AMs NR8383 cells and kidney cells (293T/17) were distributed in 96-well plates at a density of 1×10^5 cells/well in 100 µL completed medium and allowed to attach overnight. After 24 h at 37 °C and 5% CO_2, 95% humidity, the medium (100 µL) was replaced with medium containing various concentrations of drug formulations (1 to 8 µg/mL) or an equivalent concentration. After incubation for 24 h, 50 µL of a solution of MTT at 1.25 mg/mL (Invitrogen, USA) were added and incubated for a further 4 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidity, covered with an aluminum foil. After completion of the 4 h incubation, the solutions were removed from the 96 well plates and 100 µL of DMSO (Riedel-de- Haën, Seelze, Germany) added to dissolve the formazan crystals. The optical densities (OD) were measured at 570 nm in a microplate reader (Biohit BP 800, Helsinki, Finland). Control cells were incubated with medium without AmB. Four wells were used for each concentration and time-point. The percentage of surviving cells was calculated from the following formula: $(OD_{treated}/OD_{control}) \times 100$. Three independent experiments were done. The number of viable cells in the treated well was compared to those in the untreated well and estimated as percent viability.

3.10 Determination of nitric oxide release from alveolar macrophage response to AmB-lipid formulations

Alveolar macrophages are mobile phagocytic cells located within the alveolar regions and small airways of the lungs. Nitric oxide (NO) is a free radical that is produced by various cells in the lungs. NO produced by iNOS plays an important role in defense against airborne pathogens or in tissue damage associated with inflammatory processes in the lungs (Ialenti et al., 1992). These cells represent a primary defense mechanism against the adverse effects of inhalation of bacteria and foreign particles. AM are resident lung phagocytes that express high densities of immunoglobulin receptors, complement receptors, mannose receptors and several types of scavenger receptors to facilitate phagocytosis of opsonized and non-opsonized particles. AM are active producers of cytokines and leukotrienes, and have important pro-inflammatory roles in the alveolus. To determine the toxicity of AmB-lipid formulations, NO production test was carried out by an *in vitro* study with AM cells as follows.

AM NR8383 cells were added to 96 well plates at an optical cell density of $1x10^5$ cells/well and after 24 h, the supernatant was collected in a separate 96 well plates for nitric oxide production test. This test was carried out with AmB-lipid formulations (AmB-SDCS, AmB-SDC, AmB-KDC, AmB-SC and AmB-KC) and pure AmB. LPS is known as NO activator used as a positive control. The NO released by NR8383 cells after being challenged with either AmB, AmB-lipid formulations reconstituted dry powders (AmB-SDCS, AmB-SDC, AmB-KDC and AmB-SC) in concentration ranges of 1–32 μg/mL or 15–1000 ng/mL for LPS. NO was detected by the Griess reaction. This measurement relies on a diazotization reaction (Fig.3.4) of the Griess reagent. The Griess reagent was prepared by mixing 1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5% phosphoric acid in water. Equal volumes of cell supernatant (100 μL) and Griess reagent (100 μL) were mixed. After 10 min, the absorbance was measured using a microplate reader (Biohit BP 800, Helsinki, Finland) at 450 nm. The NO concentration was calculated from a sodium nitrite standard curve (Huttunen et al., 2000; Punturee et al., 2004).

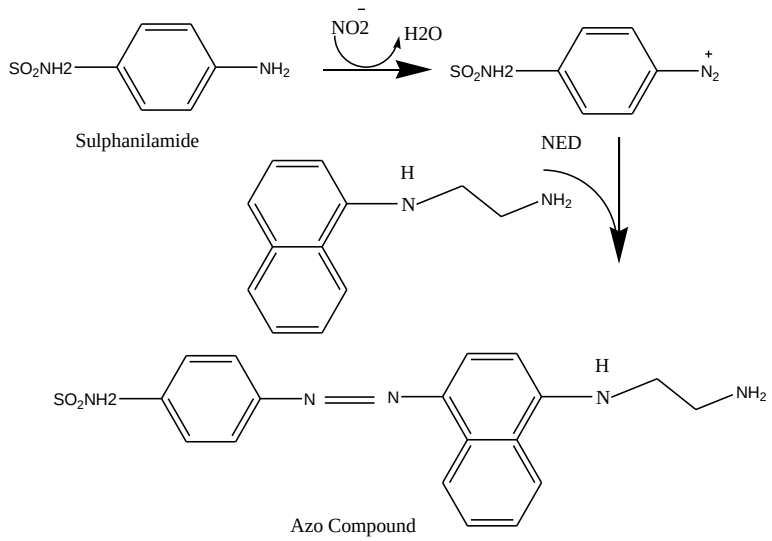


Fig. 3.4 Chemical reaction involved in the measurement of the NO^-_2 using the Griess reagent system (adapted from Promega, 1995).

3.11 Assay for anti-fungal activity

The activity (potency) of antibiotics may be demonstrated under suitable conditions by their inhibitory effect on microorganisms (USP 29-NF 24, 2006). Two general methods are employed: the cylinder-plate or ″plate″ assay and the turbidimetric or ″tube″ assay. The cylinder plate method and broth microdilution were employed for microbial assay of AmB in lipid derivatives. The cylinder plate method performs the relative potency by determining the clear zone of AmB-lipid formulation in the inoculum medium as compared with a clear zone of AmB standard. Broth microdilution method can be used to measure the minimum inhibitory concentration (MIC). The MIC is that concentration of antifungal agents which inhibits the growth of a fungus under standardized test condition. The MIC value is based upon predetermined end point, which may be interpreted as an absence of visible growth in a broth containing known concentration of AmB (adapted from Mcginnins and Rinaldi, 1991). For 1-level assay with a standard curve, dilutions were prepared representing five test levels of the standard (S1-S5) were 0.64, 0.8, 1.0, 1.25, 1.56 µg/mL, respectively and a single test level of the unknown (U3) 1.0 µg/mL corresponding S3 of the standard curve. For deriving the standard curve, alternative cylinder were filled on each of three plates with the mean test dilution (S3) of the

standard and each of remaining nine cylinders with one of the other four dilutions of the standard. The process was repeated for the three dilutions of the standard.

The microbiological method consisted of a cylinder-plate agar diffusion assay using *Saccharomyces cerevisiae* (ATCC 9763, Rockville, MD, USA) was the test microorganism as follows. *S. cerevisiae* with approximately 10^8 colony-forming units (CFU)/mL was cultivated at 30 °C on Sabouraud dextrose agar (Difco, NJ, USA) for 48 h. The yeast was then suspended in 0.9% NaCl and diluted to obtain a turbidity of 25 ± 2% at 530 nm. One milliliter portions of this suspension were added to 100 mL sterile antibiotic medium 19 at 50 °C and used as the pre-inoculated layer. Six stainless steel cylinders of uniform size (8 mm (o.d.)×6 mm (i.d.)×10 mm) were placed on the surface of the inoculated medium using a mechanical guide. The cylinders were filled with standard AmB or the AmB in the lipid derivatives reconstituted dry powder formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS). All plates were incubated at 30 °C for 16-18 h. These experiments were carried out in triplicate. The inhibition zone diameters were measured and the concentration of AmB calculated from the standard curve (USP 29, 2006).

3.12 Microdilution method for determination of MIC and MFC

The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined using *Cryptococcus neoformans* ATCC 90113 NS and *Candida albicans* ATCC 90028 (Department of Pathology, Faculty of Medicine, Songklanagarind Hospital, PSU, Thailand) as described in previous work (McGinnis and Rinaldi, 1991). Before testing, fungi were subcultured on Sabouraud dextrose agar (SDA) (Difco, NJ, USA) at 35 °C for 24-48 h to ensure cultures for inoculation were in an active phase of growth. Stock suspensions were prepared in a sterile 0.9% sodium chloride solution and adjusted to give a final concentration of 1×10^6-5×10^6 CFU/mL (90% transmittance, 530 nm). For the standard AmB and AmB-lipid formulations reconstituted dry powders preparations, these were dissolved in sterile DMSO and sterile water at a concentration 5000 µg/mL, respectively. Stock solutions were stored at -70 °C. Subsequently, the stock solutions were diluted with antibiotic medium 3 broth (Merck, Darmstadt, Germany) to a concentration of

between 0.004 and 10 µg/mL. 10 microliter of the inoculum was added to the 96-well plate and 100 µL of a two-fold serial dilutions of standard AmB or the reconstituted AmB-lipid dry powder derivatives (AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC and AmB-SC) at concentrations of 0.004-10 µg/mL. The sterile medium was used as a negative control (sterility of medium) while the sterile medium with the inoculum was used as a positive control (growth of inoculums without AmB). The plates were incubated at 30 °C for 48 h, and the absorption of the sample was recorded at a wavelength of 570 nm. All experiments were carried out in triplicate.

3.13 Determination of AmB permeation across lipid bilayer

3.13.1 Lipid bilayer and permeability

The lipid bilayer is a universal component of all cell membranes and its structural components provide the barrier that marks the cell boundaries. The cell membrane is a lipid bilayer of between 5-10 nm thick consisting of two external, a hydrophilic region (polar head groups) and an internal hydrophobic (nonpolar tail) region. Phospholipids are the major form of lipid involved. The phospholipids organize themselves into a bilayer to hide their hydrophobic tail regions and expose their hydrophilic regions to water. This organization is spontaneous, a natural process that occurs without any energy requirement. The most important properties of the lipid bilayer is the formation of a highly selective semipermeable structure and its fluidity at normal temperatures. This fluidity will provide mobility within the lipid bilayer, which is biologically important, as it influences membrane transport. The lipid bilayer component of the cell membrane helps to sustain osmotic gradients across the membrane, but it is not a perfect chemical seal. Ions, water, and other molecular compounds will invariably passively cross the membrane. Crossing the permeability barrier of the lipid bilayer is vital for targeting any drug delivery system (Mouritsen and Jørgensen, 1998). The permeation and transport characteristics of lipophilic and hydrophilic drugs across the lipid membrane are of crucial importance for allowing drugs to reach their target and action sites. During our study, all parameters were kept constant, such as the temperature of 37 ºC and the acyl chain length of the lipid bilayer membrane was the same for all experiments (phospholipid

and ergosterol concentration was a constant 67:33 mol%). The AmB or AmB-lipid formulations were also fixed at 50 μg of AmB in 5 mL phosphate buffer solution. We determined the relationship between the AmB and the AmB-lipid derivatives formulations by examining their permeation across the lipid bilayer membranes.

3.13.2 Preparation of the lipid bilayer membrane

To conduct an *in vitro* permeability test, the lipid bilayer membranes were prepared from phospholipid (L-α-phophatidylcholine) and ergosterol in a ratio of (67:33 mol %) as previously reported by Ostroumova et al., (2012). These were prepared by dissolving L-α-phosphatidylcholine 67 mol% and ergosterol 33 mol% in 30 mL of chloroform in a 100 mL round bottomed flask for uniform mixing. The solvent was removed under reduced pressure using a rotary evaporator (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at 55 °C until it formed a dried cake. The dried cake was transferred to a petri-dish and further heated at 40 °C for 48 h on a hotplate to completely remove the solvent and a dry powder mixture was obtained. The dried powder was transferred into a vial with 6 mL of distilled water and kept in a water bath (50 °C) for 1 h for hydration. The hydrated suspension (1000μL) was loaded onto each Transwell® (Corning Inc., NY, 14831, USA) and dried at 40 °C for 48 h. The dried lipid membrane on Transwell® was equipped in an Ussing chamber system (Insert for 2300 Easy Mount Diffusion Chambers, Physiologic Instruments, Harvard Apparatus Companies, USA) (Fig. 3.5).

3.13.3 Preparation of the electrodes

The electrode set consisted of two pairs of electrodes, one pair for the passing current and the other for sensing the voltage. The voltage-sensing electrode (black housing) was a small pellet formed by compressing AgCl about an Ag wire. AgCl was used for sensing the voltage because it provided a relatively stable electrode potential and was reversible. The current electrode (white housing) was a Ag wire that had been plated with chloride ions to lower its resistance. Each type of electrode was inserted into a pipette tip and a connection was made to the phosphate buffered solution in the chamber via a KCl-filled agar bridge (Fig. 3.5 A). Ag/AgCl electrodes with agarose/2M KCl bridges were used to apply the transmembrane

voltage (V) and to measure the transmembrane current (I). The electrodes were prepared by dissolving 10 mL of 2M KCl and 3% agar in a beaker and kept on water bath at 100 °C for 5 min with continuously stirring until it was well mixed and should be free from trapped air bubbles. When the agar was completely dissolved and formed a transparent solution it was then transferred inside an electrode (5 mm of length) with the help of a 2 mL syringe from the tip of the electrode and the rest of the space was filled with KCl (2M) solution. The filled pipette was placed in KCl (2M) solution and the container was sealed until used. KCl was chosen as the filling solution because K^+ and Cl^- have nearly equal free solution mobilities and, therefore, will not cause a significant diffusion potential even though the concentration gradient from the pipette to the chamber was very large.

3.13.4 Formation of lipid bilayer membrane in the Ussing chamber

The lipid bilayer Transwell® was fitted into the Ussing chamber. Phosphate buffer solution (10 mM, pH 7.4) 5 mL was added to both chambers and left for 1 h to completely hydrate and stabilize the membrane on the Transwell® in the presence of the phosphate buffer solution (Fig. 3.5 B).

3.13.5 Ussing chambers permeation across lipid bilayer model

The Ussing chamber is suitable for our experimental design in shape and size, with a charge induced by an AmB-lipid formulations in a solution form. Its basic principle was applied to measure the short-circuit current as an indicator of net ion transport taking place across the lipid bilayer membrane (from left chamber to right chamber). In the Ussing chamber, electrodes are placed on both sides of the lipid bilayer membrane to record the potential differences (PD) across the lipid bilayer. A set of two other electrodes allows for the injection of a current (short-circuit current or Isc) to nullify the PD. The intensity of the current to be injected was monitored by an electric clamp apparatus. The Ussing chamber system was filled with the experimental phosphate buffer (10 mM, pH: 7.4) 5 mL solution in both chambers and the experiment was performed at room temperature (37 ºC). To perform our study, some modifications were made to this instrument. Instead of a biological membrane, we applied a preformed lipid bilayer membrane in the Transwell® to act as a

membrane that supported for permeability of AmB or AmB-lipid formulations in the Ussing chamber (Liuqin et al., 2013).

3.13.6 Collection of samples from the Ussing chamber

Before addition of the AmB suspension into the Ussing chamber system, first, we confirmed that there was no leakage from the chambers. This was done by applying grease in the two half chambers, when the electrodes were inserted into the Transwell® holes to assist with making them air tight and leak proof. After confirming this we started the test for permeability. First of all, 50 µL of the drug solution or the AmB-lipid formulations (concentration of AmB 1000 µg/mL) was added to the left chamber and the initial current was noted, then after every 15 min the current was noted and 1 mL of sample was withdrawn from the right chamber and the reduced volume was compensated with 1 mL of phosphate buffer solution in the right chamber until the 2 h period for collecting samples were completed. All experiments were performed at 37 ºC and in triplicate. The collected sample solutions were quantified for AmB by the HPLC method to determine the percentage cumulative AmB permeation from the left chamber to right chamber.

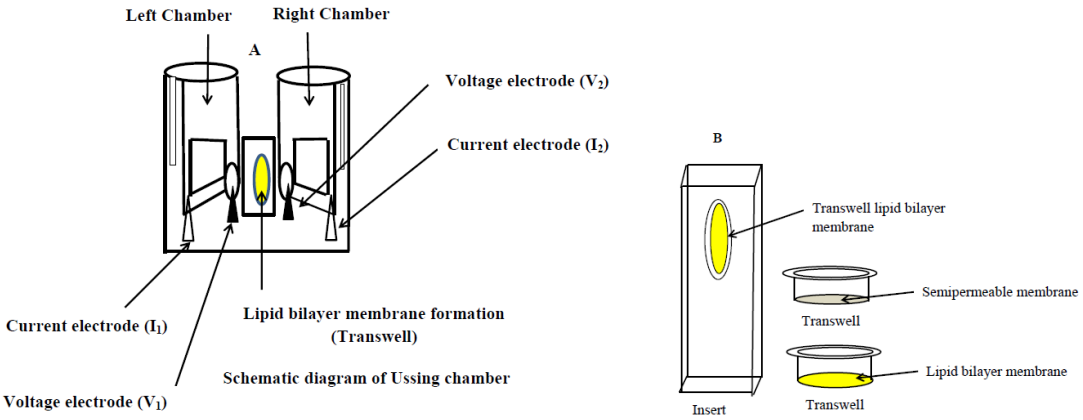


Figure 3.5 Schematic diagram of Ussing chamber (A), diagram of insert and containing lipid bilayer semipermeable membrane in transwell (B).

3.14 Phagocytosis of the reconstituted AmB-lipid formulations by macrophage cells

The AmB-lipid dry powder formulation (AmB-SDCS) was reconstituted with distilled water to obtain 1 mg/mL concentration of AmB. Quantum dot nanoparticles were employed for particle imaging. Lumidot® 640 (20 μg/mL) was added to the reconstituted formulation AmB-SDCS solution in a volume ratio 1:2. The mixture was sonicated for 5 min and reconstituted was observed with a fluorescence microscope. NR8383 cells 100 μL of 1 x 10^4 cells/mL were cultured in each well of a 96-well plate and allowed to grow and adhere overnight at 37 °C, in a 5% CO_2 and 95% humidity incubator. The AmB-lipid reconstituted dry powder formulation (AmB-SDCS) stained with Lumidot® 640 (Lumidot®, Sigma-Aldrich, St. Louis, USA) was added. Phagocytosis of anti-fungal nanoparticulate by AM NR8383 cells was observed with a fluorescence microscope (Olympus, BX61, Olympus, Tokyo, Japan).

3.15 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.

CHAPTER 4
RESULTS AND DISCUSSION

Three synthesized lipid carriers such as sodium deoxycholate sulfate (SDCS) was obtained from deoxycholic acid in three steps of reaction process known as esterification, reduction and sulfation. Similarly, 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 3.1, 3.2 and 3.3 associated with the compounds was invested by using infrared spectral analysis, ^{1}H NMR techniques, mass spectrometry and elemental analysis. The yields of SDCS, KC and KDC were obtained 78, 80 and 82% respectively. All three 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 4.3. 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.

4.1 Synthesis

4.1.1 Synthesis of deoxycholate sulfate

Sodium deoxycholate sulfate was synthesized from deoxycholic acid (**1**) as the starting material using a method reported by Burns et al. (2011) with a few modifications (**Scheme 1**) as described in the materials and methods. Deoxycholic acid was first converted to methyl deoxycholate using a reported method but with a change to the methylating reagent (Huang et al., 2011). Methyl deoxycholate ester was then successfully reduced to its corresponding alcohol, deoxycholic alcohol via a reported method (Saeed and Ashraf, 2006). The reduction was completed at reflux temperature (65 °C) for 8 h giving an alcohol yield of 70%. In this case we preferred sodium borohydride to avoid the use of strong reducing agents like lithium aluminum hydride and borane-dimethyl sulfide complex, as these are generally used at 0 °C to -78 °C. The progress of the reactions was monitored by analytical TLC.

The ^{1}H NMR spectrum showed a broad singlet at a signal of 2.5 for an alcohol proton and the disappearance of the carbonyl attached proton signal at 2.4. Sodium deoxycholate sulfate was synthesized from the deoxycholic alcohol compound using the SO_3·Pyridine complex in DMF at room temperature by a selective sulfation of the primary hydroxyl group over the secondary hydroxyl groups where both are in the same molecule (Lazar et al., 2004; Sawada et al., 2005). The crude product was purified by chromatography on a silica gel column and eluted with ethyl acetate/methanol (60:40, v/v). The sodium deoxycholate sulfate was obtained in a 78% yield. The structure of the synthesized compound sodium deoxycholate sulfate was compared with the reported authentic compound using ^{1}H NMR, IR, mass spectra its R_f and melting point. These results confirmed that the synthesized compound sodium deoxycholate sulfate was identical to the previous reports of sodium 3α, 12α-dihydroxy-5β-cholan-24-ol sulfate (Burns et al., 2011). The overall yield of the desired compound sodium deoxycholate sulfate was 78% after the 3 steps. The confirmative test result of each step was presented as follows.

Synthesis of methyl deoxycholate

The crude mixture was purified by silica gel column chromatography by elution with hexane and ethyl acetate (40:60, v/v) to obtain pure methyl

deoxycholate in a yield of 98% (3.0 g). It was a white amorphous solid; R_f = 0.8 (hexane:ethyl acetate; 60:40, v/v); m p: 57–59°C; IR(neat) v_{max}: 3435, 2940, 2861, 1721, 1445 and 1298 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$):d 0.75 (s, ^3H, 18-CH_3), 0.90 (s, ^3H, 19–CH_3), 0.95 (d, ^3H, 21–CH_3), 1.0–2.0 (m, ^{24}H), 2.40 (t, ^2H, –CO–CH_2–), 3.59–3.65 (m, ^1H, –OCH–(CH_2)2), 3.67–3.7 (s, ^3H, –OCH_3), 3.95–4.0 (d, ^1H, 12-OCH–CH_2).

Reduction of methyl deoxycholate ester to the deoxycholic alcohol

The crude mixture was purified using silica gel column chromatography by elution with hexane/ethyl acetate (20:80, v/v) to obtain the corresponding alcohol of the methyl deoxycholate in a 70% yield (1.62 g). R_f = 0.7 ($CHCl_3$:CH_3OH, 80:20); mp: 144–146 °C; IR (KBr) v_{max}: 3350, 2938, 2863, 1448 and 1237 cm^{-1}; ^1H NMR (500 MHz, DMSO-d6):d 0.63 (s, ^3H, 18−CH_3), 0.85 (s, ^3H, 19−CH_3), 0.95 (d, ^3H, 21−CH_3), 1.0–2.0 (m, ^{26}H), 2.5 (s, br, −OH), 3.2–3.4 (m, ^1H), 4.25 (t, ^1H), 4.35 (t, ^2H).

Synthesis of sodium 3α, 12α-dihydroxy-5β-cholan-24-ol sulfate

The methanol soluble fraction was dried under reduced pressure and purified by silica gel column chromatography by elution with ethyl acetate/methanol (60:40, v/v) to obtain the pure compound with a yield of 78% (0.67 g). R_f = 0.51 ($CHCl_3$: CH_3OH:satd aq NH_3, 80:18:2); mp: 173–176 °C; IR (KBr) v_{max}: 3435, 2936, 2863, 1633 and 1239 cm^{-1}; ^1H NMR (500 MHz, DMSO-d6): d 0.60 (s, ^3H, 18–CH_3), 0.85 (s, ^3H, 19–CH_3), 0.95 (d, ^3H, 21–CH_3), 1.0–1.9 (m, ^{24}H), 3.6–3.75 (m, ^1H), 3.8 (t, ^2H), 4.25 (t, ^2H); m/z (ESI) calculated for $C_{24}H_{41}SO_6Na$, 480.57; and found 480.6.

In summary, the SDCS was synthesized from deoxycholic acid by a new process with 78% yield. Due to the use of mild reaction conditions and the ease of the work up procedure, this method can be more advantages over previously reported methods. This compound is white amorphous powder and soluble in water having melting points 173-176 °C and molecular weight was found 480.6.

4.1.2 Synthesis of potassium deoxycholate (KDC)

The yield of potassium deoxycholate was obtained 82% from freeze drying process. It was white amorphous powder, very light and highly soluble in water. The final compound 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} 1641 cm^{-1}). Its molecular weight was found 430.6.

4.1.3 Synthesis of potassium cholate (KC)

The yield of potassium cholate was obtained 80% from freeze drying process. It was white amorphous powder, very light and highly soluble in water. The final compound 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}). Its molecular weight was found 446.6.

4.1.4. The synthesized lipid derivatives such as sodium deoxycholate sulfate, potassium deoxycholate and purchased from market sodium deoxycholate as drug carriers under deoxycholic acid group

Deoxycholic acid (DCA) is the bile acid and it contains two hydroxyl groups in steroid nucleus that is natural compounds consisting of an amphiphilic steroid nucleus with a hydrophilic α-side and a lipophilic β-side. These are natural body components, are less toxic, biodegradable and compatible, which are metabolic byproducts of intestinal bacteria. Cholic acid and chenodeoxycholic acid two are primary bile acids secreted in the liver. Bacteria metabolize chenodeoxycholic acid into the secondary bile acid, lithocholic acid and cholic acid into deoxycholic acid. DCA is soluble in alcohols and acetic acid. It is in white crystalline powder with a molecular weight of 392.57 g/mol. Its melting point is 174-176 °C. It has been used to dissolve and prevent gallstones since its discovery in human medicine. DCA and bile acid derivatives are actively being studied as structures for incorporation in nanotechnology. It acts as an emulsification of fats for the absorption in the intestine. As a research field, DCA is used as a mild detergent for the isolation of membrane associated proteins. The critical micelle concentration is formed around 2.4-4.0 mM. Sodium deoxycholate, the sodium salt of DCA, is often used as a biological detergent

to lyse cells and solubilize cellular and membrane components. In immunological field, it is used as a vaccine production, immunostimulant of the unspecific immune system. It can be used in a natural healing process of local inflammations, different types of herpes, and possibly cancer. The sodium deoxycholate sulfate and potassium deoxycholate were synthesized from the deoxycholic acid in the laboratory. In deoxycholic group, two hydroxyl groups containing three lipid drug carriers such as sodium deoxycholate (SDC), sodium deoxycholate sulfate (SDCS) and potassium deoxycholate are included. In this group, SDC is taken as a control compound and studied carriers effects after changing sodium ion to potassium ion and in carbonyl group, introducing the sulfate ion. Their chemical structures as shown in Fig. 4.1.

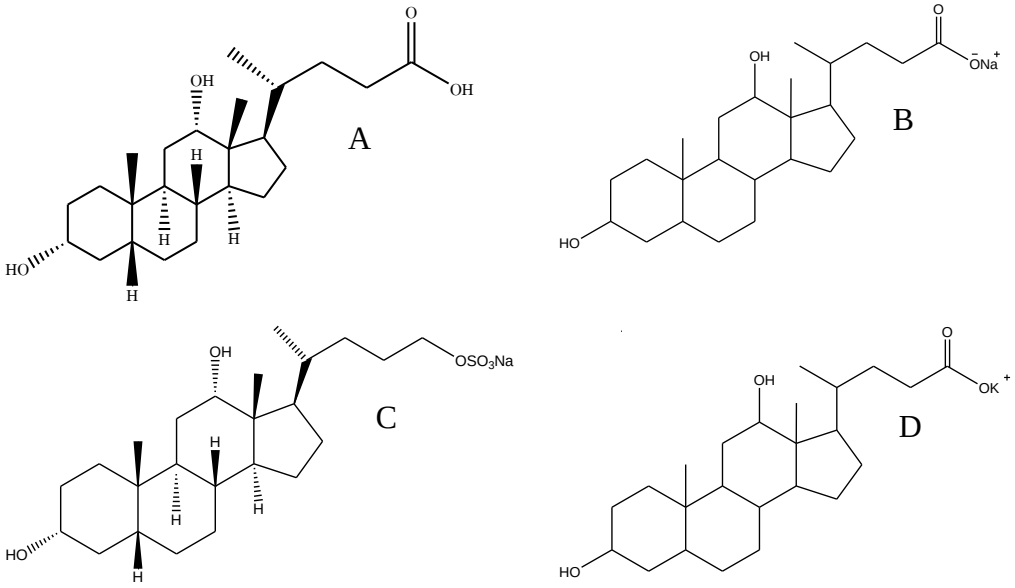


Figure 4.1 The chemical structures of deoxycholic acid (A), sodium deoxycholate (B), sodium deoxycholate sulfate (C) and potassium deoxycholate (D).

4.1.5. The synthesized lipid derivatives such as potassium cholate and purchased from market sodium cholate as drug carriers under cholic acid group

Cholic acid (CA) is also bile acid and it contains three hydroxyl groups in steroid nucleus that is natural compounds consisting of an amphiphilic steroid nucleus with a hydrophilic α-side and a lipophilic β-side. These are natural body components, are less toxic, biodegradable and compatible. It is a white crystalline substance insoluble in water and soluble in alcohol and acetic acid. It has a melting

point 200-201 °C and is synthesized from cholesterol in the liver. It is an intermediate pharmaceutical product which is used for the production of ursodeoxycholic acid, dissolution of gall stones as well as treatment and prevention of liver disease. The liver converts cholesterol into the conjugated salts of glycocholic and taurocholic acid which are excreted into the bile. Bile salts are stored in gallbladder and released into the intestine where the bile salts emulsify fats and helps for digestion of fatty meal. Sodium cholate is commercially available. In cholic acid group, it contains three hydroxyl group, included lipid derivatives drug carriers such as sodium cholate and potassium cholate. The potassium cholate was synthesized from the cholic acid in the laboratory. Here also, we want to study the effects of three hydroxyl group containing sodium and potassium ion containing carriers compare with control group SDC. Their chemical structures as shown in Fig. 4.2.

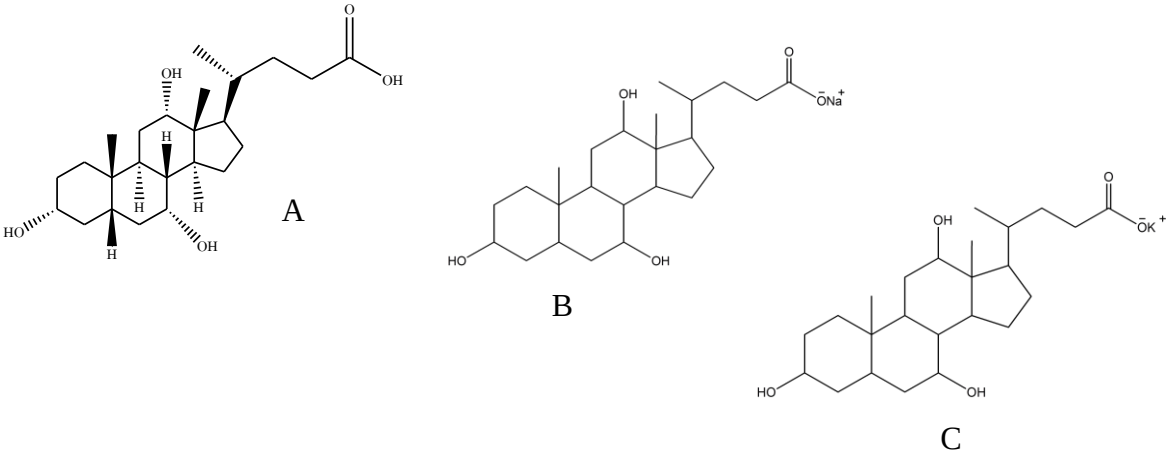


Figure 4.2 The chemical structures of cholic acid (A), sodium cholate (B) and potassium cholate (C).

4.2 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. 4.3 (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. 4.3 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. 4.4 A. Zeta potential values were found in range between – 29.17 to – 45.53 mV as shown in Fig. 4.4 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. 4.5 A and 4.5 B, respectively.

The particle size and particle distributions are crucial parameters for pulmonary applications to achieve high efficacy and a targeted administration by inhalation (Das et al., 2010). 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 (Honary and Zahir, 2013). 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 (Gharib et al., 2011).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 (Espada et al., 2008), 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).

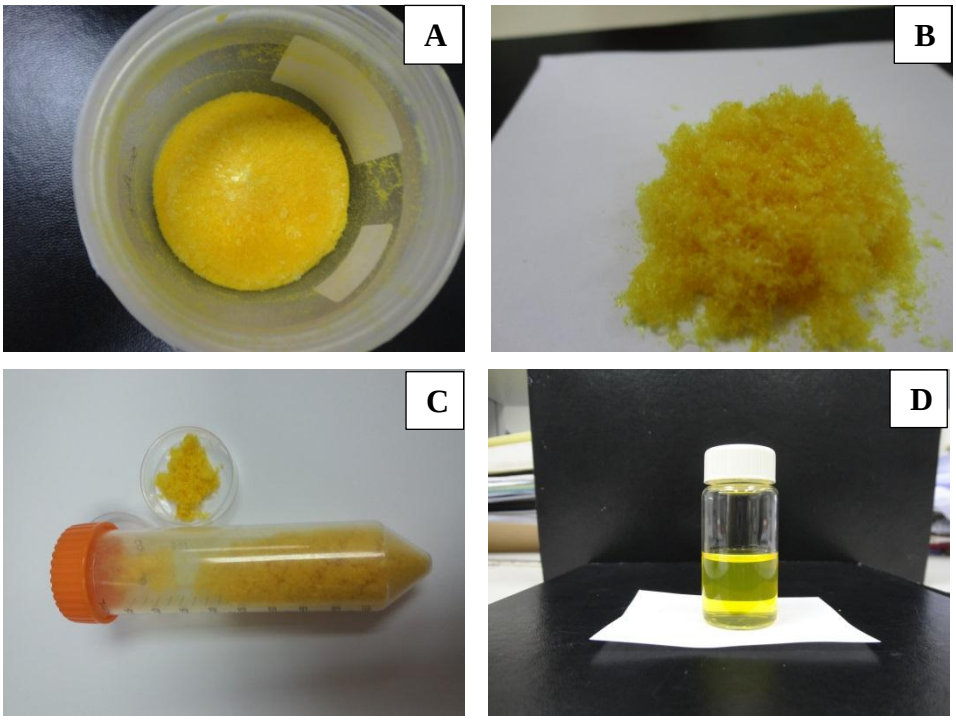


Figure 4.3 Reconstituted AmB-lipid dry powder caked formed (A), after breaking caked and formed powder (B), stored in the container (C), reconstituted dry powder into distilled water (D)

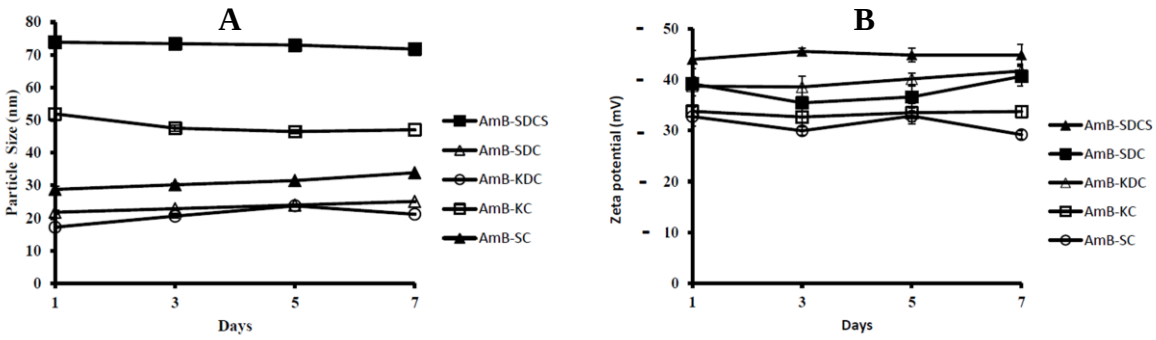


Figure 4.4 Particle size and the zeta potential of the reconstituted AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC and AmB-SC dry powder in distilled water during 7 day at a conc. 10 mg/mL of amphotericin B.

4.3 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 (Legrand et al., 1992). 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 (Barwicz et al., 1992; Nishi et al., 2007). The monomeric-AmB is less toxic than the dimeric-AmB (Mazerski and Borowski, 1996). However the water soluble aggregated state of the dimeric–AmB seems to be the more stable form (Mehta et al., 1984). 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. 4.4 A) and UV spectrophotometry as shown in Fig. 4.5. The UV spectrum of AmB in the aqueous phosphate buffers pH 7.4 exhibited an absorption maximum (Fig. 4.5). 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 1^{st} to the 7^{th} day. From the UV spectra, there was a decrease in the intensity during the first few days (2^{nd} day and 3^{rd} 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.

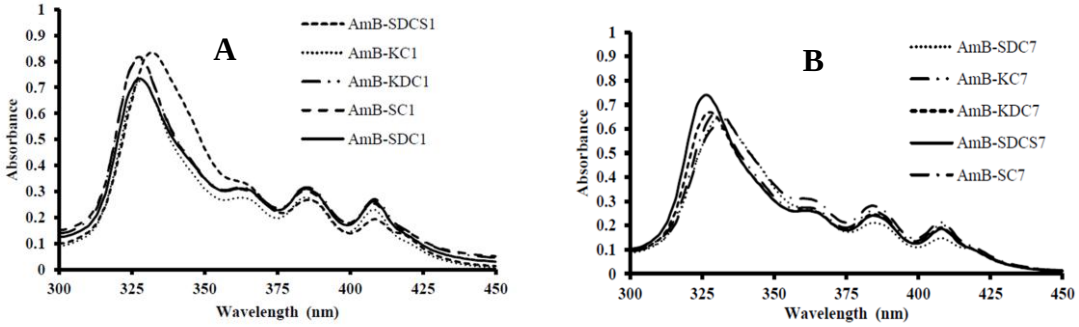


Figure 4.5 UV measurement of reconstituted AmB-lipid derivatives dry powders after reconstituted into distilled water at conc. 10 µg/mL AmB at day 1 (A) and at day 7 (B).

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 4.1. 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 4.2. 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 (ICH, 2005).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 4.2). 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. 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 (Sanna et al., 2003).

Table 4.1 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 4.2 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

4.4 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 4.3. Part of this research related to the aerosol property of the two formulations, AmB-SDCS and AmB-SDC was previously reported by Gangadhar et al., (2014). 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 (Wood and Boucher, 2000). MMADs of all the AmB-lipid formulations used in this study were between 1.70 to 2.05 μm (Table 4.3). 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 4.3 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

4.5 *In vitro* hemolysis assay

Hemolysis of human red blood cells was evaluated by a reported method as described in the materials and methods section. Erythrocytes treated with AmB-KC, AmB-KDC, AmB-SC, AmB-SDCS, AmB-SDC, and AmB showed some hemolysis as shown in Fig.4.6 In this study, we had chosen a concentration range of 1-8 μg/mL according to Yu et al. (1998).The safety level of hemolysis was reported by Yu et al., (1998), less than 5%. Similar, result was mentioned by Adams and Kwon in 2003. Percentage of hemolysis increased as the concentration of AmB increased up to 17%. AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC, AmB-SC and AmB, each at a concentration of 8 μg/mL AmB, caused some red blood cell hemolysis of 9%, 14%, 14%, 14%, 13% and 17%, respectively so all AmB-lipid formulations produced less hemolysis than AmB. Among these five formulations, AmB-SDCS was least hemolysis than others. It was due to the sulfate ions interact and stabilize micelle and low release of free AmB and free form AmB is less available to formation of dimeric form, which causes for hemolysis. These lipid derivatives may be able to stabilize AmB. Forster et al. (1988) reported that lecithin-stabilized emulsions produced low hemolysis. They explained that the reduced hemolysis of the liposome and the lecithin-stabilized emulsions was due to the strong binding between the AmB and the phospholipid. Chuealee et al. (2011) studied various cholesterol derivatives for the formulation of AmB and found that AmB was stabilized in a similar manner and hemolysis was prevented. The hemolytic effects were effectively or significantly reduced compared to conventional surfactants such as SDC and cholesterol.

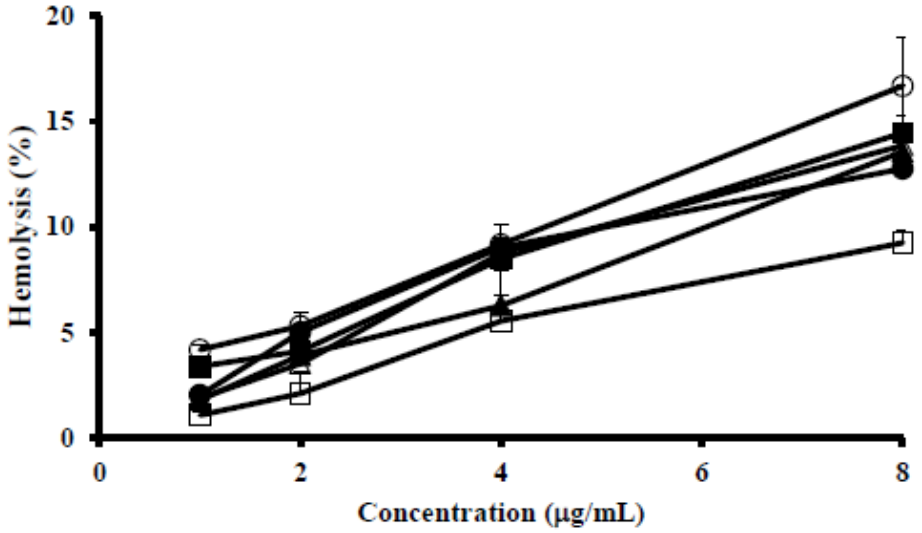


Figure 4.6 *In vitro* hemolysis after incubation with, AmB-SDC (■), AmB-KC (△), AmB-KDC (▲) AmB-SDCS (□), AmB-SC (●) and AmB (O) at 24 h (mean ± SD, n=3)

4.6 Cytotoxicity assay

The toxicity of the lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) and AmB on A549, AM NR 8383 and Calu-3 cell lines and kidney cells (293T/17) were estimated at different concentrations from 1 to 8 µg/mL by the MTT reduction assay after 24 h of exposure as shown in Fig. 4.7 Typical effective concentration of AmB in the blood circulation is 1 µg/mL so the lung tissue should have a similar concentration (Bennett, 1996; AHFS, 2009). A typical testing for the AmB concentration *in vitro* was between 8-16 times, in this case we had chosen 8 times (Yu et al., 1998). The lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) and AmB up to 8 µg/mL showed very low cytotoxicity to A549 cells. The viability of A549 cells after treatment with AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC, AmB-SC and AmB were 95, 91, 89, 94, 95 and 92%, respectively (Fig. 4.7 A). For the Calu-3 cells they showed above 92% viability with all AmB formulations up to 8 µg/mL. This indicated that the five formulations were not toxic to the lung cell lines Calu-3 (Fig. 4.7 B). In the case of the AM NR8383 cell lines, the percentage of viability was over 80% with the AmB

lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) at concentration of AmB up to 8 μg/mL and more than 99% viability was achieved at a lower concentration of 1 μg/mL. In the case of pure AmB, the viability of AM NR8383 cells rapidly declined from 93 to 56% with increasing concentrations of AmB from 1 to 8 μg/mL. There was more than 80% of cell viability at concentrations of AmB 1-4 μg/mL whereas at 8 μg/mL, it was drastically decreased by up to 56% (Fig. 4.7 C). AmB is known as a nephrotoxic drug, so its clinical application is limited due to its major toxicity (Jung et al., 2009). The incidence of nephrotoxicity was reported 49-65% in conventional AmB deoxycholate (Chavanet et al., 1997). To minimize this toxic effect, lipid derivative carriers (SDCS, KC and KDC) were synthesized in a laboratory, SDC and SC were purchased from the market and AmB-lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) were prepared by lyophilization process. This toxicity test was done *in vitro* study on human kidney cells (293T/17) as shown in (Fig. 4.7 D). The percentage viability of kidney cells was over 90% after challenging with AmB or AmB formulations at 1 μg/mL. A slight decrease of viability to around 83% at 2 μg/mL AmB was observed. However, at 4 and 8 μg/mL AmB, dramatic cell death was observed. AmB-lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) provided some protection of kidney cells as viability after treatment with 4 μg/mL was higher than 80% and except AmB-SDC, AmB-SDCS, AmB-SC, AmB-KC and AmB-KDC formulations were higher than 80% at 8 μg/mL. AmB-SDC at these concentrations was slightly less toxic than pure AmB. The micelles provided a relatively weak barrier compared to the lipid bilayers of liposomes or lipid matrix or LNPs. The release of AmB from micelles may be faster than AmBisome® or AmB entrapping LNPs (Moribe et al., 1999 a, b). Moreover, strong interaction between AmB and lipids such as phospholipids and /or cholesterol can retard release of AmB from LPNs or AmBisome® (Hac-Wydro and Dynarowicz-Lakta, 2006). Therefore, LPNs or liposomes that entrap drugs in their lipid matrix or bilayers are able to decrease cytotoxicity of the entrapped drugs (Bharma et al., 1997). In this case it is possible that AmB-SDCS, AmB-SC, AmB-KC and AmB-KDC lipid formulations can form more stable micelles than AmB-SDC. Therefore the free AmB release from

AmB-SDCS, AmB-SC, AmB-KC and AmB-KDC lipid formulations may be slower than that from AmB-SDC resulting in a lower toxicity to the kidney cells. These lipid formulations seemed to be less toxic than pure AmB. In overall toxicity studies of AmB-lipid formulations in respiratory associated three (A549, Calu-3 and NR 8383) cell lines and kidney cell lines (293T/17), they were showed that SDCS, KDC, KC and SC formed more stable micelles than SDC. Therefore the release of free AmB from AmB-SDCS, AmB-KDC, AmB-KC and AmB-SC may be slower than that from AmB-SDC resulting in less toxicity for all tested cells up to 8 µg/mL concentration of AmB.

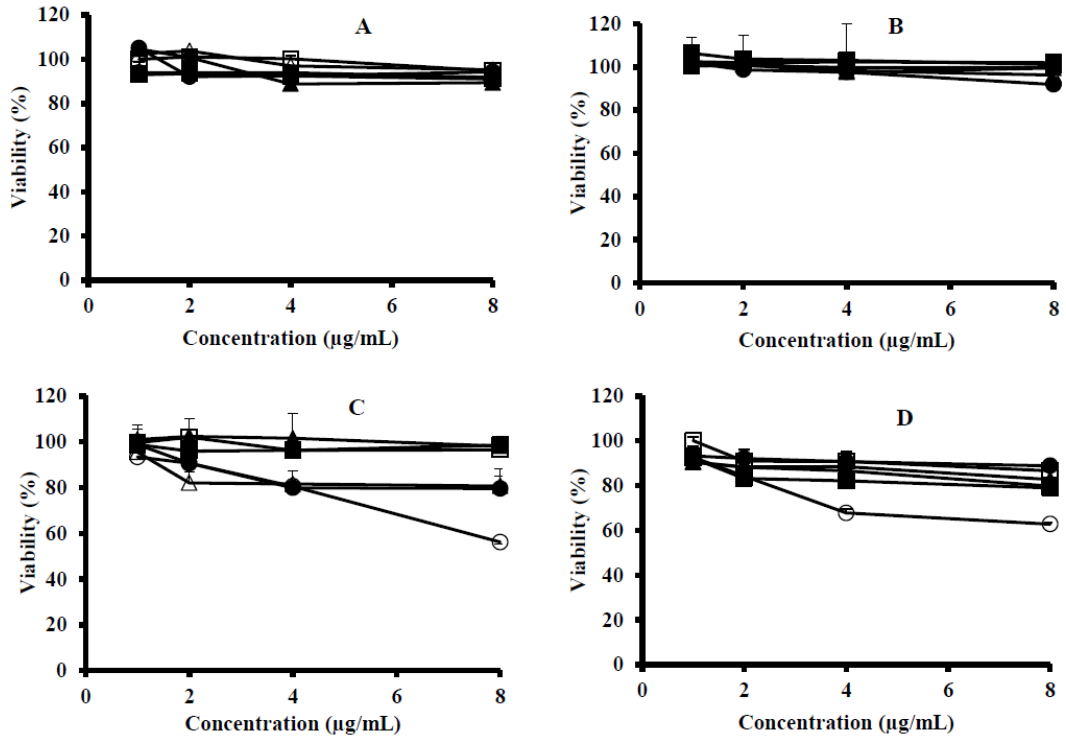


Figure 4.7 Viability of A549 cell line (**A**), calu-3 cell line (**B**) , AMs NR8383 (**C**) and kidney cells (293T/17) (**D**) after they have been incubated with AmB (○), AmB-SDCS (□), AmB-SDC (■), AmB-KDC(▲), AmB-KC (Δ), AmB-SC(●); (mean ± SD, n=4).

4.7 Nitric oxide release from the alveolar macrophage response to AmB-lipid formulations

Alveolar macrophages are phagocytic cells and play an important role in pulmonary defense and are known to undergo increased oxidative metabolism (Fantone and Wards, 1982). Accordingly, the response of AmB-lipid formulations may reflect the potential safety/toxicity of such formulations when delivered to the peripheral airways.

Lipopolysaccharides (LPS) also known as lipoglycans and endotoxin, are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond. They are found in the outer membrane of Gram-negative bacteria *(E. coli*.). The toxic activity of LPS was first discovered and termed endotoxin by Richard Friedrich and Johannes Pfeiffer. LPS was stimulated AMs and released nitric oxide from the cells, which was compared to the pure AmB and AmB-lipid formulations. The reconstituted solutions (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) were delivered to the peripheral lungs via nebulization to determine the safety or toxicity of such formulations. Despite the potential concerns, AM NR 8383 cells produced only low levels of nitric oxide after exposure of the AmB-lipid formulations when compared to LPS (Fig. 4.8). The concentration of LPS (15-1000 ng/mL, data not shown), that stimulated AM NR8383 cells to produce amounts of NO levels, that were 1000-fold greater than any of the concentration of the AmB-lipid formulations (1-32 μg/mL of AmB). Davidson et al., (1998) was reported that the safety level of nitric oxide was less than 80 μM. The AmB-lipid formulations at concentrations of AmB 1-32 μg/mL were safe for the AM cell lines.

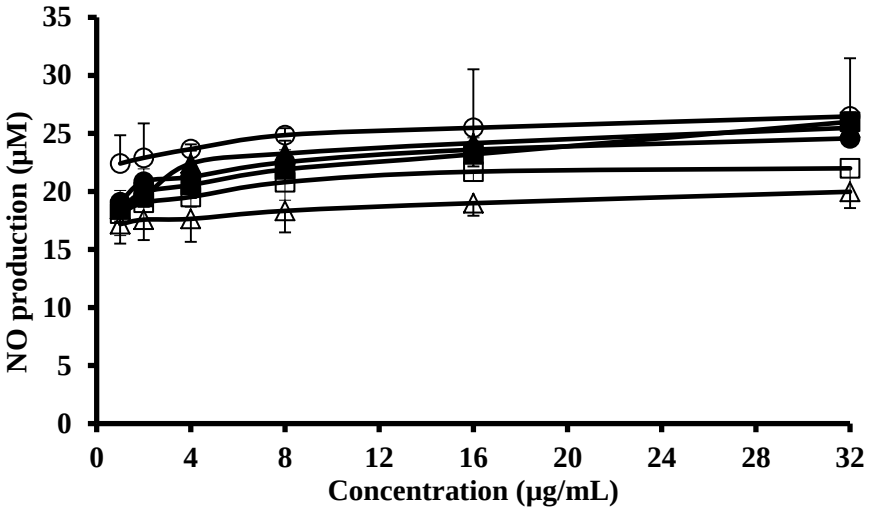


Figure 4.8 The level of nitric oxide produce from NR 8383 cell lines after exposure different concentrations of AmB (O), AmB-SDCS (□), AmB-KDC (▲), AmB-SDC (■), AmB-KC (△) and AmB-SC (●) respectively. For 24 h (Mean ± SD, n=4).

4.8 Anti-fungal studies

A comparison of the potency of the five AmB-lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) with those of the same concentrations of standard AmB are shown in table 4.4. The lipid derivatives carriers (KC, KDC, SC SDC and SDCS) without AmB did not inhibit the growth of yeast (this data is not presented). The lipid carriers did not inhibit the growth of fungi in culture plate, which was similar as an inoculum media contained the culture plate. AmB-lipid formulations of AmB-SDC, AmB-SDCS, AmB-KDC, AmB-KC and AmB-SC in the reconstituted dry powders had potency ranges of 102%, 103%, 102%, 104% and 98%, respectively. These values were equivalent to that of the standard AmB (100%). These results indicated that the lipid did not affect the potency of the AmB when compared to the standard. It also did not contribute to enhancing the potency of AmB by transporting it into fungal cells and / or facilitating a greater penetration into the fungus membrane (Chuealee et al., 2011). However, the lipid derivatives do play important roles for dissolving the poorly soluble drug AmB in water.

Table 4.4 Potency, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of AmB in lipid derivatives reconstituted dry powder (n=3)

Material	**Lipid :AmB** (mole ratio)	**Potency** %	***C. albicans***		***C. neoformans***	
			MIC (μg/mL)	MFC (μg/mL)	MIC (μg/mL)	MFC (μg/mL)
Pure AmB	0:1	100	0.32	0.63	0.32	0.63
AmB-SDC	2:1	102	0.16	0.32	0.16	0.32
AmB-SDCS	2:1	103	0.16	0.32	0.16	0.32
AmB-KDC	2:1	102	0.16	0.32	0.16	0.32
AmB-KC	2:1	104	0.16	0.32	0.16	0.32
AmB-SC	2:1	98	0.16	0.32	0.16	0.32

4.9 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

MIC and MFC were evaluated of five formulations (AmB-KDC, AmB-KC, AmB-SC, AmB-SDC and AmB-SDCS) and pure AmB against *C. albicans* and *C. neoformans*. The MIC and MFC values for the AmB are shown in Table 4.4. For AmB-lipid formulations (AmB-KDC, AmB-KC, AmB-SC, AmB-SDC and AmB-SDCS), the MIC and MFC exhibited a lower value than the pure AmB against *C. neoformans and C. albicans* when compared to that of the pure AmB. The obtained MIC and MFC results were almost similar to that of previous reported studies against *C. albicans and C. neoformans* in the range of 0.015 to 2 µg/mL (Aller et al., 2000; Archibald et al., 2004; Chuealee et al., 2011; Lopez-Jodra et al., 2000; Lozano-Chiu et al., 1988; Park et al., 2006; Van Eldere et al., 1996). They were essential for the solubility of the AmB.

4.10 Permeation of AmB and AmB-lipid formulations across lipid bilayer

The permeability of AmB transported through ion-permeable channels was studied using the Ussing chambers in a phosphate buffer solution (10 mM, pH 7.4) with different AmB-lipid formulations (AmB-KDC, AmB-KC, AmB-SC, AmB-SDC and AmB-SDCS) and AmB. The percentage of the cumulative AmB that was transferred from the left chamber to right chamber in a 2 h period is shown in Fig. 4.9. The cumulative drug transferred from the left to the right chamber using the AmB-lipid formulations were 10.6, 4.3, 3.6, 5.6 and 4.0 %, respectively. Whereas, AmB only was cumulatively transferred 2.0%. During permeability study period, we had measured the current. At the initial period (before addition of sample) it was 0.6 µA. After an addition of 50 µL AmB suspension into the left chamber, the current was increased to 1.1 µA at 15 min. At every 15 min interval, current was gradually increased with time, reached 1.9 µA at the end of sampling time period (2 h). Similarly, the current of AmB-lipid formulations was found to be 0.3-1.5 µA. This indicated that AmB, and AmB-lipid formulations permeated across lipid bilayer membrane from the left chamber to the right chamber. AmB has a very low water solubility (<1 µg/mL at pH 6-7) and the AmB was in an aggregated form in solution

and the lowest amount of AmB was able to cross the membrane. On the other hand the AmB-lipid formulations have counter ions that interacted with the lipid bilayer and facilitated the permeation of AmB. In the case of AmB, there were no charges available to counteract the lipid bilayer membrane. AmB may form a complex with the lipid bilayer resulting in low permeability. This suggestion has been supported by the work of Herec et al. (2005) who proposed that hydrogen bonding between the horizontally oriented AmB and the polar groups of the lipids makes the membrane more compact and less permeable to ions. AmB-SDCS had the highest zeta potential (-45.53 mV), (Gangadhar et al., 2014) and it produced the highest permeability (10.6%). This was due to the replacement of a carbonyl group of the deoxycholic acid ring with the sulfate group, which developed a highly negative charge. This sulfate group has a vital role to interact with the lipid bilayer to increase the pore size and facilitate the penetration of more AmB. Similar phenomena have also occurred with other formulations. These negatively charged ions facilitated 2-5-fold more AmB from the formulations to cross the lipid bilayer as compared with pure AmB. Although, only a small amount of AmB was transferred from the left to the right chamber. It was postulated that this amount was enough to kill the fungi as the MIC was 0.16 µg/mL and the MFC was 0.32 µg/mL (Table 4.4). It is possible that the AmB and lipid bilayer might form a complex channel with the lipid bilayer and therefore reduce the amount of AmB available for permeation.

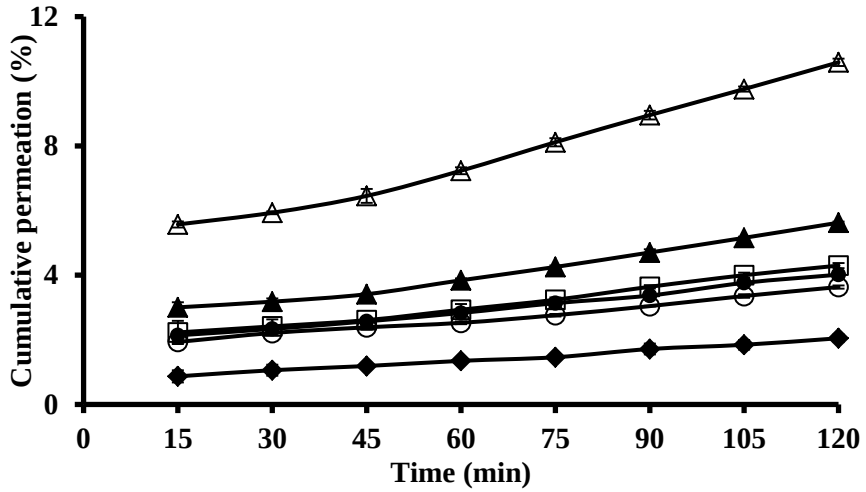


Figure 4.9 Permeability determination of AmB (◆), AmB-KDC (▲), AmB-SDC (□), AmB-SDCS (△), AmB-KC (●) and AmB-SC (O) from 15 min to 120 min, respectively (Mean ± SD, n=3).

4.11 Phagocytosis of anti-fungal reconstituted dry powder particles by macrophage cells

AmB-SDCS formulation was reconstituted in distilled water and stained with Lumidot®640. AmB-SDCS lipid formulation photographs were taken in bright field mode; fluorescence mode and overlay mode are shown in Figure 4.10 (A-C). Our AmB-lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS), particles size were obtained less than 100 nm (range 17-74 nm). These data were harmonized with the size measurement after the reconstitution study. The AmB-SDCS in liquid dispersion, it was in a round shape with narrow size distribution. Uniform nanoparticles gave a high intensity of light with stable images.

AM NR8383 cells were bright green cells when the cell photographs were taken in bright field mode. To observe the phagocytosis, AmB-SDCS formulation was reconstituted to distilled water and stained with Lumidot®640 because it could be read in the fluorescence mode. It took shorter than 2 min to undergo phagocytosis by the AM NR8383 cell. In Figure 4.10 (D-E) shown phagocytosis of AM NR8383 cells taken in different modes such as bright field, fluorescence and overlay, respectively. In fluorescence mode, the intensity of the color was glowing and visible producing very clear pictures and showed that the AmB-SDCS had entered the cells. Phagocytosis was observed in AM NR8383 cells. It indicated that AM NR8383 could phagocytose the particles stained with Lumidot® 640. Similarly, in the overlay mode, the intensity of the color increased and AmB-SDCS was clearly visible inside the cells. The phagocytosis was observed in NR8383 cells, some of cells were changed their shape and sizes also. These results were correlated with the size measurement after the reconstitution study. Kanchan and Panda (2007) reported that the nanoparticles of 200-600 nm in size were efficiently phagocytosed and in our case, nanoparticles size were less than 100 nm efficiently phagocytosed. Similar result was reported by Rojanarat et al. (2012). These results supported the view that AmB-SDCS lipid formulation could be phagocyted by AM NR8383 cells in an *in vitro* study.

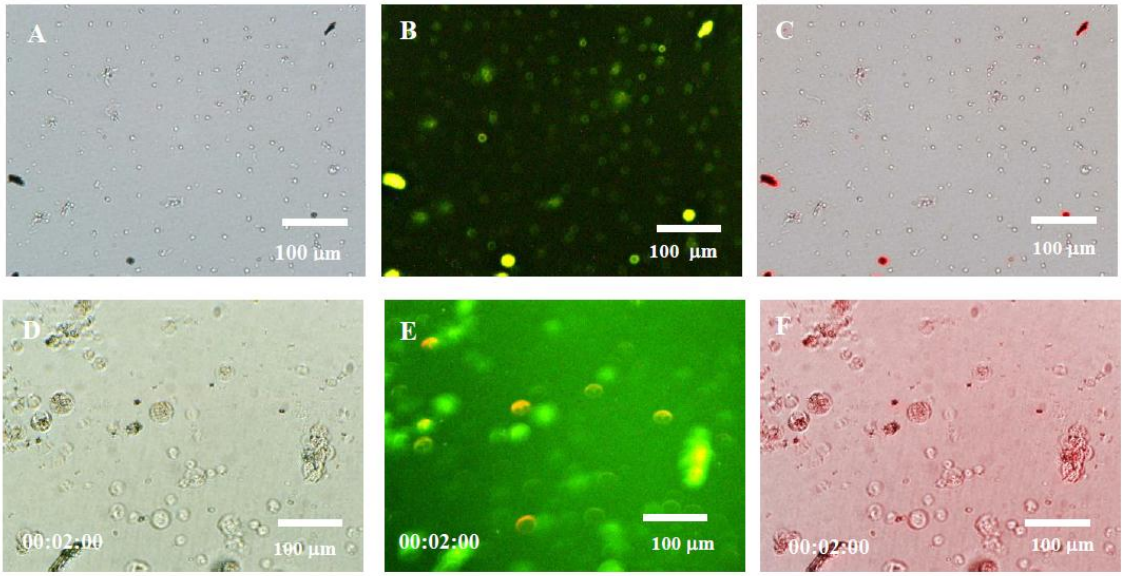


Figure 4.10 Phagocytosis of AM NR 8383 cells incubated with reconstituted dry powder AmB-lipid formulation (AmB-SDCS) stained with Lumidot®640.Pictures (A), (B) and (C) represent AmB-SDCS with Lumidot®640 in bright field, fluorescence mode and overlay mode, respectively. Pictures (D), (E), and (F) represent AM NR 8383 cells with AmB-lipid formulation containing Lumidot®640 in 2 min in bright field, fluorescence mode and overlay mode, respectively (bar=100 µm).

CHAPTER 5
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, 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. Aerosolization characteristics such as MMADs were obtained ranges 1.70- 2.05 µm with high FPF 70-80%. The potency and susceptibility testing against yeasts, *S. cerevisiae*, *C. albicans* and *C. neoformans*, the MICs and MFCs of all formulations was 2 fold less than that of standard AmB. The potency these formulations were an equivalent to pure AmB (100%). Although, these lipid drug carriers are vital role play to dissolve the poorly soluble AmB into highly soluble and stable solution form. The cytotoxicity examinations of respiratory associated cell lines (A549, calu-3 and AM NR 8383), kidney cells and human RBC hemolysis were shown higher viability with AmB-lipid formulations as compared to pure AmB. In addition, AM NR 8383 cell lines were not released toxic level of nitric oxide with exposures to AmB-lipid formulations as compared to LPS. The AM NR 8383 cells

were phagocyted with AmB-lipid formulation was observed under fluorescence microscope. This was possible for targeting the AM in lung fungal infections. The permeation across the lipid bilayer was determined using the Ussing chamber. AmB-lipid formulations were obtained 2-5 fold higher than AmB. Therefore, we carried out all the relevant study of AmB-lipid formulations, concluded that they were suitable for the treatment pulmonary aspergillosis by targeting AM via jet nebulization. Among five AmB-lipid formulations, it can be concluded that SDCS was the best lipid drug carrier in terms of its physical and chemical properties, aerosol property, toxicity tests, and permeability. Finally, the success in pre-clinical study of AmB-lipid formulations via nebulization further into clinical studies in systemic fungal infectious patients. This will be compared with a conventional treatment.

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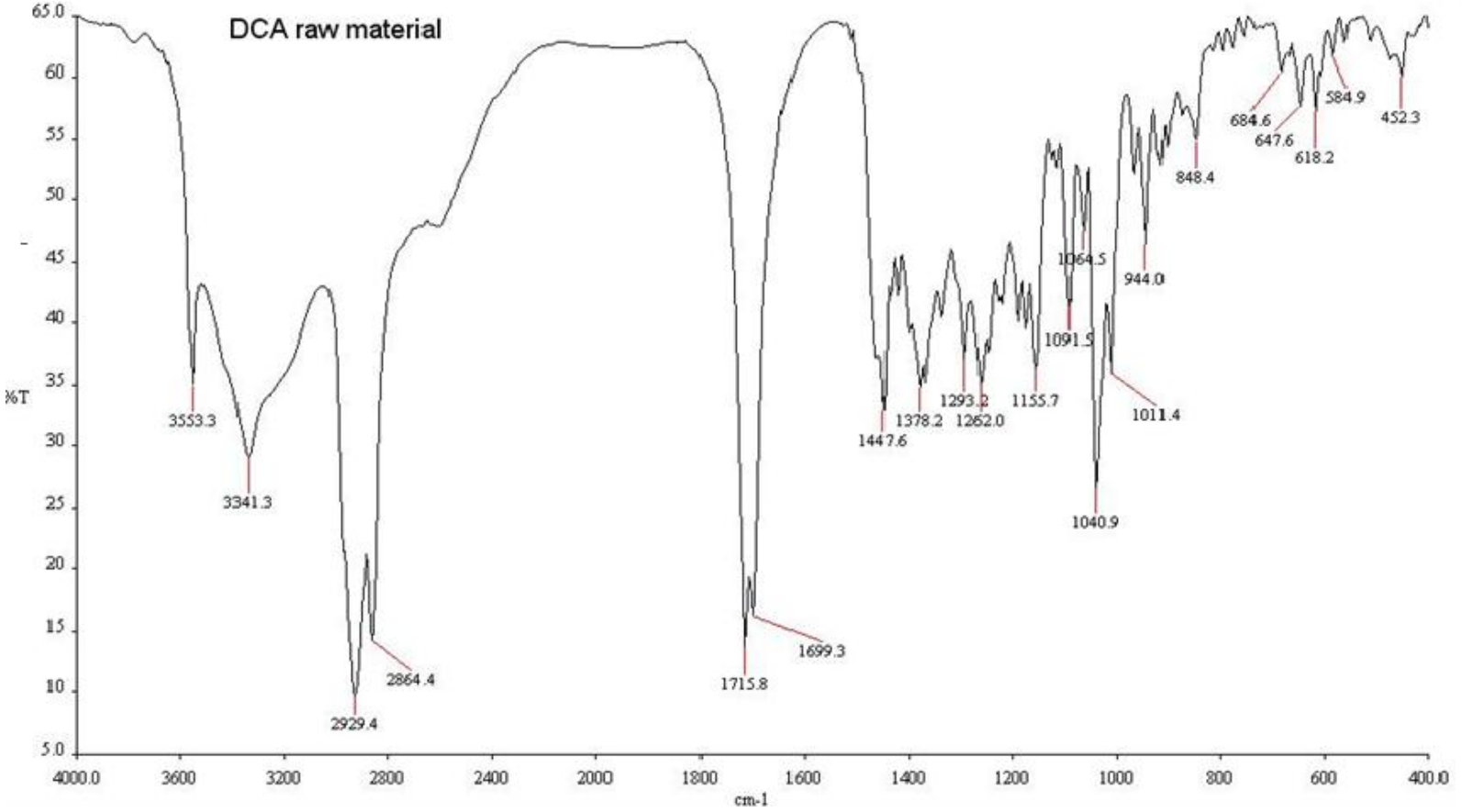
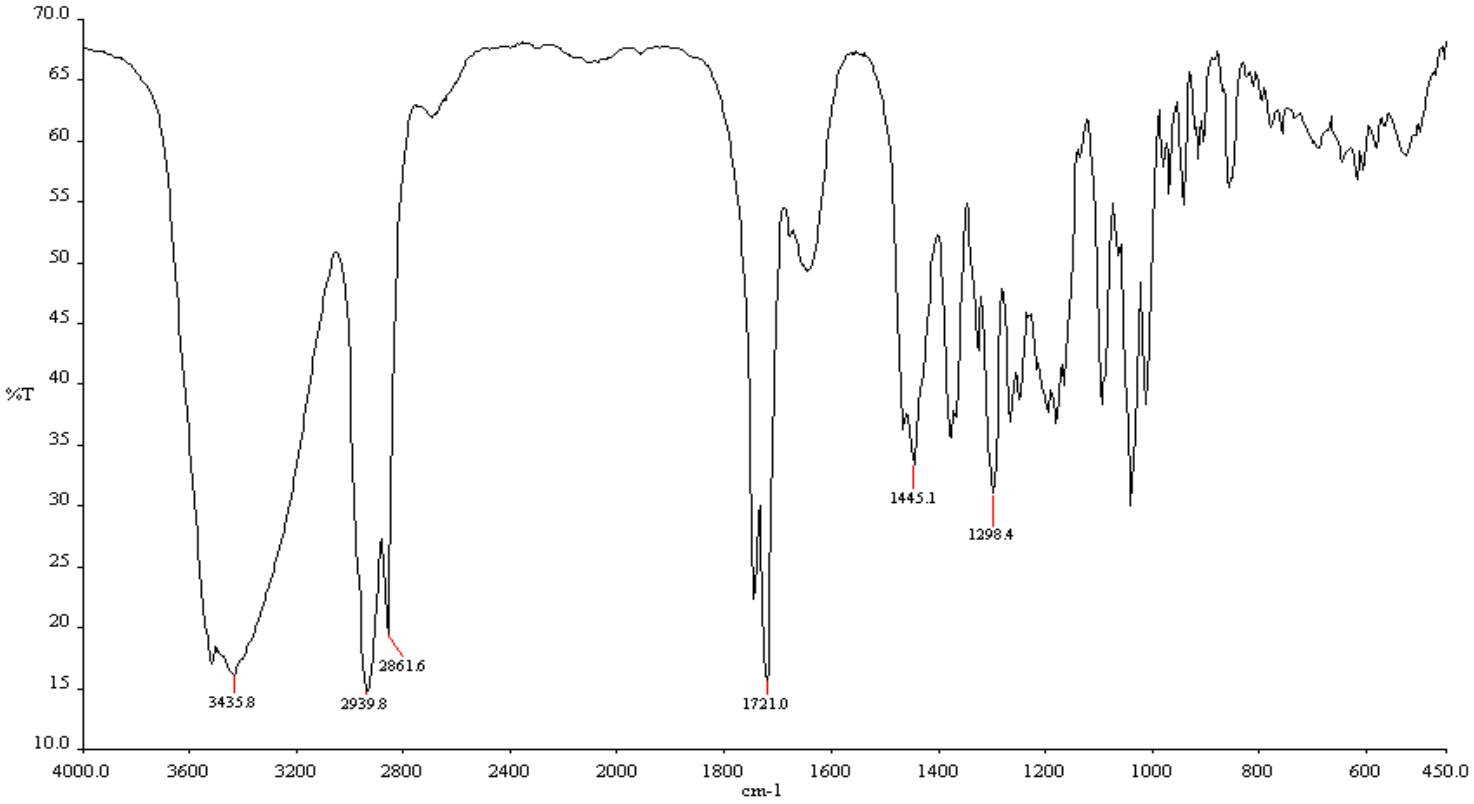
APPENDICES

FTIR AND ^{1}H NMR SPECTRA



FTIR spectra of deoxycholic acid (DCA)



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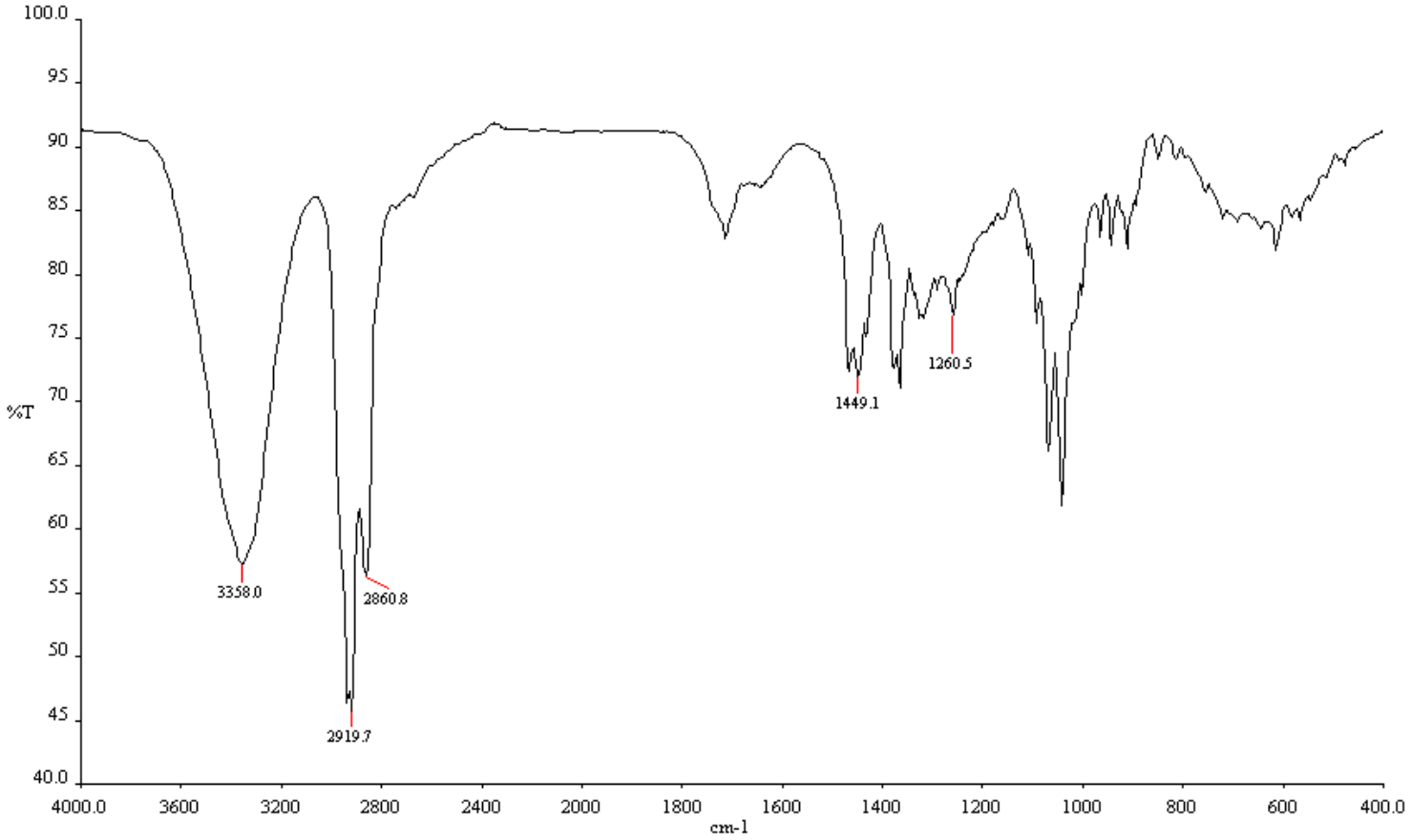
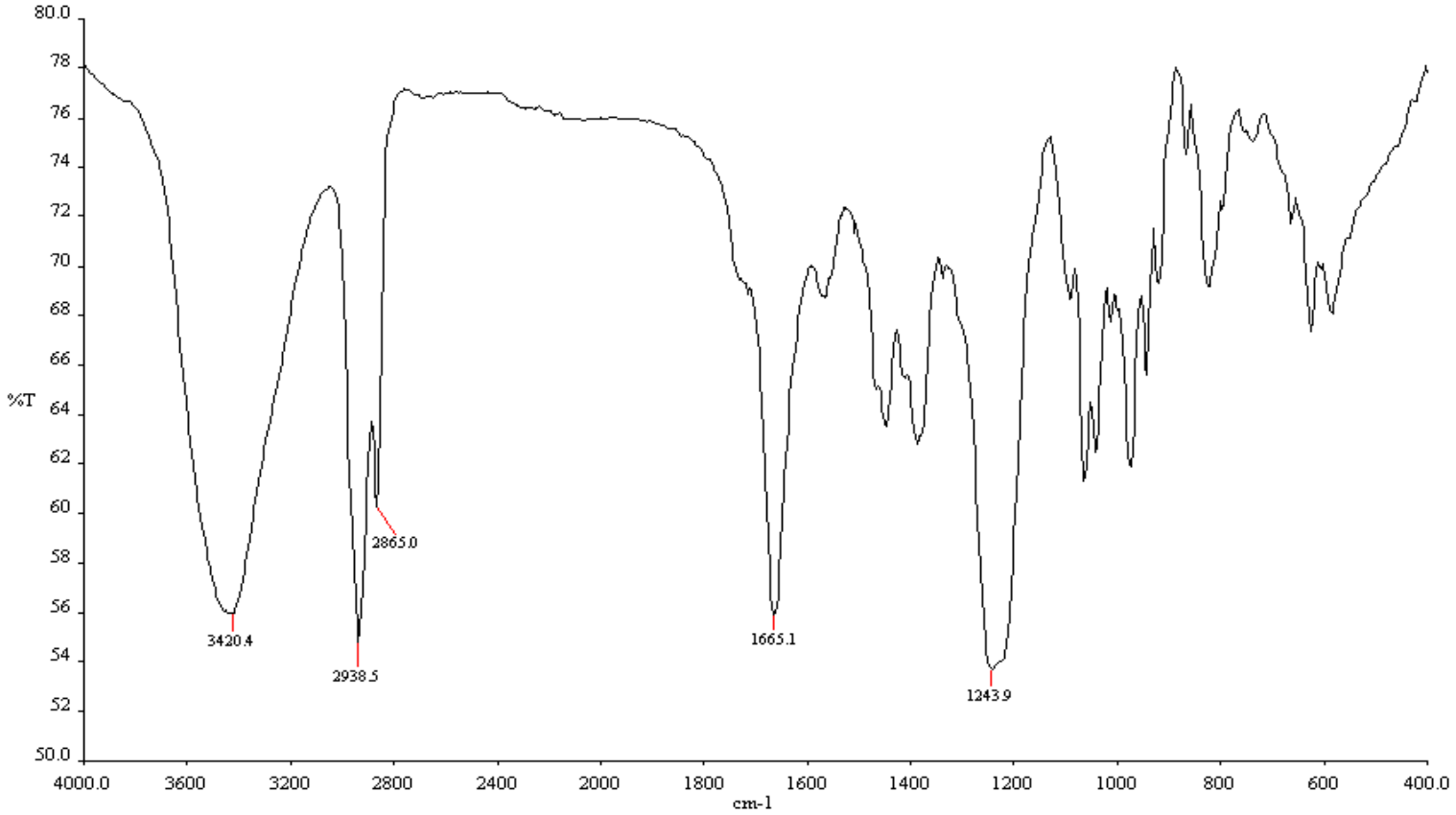
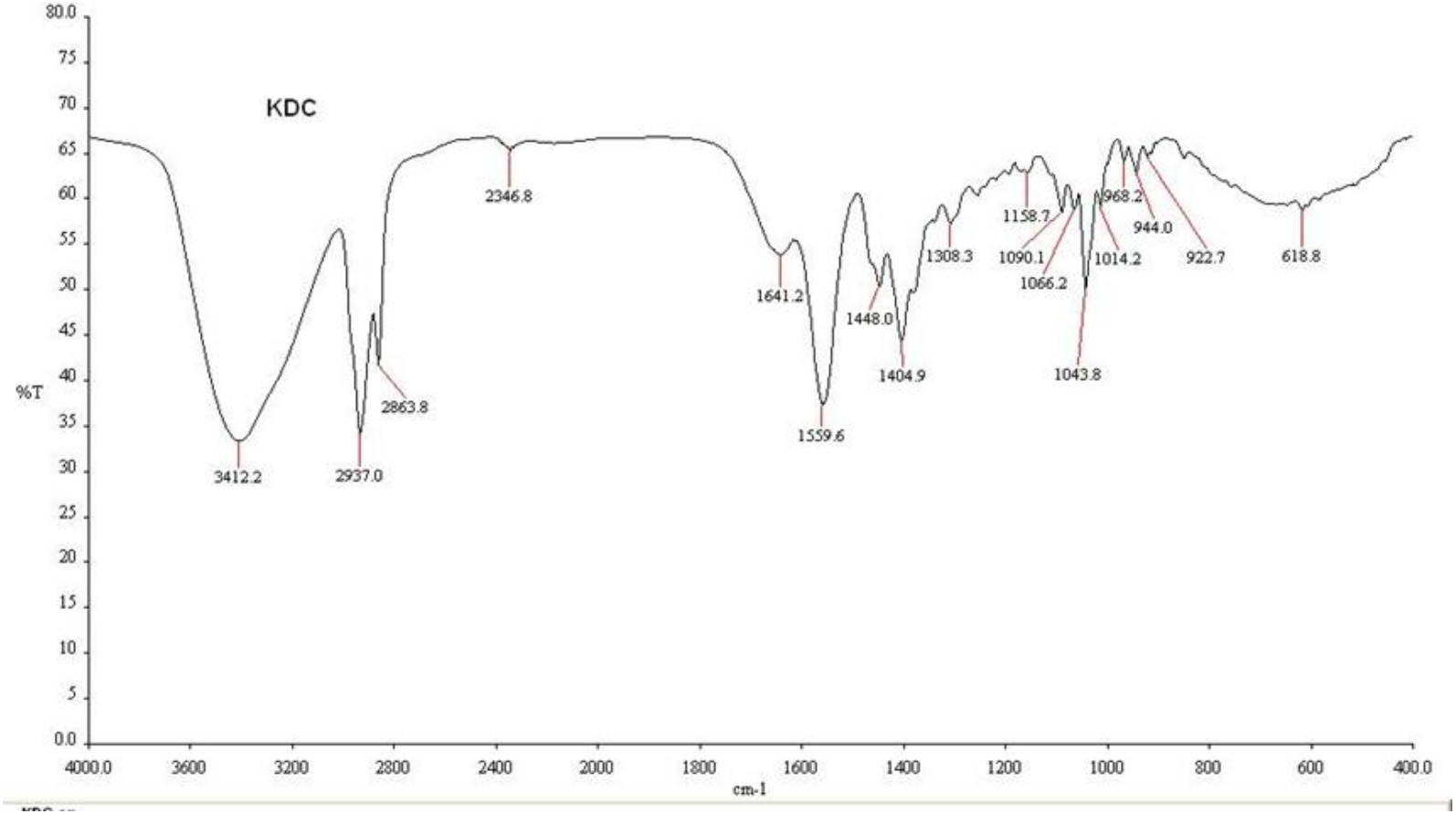


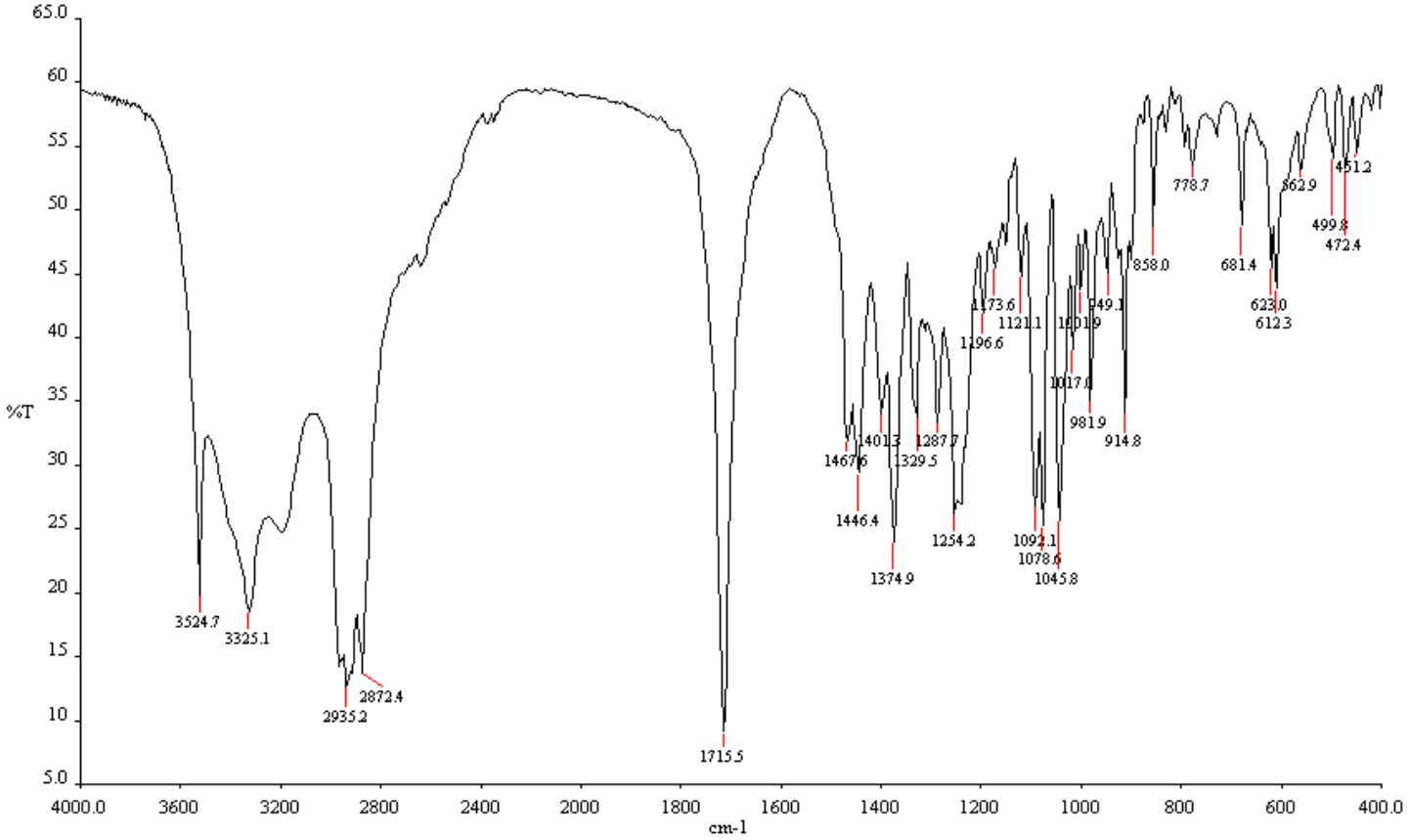
FTIR spectra of deoxycholic alcohol (DAL)



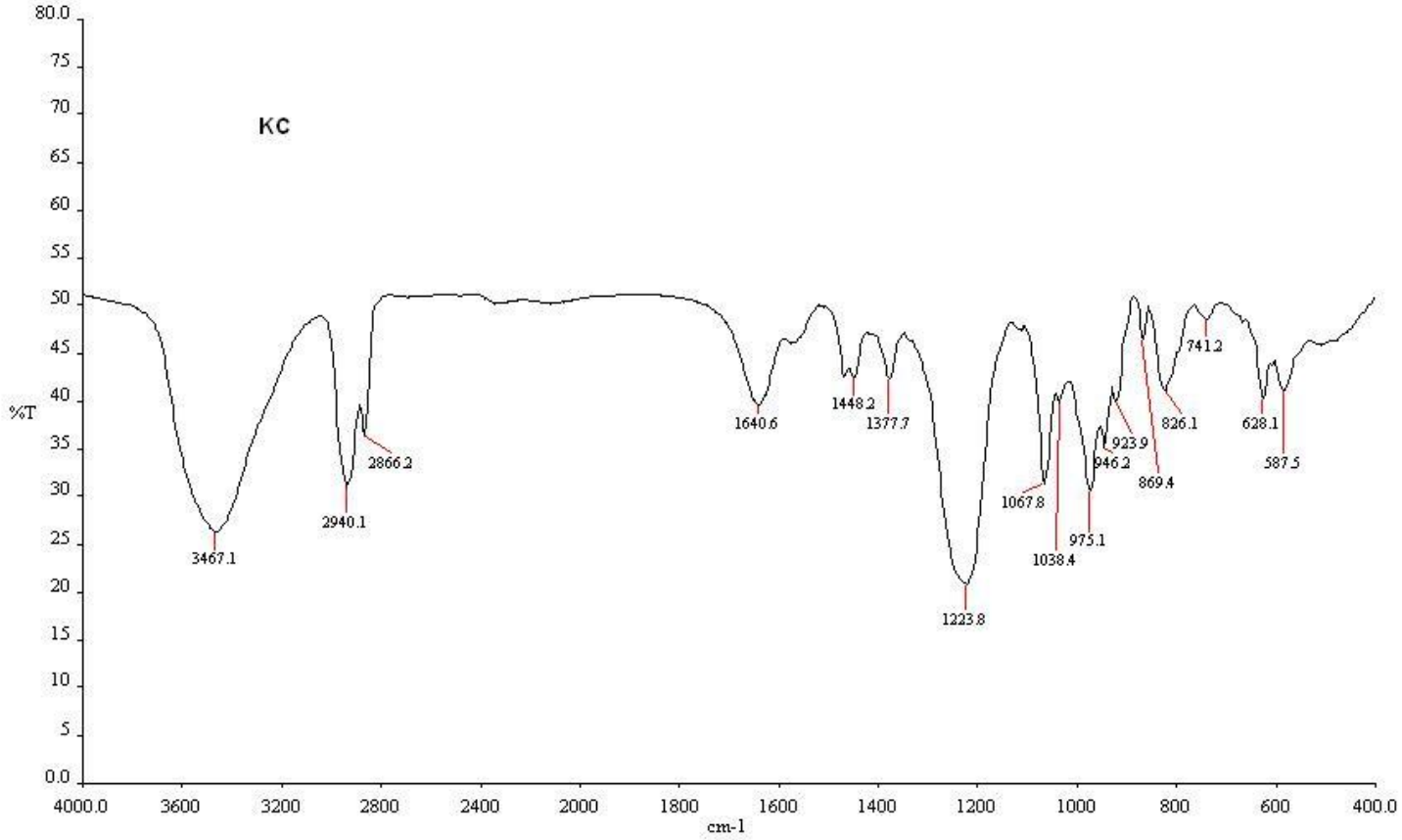
FTIR spectra of sodium deoxycholate sulfate (SDCS)



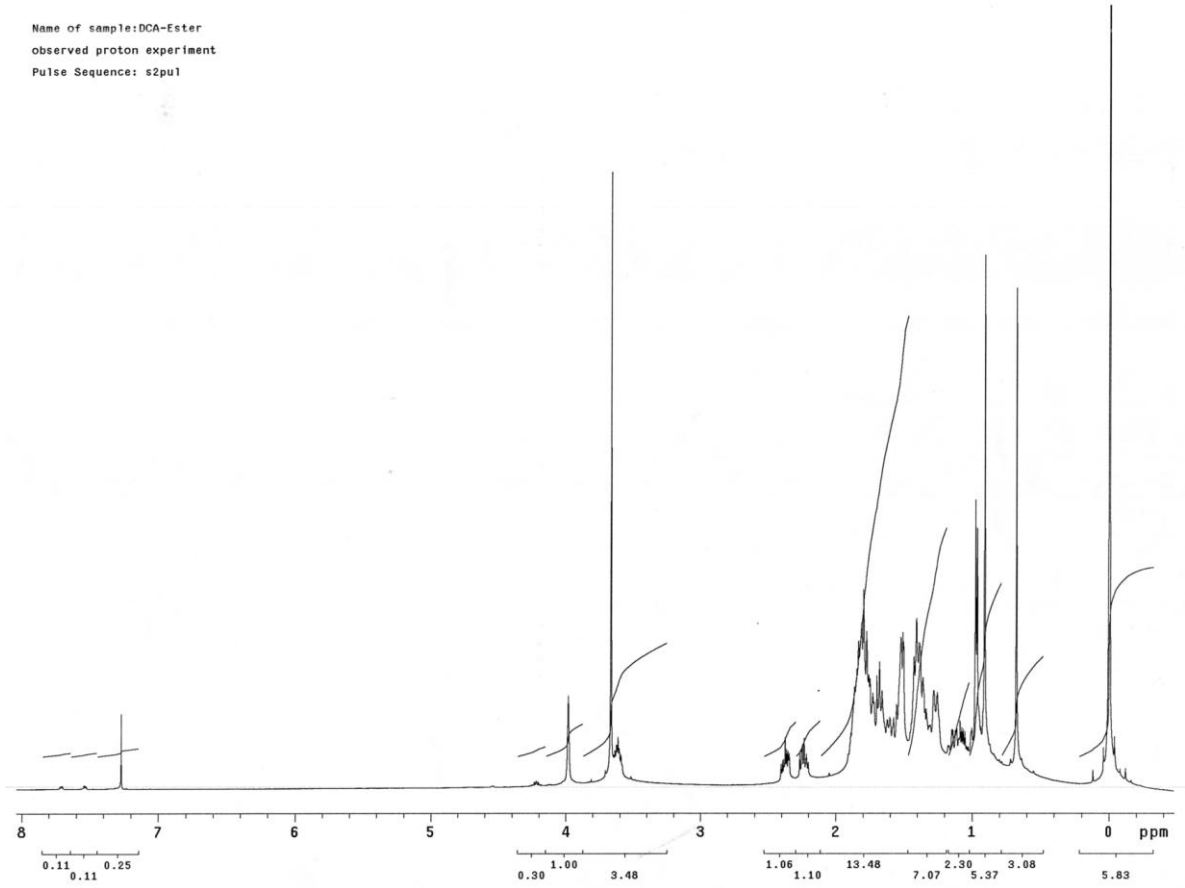
FTIR spectra of potassium deoxycholate (KDC)



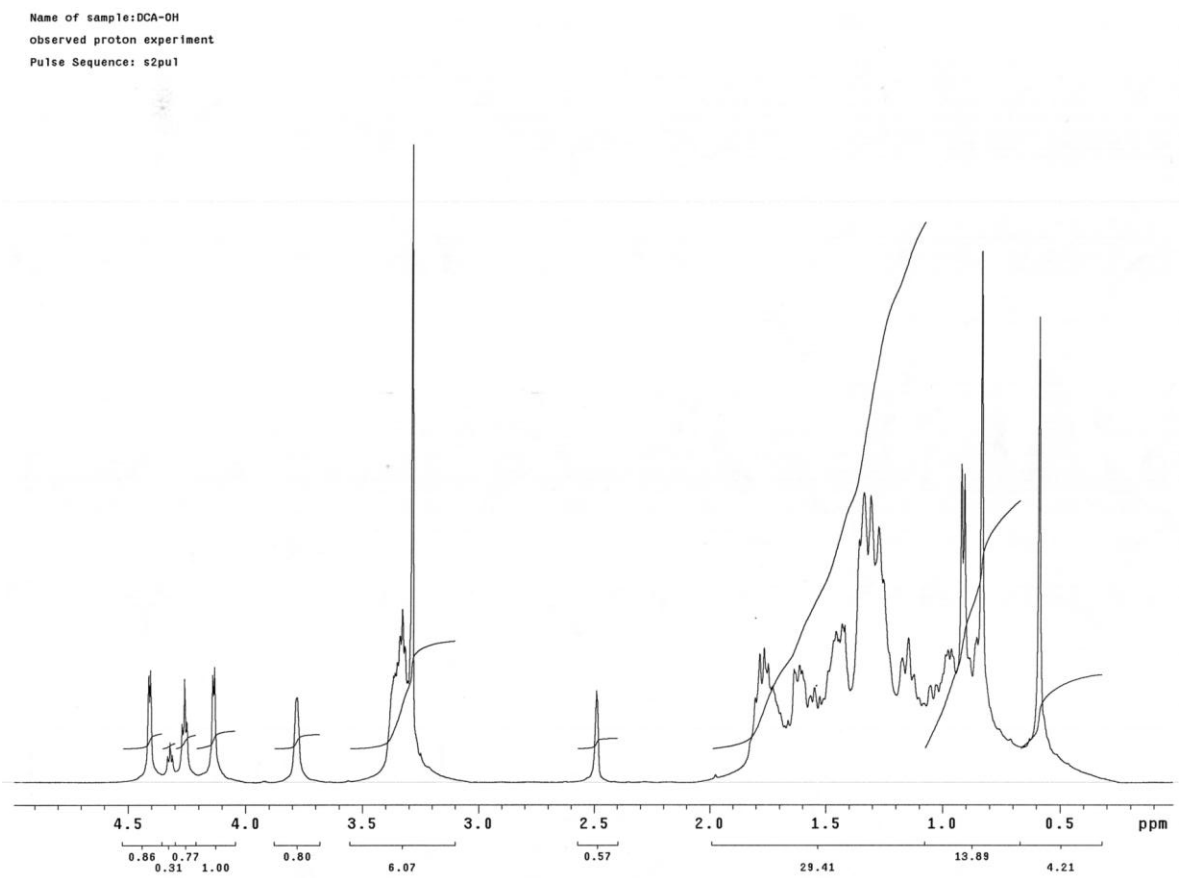
FTIR of cholic acid (CA)



FTIR of potassium cholate (KC)



^{1}H NMR of methyl deoxycholate



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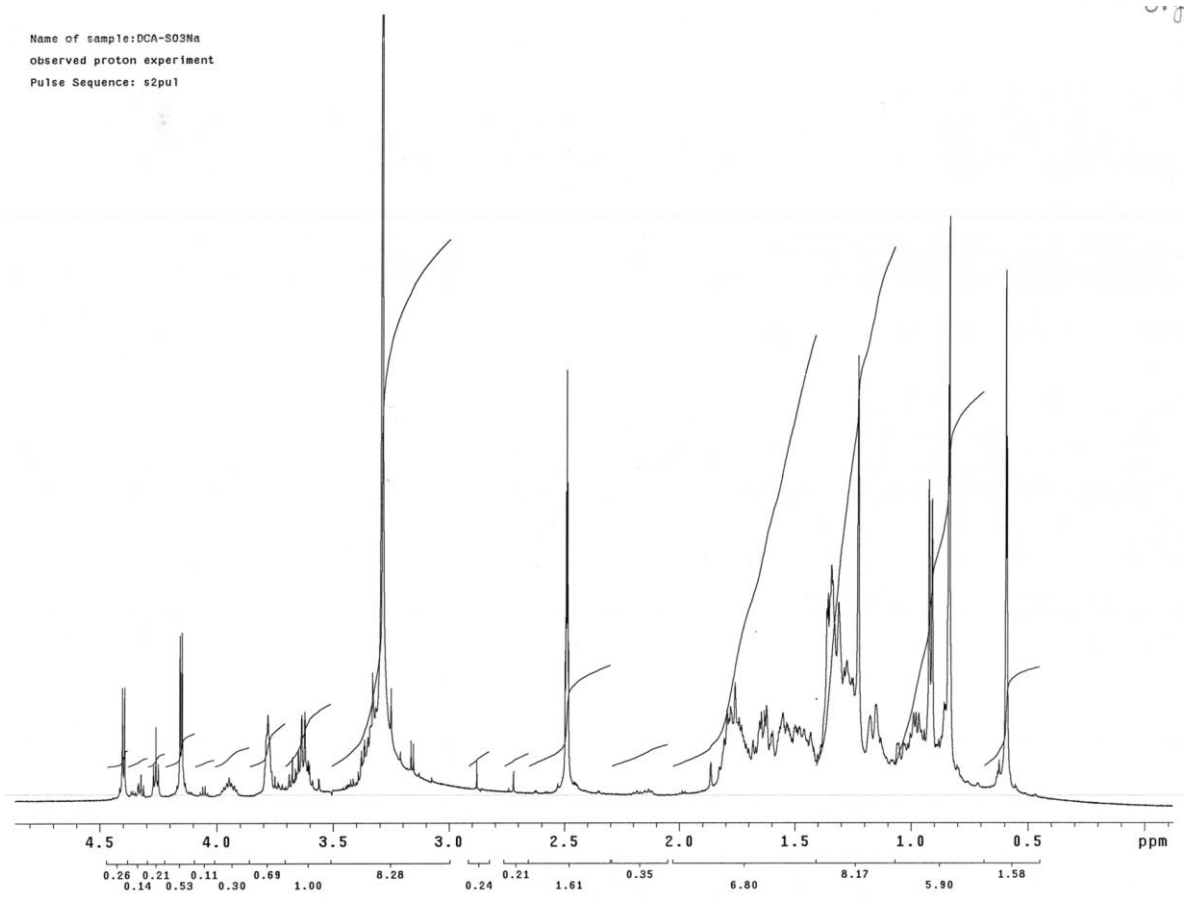

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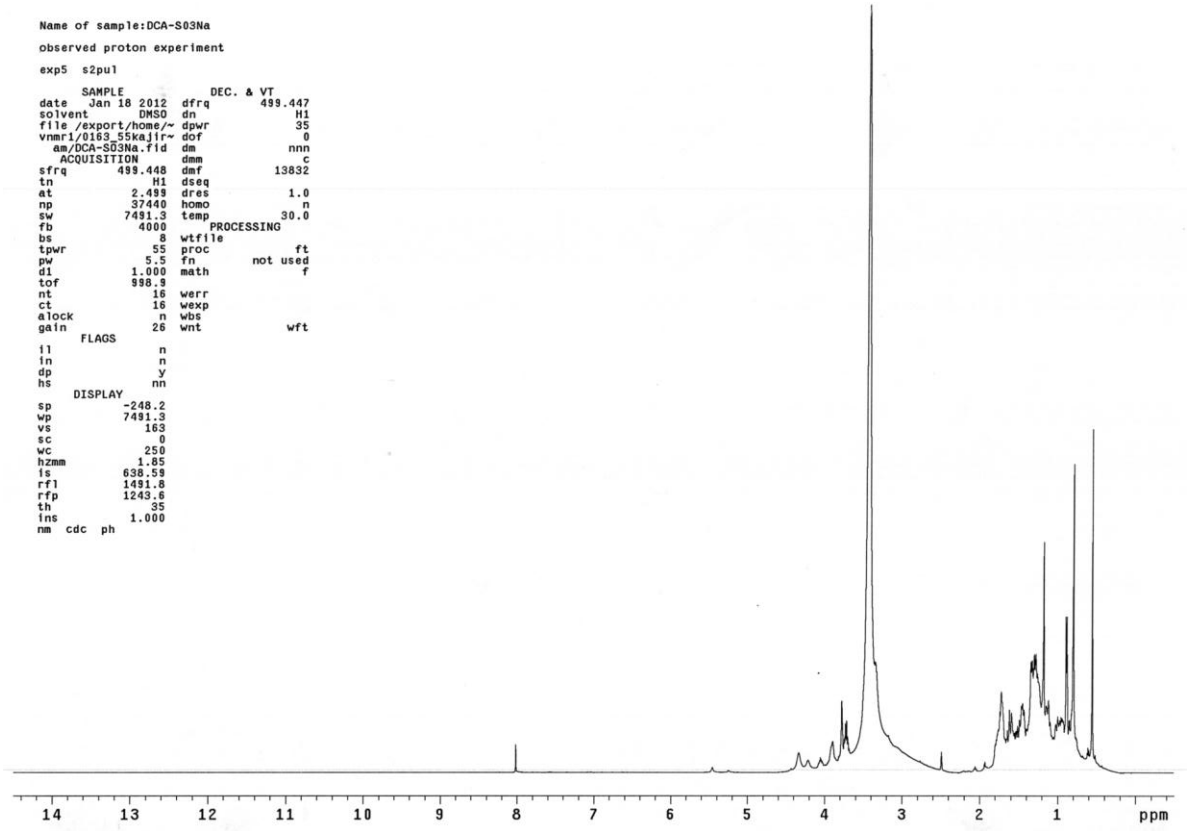

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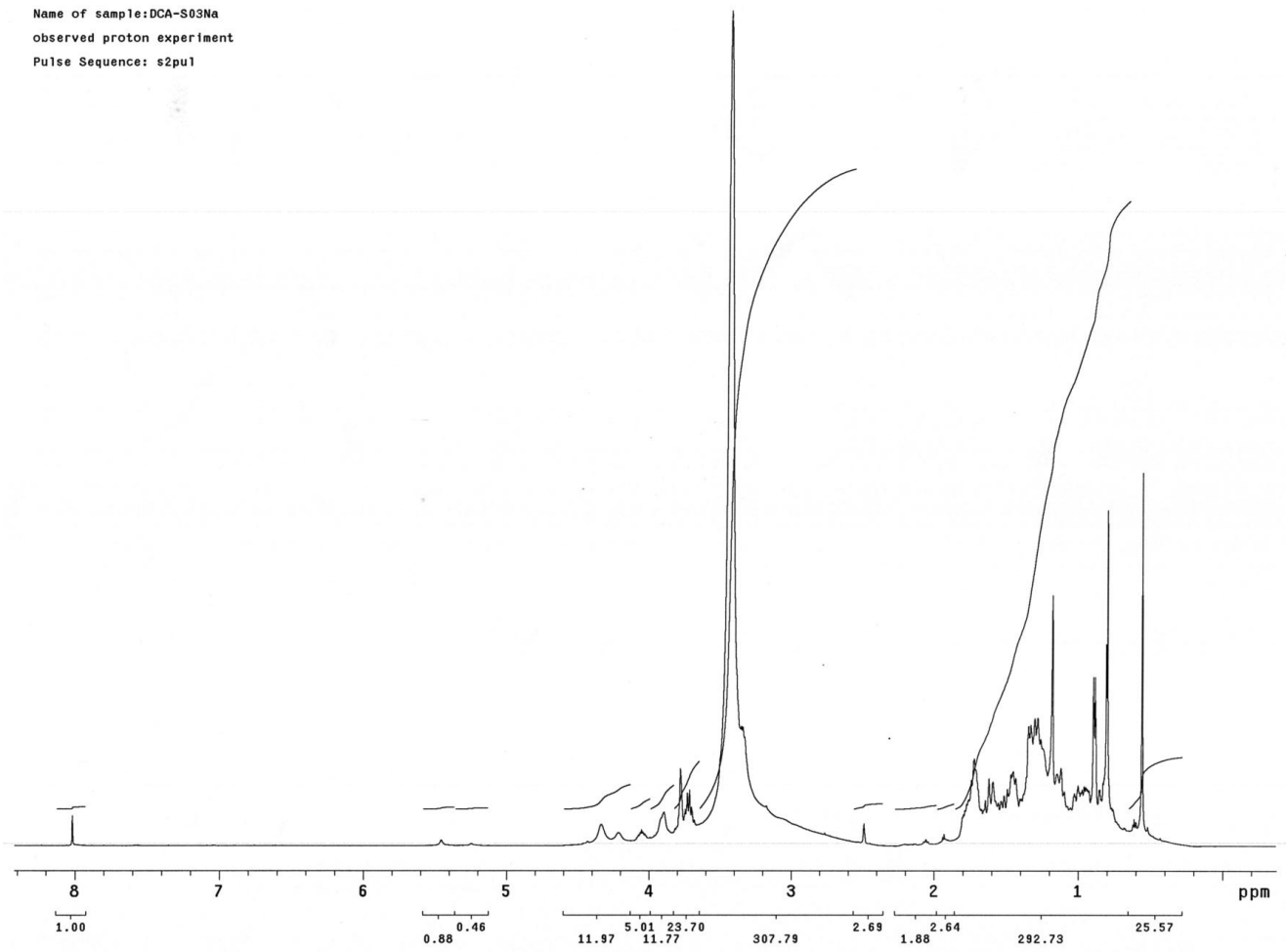

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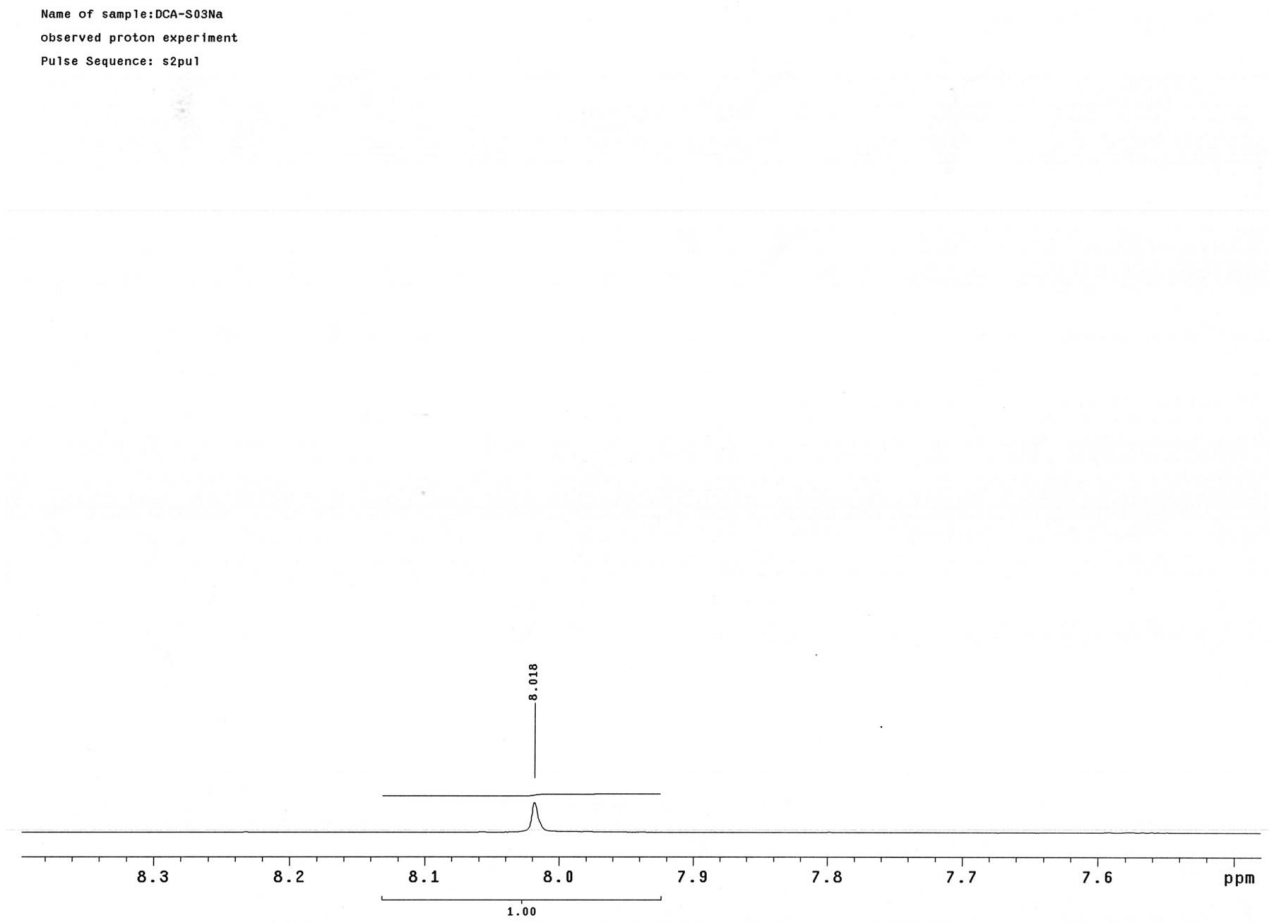

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