Preparation of Pyrazinamide and Levofloxacin Proliposomes and Feasibility in Tuberculosis Treatments

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ชื่อเรื่องวิทยานิพนธ์ การเตรียมไพราซินาไมด์และลีโวฟลอกซาซินโปรลิโปโซม และความเป็นไปได้ในการรักษาวัณโรคปอด

ผู้เขียน นางสาววิภาพร โรจนรัตน์

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บทคัดย่อ

การนำส่งยาสู่ทางเดินหายใจเป็นที่นิยมอย่างกว้างขวางเพื่อผลเฉพาะที่และทั่วร่างกาย สูตรตำรับการนำส่งยารูปแบบดังกล่าวต้องสามารถนำส่งยาสู่ปอดในส่วนที่ต้องการ ในขนาดยาและเวลาที่ถูกต้อง ยาสูดชนิดผงแห้งเป็นหนึ่งในแนวทางสำหรับการนำส่งยาสู่ทางเดินหายใจ ในการศึกษานี้ ใช้สเปรย์ดรายแมนนิทอลที่มีรูพรุนเป็นตัวพา ซึ่งแมนนิทอลที่มีรูพรุนถูกเตรียมจากการเติมแอมโมเนียมอะซิเตท หรือแอมโมเนียมคาร์บอเนตก่อนการทำแห้งแบบพ่น วัดขนาดและลักษณะทางกายภาพของสเปรย์ดรายแมนนิทอลด้วยกล้องจุลทรรศน์ชนิดอิเล็กตรอนแบบส่องกราด หาความหนาแน่นของอนุภาคโดยใช้วิธีการแทนที่ของเหลว และวิเคราะห์แอมโมเนียมที่หลงเหลือในแมนนิทอลโดยใช้ energy dispersive X-ray spectrometer (EDX)

แมนนิทอลที่เตรียมได้เป็นอนุภาคกลมขนาด 3 - 5 ไมโครเมตร มีรูพรุน พบว่าความหนาแน่นของแมนนิทอลลดลงเมื่อเปอร์เซ็นต์การมีรูพรุนสูงขึ้น แมนนิทอลที่มีรูพรุนซึ่งเตรียมจากการเติมแอมโมเนียมคาร์บอเนต 20% มีความหนาแน่นน้อยที่สุดในการศึกษานี้ ผลจากการวิเคราะห์ EDX ไม่พบแอมโมเนียมคาร์บอเนตหลงเหลือในแมนนิทอลที่มีรูพรุน ดังนั้น แมนนิทอลที่มีรูพรุนอาจเป็นทางเลือกสำหรับใช้เป็นตัวพาในยาสูดชนิดผงแห้ง

ในการศึกษานี้ใช้ไพราซินาไมด์และลีโวฟลอกซาซินเป็นยาต้านวัณโรคต้นแบบ ไพราซินาไมด์ซึ่งเป็นยาต้านวัณโรคกลุ่มที่หนึ่ง ถูกเลือกเป็นตัวแทนของยาที่ละลายในน้ำได้ดี สำหรับยากลุ่มที่สอง ลีโวฟลอกซาซินถูกเลือกเป็นตัวแทนของยาที่ละลายน้ำน้อย ไพราซินาไมด์โปรลิโปโซมและลีโวฟลอกซาซินโปรลิโปโซมในรูปแบบยาสูดชนิดผงแห้งได้ถูกพัฒนาเพื่อนำส่งยาสู่ alveolar macrophages (AMs) ที่ติดเชื้อไมโคแบคทีเรีย โปรลิโปโซมที่ประกอบด้วยยาต้านวัณโรค ฟอสฟาทิดิลโคลีนจากถั่วเหลือง โคเลสเตอรอล และแมนนิทอลที่มีรูพรุน ถูกเตรียมโดยวิธีการทำแห้งแบบพ่น ทำการทดสอบคุณสมบัติทางกายภาพและทางเคมี โดยใช้ cascade impactor, X-ray diffraction (XRD), differential scanning calorimetry (DSC) และ fourier transform-infrared spectroscopy (FT-IR) ทดสอบความเป็นพิษของโปรลิโปโซมต่อเซลล์ทางเดินหายใจ และการกระตุ้นการตอบสนองของระบบภูมิคุ้มกันจาก AMs นอกจากนี้มีการประเมินประสิทธิภาพในการฆ่าเชื้อ *Mycobacterium bovis* (*M. bovis*) และ *M. tuberculosis* และความเป็นพิษในสัตว์ทดลองทั้งการได้รับยาครั้งเดียวและได้รับยาติดต่อกันของยาทั้งสองชนิดดังกล่าว

ไพราซินาไมด์โปรลิโปโซมและลีโวฟลอกซาซินโปรลิโปโซมที่เตรียมได้มีขนาดอนุภาคแอโรไดนามิกส์ของไพราซินาไมด์โปรลิโปโซมเป็น 4.26 - 4.39 ไมโครเมตร มีค่าสัดส่วนของอนุภาค (fine particle fraction, FPF) ที่มีเส้นผ่าศูนย์กลางน้อยกว่า 4.4 ไมโครเมตร คิดเป็น 20-30 เปอร์เซ็นต์ เมื่อวัดที่แรงดูดอากาศ 60 ลิตรต่อนาที และสามารถกักเก็บยาไพราซินาไมด์ในลิโปโซมได้ประมาณ 26 - 45 เปอร์เซ็นต์ ขนาดอนุภาคลีโวฟลอกซาซินโปรลิโปโซมมีค่า 4.15 - 4.44 ไมโครเมตร และ FPF มีค่า 13 - 38 เปอร์เซ็นต์ ที่แรงดูดอากาศ 60 ลิตรต่อนาที และสามารถกักเก็บยาลีโวฟลอกซาซินได้ประมาณ 15-24 เปอร์เซ็นต์ ผลจาก XRD, DSC และ FT-IR แสดงว่ามีอันตรกริยาเกิดขึ้นระหว่างยากับยา และยากับสารตัวพา ไพราซินาไมด์

ความเป็นพิษในสัตว์ทดลองทั้งการได้รับยาครั้งเดียวและได้รับยาติดต่อกันในหนูขาวเพศผู้ พบว่า หลังการให้ยาครั้งเดียว ระดับ blood urea nitrogen (BUN) และ creatinine (Cr) ในหนูมีค่า 17 - 33 และ 0.5 - 0.7 มิลลิกรัมต่อเดซิลิตร ตามลำดับ สำหรับความเป็นพิษในสัตว์ทดลองที่ได้รับยาติดต่อกัน 28 วัน ค่า BUN, Cr, alanine aminotransferase และ asparatate aminotransferase มีค่าเป็น 17 - 25 มิลลิกรัมต่อเดซิลิตร, 0.6 - 1.0 มิลลิกรัมต่อเดซิลิตร, 53 - 90 ยูนิตต่อลิตร และ 87 - 220 ยูนิตต่อลิตร ตามลำดับ จากผลการศึกษาสามารถสรุปได้ว่าการให้ยาไพราซินาไมด์โปรลิโปโซม และลีโวฟลอกซาซินโปรลิโปโซม ไม่มีความเป็นพิษในสัตว์ทดลองทั้งการได้รับยาครั้งเดียวและได้รับยาหลายครั้ง ดังนั้นผงยาไพราซินาไมด์โปรลิโปโซม และลีโวฟลอกซาซินโปรลิโปโซมมีศักยภาพในการรักษาการติดเชื้อวัณโรคปอด

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ABSTRACT

The pulmonary route is widely used for local and systemic drug delivery. To deliver a drug via inhalation, the formulations must flow to the desired part of the lung with the correct amount of drug over an appropriate time. Use of a dry powder inhaler (DPI) is one strategy for pulmonary delivery. In this study, porous spray-dried mannitol was used as a carrier. Porous mannitol was prepared by adding ammonium acetate or ammonium carbonate prior to spray drying of the formulation. The sizes and morphologies were determined with a scanning electron microscope (SEM). The liquid displacement method was used to investigate the particle density. The ammonium residue was analyzed using an energy dispersive X-ray spectrometer (EDX). The porous microspheres of mannitol exhibited a smooth surface of 3-5 μm size. The bulk density of the porous mannitol was decreased with increased % porosity. In this study, the porous mannitol using 20% ammonium carbonate had the lowest density. EDX analysis data revealed that there was no residue of ammonium carbonate in the porous mannitol. Porous mannitol may be a good candidate for a DPI carrier.

In this study, pyrazinamide (PZA) and levofloxacin (LEV) were used as model antituberculosis (anti-TB) drugs. PZA, as a first line medication drug was chosen for the hydrophilic model drug. For the second line medication drug, LEV was selected as a hydrophobic model drug. PZA-proliposomes and LEV-proliposomes in dry powder aerosol forms were developed for delivering drugs to alveolar macrophages (AMs) infected with mycobacteria. Proliposomes consisting of the anti-TB drug, soybean phosphatidylcholine, cholesterol and porous mannitol were prepared by a spray drying method. The proliposome physicochemical properties were determined using a cascade impactor, X-ray diffraction (XRD), differential scanning calorimetry (DSC) and fourier transform-infrared spectroscopy (FT-IR). The toxicity of the proliposomes to respiratory-associated cell lines and its potential to provoke immunological responses from AMs were determined. The antimycobacterial activities of the proliposomes against *M. bovis* and *M. tuberculosis* were tested. *In vivo* single and repeated dose toxicity in male Wistar rats were evaluated.

PZA-proliposomes and LEV-proliposomes were successfully prepared. For the aerosolization properties of the PZA-proliposomes at 60 L/min, the powders showed mass median aerodynamic diameters (MMAD) of 4.26 - 4.39 μm, with fine particle fractions (FPF) (aerosolized particles less than 4.4 μm) of 20 - 30%. Encapsulation of PZA was 26 - 45%. The MMAD of the LEV-proliposomes was 4.15 - 4.44 μm with a FPF of 13 - 38% at 60 L/min. Encapsulation of LEV was 15 - 24%. The results from XRD, DSC and FT-IR showed that there were some chemical interactions in the PZA-proliposomes and LEV-proliposomes. The PZA-proliposomes and LEV-proliposomes were less toxic to respiratory-associated cells,

and did not activate AMs to produce inflammatory mediators, including interleukin-1β, tumor necrosis factor-α, and nitric oxide at a toxic level. PZA and PZA-proliposomes were inactive against *M. bovis* and *M. tuberculosis*, while the minimum inhibitory concentrations (MICs) against *M. bovis* of the LEV and LEV-proliposomes containing 10% LEV were 1 and 0.5 μg/mL, respectively. The efficacy of the LEV-proliposomes against *M. bovis* was significantly higher than that of the free LEV ($p < 0.05$). The efficacy of the LEV-proliposomes against *M. tuberculosis* was equal to that of the free LEV (MIC = 0.195 μg/mL).

The tests for single and repeated dose toxicity *in vivo* in rat studies were carried out for one hour and for 28 successive days, respectively. In this study, the single and repeated dose of PZA-proliposomes and levofloxacin-proliposomes were investigated in male Wistar rats after intratracheal administration. Blood urea nitrogen (BUN) and creatinine (Cr) for the single dose toxicity study were in the range of 17 - 33 and 0.5 - 0.7 mg/dL, respectively. For the repeated dose toxicity study, BUN, Cr, alanine aminotransferase and asparatate aminotransferase were 17 - 25 mg/dL, 0.6 - 1.0 mg/dL, 53 - 90 U/L and 87 - 220 U/L, respectively. There were no significant differences in toxicity observed when the 4 mg/kg per day proliposome formulations were administered to rats either in the single or repeated dose. Situation based on these results, it can be concluded that there are no single and repeated dose toxicity effects after PZA- proliposomes and levofloxacin-proliposomes were administered. PZA-proliposomes and LEV-proliposomes are potential candidates for an effective treatment of pulmonary tuberculosis.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Rationale

Tuberculosis (TB) is the contagious disease that has caused the problem for developing economy and social status of the country. In the past, controlling tuberculosis was successful in Thailand. Economic crisis in 1997, poverty, immigration and acquired immunodeficiency syndrome (AIDS) spread cause unsuccessful control of TB (WHO, 2010). Das and Horton (2010) reported that globally 2 million TB patients died per year. TB is the important opportunistic disease of AIDS, about 20-25% of AIDS persons are pulmonary and extra-pulmonary tuberculosis infected (Ghebreyesus et al., 2010). Therefore the TB control is complicated and risk in multiple drug resistance - tuberculosis (MDR-TB), at least two first line medication drugs are resistant.

TB is caused by tubercle bacilli of Mycobacteriaceae family. Mycobacteria can cause a variety of diseases, and those that cause TB are grouped under the term *Mycobacterium tuberculosis* complex. In human beings, TB is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). Other tuberculosis mycobacteria, namely, *M. bovis* and *M. africanum*, are rarely implicated in human disease (Kim, 2002). Pulmonary tuberculosis comprises more than 80% of all cases. Extra-pulmonary TB, or tuberculosis of other parts of the body, make up the remaining 20%

(Nair, 2002). TB bacilli can remain viable in the body without spread from one person to another, this phase is called primary tuberculosis. Only 10% of primary tuberculosis becomes developed to post - primary tuberculosis that spread from one person to another (Weinberger, 1992; Piessens and Nardell, 2000; Frieden et al., 2003).

Nowadays, antituberculosis drugs are divided into two groups; first and second line medication. First line medication drugs such as isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin have high efficacy. The second line medications are ethionamide, cycloserine, para-amino-salicylic acid (PAS), capreomycin, levofloxacin, gatifloxacin and moxifloxacin. New generation in this group has good antimycobacterial activity (gatifloxacin and moxifloxacin) although some of older medicines have high side effects and expensive. The second line therapy is useful in case of MDR-TB patients (Frieden et al., 2003). World health organization (WHO) recommends short course drug therapy for new infected TB into two phases. Initial phase is an intensive course, the patients have to take three to four drugs (isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin) for at least two months. The continuous phase, two drugs are used together for at least four months (usually isoniazid and rifampicin) (Weinberger, 1992; Piessens and Nardell, 2000; Frieden et al., 2003). Although the first line medication drugs have high efficacy, taking many drugs for long time (at least six months) is the cause of liver and renal toxicity. In addition, it is the cause of drug resistant TB especially MDR-TB from non-adherence. This is why second line medication drugs are introduced and the

treatment takes longer duration (Reichman and Hershfield, 2000; Frieden et al., 2003).

To date, there is no introduction of new first line medication drug therefore increasing efficacy of existing antituberculosis drugs is achieving importance. Alveolar delivery system is attracting approach to deliver drugs to TB infected lung especially infected alveolar macrophage (AM) (Alving, 1988; Ahsan et al., 2002). This system by inhalation has many benefits such as target oriented, low dose, less side effect and decreased administration frequency. The important outcomes are better drug compliance, reduced drug resistance and short course therapy.

Pulmonary administration is appropriate route for drugs having a local action in the respiratory tract, resulting in rapid localized drug action. A major advantage of liposomes as pulmonary drug delivery systems is that they can be prepared from phospholipids that are endogenous to the lungs as components of lung surfactant. It is assumed that pulmonary-delivered vesicles will thus be biocompatible (Taylor and Elhissi, 2006). Antituberculosis delivery in liposome form has been developed (Dutta et al., 2000; Swarbrick and Boyland, 2007). Couvreur and co-workers (1991) reported that antituberculosis drug entrapped in liposome is more effective against TB than drug alone. Drug encapsulated in liposome coated with polyethylene glycol (stealth liposome) was developed. It could sustain the drug in blood circulation (Dutt and Khuller, 2001). Liposome linked with *O*-steroyl amylopectin could be specific for lungs and increased efficacy (Deol and Khuller, 1997; Deol et al., 1997). Labana (2002) reported that TB could be decreased in the animals treated with antituberculosis drug encapsulated in liposome once a week for six weeks when

compared with control group. Previous research works employed intravenous injection as an administrative route (Dutta et al., 2000; Swarbrick and Boyland, 2007). Vyas (2004) prepared liposome in pressurized metered dose inhaler formulation and determined the effect against TB. The results showed that liposome formulation was more effective than drug alone.

Liposomes are relatively unstable colloidal systems. Physical instability is manifested in vesicle aggregation and fusion, which are associated with changes in vesicle size and loss of entrapped hydrophilic materials (Elhissi et al., 2007). Chemical instability, particularly ester bond hydrolysis, and oxidation of unsaturated acyl bonds accelerates liposome breakdown and alters drug-release characteristics (Heurtault et al., 2003). To overcome instability problems, proliposome approaches to liposome formation have been described as a mean of enhancing stability. Freeze dried and spray dried liposomes can be a useful tool to prepare proliposome (Taylor and Elhissi, 2006). Proliposomes are defined as dry, free-flowing particles that immediately form a liposomal dispersion on contact with water, so it has a potential for pulmonary delivery as dry-powder inhalers (DPIs) (Elhissi et al., 2010; Wagner and Vorauer-Uhl, 2011). Antituberculosis in proliposome has been studied (Changsan et al., 2009). Rifampicin proliposome was prepared by freeze-drying with mannitol as a cryoprotectant (Changsan et al., 2009). Rifampicin-encapsulated liposomes were evidently nontoxic to respiratory associated cells, including bronchial epithelial cells, small airway epithelial and AMs. The minimum inhibitory concentrations against *M. bovis* was 0.2 and 0.8μM for proliposome containing rifampicin and free rifampicin,

respectively. The authors indicated that antituberculosis drugs entrapped in proliposome enhanced drug bioactivity (Changsan et al., 2009).

Antituberculosis mixing with lactose carrier can improve aerodynamic properties. Isoniazid mixing with the different size of carrier particles strongly affected the deposition of isoniazid to the lower airways *in vitro* (Sawatdee et al., 2006). Particle density is an important parameter in aerosol physics. It involves in aerodynamic diameter. The aerodynamic diameter of a particle, or d_{ae}, is the diameter of a unit density sphere that has the same settling velocity (Crowder et al., 2002). It can be calculated using equation.

$$d_{ae} = d_g (\rho_p)^{1/2}$$

where d_g is the particle's geometric diameter and ρ_p is the particle density. This will make low density of smaller size particle that have low aerodynamic diameter. Low density particle was explored as a mean of improving the delivery of inhaled therapeutics (Crowder et al., 2002).

Besides mixing with carrier, spray drying with pore forming agent method is also an alternative approach (Yang et al., 2009; Nolan et al., 2009). Poly(lactic-co-glycolic acid) (PLGA) microparticles were made by the double-emulsion method and an effervescent salt ammonium bicarbonate was included in the internal aqueous phase. This method was able to produce mass median aerodynamic diameter (MMAD) from 6.2 ± 1.2 to 4.0 ± 1.2 μm and fine-particle fraction (FPF) from 10.3 ± 4.8 % to 32.0 ± 9.1 % (Yang et al., 2009). Low MMAD with high FPF gained benefit the drug targeting to the lower airways. When budesonide was spray dried with ammonium carbonate, it showed good potential for drug delivery by oral inhalation

with improved *in vitro* deposition properties compared to non-porous particles (Nolan et al., 2009).

In this study, pyrazinamide and levofloxacin were used as model antituberculosis drugs. Pyrazinamide, the first line medication drug was chosen for hydrophilic model drug. For the second line medication, levofloxacin was selected as hydrophobic model drug.

Proliposomes in this study were prepared by spray drying method. Lipid part containing soybean phosphatidylcholine, cholesterol and drug were dissolved in ethanol to obtain clear solution. Mannitol as a carrier was added to solution. The suspension was spray dried to obtain proliposome. To improve aerodynamic properties of the products, mannitol was modified by pore forming agents either ammonium acetate or ammonium carbonate. The morphology, bulk density, % porosity and ammonium residue of porous mannitol were determined to optimize the amount of pore forming agent that produces suitable morphology, high porosity with no ammonium residue.

Pyrazinamide-proliposomes and levofloxacin-proliposomes were prepared by spray drying. Physico-chemical and aerodynamic properties were determined. Toxicity of proliposome formulations which had good aerodynamic properties on respiratory and macrophage cell lines were investigated. Phagocytosis of macrophage cells incubated with proliposomes was observed to ensure that reconstituted products can be phagocyted. Minimum inhibitory concentration of the antituberculosis dry powder inhaler formulations on mycobacteria both extracellular and intracellular macrophage cells were determined by flow cytometric method (Sawatdee et al.,

2006). *In vivo* single and repeated dose toxicity of PZA and LEV proliposome was investigated in Wistar rats administered with the proliposomes by intratracheal instillation.

1.2 The objective of the studies

The aims of the studies are:

1.2.1 To prepare and develop pyrazinamide and levofloxacin dry powder inhaler formulations.

1.2.2 To study toxicity of formulations on respiratory and macrophage cell lines.

1.2.3 To observe the phagocytosis of antituberculosis dry powder particles by macrophage cells.

1.2.4 To determine antituberculosis dry powder inhaler formulation efficacy and minimum inhibitory concentration of the formulations on mycobacteria both extracellular and intracellular macrophage cells.

1.2.5 To study *in vivo* single and repeated dose toxicity of the formulations.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Pulmonary tuberculosis

Tuberculosis (TB), a chronic infectious disease, is caused by tubercle bacilli of Mycobacteriaceae family. Mycobacteria can cause a variety of diseases, and those that cause TB are grouped under the term *Mycobacterium tuberculosis* complex. In human beings, TB is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) (Figure 2.1). Other tuberculous mycobacteria, namely, *M. bovis* and *M. africanum*, are rarely implicated in human disease. In 1999, a few cases of human infection by *M. tuberculosis subsp. caprae*, a mycobacterium that infects goats, were reported. Another member, *M. microti*, that usually infects small mammals, has not been isolated in human beings (Grosset et al., 2000; Kim, 2002; Grange and Zumla, 2002).

There are about 30 other species of mycobacteria, known as 'Nontuberculous mycobacteria (NTM), which neither cause TB nor leprosy. NTM are present in soil and water, cattle and birds or as normal pharyngeal flora in humans. People get infected by nontuberculous mycobacteria through inhalation or inoculation, but person to person spread of NTM is rare. On the other hand, NTM are becoming increasingly important as opportunistic infections.

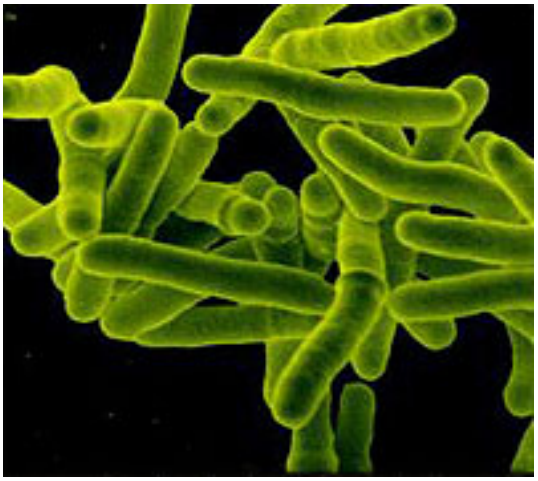


Figure 2.1 *Mycobacterium tuberculosis* (Institute of chemical biology and drug discovery at Stony Brook University, 2005)

Unsuccessful control TB might be from economic crisis in 1997, poverty, immigration and acquired immunodeficiency syndrome (AIDS) spread. Twenty-two high burden TB countries including Thailand are shown in Figure 2.2 (WHO, 2010). Das and Horton (2010) reported that globally 2 million TB patients died per year. The occurrence of TB is most often due to *M. tuberculosis* infection and the lung is the primary site of infection; 80% of TB cases are of pulmonary TB alone (Nair, 2002; Pandey and Khuller, 2005). Extensively drug-resistant TB (XDR-TB) is defined as an MDR-TB with resistance to a fluoroquinolone or at least one second-line injectable agent (amikacin, kanamycin and/or capreomycin).

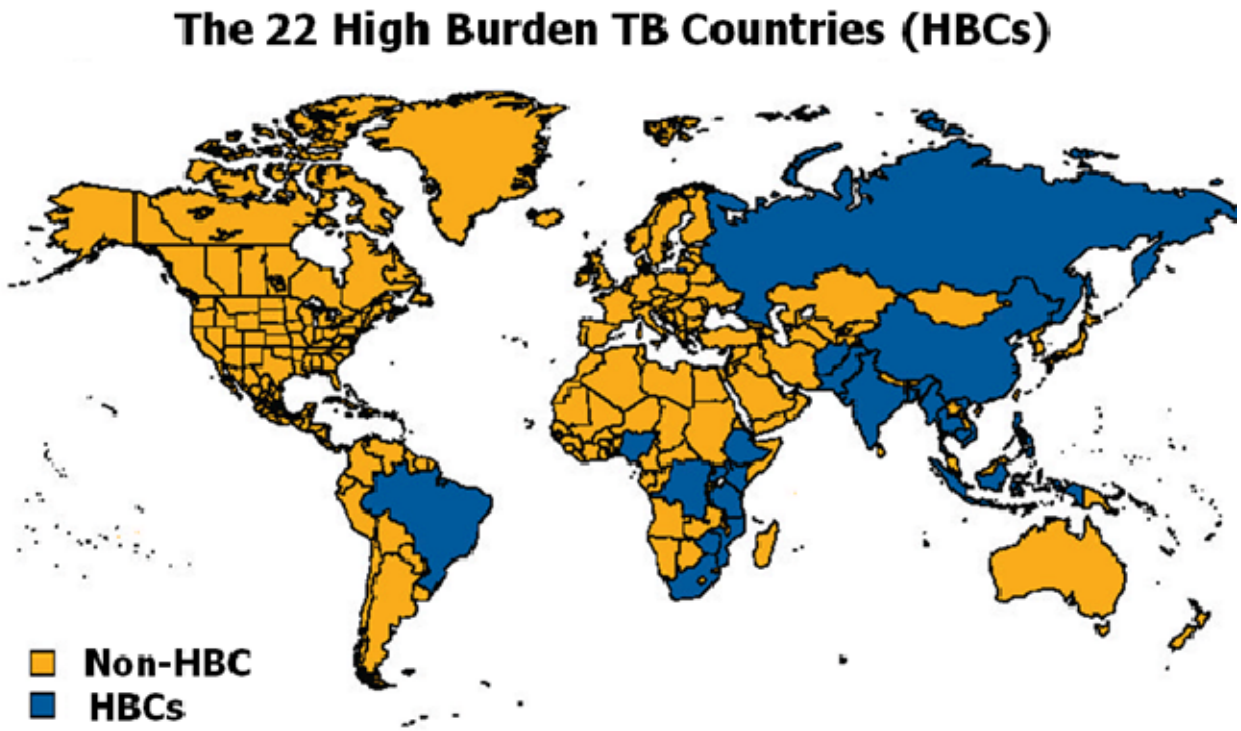


Figure 2.2 Twenty-two high burden TB countries (WHO, 2010)

M. tuberculosis is an intracellular parasitic bacterium that mainly infects the respiratory tract and hides in alveolar macrophages (AMs). AMs are one type of immunocompetent cell present in the lungs. When foreign substances such as a disease organism are inhaled and reach an alveolus, they are endocytosed by AMs and eventually transported to phagosomes. The endocytosed foreign substance is then degraded by the digestive enzymes in the phagosome. However, when *M. tuberculosis* is endocytosed, it inhibits formation of phagolysosomes and avoids degradation in the AMs, resulting in a latent infection (Pethe et al., 2004).

2.1.1 Tuberculosis infection

TB is spread through the air from one person to another. When a person with TB of the lung or throat coughs, sneezes, talks or even sings, the bacteria are sprayed out into the air as infectious droplets. These droplets dry up rapidly and the smallest of them remain suspended in the air for several hours. People nearby may inhale these bacteria and become infected. (Piessens and Nardell, 2000). When a person breathes in TB bacilli, the organisms could settle in the lungs and begin to multiply. From the lungs, they could journey through the circulatory system to various parts of the body, such as the kidney, the spine, and the brain. TB bacilli can remain viable in wet sputum for months and in dried sputum for weeks. Most TB infections are acquired by continued exposure rather than casual contact, because respiratory defense mechanisms can kill small numbers of mycobacteria.

Once a person breathes in the air that contains droplets contaminated with TB bacilli, most of the larger droplets settle in the nose or the throat where they are

unlikely to establish infection. However, TB bacilli may reach the alveoli. Within the alveoli the bacilli are ingested by AMs and other phagocytes. Live virulent bacilli multiply within the macrophages, which get destroyed in the process. The bacilli are subsequently ingested by other tissue macrophages or by macrophages infiltrating the area from blood vessels. Bacterial multiplication cannot be stopped within those nonactivated macrophages. Within 2-4 weeks after infection, the host mounts a specific cell-mediated immunity that will arrest TB infection in more than 90% of infected individuals. TB infection means that tubercle bacilli are viable within the body but are kept from multiplying and causing disease by the body's immune system. People who have TB infection but not TB disease are not infectious; in the other words, they cannot spread the infection to other people (Figure 2.3) (Piessens and Nardell, 2000).

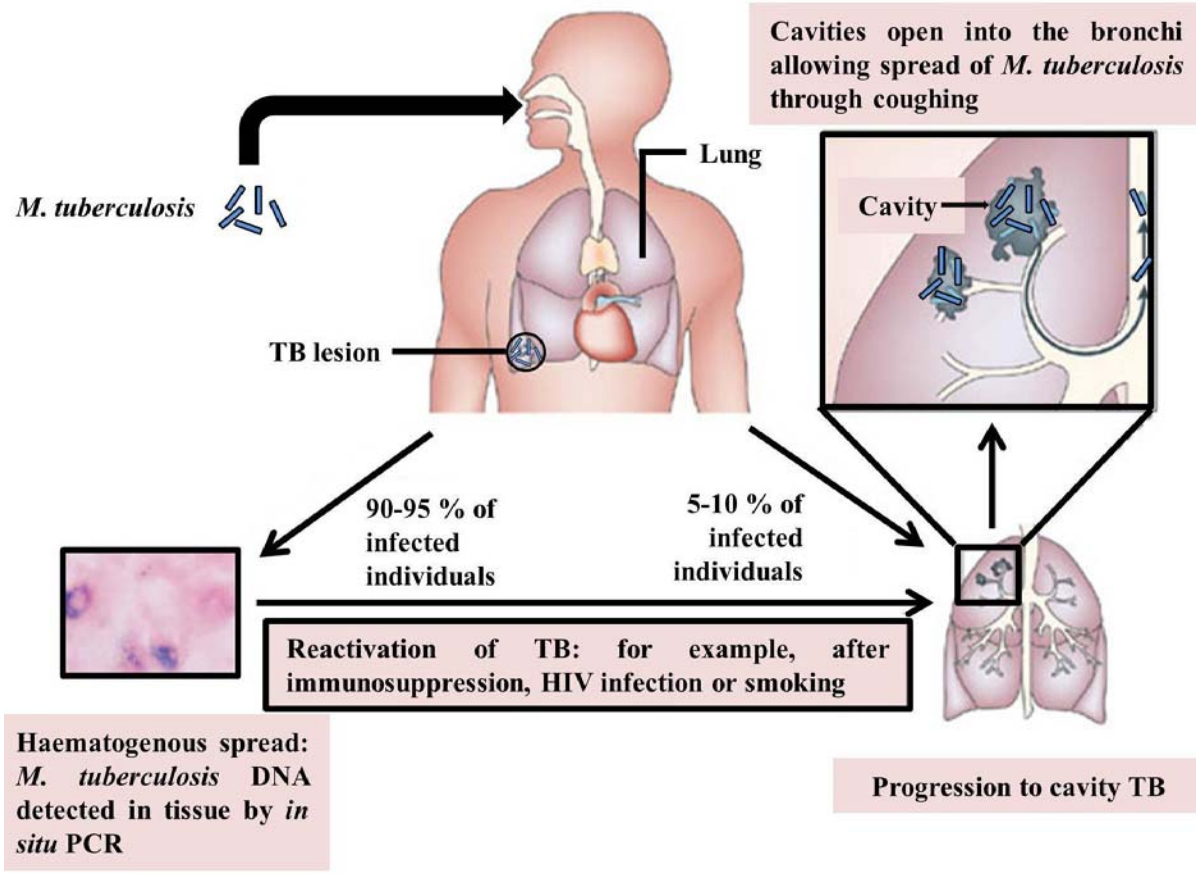


Figure 2.3 TB infection (Rook et al., 2005)

2.1.2 Tuberculosis disease

Not all infected individuals develop TB; approximately 10% develop the disease. During the first two years following the infection, the risk of developing TB disease is the highest approximately half of the cases develop TB during this period. As mentioned above, the risk of progression to the TB disease is determined by innate and acquired defence mechanisms (immunity) against the invading organisms. On average, the possibility that a person with no risk factors will develop TB disease is about 10%, and is the highest in the first two years after infection as early as possible helps prevent new cases of TB. Once infected, a person could develop TB disease at any age. Certain conditions increase the risk of progression. For example, diabetics with TB infection are three times more likely to progress to the disease, and people with HIV are over 100 times more likely to develop infection.

Primary TB, as the first manifestation of TB disease is called, begins with a lesion known as Ghon's focus in the mid-zone of the lung. TB bacilli here are ingested by macrophages and carried to other parts of the lung. The bacilli also spread through the lymphatics to trachea-bronchial and mediastinal nodes. The Ghon's focus and hilar node involvement are together termed as primary complex (Grange and Zumla, 2002).

Primary TB usually heals, leaving behind a completely fibrosed Ghon's focus, or a larger calcified focus. If there is inflammation of the adjacent lung tissue, pleural effusion could result. A tuberculous empyema could develop if the pleura becomes infected. Hilar node enlargement could cause brochial obstruction due to pressure on bronchi and, in turn, lead to collapse, retention of secretions and consolidation. Or,

tubercles could spread along bronchial lymphatics leading to the ulceration of the bronchial wall, bronchiectasis or brocho-pneumonia. If TB bacilli enter the bloodstream, they spread throughout the body, establishing TB foci in various organs, causing miliary TB.

Secondary or post-primary TB is the recurrence of TB in later life due to the reactivation of latent foci of the primary episode or reinfection with TB. Tubercles may appear in the wall of a bronchiole or in the lung tissue in the posterior sub-apical zone or the upper part of the lower lobes. The tubercles eventually merge and undergo caseous necrosis due to cell-mediated immunity. The necrosis kills many macrophages and bacilli and, thus, the lesion expands slowly and hilar spread does not occur. The lesion is encapsulated by fibrosis and (i) heal with a calcified, thick, residual scar (ii) become encysted with a few bacilli or ‘persisters’ or (iii) may gradually expand by necrosis of the fibrous capsule. Once the lesion necroses into a bronchiole or brochus, the caseum could drain out, leaving behind a cavity (Grange and Zumla, 2002).

2.2 Treatment of pulmonary tuberculosis

The effective treatment of TB is critical to the well-being of the patients, their families and the communities. Treatment benefits the patient by preventing disability and death; at the same time it arrests the chain or transmission to the community.

2.2.1 Directly Observed Treatment (DOTS) strategy

Effective anti-TB drugs have been available for nearly 50 years. Yet TB remains a major health concern world-wide especially in developing countries. Treating TB entails months of combination therapy with 3-4 drugs. When a single drug is used to treat TB, the tubercle bacilli quickly develop resistance to that drug. Examples of anti-TB drugs are shown in Table 2.1.

Properly applied short course chemotherapy is now the recommended standard for the treatment of TB. However, prescribing anti-TB medication correctly is not enough. Each patient must receive the prescribed drugs at the correct dosage on a regular basis for the required duration. If patients fail to take their combination of drugs regularly, the bacilli may become drug-resistant. It is essential for patients to receive and to adhere to the recommended course of treatment, usually 6-8 months, in order to get cured. This becomes somewhat of a challenge in various health settings around the world.

a) First-line treatment

Standardised short-course chemotherapy containing rifampicin and isoniazid for 6 months, supplemented with pyrazinamide (PZA) and ethambutol in the first 2 months which is effective against drug-susceptible TB under controlled conditions. The most common treatment for TB involves oral administration of high systemic doses of single or combined antibiotics (Singh et al., 2001; Panchagnula et al., 2004). The recommended daily doses of four anti-TB drugs are 5, 10, 25 and 15 mg/body weight (kg) for rifampicin, isoniazid, PZA and ethambutol, respectively. However, it

has been reported that inappropriate monotherapy can cause the development of TB that resistance to the first-line drugs (Singh et al., 2001; Panchagnula et al., 2004). To avoid problems associated with monotherapy, such as, over-dose or under- dosing, the first-line drugs have been recommended to be taken in combination (Panchagnula et al., 2004). Several fixed-dose combination (FDC) products containing rifampicin, isoniazid, PZA, and ethambutol are in use today (Singh et al., 2001; Bhutani et al., 2004). However, its effectiveness is compromised by the long treatment, which necessitates structured programmes to improve adherence. Although rates of treatment-limiting side effects vary, mild adverse effects are common (Yee et al., 2003). The potential for drug–drug interactions is high, largely due to the induction of the cytochrome P450 system, mediated through the activation of the pregnane-X receptor by rifampicin, which increases oral clearance of concomitant medications (Piscitelli et al., 2001). Intermittent dosing, reduced rifampicin exposure, low drug concentrations, poor adherence to treatment, advanced disease and immunosuppression predispose patients to disease relapse (Nahid et al., 2007; Cox et al., 2008) and development of drug resistance (Weiner et al., 2005). The limitations of control programmes are most evident in settings that are frequently characterised by poverty, high levels of HIV co-infection, and poor access to a high standard of treatment (Cox et al., 2008; Lawn et al., 2006). Short and simple regimens that are effective, safe, and robust during routine programmatic conditions are urgently needed.

However, concern has been expressed on the poor bioavailability and the unstable chemical potency of rifampicin from FDC products containing isoniazid

and/or PZA (Pillai et al., 1999; ShiShoo et al., 2001). The existence of rifampicin in different crystal forms and the change from one form to another during processing are repeatedly quoted as the reasons for the variable bioavailability of rifampicin from FDC products (Pelizza et al., 1977; Henwood et al., 2000; Henwood et al., 2001; Agrawal et al., 2004). The rifampicin is an important component in anti-TB therapy, thus using FDC tablets with poor rifampicin bioavailability could lead directly to treatment failure and may encourage drug resistance. Furthermore, clinical and bacteriological investigations have revealed that the anti-bacterial activity of rifampicin is dose-dependent (Gumbo et al., 2007). Therefore, a good quality FDC tablet with demonstrated bioavailability of rifampicin is an absolute requirement for successful TB treatment.

b) Second-line treatment

MDR-TB has become increasingly prevalent, and the XDR-TB form is emerging (Gandhi et al., 2010). Combinations of first-line and second-line drugs are used for the treatment of MDR-TB and XDR-TB according to results of drug susceptibility testing. Second-line drugs include aminoglycosides (kanamycin and amikacin), cycloserine, terizidone, ethionamide, protionamide, capreomycin, aminosalicylic acid, and fluoroquinolones (including ofloxacin, levofloxacin, gatifloxacin, and moxifloxacin). Treatment regimens for MDR-TB and XDR-TB are longer, less effective, less tolerable, and more expensive than is standardised short-course chemotherapy and include the use of injectable drugs. The percentage of patients with MDR-TB who are cured is estimated to be no more than 69% on the

basis of results from retrospective cohort studies, even when treated for more than 18 months with directly observed treatment (Orenstein et al., 2009). Toxicity frequently leads to drug discontinuation (Prasad et al., 2006) and mortality in patients with HIV infection is particularly high (Gandhi et al., 2006; Kliiman et al., 2009; O'Donnell et al., 2009). In 2008, only about 1% of cases of MDR-TB were estimated to have received proper treatment according to WHO's recommended standards (WHO, 2009). Containment of the spread of the MDR-TB and XDR-TB will be extremely difficult without treatment regimens that are shorter, safer, more effective, and less expensive than are those that are available. New drugs with novel mechanisms of action are needed for the effective management of MDR-TB and XDR-TB.

c) Third-line treatment

These drugs may be considered "third-line drugs" and are listed here either because they are not very effective (e.g., clarithromycin) or because their efficacy has not been proven (e.g., linezolid). Rifabutin is effective, but is not included on the WHO list because for most developing countries, it is impractically expensive (Anon, 2012).

Table 2.1 Anti-TB drugs

Anti-TB drugs		
First-line	**Second-line**	**Third-line**
Ethambutol	-Aminoglycosides e.g., Amikacin, Kanamycin	Rifabutin
Isoniazide	-Polypeptides e.g., Capreomycin, Viomycin	Linezolid
PZA	-Fluoroquinolones e.g., Ciprofloxacin,	Clarithromycin
Rifampicin	Levofloxacin, Moxifloxacin	Thioacetazone
	-Thioamides e.g., Ethionamide, Prothionamide	Thioridazine

2.2.2 Anti-tuberculosis inhaled therapy

The pulmonary route of drug administration delivers adequate therapeutic levels of potent bronchodilators in respiratory tract and provides a better clinical response whilst reducing their distribution to other organs. This route provides an excellent example of targeted drug therapy. Indeed, aerosol delivery has long been viewed as a promising technique in the treatment of lung cancer (Koshkina et al., 2004). The lung has a large surface area (up to 100 m^2), thin absorptive barrier, low enzymatic metabolic activity, and good blood supply. These characteristics make the lung an attractive target for the non-invasive administration of aerosolized systemically-active peptide and protein drugs. The pulmonary delivery route as well as nasal, rectal, and oral routes, have attracted much attention, in attempts to improve the quality of patient lives, because no repeated injections are required (Kawashima et al., 1999). Contrary to the oral route of drug administration, pulmonary inhalation is not subject to first pass metabolism. Therefore, expensive biotechnology drugs like

toxic chemotherapeutics are ideal drug candidates for local pulmonary administration (Sharma et al., 2001). The main advantage of the treatment of the respiratory tract diseases via inhalation aerosols therapy is that a relatively low drug concentration reaches systemic circulation. Consequently, the intensity and incidence of the side effects of inhaled drugs is, in many instances, markedly reduced compared to administration via the oral route. Several drugs such as bronchodilators, anti-inflammatory agents, mucolytics, antiviral agents, antibiotics agents, and pentamidine are all routinely given as aerosolized formulations. In addition, a number of drugs for example insulin, cyclosporin, interferon, antitrypsin, protease inhibitors, deoxyribonucleases, recombinant adenoviruses and others have been reported to have high potential for delivery via the respiratory route (Karathanasis et al., 2005).

A Greek pharmacist, Pedanus Discorides, introduced the concept of inhaled fumigation during the first century. Antiseptic aerosol therapy, e.g. boiling tar vapours, became a popular antitubercular medication in the middle of the 20th century, although it hardly had any therapeutic value. Since then, antitubercular inhaled therapy has come a long way to a stage of experimental reality with potential clinical applications. The importance of the subject stems from the fact that TB continues to be a leading killer disease causing 3 million deaths annually and has emerged as an occupational disease in the health care system.

Oral therapy using the currently employed antitubercular drugs is very effective, but is still associated with a number of significant drawbacks. More than 80% of TB cases are of pulmonary TB alone and high drug doses are required to be administered because only a small fraction of the total dose reaches the lungs after

oral administration. Even this small fraction is cleared in a matter of a few hours thus explaining the necessity to administer multiple antitubercular drugs on a regular basis, a regimen which the majority of TB patients find difficult to adhere to. Clearly, antitubercular drugs delivery systems which can be administered via the pulmonary route and can avoid the daily dosing, would be a vast improvement because they would help in: (i) direct drug delivery to the diseased organ; (ii) targeting to AMs which are used by the mycobacteria as a safe site for their prolonged survival; (iii) reduced systemic toxicity of the drugs; and (iv) improved patient compliance (Pandey and Khuller, 2005).

The lung is the primary portal of entry for mycobacteria that cause pulmonary TB. It has therefore been of interest to deliver drugs used in the management of TB through the same route. In recent years, there is renewed interest in formulating drugs for pulmonary delivery for reasons that remain significant. First, the lung mucosa represents a large surface from which drugs may be systemically absorbed into the bloodstream, without having to undergo first-pass metabolism (Mathias and Hussain, 2010). During the process of systemic absorption from the lung, drugs introduced are likely to provide early and high concentrations within it. This is advantageous if, as in pulmonary TB, the lungs are the intended target site of drug delivery. Second, lung macrophages are efficient at fulfilling their evolutionary role of phagocytosing material entering the lungs. It has long been established that macromolecular drugs (Lombry et al., 2004) and particulate (Moller et al., 2001; Yadav et al., 2009) or vesicular (Myers et al., 1993) drug delivery systems introduced into the deep lung are likely to be taken up by AMs. Finally, it has been argued that uptake of drug delivery

systems by infected macrophages effects rescue of the macrophage from ‘alternative activation, enabling the elaboration of innate bactericidal responses that could help in killing or containing TB bacilli (Yadav et al., 2009; Kahnert et al., 2006; Sharma et al., 2007).

To date, no aerosol products containing anti-TB drugs have been developed yet. However, several current studies are under investigation for administration of anti-TB drugs to the primary infection site, the lung, with the idea of increasing the local therapeutic effect and reducing overall systemic exposure (Ohara and Hickey, 2000; Suarez et al., 2001(a); Suarez et al., 2001(b); Sharma et al., 2001; Vyas et al., 2004; Tewes et al., 2008; Sung et al., 2009). Specifically, pulmonary delivery of rifampicin has been widely studied (Ohara and Hickey, 2000; Suarez et al., 2001(a); Suarez et al., 2001(b); Vyas et al., 2004; Ito and Makino, 2004; Sung et al., 2009) as it is the first drug of choice in the treatment of TB (Becker et al., 2009). Furthermore, pulmonary delivery bypasses drawbacks seen in current oral delivery systems, specifically, delivery of rifampicin to the neutral milieu of the lung avoids severe chemical decomposition of rifampicin in the acidic conditions of the stomach when taken orally (Shishoo et al., 1999; Singh et al., 2001; Sankar et al., 2003). Several respirable forms of rifampicin, such as nano/microparticles (Ohara and Hickey, 2000; Suarez et al., 2001(b)), liposome (Vyas et al., 2004; Sung et al., 2009) and liquid (Tewes et al., 2008) have been introduced to localize the rifampicin in the lung.

Recently, there has been an increased interest in research focusing on dry powder inhaler formulations to prolong drug residence time in the lung (Ohara and Hickey, 2000; Cook et al., 2005; Learoyd et al., 2008; Salama et al., 2008; Sung et al.,

2009). Generally, the phagocytic uptake of inhaled particles by AMs has been known to be the primary hurdle in extending drug residence time in the lung (Hardy and Chadwick, 2000; Salama et al., 2008). However, in pulmonary anti-TB therapy, the phagocytic activities play a useful role in terms of targeting anti-TB drugs at the main site of the *M. tuberculosis* proliferation (Hirota et al., 2007; Onoshita et al., 2010). Several formulations that encapsulate anti-TB drugs into the polymeric microspheres have been introduced to target the AMs (Ohara and Hickey, 2000; Muttil et al., 2007; Mizoe et al., 2008; Sung et al., 2009). Respirable poly(lactide-co-glycolide) (PLGA)/ polylactic acid (PLA) microspheres showing up to 3 days of *in vitro* drug release capability were manufactured by means of a solvent evaporation and a spray drying method (Ohara and Hickey, 2000; Hirota et al., 2007; Onoshita et al., 2010). A formulation consisting of PLGA nanoparticle aggregates was also introduced by Sung and co-workers (2009). However, there are still numerous limitations in manufacturing the dry powder forms to have good aerosolization properties with controlled drug release capabilities due to their fine particle size.

Another issue in delivering the dry powder form of anti-TB antibiotics to the lung is that there is no dry powder inhaler device specifically designed to carry a large amount of antibiotic formulation. Currently available devices are all designed for delivering small amounts (microgram dose) of bronchodilators or steroids (Son and McConville, 2008). Only single-dose capsule devices have a capability to hold the substantial amount of (milligram dose) formulations among the current devices. However, those devices are relatively old type and show poor aerosolization properties compared to novel active devices. Thus, the development of formulations

that show a low device dependency and good aerosolization performances is required to deliver a maximum potency formulation of the antibiotic directly to the lung.

2.3 Pyrazinamide

2.3.1 Pyrazinamide properties

PZA or pyrazinecarboxamide (Figure 2.4), an important frontline TB drug, plays a key role in shortening TB therapy from 9 to 12 months to the current 6 months (Zhang and Mitchison, 2003). The ability of PZA to shorten the TB therapy is related to its activity against a population of non-growing, persisted tubercle bacilli residing in an acid pH environment that are not killed by other TB drugs (Zhang and Mitchison, 2003). Its solubility is 15 mg/mL in water, 5.7 mg/mL in absolute ethanol and 3.8 mg/mL in isopropanol (Merck, 1997). PZA is an extremely weak base. A pKa value of 0.5 and log P of -0.68 have been reported (Kasim et al., 2004; Becker et al., 2008). The mechanism of action of PZA is poorly understood: pyrazinoic acid (POA), the active moiety of PZA, has been shown to inhibit various functions at acid pH in *M. tuberculosis* (Boshoff et al., 2002; Zhang et al., 2003). The hypothesis for the mode of action of PZA according to Zhang and co-workers (2003) is shown in Figure 2.5. Experimental evidence suggests that PZA diffuses into *M. tuberculosis* and is converted into POA by pyrazinamidase (PZAase); the *in vitro* susceptibility of a given strain of the organism corresponds to its PZAase activity. PZAase is also called nicotinamidase and metabolizes both PZA and nicotinamide. Once converted, a portion of the POA exits the cell and, providing the media pH is acidic, it re-enters as

protonated POA, which may help to disrupt membrane potential (Zhang and Mitchison, 2003). An inefficient efflux system causes protonated POA to diffuse in at a faster rate than the efflux of POA. In fact, resperine an inhibitor of a multidrug resistant efflux pump can sensitize the cells to PZA (Zhang et al., 2003). The accumulation of POA and protonated POA lowers the intracellular pH to a suboptimal level that may inactivate many pathways including fatty acid synthase and membrane transport function. POA was thought to inhibit the enzyme fatty acid synthase (FAS) I, which is required by the bacterium to synthesized fatty acids (Zimhony et al., 2000) although this has been discounted (Boshoff et al., 2002). It was also suggested that the accumulation of pyrazinoic acid disrupts membrane potential and interferes with energy production, necessary for survival of *M. tuberculosis* at an acidic site of infection. The studies reproduced the results of FAS I inhibition as the putative mechanism first in whole cell assay of replicating *M. tuberculosis* bacilli which have shown that pyrazinoic acid and its ester inhibit the synthesis of fatty acids (Zimhony et al., 2007). That study was followed by *in vitro* assay of tuberculous FAS I enzyme that tested the activity with PZA, pyrazinoic acid and several classes of PZA analogs. PZA and its analogs inhibited the activity of purified FAS I. Pyrazinoic acid binds to the ribosomal protein S1 (RpsA) and inhibits translation. This may explain the ability of the drug to kill dormant mycobacteria. However it is widely accepted that POA may not have a specific target, but rather that cellular acidification causes inhibition of major processes (Zhang and Mitchison, 2003). Weak acids, UV and respiratory chain inhibitors (e.g. sodium azide) enhance the action of PZA (Wade and Zhang, 2004; Wade and Zhang, 2006). Continuing studies on individual targets such as the

nicotinic acid pathway may lead to alternate mechanisms. No target-specific mutants have been isolated to date. PZA-resistant mutations are usually found in the converting enzyme PZAase (Davies et al., 2000). The mutations are unusually located, spread throughout the gene, but there are three areas of clustered mutations around amino acids 3-71, 61-85 and 132-142 (Scorpio et al., 1997). A crystal structure of PZAase is now available from *Pyrococcus horikoshii* and, although it only shares 37% identity with the *M. tuberculosis* enzyme, it may help in understanding the PZAase mutations in *M. tuberculosis* (Du et al., 2001).

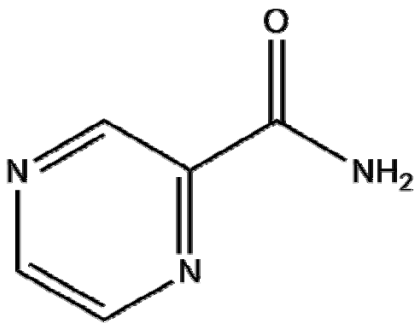


Figure 2.4 PZA structure

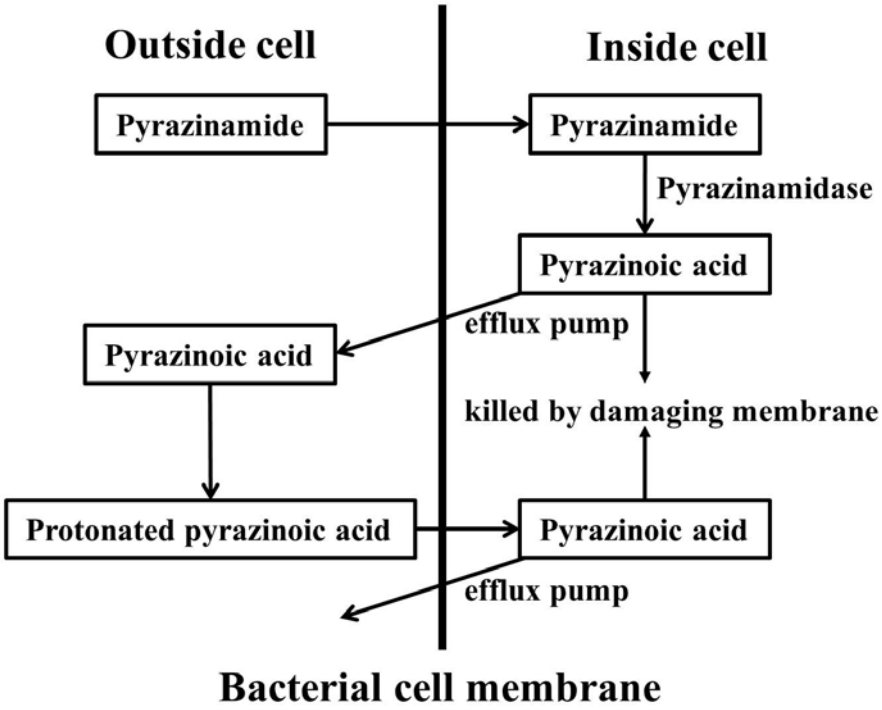


Figure 2.5 The hypothesis for the mode of action of PZA (Zhang et al., 2003)

Minimum inhibitory concentrations (MICs) of PZA against *M. tuberculosis* are reported as 6-50 µg/mL at pH 5.5 (Zhang and Mitchison 2003) but >2000 µg/ml for *E. coli* and *M. smegmatis* at the same pH. Careful monitoring is required during MIC measurements as bacterial density and serum albumin can affect results (Zhang and Mitchison, 2003; Salfinger and Heifets, 1988). Specifically, MIC_{90} at pH 5.5 is 50 µg/mL, at pH 5.8 is 100 µg/mL, at pH 5.95 is 200 µg/mL (Salfinger and Heifets, 1988). PZA is presumed to be specific for *Mycobacterium* species, exerting its antibacterial properties under specific conditions (acidic pH). *M. bovis* and *M. leprae* are innately resistant to PZA. PZAase is widely distributed in bacteria yet efficacy of PZA is limited to *M. tuberculosis* and few other organisms. All bovine mycobacterial strains lack PZAase activity due to a point mutation in the *pncA* gene. *M. smegmatis*

is also PZA resistant probably due to a very efficient efflux system which does not allow POA to accumulate within the cell (Zhang and Mitchison, 2003). Anaerobic conditions enhanced the activity of PZA (Wade and Zhang, 2004). Older cultures of *M. tuberculosis* appear to be somewhat more sensitive to PZA (Hu et al., 2006) and have a weaker membrane potential compared to fresh cultures (Zhang et al., 2003). Under some conditions bactericidal activity is inversely proportional to [3H] uridine uptake, reflecting the activity of PZA against persistent bacilli *in vivo* (Hu et al., 2006). An elegant experiment showed PZA activity against *E. coli* persistors following treatment with ampicillin (Wade and Zhang, 2006). PZA activity against *M. tuberculosis* in macrophages is controversial although many authors reported some activity (Zhang and Mitchison, 2003). PZA has been shown to be effective in whole blood assays where blood harvested from a drug-treated donor was used as a medium in which to grow the bacilli (Wallis et al., 2001).

2.3.2 Pyrazinamide inhaled therapy

PZA inhaled formulations in previous studies were administered using nebulizer (Pandey et al., 2003; Zahoor et al., 2005). Rifampicin, isoniazid and PZA encapsulated in biodegradable PLGA nanoparticles were administered to the lungs of guinea pigs using nebulization (Pandey et al., 2003). They showed that the aerosolized formulation maintained therapeutic drug concentrations in the lungs for up to 11 days. Zahoor and co-workers (2005) performed similar aerosolisation studies in *M. tuberculosis* infected guinea pigs using isoniazid, rifampicin and PZA-encapsulated alginate-based nanoparticles. Interestingly, the efficacy of drug-loaded

alginate nanoparticles nebulised every 15 days until 45 days was demonstrated to be comparable to 45 daily oral doses of free drugs (Zahoor et al., 2005).

Although, many researchers developed PZA inhaled formulations, no PZA formulation for inhalation is available. From previous studies, PZA was administered to the lung using nebulizer (Pandey et al., 2003; Zahoor et al., 2005).

2.4 Levofloxacin

2.4.1 Levofloxacin properties

Levofloxacin (LEV) is the S-(-) form of ofloxacin and it is an active antimicrobial agent (Figure 2.6). It is substantially more active than ofloxacin (8 to 125 times depending on the bacterial species) (Okeri and Arhewoh, 2008). It is freely soluble in glacial acetic acid and sparingly soluble in water (Merck, 1997). LEV is a zwitterion at physiological pH, possessing a carboxylic group with p*K*a = 5.5, a piperazinyl group with p*K*a = 8.0 and another proton accepting function with p*K*a = 6.8. Log P value of 1.49 has been reported (Koeppe et al., 2011). LEV is described as a second-generation quinolone along with ciprofloxacin and ofloxacin.

MICs of LEV against *M. tuberculosis* H37Rv were reported to be 0.5 µg/mL (Rastogi et al., 1996) and 0.2 µg/mL (Drlica et al., 2003).

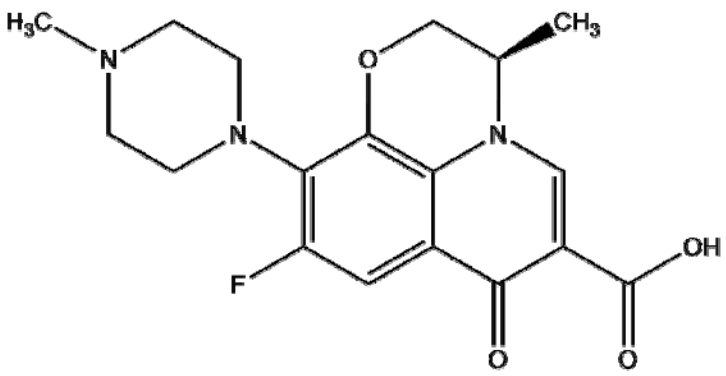


Figure 2.6 Levofloxacin structure

2.4.2 Levofloxacin inhaled therapy

LEV inhalation solution (MP-376) is a novel formulation of LEV that is currently being evaluated in clinical trials and that may be safely and rapidly administered by the aerosol route using nebulizer. Following MP-376 administration, high LEV concentrations were achieved in the sputum of cystic fibrosis patients, resulting in the high PK-PD exposures associated with bactericidal activity and a reduced possibility for bacterial resistance (Geller et al., 2002; Drusano, 2004; Geller et al., 2008; King et al., 2010).

CHAPTER 3

VALIDATION OF HPLC METHOD FOR DETERMINATION OF PYRAZINAMIDE AND LEVOFLOXACIN

3.1 Introduction

Validation of analytical method for pyrazinamide (PZA) and levofloxacin (LEV) determination was performed to ensure that the method is precise and reliable. High performance liquid chromatography (HPLC) was employed as a tool for drug analysis in this study.

3.2 Materials

Acetonitrile (Lab-scan Analytical Sciences, Bangkok, Thailand)

Methanol (RCI Lab-scan Limited, Bangkok, Thailand)

Polyamide membrane 0.45 μm (Sartorius Stedim, Goettingen, Germany)

Potassium dihydrogen orthophosphate (Ajax Finechem Pty Ltd, NSW, Australia)

Pyrazinamide (PZA) (Aldrich, USA)

Levofloxacin (LEV) (Sigma, Germany)

3.3 Equipment

Chromatographic experiments were performed using HPLC system consisting of a Spectra System SCM 1000 and Spectra System Pump P2000 with Spectra System AS3000 autosampler equipped with a Spectra System UV1000 detector (Thermo Scientific, United Kingdom). Luna 5 µm C18 column (Phenomenex, USA) (150 x 4.6 mm id, 5 µm) was used in this study.

3.4 Chromatographic Conditions

The mobile phases consisted of 0.2 M potassium phosphate and acetonitrile (97:3 v/v for PZA and 85:15 v/v for LEV). These mobile phases were set at a flow rate of 1 ml/minute at ambient temperature. The UV detector was operated at 254 and 288 nm for PZA and LEV, respectively. The injection volume was 50 µl.

3.5 Methods

3.5.1 Preparation of stock solution and standard solution

The stock solution was prepared. Ten mg of PZA or LEV was weighed and dissolved in 200 ml of methanol. This solution was diluted with mobile phase as needed to prepare various concentrations of standard solution.

3.5.2 Determination of linearity

The standard solutions including 0.5, 1, 3, 5 and 10 µg/ml of PZA and 0.5, 1, 5, 10 and 20 µg/ml of LEV were prepared. They were analyzed for the concentration of PZA or LEV by injection each standard solution into HPLC system in triplicates. Peak areas were plotted against theoretical concentrations of standard. The linearity was expressed as a correlation coefficient (r^2).

3.5.3 Determination of accuracy

The intra-day accuracy was performed by injection five standard solutions for five times on the same day, and inter-day accuracy was obtained from the injection of each standard solution on five consecutive days. The accuracy of an analysis was determined by comparing the amount of drug measured by this analytical method and the theoretical amount. The data were presented as a percentage recovery.

3.5.4 Determination of precision

The intra-day precision was determined by performing five repeated analyzes of five standard solutions on the same day. The inter-day precision was received from an analysis of the same set of standard solution for five consecutive days. The system precision was expressed as percentage relative standard deviation (% RSD). The results must be less than 2%.

3.5.5 Determination of limit of detection and quantitation

The limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The limit of quantitation (LOQ) is the lowest amount of analyte which can be quantitatively determined with suitable precision.

In this study the LOD and LOQ were determined by using an approach based on the standard deviation of the response and the slope. The LOD and LOQ may be expressed as equation (1) and (2), respectively.

$$LOD = 3.3\sigma / S \quad (1)$$

$$LOQ = 10\sigma / S \quad (2)$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve.

To obtain the LOD and LOQ the series of standard solution of PZA ranged 0.5-10 µg/ml and LEV ranged 0.5-20 µg/ml were injected into HPLC system, in triplicates of each concentration. The calibration curve was obtained. The S was obtained from the slope of regression line, and the σ was the standard deviation of y-intercepts of regression lines (ICH, 2005).

3.6 Results and discussion

3.6.1 Linearity

Figure 3.1 shows the linear regression analysis of standard curve. It was observed that standard curves were linear over the investigated range (0.5-10 µg/ml

for PZA and 0.5-20 μg/ml for LEV) with the correlation coefficient (r^2) over 0.9999. Therefore, these ranges of concentration were suitable for preparing the standard solution and sample for analysis.

3.6.2 Accuracy and precision

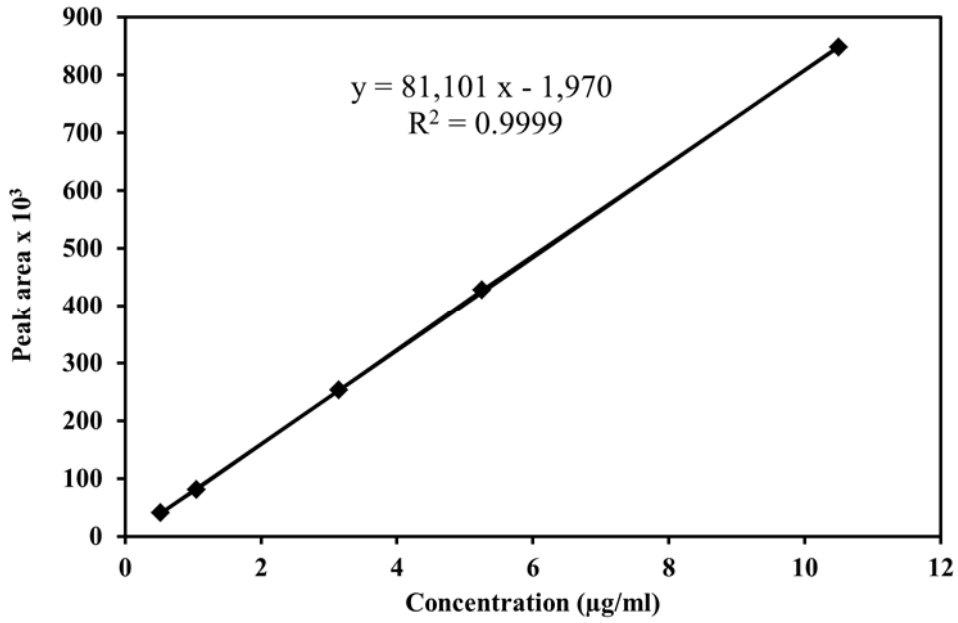
The results of accuracy and system precision tests performed on each concentration of PZA and LEV are shown in Table 3.1 and 3.2, respectively. The accuracy and precision were presented as % recovery and % RSD, respectively.

For accuracy, both intra- and inter-day run gave the values of % recovery varied between 99-100% which were 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- and inter-day run (ICH, 2005).

3.6.3 Limit of detection and quantitation

Under the HPLC conditions, the limit of detection of PZA and LEV were found to be 13 and 16 ng/ml and the limit of quantitation were determined to be 41 and 48 ng/ml, respectively.

(a)



(b)

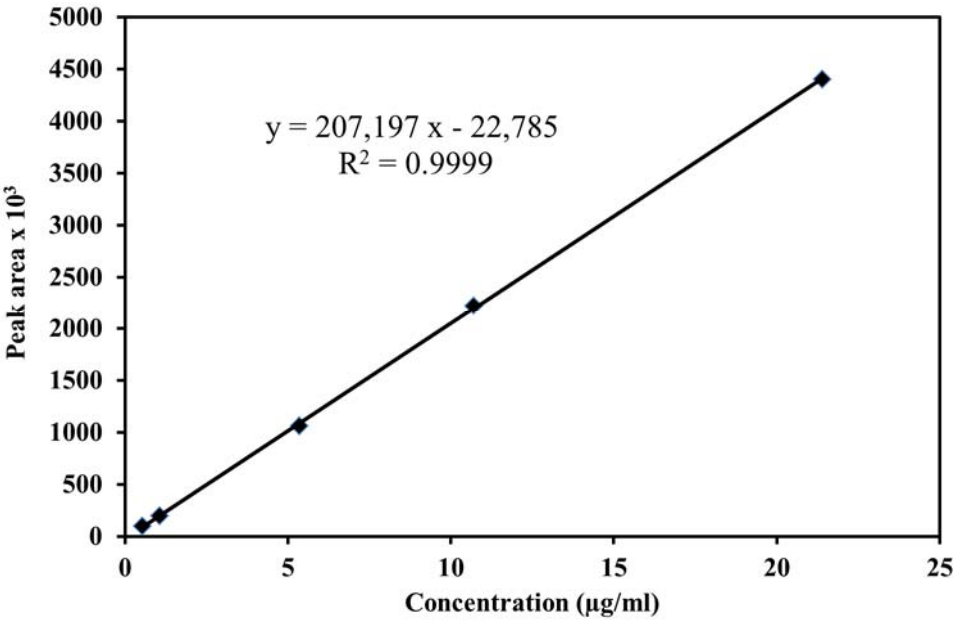


Figure 3.1 Standard curve of (a) PZA and (b) LEV (mean ± SD, n=5)

Table 3.1 Percent recovery and RSD for accuracy and precision of PZA (n=5)

Concentration	Accuracy (%Recovery ± SD)		Precision (%RSD)	
(μg/ml)	Intra-day	Inter-day	Intra-day	Inter-day
0.53	99.8 ± 1.5	99.7 ± 1.0	1.52	0.98
1.05	99.1 ± 0.9	99.3 ± 1.5	0.94	1.53
3.15	99.7 ± 0.7	99.3 ± 0.9	0.68	0.92
5.25	100.7 ± 1.5	100.9 ± 1.3	1.49	1.31
10.5	99.9 ± 0.8	99.8 ± 0.6	0.80	0.59

Table 3.2 Percent recovery and RSD for accuracy and precision of LEV (n=5)

Concentration	Accuracy (%Recovery ± SD)		Precision (%RSD)	
(μg/ml)	Intra-day	Inter-day	Intra-day	Inter-day
0.54	100.3 ± 0.8	99.7 ± 1.1	1.01	1.73
1.07	100.5 ± 1.1	100.2 ± 0.8	1.29	0.96
5.35	99.1 ± 0.9	100.9 ± 1.4	0.93	1.49
10.7	100.5 ± 1.9	99.3 ± 0.7	1.96	0.69
21.4	99.9 ± 0.7	100.1 ± 0.6	0.70	0.64

3.7 Conclusion

In summary, the established analytical methods are rapid, sensitive, reliable, and reproducible. The proposed HPLC methods allowed successful determination of PZA and LEV with the total time of the analysis not more than 15 minutes. The RSD values indicated a good precision and accuracy of the method. The presented methods are selective, sensitive, and reproducible with good recovery values and therefore can be used for simultaneous quantification of PZA and LEV in different pharmaceutical formulations.

CHAPTER 4

PREPARATION AND CHARACTERIZATION OF POROUS MANNITOL MICROPARTICLES FOR PROLIPOSOME CORE CARRIER

4.1 Introduction

Lung becomes interesting organ for local and systemic drug delivery because it has a lot of absorption area and could reduce first pass effect and administration dose. Treating with inhalers requires delivering sufficient drug to the lungs for therapeutic response. The design of dry powder inhaler (DPI) formulation is a strategy to achieve the delivery goals. One factor that affects the properties of DPI formulation is the carrier. Lactose monohydrate is a carrier of choice for DPI. The advantages of lactose monohydrate are well-investigated with broad availability and low price. Their surface morphology was desirable to improve inhalation property of DPI (Kawashima et al., 1998). Several other compounds such as mannitol (Tee et al., 2000), sucrose and sorbitol (Tang et al., 2006), glucose (Steckel and Bolzen, 2004) and more recently cyclodextrin, raffinose, trehalose and xylitol (Hooton et al., 2006) have been suggested as possible alternatives to lactose. Alternatives are required if the drug is chemically incompatible (reacts) with lactose. In previous study, alternative carriers like mannitol, glucose, sorbitol, maltitol and xylitol had been evaluated for their potential use in DPI formulations. They were mixed with budesonide and tested for

their aerodynamic property. Mannitol showed potential as a drug carrier to be used in DPI formulations (Steckel and Bolzen, 2004).

Porous microparticles are potential candidate for pulmonary drug delivery. Pore forming agent was employed to obtain porous microparticles in this study. Bernstein and co-workers (2003) suggested that pore forming agents can be added in range of 0.01-90% w/v, to increase matrix porosity and pore formation during the production. The pore forming agent can be added as solid particles to the polymer solution or melted polymer or added as aqueous solution. Bendroflumethiazide was prepared successfully in porous microparticles by spray drying with ammonium carbonate (Healy et al., 2008). Nolan and co-workers (2009) prepared nanoporous microparticles of budesonide using ammonium carbonate. Both studies demonstrated that *in vitro* deposition properties were improved compared to non-porous particles, so it has potential use for drug delivery by inhalation.

Mannitol, alcohol sugar, becomes interesting DPI carrier. The aim of this study was to prepare porous mannitol for DPI carrier. Morphology, bulk density and porosity of raw material, porous mannitol generated by ammonium acetate or ammonium carbonate at different weight ratios was assessed. Porous mannitol with high porosity was determined in ammonium residue using energy dispersive X-ray spectrometry analysis.

4.2 Materials

Mannitol, L-α soybean phosphatidylcholine (SPC), and cholesterol from lanolin (CH) were obtained from Fluka (Buchs, Switzerland). Ammonium acetate and ammonium carbonate were purchased from Ajax Finechem (NSW, Australia). Pyrazinamide (PZA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were analytical grade.

4.3 Methods

4.3.1 Production of porous microparticulate mannitol

Mannitol with either ammonium acetate or ammonium carbonate was dissolved in distilled water. The total solid concentration in solution was 10 mg/mL. Pore forming agent was added in different percent of the total weight of solids as shown in Table 4.1. The solution was sprayed through a 1.5 mm nozzle using a mini spray dryer B-290 (Büchi, Flawil, Switzerland) under an inlet temperature of 110°C, an atomizing pressure of 800 kPa, and a feeding rate of 3 ml/minute. The product was separated and collected by the cyclone and then directed into the collecting chamber. The microparticles of mannitol were obtained.

Table 4.1 Composition of non-porous and porous mannitol preformed microparticles

Formulation	Mannitol (% w/v)	Ammonium acetate (% w/v)	Ammonium carbonate (% w/v)
Non-porous mannitol	10	-	-
AA1	10	1	-
AA2	10	2	-
AC1	10	-	1
AC2	10	-	2
AC4	10	-	4
AC6	10	-	6

4.3.2 Morphology of the microparticles

An adhesive tape was stuck on an aluminum stub. A sample was sprinkled on the adhesive tape and coated with gold by a sputtering technique using a Sputter Coater (SPI supply, USA) for 120 s. The surface morphology of the microparticles was examined by scanning electron microscopy (SEM) (FEI Quanta 400 FEG, FEI Company, USA) at 20 kV.

4.3.3 Bulk density and % porosity

Bulk density was determined by filling the particles in a 10 ml graduated cylinder with a funnel until the particles reached 5 ml (bulk volume). The weight of the particle was recorded to calculate bulk density. Porosity (%) was then measured using liquid displacement method (Martin, 1993). Absolute ethanol (5 ml) was poured

into the cylinder and sonicated until there was no air bubble. The volume over 5 ml was recorded (true volume). Porosity was calculated following the equation:

$$\text{Percent porosity} = \frac{\text{Bulk voume - True volume}}{\text{Bulk voume}} \times 100$$

Each measurement was performed in triplicate.

4.3.4 Ammonium residue

Energy-dispersive X-ray spectroscopy (EDX) was used to investigate the ammonium residue in the porous mannitol. An adhesive tape was stuck on an aluminum stub. The particles were loaded on the adhesive tape and evaluated under SEM-EDX. The analysis was performed qualitatively.

4.3.5 Statistical analysis

Data, when applicable, are 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) with Tukey's multiple group comparison procedure. All statistical comparisons were calculated using SPSS software version 17.0 (SPSS Inc., Chicago, IL). A p-value < 0.05 was considered statistically significant.

4.4 Results and discussion

4.4.1 Morphology of the microparticles

Microparticles of non-porous and porous mannitol were successfully prepared in this study. They exhibited smooth surface and spherical particles with 3-5 μm in size (Figure 4.1). For spray drying, it is usual to pump solution or suspension to the atomizing device where small droplets are produced. These droplets suspending in the drying air lose their moisture very rapidly when they meet a stream of hot air. The dry particles are separated from the moist air in cyclones by centrifugal action. The dense powder particles are forced toward the cyclone walls while the moist air is directed away through the exhaust pipes. The spherical particles are produced when they cash the cyclone walls. The powder then settles to the bottom of the cyclone.

Porous mannitol microparticles were obtained by adding pore forming agents into the solution prior spray-drying. In this study, ammonium acetate and ammonium carbonate were compared. From Figure 4.1a-4.1f, porous mannitol generated by either ammonium acetate or ammonium carbonate had the macropores, micropores and nanopores. At the same ratio of pore forming agent, there were different observed pores. AC2 was observed to have large hollow, while AA2 had smaller pores (Figure 4.1b and 4.1d). Hollow particles were clearly observed when increased in amount of pore forming agent (Figure 4.1c-4.1f). It was the result from adding pore forming agents which was volatilized. Nolan and co-workers (2009) found that a spray-drying process could be employed to produce nanoporous budesonide microparticles. The adding of ammonium carbonate was necessary to obtain porous particles in

methanol/water solvent condition. The density of porous particles was significantly lower and the surface area significantly higher than non-porous spray dried. The porous micropartcles were improved *in vitro* deposition property.

4.4.2 Bulk density and % porosity

In spray-drying process, the inlet temperature was 110°C while the outlet temperature was recorded. It was in range of 78-84°C that different from the inlet temperature about 30°C. Porous mannitol generated by ammonium acetate gave higher % yield (78-80%) than porous mannitol generated by ammonium carbonate (73-76%) (Table 4.2). This may due to the fact that porous mannitol generated by ammonium carbonate was lost during spray drying process. The fine particles were blown partially to the outlet filter instead of collecting chamber.

The bulk density and % porosity of mannitol microparticles are shown in Table 4.2. Non-porous mannitol displayed the highest bulk density (0.53 ± 0.02 g/ml) and the lowest % porosity (62.7 ± 2.3%) (p<0.05). AC1, AC2 and AC4 gave the lowest bulk density between groups (0.21 ± 0.01, 0.22 ± 0.02 and 0.22 ± 0.01 g/ml) (p<0.05). Only AC2 had the highest % porosity (88.0 ± 4.0%) (p<0.05). It was found that pore forming agent was added to increase pore formation during the process. First, pore forming agent and mannitol was dissolved in water. This solution was then spray dried. In spray drying process (inlet temperature = 110°C), water was evaporated and pore forming agent, volatile salt, was decomposed by volatilization when heated. The volatilization of pore forming agent enhanced pore formation in the particles. Ammonium acetate melts and decomposes at 114 °C while ammonium

carbonate melts and decomposes at 58 °C (McGraw, 1993). Both pore forming agents decomposed when they were heated as shown in equations (1) and (2). However, in this case ammonium carbonate decomposed much easier than ammonium acetate and the decomposition yielded ammonia, carbon dioxide and water.

$$CH_3COONH_4 \xrightarrow{\Delta} CH_3COOH + NH_3 \quad (1)$$

$$(NH_4)_2CO_3 \xrightarrow{\Delta} 2NH_3 + CO_2 + H_2O \quad (2)$$

Their stabilities vary according to the nature of the acid. Salts of weak acids decompose at lower temperatures than salts of strong acids (McGraw, 1993). Porous mannitol generated by ammonium carbonate had higher porosity than that of ammonium acetate because ammonium carbonate decomposed at low temperature. It could be volatilized easier than ammonium acetate when heated.

4.4.3 Ammonium residue

From the structure of mannitol, it contains carbon, hydrogen and oxygen, so the EDX image of mannitol shows only carbon and oxygen. Ammonium residue could be detected as nitrogen presenting in EDX of sample containing ammonium. From Figure 4.2, there was no nitrogen in AC2, AC4 and AC6. This confirmed the ammonium carbonate was completely removed during the spray-drying process.

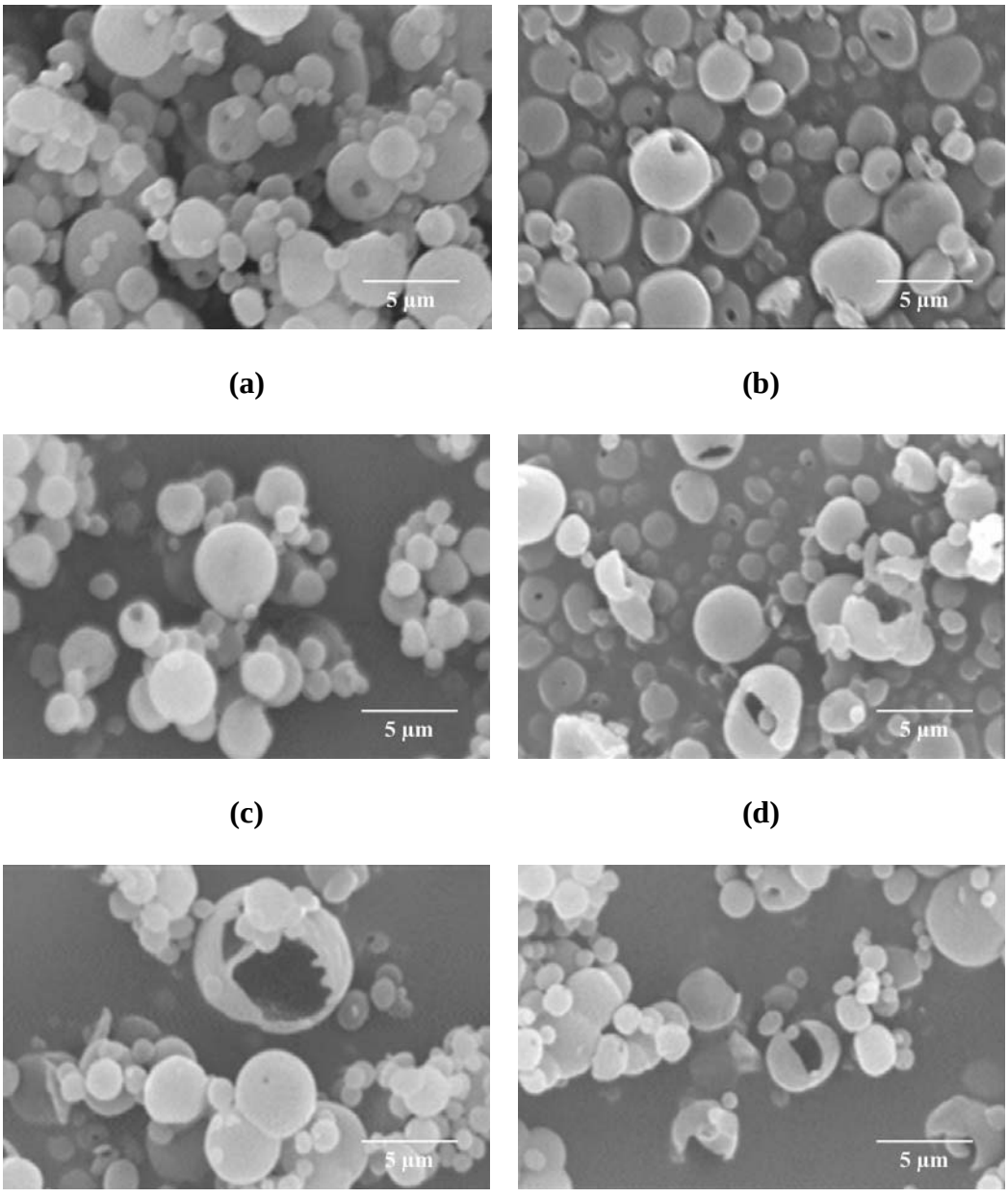


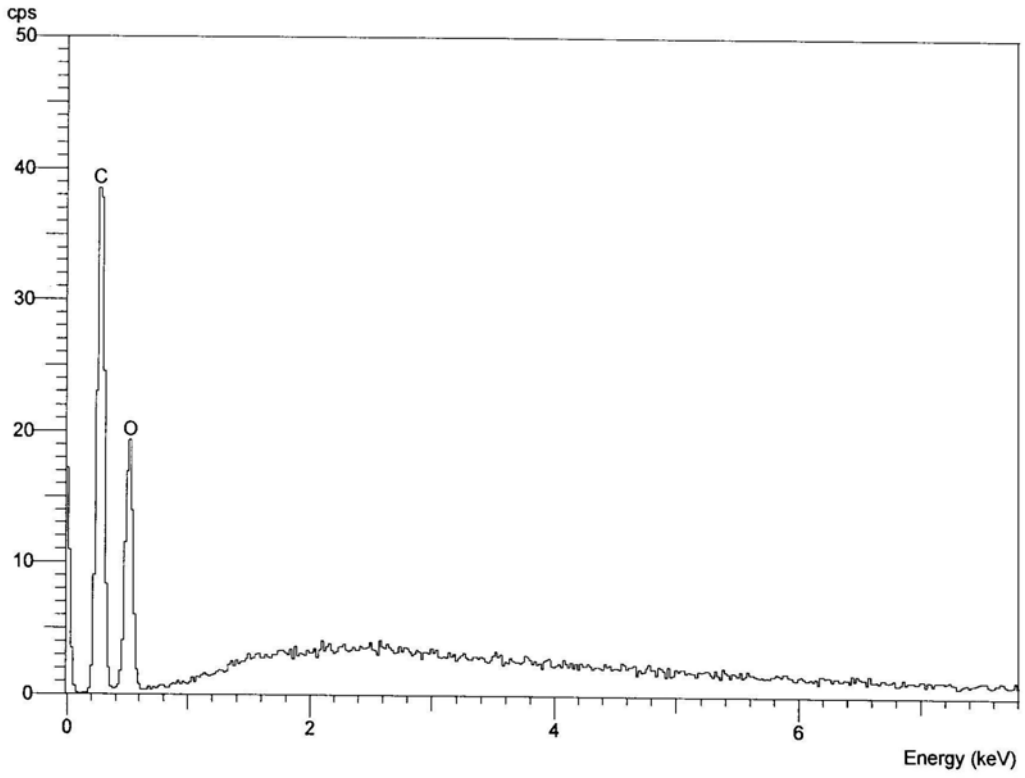
Figure 4.1 The SEM images of porous mannitol: AA1 (a), AA2 (b), AC1 (c), AC2 (d), AC4 (e) and AC6 (f) (bar=5 µm)

Table 4.2 Bulk density and % porosity of non-porous and porous mannitol microparticles (mean ± SD, n=3)

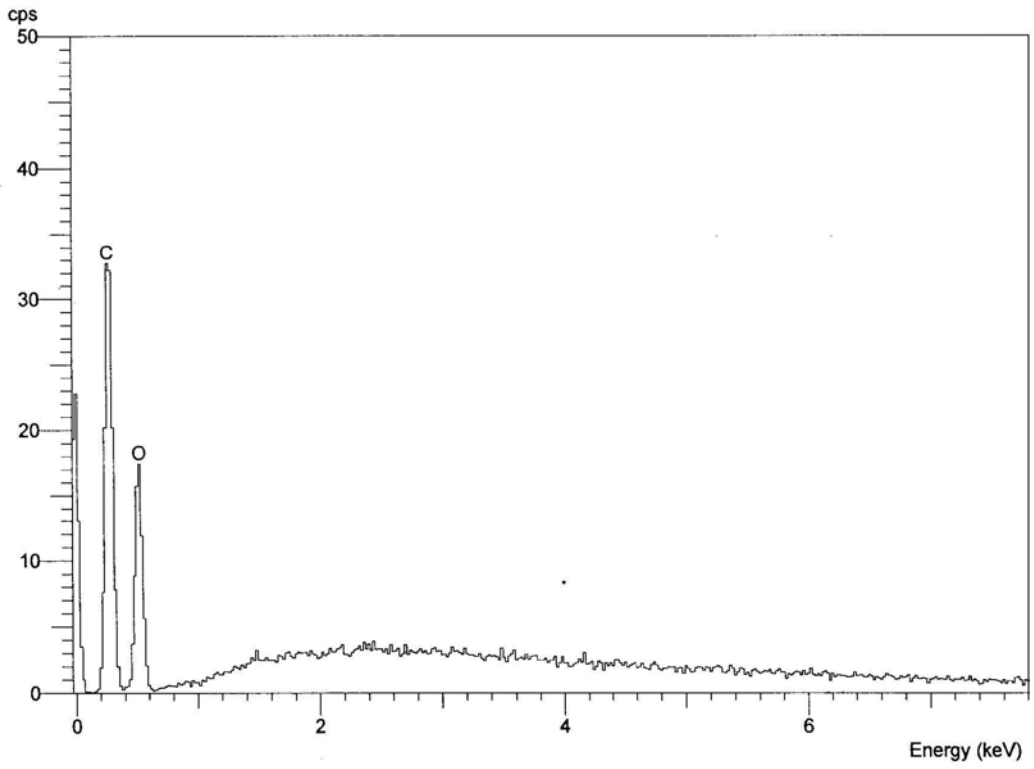
Formulation	% Yield	Bulk density (g/ml)	% Porosity
Non-porous mannitol	93.28 ± 2.90	0.53 ± 0.02	62.7 ± 2.3
AA1	80.31 ± 3.49	0.26 ± 0.01*	81.3 ± 2.3*
AA2	78.52 ± 0.35	0.25 ± 0.01*	82.7 ± 1.2*
AC1	76.89 ± 0.98	0.21 ± 0.01*,**	81.3 ± 1.2*
AC2	77.23 ± 1.49	0.22 ± 0.02*,**	88.0 ± 4.0*,**
AC4	75.46 ± 2.61	0.22 ± 0.01*,**	84.7 ± 1.2*
AC6	73.28 ± 2.12	0.23 ± 0.01*	85.3 ± 2.3*

*Significantly different ($p<0.05$) when compared with mannitol (raw material)

**Significantly different ($p<0.05$) when compared between groups

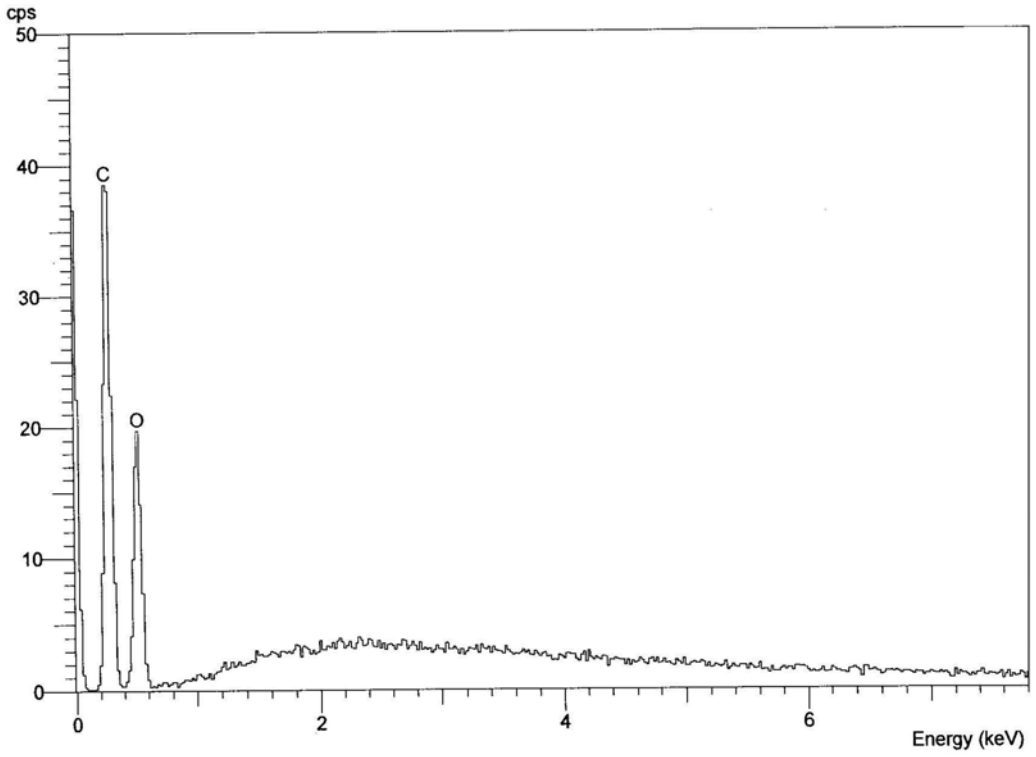


(a)



(b)

Figure 4.2 The EDX images of AC2 (a), AC4 (b) and AC6 (c)



(c)

Figure 4.2 The EDX images of AC2 (a), AC4 (b) and AC6 (c) (Continued)

4.5 Conclusion

Porous mannitol exhibited sphere and smooth surface. From SEM, they were 3-5 μm in size. They exhibited lower bulk density and higher porosity than non-porous one. It indicates that porous mannitol is a good carrier for DPI formulation. In this study, the porous mannitol using 20% ammonium carbonate had the lowest density with the highest porosity. EDX results indicated that there was no residue ammonia in the formulation using ammonium carbonate although there was ammonium carbonate up to 60% w/w in the pre-formulation. Porous mannitol using ammonium carbonate as pore forming agent may be a good candidate for DPI carrier.

CHAPTER 5

INHALED PYRAZINAMIDE PROLIPOSOME FOR TARGETING ALVEOLAR MACROPHAGES

5.1 Introduction

The use of alveolar delivery systems are an attractive approach for delivering drugs to lungs of tuberculosis (TB) patients especially to access and inhibit infected alveolar macrophages (AMs) (Alving, 1998; Ahsan et al., 2002). These systems have many benefits such as being target oriented, low dose, with low side effects and less frequent administration (Mitchison and Fourie, 2010). The important outcomes are better drug compliance, reduced drug resistance and shorter courses of therapy. Pulmonary administration is an appropriate route for drugs required to have a local action in the respiratory tract as it results in rapid localized drug action. One major advantage of using liposomes for pulmonary administration is that they can be prepared from phospholipids that are normal components of lung tissues. It can therefore be assumed that pulmonary-delivered liposomal vesicles will lack inherent toxicity (Taylor and Elhissi, 2006). The technology for lung specific drug delivery systems is now at a point where aerosolized liposomes and even timed release methodology may offer advantages for a more effective treatment and prevention of TB (Khuller et al., 2004). Nebulization of liposomes would be perhaps more acceptable when compared with I.V. liposomes. Pandey and co-workers (2004) have

suggested that the controlled drug release together with macrophage targeting may obviate the need for daily anti-TB drug dosing and consequently, liposome based inhaled therapy would be a novel approach towards the management of pulmonary TB. Liposomes are relatively unstable colloidal systems. Physical instability is manifested in vesicle aggregation and fusion, associated with changes in vesicle size and loss of entrapped hydrophilic materials (Elhissi et al., 2007). Chemical instability, particularly ester bond hydrolysis, and oxidation of unsaturated acyl bonds accelerates liposome breakdown and alters the drug-release characteristics (Heurtault et al., 2003). Since, the therapeutic efficacy of liposomes is dependent upon the physical integrity and stability of lipid bilayer structures; it may become imperative that the chemical and physical stability of liposomes is maintained (Khuller et al., 2004). To overcome instability problems, proliposome approaches to liposome formation have been described as a means for enhancing stability. Freeze dried and spray dried liposomes can be a useful tool to prepare proliposomes (Taylor and Elhissi, 2006; Agnihotri et al., 2010). Proliposomes are defined as dry, free-flowing particles that immediately form a liposomal dispersion on contact with water, so they have a potential for pulmonary delivery as dry-powder inhalers (DPIs) (Elhissi et al., 2010; Wagner and Vorauer-Uhl, 2011). Anti-TB proliposomes have been studied (Changsan et al., 2009). Rifampicin proliposomes were prepared by freeze-drying with mannitol as a cryoprotectant (Changsan et al., 2009). These encapsulated rifampicin liposomes were evidently nontoxic to respiratory associated cells, including bronchial epithelial cells, small airway epithelial cells and AMs. The minimum inhibitory concentration against *M. bovis* was 0.2 μM for proliposomes containing rifampicin and 0.8 μM for

free rifampicin. In addition to freeze-drying, using a spray drying method is an alternative approach (Rojanarat et al., 2011). Isoniazid (INH) proliposomes in a dry powder aerosol form were prepared by a spray drying method. These INH proliposomes were nontoxic to respiratory-associated cells, and did not activate AMs to produce inflammatory mediators, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and nitric oxide, at a toxic level. The efficacy of INH proliposome against AMs infected with *M. bovis* was significantly higher than that of free INH. The authors indicated that anti-TB drugs entrapped in proliposome enhanced drug bioactivity and were potential candidates for an alternative TB treatment (Rojanarat et al., 2011).

In this study, pyrazinamide (PZA) -proliposomes were prepared by a spray drying method using porous mannitol which had a low density and high porosity. The toxicity of proliposome formulations with good aerodynamic properties against respiratory and macrophage cell lines was investigated. Phagocytosis by macrophage cells incubated with proliposomes was observed. The MIC of the anti-TB dry powder inhaler formulations on mycobacteria was determined by a flow cytometric method (Sawatdee et al., 2006).

5.2 Materials

Mannitol, L-α soybean phosphatidylcholine (SPC), and cholesterol from lanolin (CH) were obtained from Fluka (Buchs, Switzerland). Ammonium carbonate was from Ajax Finechem (NSW, Australia). Pyrazinamide (PZA) was from Sigma-Aldrich (St. Louis, MO, USA), and dimethyl sulfoxide (DMSO) from Riedel-de Haën (Seelze, Germany). All other reagents were analytical grade.

All solutions (reagent packs) used for the maintenance and culture of human bronchial epithelial cells (Calu-3) and human lung adenocarcinoma cell line (A549) were acquired from Gibco (Grand Island, NY, USA). Samples of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fluorescein diacetate (FDA) were from Sigma-Aldrich. Quantikine® RTA00 and Quantikine® RLB00 kits for rat TNF-α and IL-1β, respectively, were from R&D Systems Inc. (Minneapolis, MN, USA). The BCG vaccine of *M. bovis*, Middlebrook 7H9 culture medium, *M. tuberculosis* H_{37}Ra (ATCC 25177) cells and alamar blue solution were supplied by the Queen Saovabha Memorial Institute (Bangkok, Thailand), Becton Dickinson and Co. (Franklin Lakes, NJ, USA), American Type Culture Collection (Rockville, MD, USA) and Alamar Biosciences/Accumed (Westlake, OH, USA), respectively.

5.3 Methods

5.3.1 Production of porous mannitol

Porous mannitol microparticles (Formulation AC2) were prepared as previously described in section 4.3.1.

5.3.2 Production of PZA-proliposome by spray-drying technique

To prepare the PZA-proliposome formulations, the ingredients were divided into two parts. The lipid part was composed of a mixture of SPC (0.06 mmole) and CH (0.06 mmole). The porous mannitol used as a core carrier and PZA was the powder part. The powder part was calculated to be 10 mg/mL and the weight ratio of PZA: porous mannitol was 1:9 (Formulation I), 2:8 (Formulation II), 4:6 (Formulation III), 6:4 (Formulation IV) and 8:2 (Formulation V). PZA-proliposome without mannitol and PZA-proliposome with non-porous mannitol (PZA: non-porous mannitol = 1:9) were prepared.

The lipid part was weighed and dissolved in 100 mL of 95% ethanol to obtain the ethanolic lipid solution. PZA was added to the ethanolic lipid solution and sonicated until a clear solution was obtained. The maximum concentration of PZA in this study was only 0.1% and was completely dissolved. Porous mannitol was dispersed in the solution, and the suspension was sonicated for 15 minutes in order to deaggregate mannitol particles before the spray drying process began. The suspension was continuously stirred to provide homogeneity of the suspension during spray drying. The inlet temperature was 90°C and the atomizer pressure was 800 kPa, with

a feed rate of 3 mL/minute. The proliposome powder was collected from the collecting chamber and kept in a desiccator until used.

5.3.3 Morphology of the microparticles

The employed method was previously described in section 4.3.2.

5.3.4 Content uniformity of PZA in the proliposome powder

PZA-proliposome powder (10 mg) was randomly sampled and weighed. The powder was suspended in 10 mL of methanol to dissolve the lipid content. The volume was adjusted to 25 mL with distilled water, followed by sonication to obtain a clear solution. The PZA content from the clear solution was analyzed by high performance liquid chromatography (HPLC) as previously described in section 3.3 and 3.4.

5.3.5 *In vitro* evaluation of aerosol performance of the PZA-proliposome dry powder using a cascade impactor

The PZA-proliposome dry powder was aerosolized using a delivery device made in-house (Srichana et al., 2003). The aerosolized parameters of the products, including mass median aerodynamic diameter (MMAD), fine particle fraction (FPF) and emitted dose (ED), were determined using an Andersen cascade impactor (ACI) (Andersen Instruments, Atlanta, GA, USA) equipped with preseparator and glass throat. The ACI used a vacuum pump with a flow rate of 60 L/minute for 10 s. Triton X-100 (0.1%) in methanol was used to rinse particles deposited on each stage. The

PZA content was determined by HPLC, as described in the content uniformity section. The cumulative percentage of deposition was transformed to a Z-value and plotted against the log cutoff diameter of each stage. MMAD was obtained from the particle diameter at a Z-value of zero (Atkins et al., 1992). The emitted dose (ED) is the amount of drug propelled from the delivery device. The FPF is the fraction of particles smaller than 4.4 μm.

5.3.6 Encapsulation and size measurement after reconstitution of PZA-proliposome into a liposome suspension

To determine the percent encapsulation, 10 mg of proliposome powder was reconstituted with distilled water (4 mL), and then centrifuged with a SW 60 Ti rotor (Beckman Coulter Inc., Palo Alto, CA) at 100,000 *g* for 20 minutes at 25 °C (Saetern et al., 2004). The supernatant was analyzed as the unencapsulated PZA by HPLC, as described in the content uniformity section. The percent encapsulation was obtained using the following equation:

$$\% \text{ encapsulation} = \frac{\text{Initial drug incorporation - Unencapsulated drug amount}}{\text{Initial drug incorporation}} \times 100$$

The size of the liposome after reconstitution was measured using ZetaPALS (Brookhaven, New York, USA) at 25°C. The proliposome powder was reconstituted with milliQ water to obtain nanovesicles while the undissolved particles were removed by centrifugation. The centrifugation conditions were 10,000 *g* for 20

minutes at 25 °C. Sizes were determined immediately after obtaining the supernatant from the centrifugation.

5.3.7 X-ray diffraction measurement of PZA-proliposome

An X-ray diffraction (XRD) of the porous mannitol, PZA and PZA-proliposome was performed with a Siemens D 5000 (Siemens AG, Berlin, Germany) equipped with a diffracted-beam monochromator, using Cu radiation. The proliposome powder samples were spread over glass sample holders, each in an area of 4 cm^2 with a depth of 1 mm. The powder surfaces were pressed and smoothed with a glass slide. The diffraction intensity was recorded at an angle of 2θ from 5 to 60° with a step size of 0.05° and step time of 1 s. The total time of the diffraction scan was 19 minutes, and each sample was examined in three separate experiments. The voltage and current generator were set at 40 kV and 30 mA, respectively. The obtained data were analyzed by EVA software. Relative crystallinity was determined from the XRD results by the ratio of the intensity of a characteristic crystalline peak to that of the amorphous halo for each powder sample. If the ratio of the peak to the amorphous halo around it decreased, then the crystallinity was proportionately lower (Morris et al., 1993).

5.3.8 Differential scanning calorimetry of PZA-proliposome

A differential scanning calorimeter (DSC) model 2920 (TA Instruments, Newcastle, DE, USA) was used to investigate the interaction of the PZA-mannitol-SPC in the proliposome powder produced by spray drying. A powder sample was

placed in an aluminum pan, hermetically sealed, and then assessed by DSC from 30°C to 200°C at a rate of 10 °C/minute. The DSC thermograms were analyzed using the Universal Analysis 2000 program, version 3.4c.

5.3.9 Fourier transform-infrared spectroscopy (FT-IR) of PZA-proliposome

Infrared spectroscopy was used to characterize the functional groups of the samples. A dried sample was carefully mixed with dry KBr and pressed into a pellet. The solid pellet was placed in a magnetic holder. FT-IR spectra between 4000 and 400 cm^{-1} were determined after an accumulation of 16 scans using a Spectrum One (Perkin-Elmer, MA, USA).

5.3.10 Cell cultures: Growth of Human bronchial epithelial cells (Calu-3), Human lung adenocarcinoma cell line (A549) and Alveolar macrophage cell line (NR8383)

The Calu-3 cell line (ATCC HTB-55, Rockville, MD, USA) was cultured in Modified Eagles Media supplemented with 10% fetal bovine serum (FBS), and 50 units/mL penicillin and 50 µg/mL streptomycin. The A549 cell line (ATCC CCL-185, Rockville, MD, USA) was cultured in Ham's F12K supplemented with 10% FBS, 50 units/mL penicillin and 50µg/mL streptomycin. Both cell lines were maintained at 37°C in a fully humidified atmosphere at 5% CO_2 in air and the media were changed every 2-3 days. When the cells reached 60-80% confluency, they were rinsed with phosphate buffered saline solution (PBS) to wash off any remaining complex proteins

that might neutralize the trypsin activity. PBS was aspirated, and the cells were covered with 2 mL of trypsin/EDTA solution. The cells were then detached from the plate by the trypsin/EDTA. Cells were centrifuged, resuspended and then transferred to a new culture flask. The medium was replaced with fresh medium two or three times weekly.

The NR8383 cells (ATCC CRL-2192, Rockville, MD, USA) had been isolated from normal rat lung lavage. The cells were cultured in F-12 Kaighn's cell culture medium supplemented with 15% (v/v) heat-inactivated FBS, 50 units/mL penicillin, 50 μg/mL of streptomycin, and then incubated at 37 °C, in 5% CO_2 and 95% humidity. The cells were maintained by transferring floating cells to new flasks. Adherent cells could be harvested by scraping. Upon reseeding, about half of the cells re-attached. The medium was replaced with fresh medium two or three times weekly.

5.3.11 Determination of cytotoxicity of PZA-proliposome to cells in the respiratory tract

The viabilities of Calu-3, A549 and NR8383 cells were evaluated using the MTT assay to detect functioning mitochondria. Live mitochondria transform MTT to insoluble purple formazan crystals, which was measured with a spectrophotometer. Briefly, 100 μL of 1 x 10^5 cells/mL was cultured in each well of a 96-well plate and allowed to adhere and grow overnight at 37°C, in a 5% CO_2 and 95% humidity incubator. The following day, the media (100 μL) was replaced with fresh media and 100 μL of cell culture media containing either PZA or PZA-proliposome was added. The plate was then incubated for 24 h. A filtered sterilized stock MTT solution (50 μL

of 5 mg/mL in Dulbecco's phosphate buffer saline, DPBS, Gibco, Grand Island, NY, USA) was added into each well containing 150 μLof fresh media and incubated for 4 h at 37 °C. After that, the supernatant was carefully removed and the resulting formazan crystals were dissolved by adding 200 μL of dimethylsulfoxide (DMSO, Riedel-de Haën, Seelze, Germany) and thoroughly mixed. The absorbance was recorded at 570 nm with the microplate reader (Biohit BP 800, Helsinki, Finland). The proportion of viable cells in the treated well was compared to the untreated well.

5.3.12 Determination of the alveolar macrophage response to PZA-proliposome

a) Production of inflammatory cytokines

An enzyme linked immunosorbent assay (ELISA) method was used to measure the levels of the inflammatory cytokines TNF-α and IL-1β generated from NR8383 cells responding to PZA, PZA-proliposome or lipopolysaccharide (LPS) from *E.coli* (positive control). Commercial ELISA kits (Quantikine® RTA00 and Quantikine® RLB00 for rat TNF-α and IL-1β, respectively, R&D systems Inc., MN, USA) were used as described in the product assay procedures. The detectable amount of both TNF-α and IL-β was less than 5 pg/mL. Standards, controls and samples were pipetted into the wells and any rat TNF-α or IL-β present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TNF-α or IL-β was added into the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate

solution was added to the wells. The enzyme reaction yielded a blue product that turns yellow when the stop solution was added. The intensity of the color measured was in proportion to the amount of rat TNF-α or IL-β bound in the initial step. The sample values were then read from the standard curve. The detectable dose of both TNF-α and IL-β was in the range of 12.5-400 pg/mL.

b) Nitric oxide assay by the Griess reaction

Nitric oxide (NO) released by NR8383 cells after being challenged with PZA, PZA-proliposome or LPS in a concentration range of 25-500 μg/mL for PZA and PZA-priliposome and 25-500 ng/mL for LPS was detected by the Griess reaction. Nitric oxide in the form of nitrite (NO_2^-), which is one of the two primary, stable and nonvolatile products of NO was investigated. This measurement relies on a diazotization reaction of the Griess reagent. 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 mixing for 10 minutes, the absorbance was determined using a microplate reader at 450 nm. The NO concentration was calculated from a sodium nitrite standard curve (Huttunen et al., 2000; Punturee et al., 2004).

5.3.13 Assessments of the antimycobacterial activity of PZA and PZA-proliposome

a) Culture of *M. bovis* from BCG vaccine

The lyophilized BCG vaccine of *M.bovis* was reconstituted with 1 mL of sterile deionized water for injection. Reconstituted BCG vaccine (200 µL) was grown in Middlebrook 7H9 broth (pH 5.5) containing 0.5% Tween 80 and 10% oleic acid-albumin-dextrose catalase (OADC) enrichment (Ritelli et al., 2003). The bacilli were incubated at 37 °C and subcultured every 3 weeks. The obtained *M. bovis* suspension at 3 weeks old was used in this experiment.

b) Determination of minimum inhibitory concentration against *M. bovis*

The minimum inhibitory concentrations (MIC) of PZA and PZA-proliposome (Formulation I and V) in a drug concentration range of 25-400 µg/mL were determined. An inoculum of *M. bovis* was prepared from a suspension of a 3-week-old organism culture in Middlebrook 7H9 broth (M7H9) (pH 5.5) supplemented with Middlebrook OADC enrichment. The suspension of bacilli was adjusted with normal saline solution to obtain a turbidity of a McFarland standard of 1 (3×10^8 CFU/mL). Tenfold serial dilution (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of the above inoculum suspension was made in M7H9 medium. Each proliposome concentration (100 µL) was added into wells containing the diluted bacilli suspension (900 µL of 3×10^8 CFU/mL bacilli suspension), and incubated at 37 °C. The final concentrations of PZA and PZA-proliposome were 25, 50, 100, 200 and 400 µg/mL. Every day until the 7^{th} day, 500

μL of sample was taken from each well and placed in a sterile screw-cap micro-tube containing 500 μL of FDA prepared at 500 ng/mL in PBS at pH 7.4. Samples were then incubated at 37°C for 30 minutes before being analyzed using a flow cytometer (FACSCalibur, Becton-Dickinson, California, USA) and CellQuestTM software for data acquisition and analysis (Sawatdee et al., 2006).

c) Determination of the minimum inhibitory concentration against *M. tuberculosis*

The antimycobacterial activity of PZA and PZA-proliposome (Formulation I and V) against *M. tuberculosis* H37Ra (ATCC 25177) was determined. *M. tuberculosis* was grown in 100 mL M7H9 broth supplemented with 10% OADC enrichment and 0.05% Tween 80. Antimicrobial susceptibility testing was carried out in a dark room using clear-bottomed, 96-well microplates in order to minimize background fluorescence. The outer wells of the microplates were loaded with sterile water to prevent dehydration in the experimental wells. First, samples were diluted with distilled deionized water; two fold dilutions were then performed in 0.1 mL of M7H9 (without Tween 80). The final colony-forming unit (CFU) bacterial density of 5 x 10^4 CFU/mL was added into the wells. The sample without bacteria as control wells was used to detect the autofluorescense of the compounds. Plates were incubated at 37°C. After 4 days of incubation, 20 μL of 10x Alamar Blue solution and 12.5 μL of 20% Tween 80 were added to either test wells (containing bacilli) or control wells, and plates were incubated at 37°C. A color change from blue to pink was observed at 12 and 24 h. If a well containing bacterium became pink by 24 h,

reagent was added to the entire plate. If the well remained blue, additional bacteria was loaded and tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C, and results were recorded at 24 h post-reagent addition. Visual MICs were defined as the lowest concentration of drug that prevented a color change (Collins and Franzblau, 1997).

5.3.14 Phagocytosis of anti-TB dry powder particles by macrophage cells

Proliposome dry powder (Formulation I) was reconstituted with distilled water to obtain 10 mg/mL. Quantum dot nanoparticles were employed for particle imaging (Lumidot®, Sigma-aldrich, St. Louis, USA). Lumidot® 510 (20 μg/mL) was added to the liposome suspension in a volume ratio 1:2. The mixture was sonicated for 3 minutes and observed with a fluorescence microscope (Olympus, BX61, Olympus, Tokyo, Japan).

One hundred μL of 1 x 10^4 cells/mL NR8383 cells was 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. Liposome (Formulation I) stained with Lumidot® 640 was added. Phagocytosis of anti-TB particles by NR8383 cells was observed with a fluorescence microscope.

5.3.15 Statistical analysis

Data, when applicable, are presented as a mean ± standard deviation (SD) from at least three samples unless indicated. The data were compared using analysis of variance (ANOVA) followed by a One-Way ANOVA to determine the difference

between data sets. All statistical comparisons were calculated using SPSS software version 17 (SPSS Inc., Chicago, IL). A p-value < 0.05 was considered statistically significant.

5.4 Results and discussion

5.4.1 Morphology of the microparticles

Porous particles had a similar geometric size in comparison to non-porous particles (Figure 5.1a) but differed with respect to density. Therefore it is expected that the aerodynamic properties of the porous particles must be better than those of the non-porous particles. Spherical particles of porous mannitol with a size of around 1-3 μm (Figure 5.1b) were obtained from the spray-dried mannitol and ammonium carbonate as a pore forming agent.

PZA appeared to be incorporated into the pores and on the surface of the core particles. PZA-proliposome powders were successfully prepared using the spray drying technique. The surface and morphology of the proliposomes obtained from formulations I to V are shown in Figure 5.1c-5.1g. Spherical microparticles were observed in formulation I, II and III (Figure 5.1c-5.1e) with a high (60-90%) content of porous mannitol. Other PZA-proliposomes containing porous mannitol less than 60% were irregular in shape, with some tiny particles (less than 1 μm) or elongated particles (Figure 1f and 1g) adhering to large aggregated particles. In the spray drying process, the porous mannitol was dispersed in 95% alcohol containing PZA and the lipid part. PZA could be filled or adsorbed onto the porous mannitol and the particles

became coated with the lipid. Formulation I, II and III had sufficient porous mannitol, so they were spherical in shape, while the formulation of IV and V were irregular. This might be due to insufficient amounts of porous mannitol in the formulation to produce spherical PZA-proliposome particles. The remaining drugs might be irregular in shape after the spray drying process. It could be expected that the liposomal properties of the formulation IV and V might be unsuitable, although the drug delivery to the lower airways was increased. Porous mannitol is an important ingredient to obtain spherical particles by the spray drying method. PZA-proliposome formulations were different in shape from the pure PZA (Figure 5.1h). This might be affected by the spray drying process that changed crystallinity and morphology. We have suggested that the ideal formulation should contain porous manitol of not less than 60%, to obtain perfect spheres. And other factors have to be taken into account such as MMAD, % encapsulation and FPF.

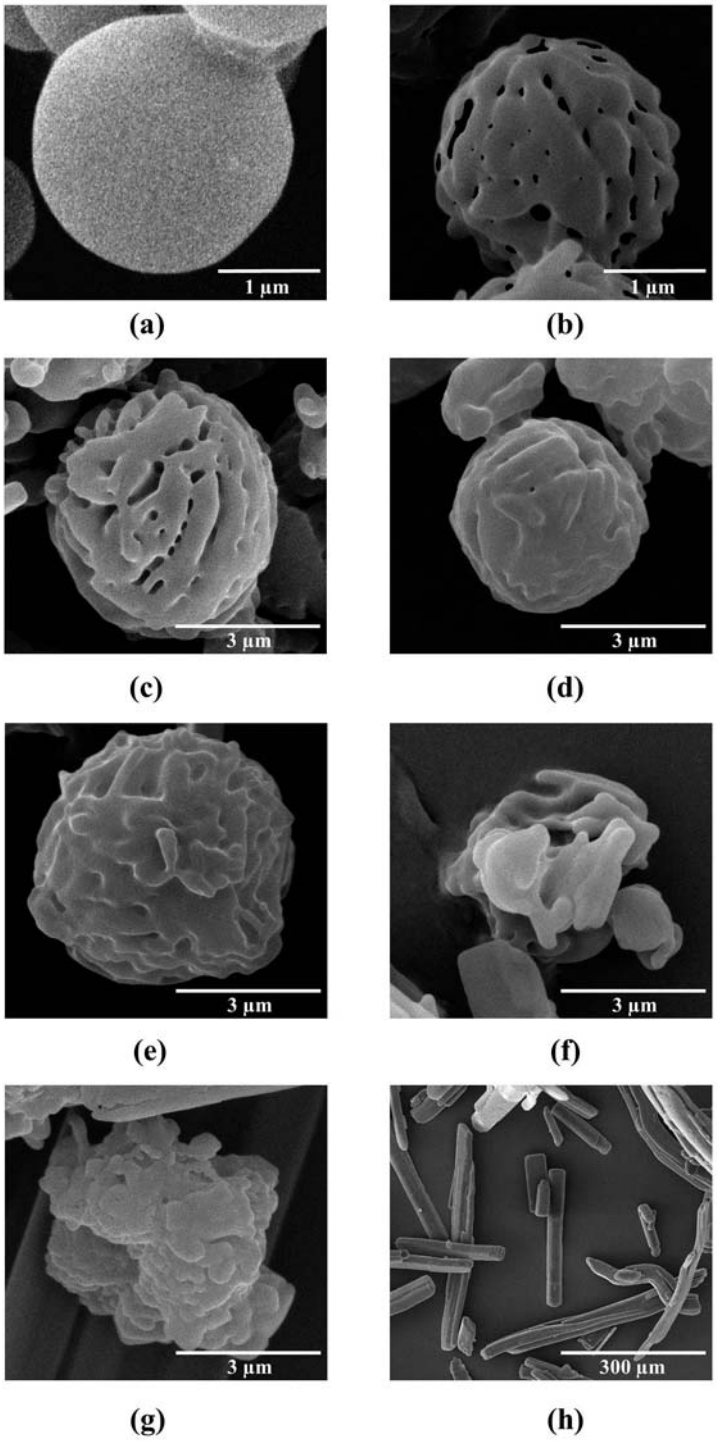


Figure 5.1 The SEM images of (a) spray dried mannitol (bar =1 µm), (b) porous mannitol (bar =1 µm), (c-g) PZA- proliposome formulation I to V (bar =3 µm) and (h) PZA (bar =300 µm)

5.4.2 *In vitro* evaluation of the aerosol performance of the PZA-proliposome dry powder using the cascade impactor

PZA-proliposome was successfully prepared by spray drying method. Production yield of PZA-proliposomes containing manntiol particles was higher than that without mannitol (Table 5.1). While % yield of PZA-proliposomes of 10%PZA with non-porous mannitol was 88% and with porous mannitol it was found to be 89%. Yields of PZA-proliposomes formulation I to V tend to decrease when PZA ratio was increased from 89 to 64%. From the results as shown in Table 5.1, content uniformity of all formulations was acceptable (% RSD less than 6, USP 30 and NF 25; USP-NF, 2007). This indicated that spray dry method could be used for proliposome production because it was able to produce a uniform distribution of the active ingredient. All formulations gave % ED more than 80% and % FPF more than 19%. The MMAD of formulation I containing porous mannitol (PZA: mannitol = 1:9) was lower (4.26 ± 0.06 μm) than that of PZA-proliposome with non-porous mannitol (PZA: mannitol = 1:9) (5.03 ± 0.07 μm) and PZA-proliposome without mannitol (5.33 ± 0.13 μm). It might be the result from the properties of porous mannitol which has low density. From the calculation of a particle's aerodynamic diameter, particle density was an important parameter in aerosol physics (Crowder et al., 2002). Particle's aerodynamic diameter was directly proportional to square root of particle density. As particle density decreased, particle's aerodynamic diameter decreased. Hence, aerosolization properties of PZA-proliposome could be improved using porous mannitol as a core carrier.

The PZA-proliposome formulation I to V showed no difference in their MMAD values (4.20-4.60 μm, $p > 0.05$). The MMAD values were correlated with a high FPF (the fraction of particles smaller than 4.4 μm) in the range of 20-30%. An ED of 80-98% was obtained from the formulations. FPF decreased when the porous mannitol increased until the porous mannitol reached 40%. In formulation IV and V containing low porous mannitol (40 and 20%, respectively), FPF was slightly increased. This might be from the lower size of the particles obtained from the spray drying. ED values decreased to lower than 90% when the porous mannitol ratio decreased to 40% or lower. This might be due to a greater aggregation of particles that caused difficulty in de-aggregating. Formulation I gave a higher FPF, with the highest ED value of 98%. To deliver the dry powder inhaler to the lower part of the respiratory tract, the MMAD of the formulation should be less than 5 μm and prepared formulation I to V met this requirement.

Table 5.1 Aerosolization properties of PZA-proliposome formulations and properties of PZA-liposomes (mean ± SD, n=3)

Formulation (PZA: porous mannitol)	% Yield	Content uniformity (%)	MMAD (µm)	FPF (%)	Emitted dose (%)	Encapsulation (%)	Size measurement (nm)	Polydispersity index
PZA-proliposome without mannitol	58.9 ± 2.3	102.3 ± 3.9	5.33 ± 0.07	27.7 ± 6.9	98.6 ± 1.1	-	-	-
PZA-proliposome with non-porous mannitol	88.7 ± 1.5	99.9 ± 4.1	5.03 ± 0.07	24.5 ± 7.1	96.4 ± 3.1	-	-	-
I (1:9)	89.2 ± 2.7	97.8 ± 4.8	4.26 ± 0.06	29.0 ± 4.1	97.8 ± 1.0	44.6 ± 0.5	216 ± 11	0.319
II (2:8)	88.5 ± 1.8	108.6 ± 0.6	4.26 ± 0.04	22.5 ± 6.9	94.5 ± 1.0	44.4 ± 1.1	359 ± 13	0.317
III (4:6)	70.2 ± 2.7	94.8 ± 0.4	4.26 ± 0.04	19.4 ± 5.1	94.4 ± 7.6	44.0 ± 1.9	516 ± 20	0.341
IV (6:4)	65.9 ± 3.7	94.3 ± 0.4	4.29 ± 0.03	25.8 ± 1.2	89.9 ± 4.1	37.3 ± 2.6	449 ± 15	0.327
V (8:2)	63.9 ± 4.3	93.4 ± 0.8	4.39 ± 0.21	23.7 ± 2.4	80.2 ± 7.3	26.7 ± 2.4	382 ± 12	0.313

5.4.3 Encapsulation and size measurement after reconstitution of PZA-proliposome into a liposome suspension

After PZA-proliposomes were reconstituted with distilled water, vesicle sizes (Table 5.1) showed a normal distribution. The mean size ranges were 200-520 nm from all formulations and polydispersity index was about 0.3. It indicated that the size distribution was narrow. The Formulation I gave the lowest vesicle size (200 nm). Nanoparticles in these ranges (200-600 nm) were efficiently taken up by macrophages (Kanchan and Panda, 2007). It is expected that the PZA-liposome in this study is suitable for targeting AM.

The encapsulation efficiency of PZA was in the range of 26-45% (Table 5.1). In this study, the encapsulation efficiency was determined using an indirect method. PZA-proliposome was reconstituted with distilled water, whereas free PZA would dissolve instantly. After ultracentrifugation, PZA-encapsulated liposomes were packed into pellets at the bottom of the centrifuge tube. The supernatant was analyzed to determine free PZA. The encapsulation efficiency was calculated from the difference between the PZA content and the free PZA values. Formulation I had the highest PZA encapsulation, while formulation V had the lowest. This could be explained by a low PZA loading amount in the formulation providing for high encapsulation efficiency. In the spray-drying process, PZA could be incorporated into the porous mannitol particles before the lipid coating. When the PZA loading was high (formulation IV and V), therefore the lipid could not coat all the particles. A PZA:porous mannitol ratio of 1:9 seemed to give the highest encapsulation efficiency.

5.4.4 X-ray diffraction measurement of PZA-proliposome

The XRD pattern of the porous mannitol, PZA-proliposome formulation I to V and PZA are shown in Figure 5.2. The porous mannitol diffractogram showed sharp reflections at 2θ values of 14.58°, 16.76°, 18.70°, 20.38°, 21.06°, 21.67°, 23.35° and 29.43° (Figure 5.2a). In a similar way, the diffractogram of PZA showed reflections at 2θ values of 7.80°, 13.74°, 15.32°, 15.66°, 17.67°, 27.11°, 27.37° and 35.80° (Figure 5.2g). It was identified as polymorph α (Castro et al., 2010). The sharp reflections of the diffractogram of the porous mannitol and PZA confirmed the crystallinity of the ingredients.

PZA-proliposome formulation I showed intense peaks of crystallinity at 14.58°, 16.76°, 18.71°, 20.39°, 21.07°, 21.67°, 23.35° and 29.44° (Figure 5.2b). This was the same XRD pattern for porous mannitol. Formulation II (Figure 5.2c) exhibited intense peaks of crystallinity like the XRD pattern of porous mannitol (14.57°, 16.75°, 18.71°, 20.38°, 21.07°, 23.35°, 29.44° and 33.52°). Formulation III showed reflections at 2θ values of 14.57°, 16.75°, 18.71°, 20.39°, 21.06°, 23.36°, 27.64° and 29.43° (Figure 5.2d). The XRD pattern of formulation IV showed intense peaks of crystallinity at 14.58°, 17.25°, 18.22°, 18.70°, 20.38°, 21.07°, 23.38° and 27.65° (Figure 5.2e). The diffractogram of formulation V showed reflections at 2θ values of 16.31°, 17.23°, 18.24°, 18.69°, 19.79°, 20.37°, 21.31° and 27.65° (Figure 5.2f). Several new reflections appeared in the spectra (calculated $d_{spacing}$ in Angstroms) for example 33.52° (2.67 °A) for formulation II; 27.64° (3.22 °A) for formulation III; 17.25° (5.14 °A), 18.22° (4.87 °A) and 27.65° (3.23 °A) for formulation IV and

16.31° (5.44 °A), 17.23° (5.15 °A), 18.24° (4.86 °A), 19.79° (4.49 °A), 21.31° (4.17 °A) and 27.65° (3.23 °A) for formulation V.

The X-ray diffraction patterns of PZA-proliposome formulations II, III, IV and V were not matched to either mannitol or PZA. PZA-proliposome formulations II - V was unlikely to be polymorph α, β, δ or γ (Castro et al,. 2010). It may indicate that some interactions had occurred during formulation production.

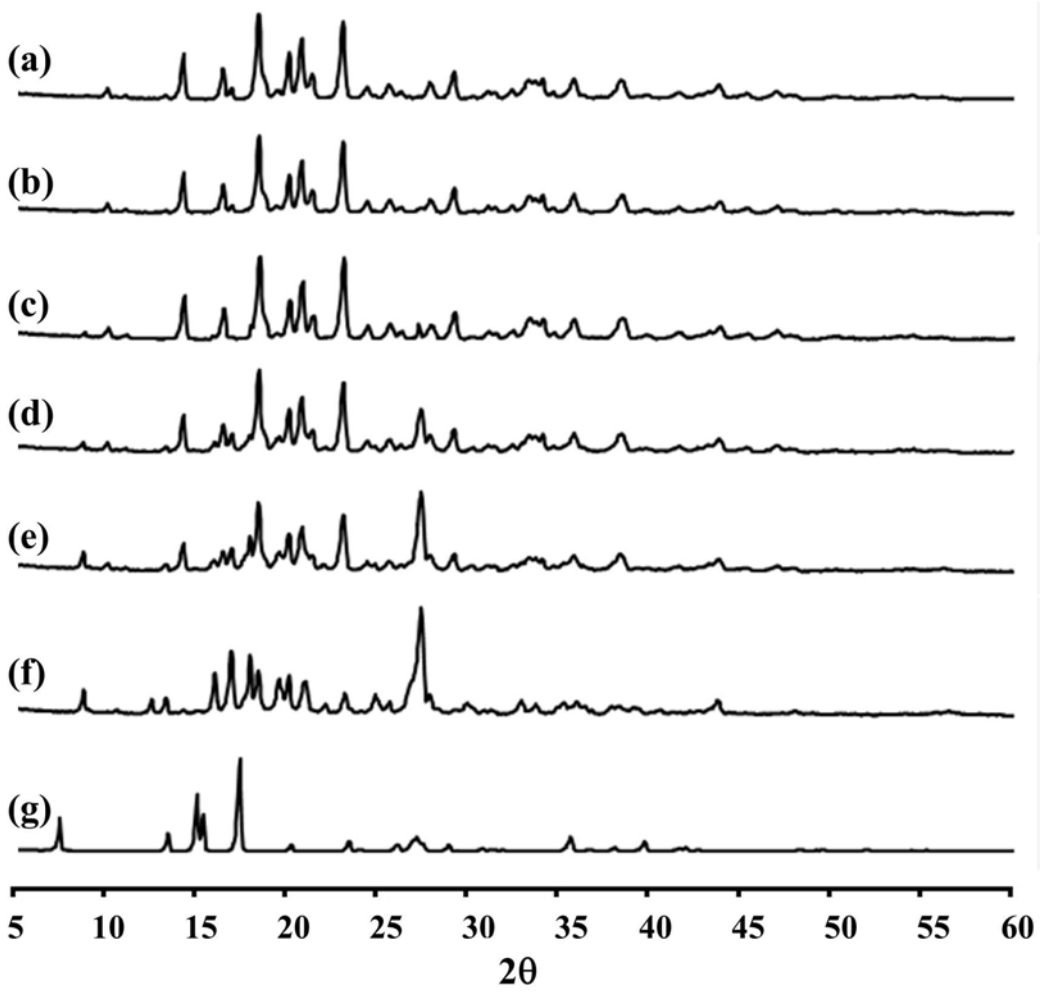


Figure 5.2 The X-ray diffraction patterns of (a) porous mannitol, (b-f) PZA-proliposome formulation I to V and (g) PZA

5.4.5 Differential scanning calorimetry of PZA-proliposome

DSC experiments were carried out to evaluate the thermal behavior of PZA-proliposomes in relation to the individual ingredients. The DSC thermograms and thermal data for porous mannitol, PZA-proliposome formulation I to V and PZA are presented in Figure 5.3 and Table 5.2. Porous mannitol exhibited an endothermic peak at 163.5°C and a melting point at 151.8°C. Its enthalpy ($\triangle H_f$) was 266.5 J/g (Figure 5.3a and Table 5.2). PZA displayed an endothermic peak at 187.3°C, a melting point at 182.7°C and $\triangle H_f$ at 200.5 J/g (Figure 5.3g).

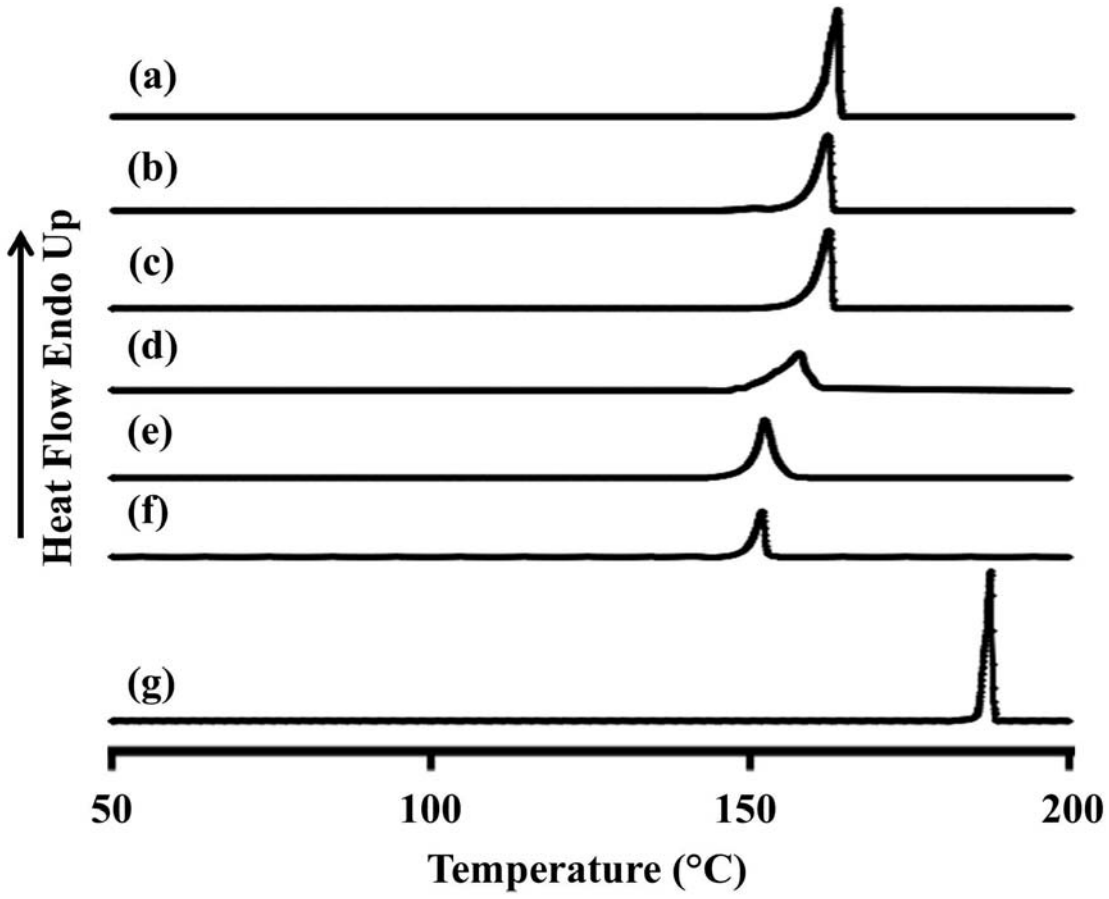


Figure 5.3 Differential scanning calorimeter thermogram of (a) porous mannitol, (b-f) PZA-proliposome formulation I to V and (g) PZA

The endothermic peak, melting point and $\triangle H_f$ of PZA-proliposome formulation I to V are shown in Figure 5.3b-5.3f and Table 5.2. They were less than that of porous mannitol and PZA. They tended to decrease when the PZA ratio increased. The DSC curves of PZA-proliposome formulations III to V that showed a broadening of the endothermic peak and shifts to a lower temperature could indicate that there was some interaction between PZA itself or with the porous mannitol. These results also correlated with the X-ray diffraction patterns of PZA-proliposome formulation II, III, IV and V. This point will be discussed in the next section.

Table 5.2 Differential scanning calorimeter data of porous mannitol, PZA-proliposome formulations and PZA.

Material or formulation	Peak (°C)	Onset (°C)	End (°C)	Peak area (J/g)
Porous mannitol	163.5	151.8	166.5	266.5
I	161.9	149.3	165.4	249.9
II	162.3	150.3	166.0	234.3
III	157.8	145.4	162.9	221.3
IV	152.3	143.4	158.9	208.3
V	151.9	144.1	154.3	104.6
PZA	187.3	182.7	189.3	200.5

5.4.6 FT-IR of PZA-proliposome

To investigate the relationships between the components of the proliposome, FT-IR studies were conducted. It is well known that the hydroxyl group of the sugar may interact with the amine group of drugs (Ross and Rekharsky, 1996). Therefore PZA may interact with mannitol or with PZA itself. It was observed that there were changes in the FT-IR spectra in the absorption bands of the amine groups and hydroxyl groups.

The mannitol spectrum (Figure 5.4a) showed characteristic bands of hydroxyl in the plane vibration (1419 cm^{-1}) and hydroxyl stretching vibration (1080 and 1019 cm^{-1}). The characteristic peaks for pyrazinamide (Figure 5.4g) was seen at 1716 cm^{-1}, 1610 cm^{-1} and 1580 cm^{-1} corresponding to amide I (C=O stretching vibration), amine bending and amide II (N-C=O symmetric stretching vibration), respectively. In the spectrum of the formulations of I to V (Figure 5.2b, 5.2c, 5.2d, 5.2e and 5.2f), the amine peak shifted from 1610 cm^{-1} to 1637 cm^{-1} for formulation I and II, to 1638 cm^{-1} for formulation III and IV and to 1672 cm^{-1} for formulation V, respectively. The peak of amide I (1716 cm^{-1}) and amide II (1580 cm^{-1}) were absent. These results indicated that some function groups were altered.

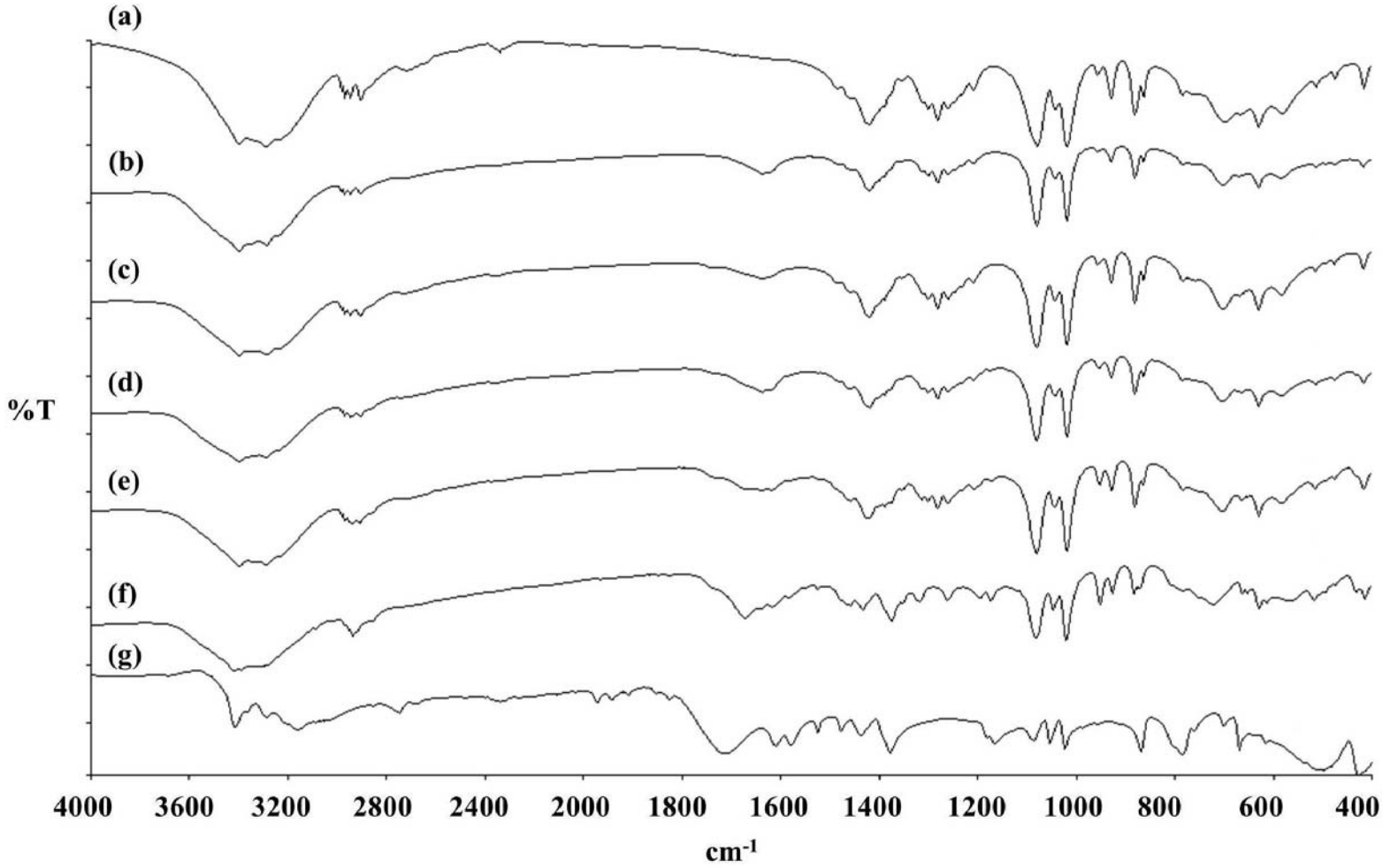
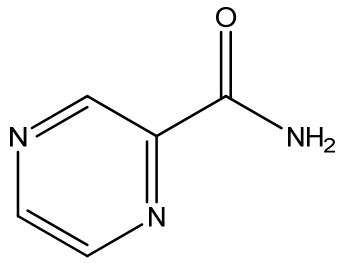
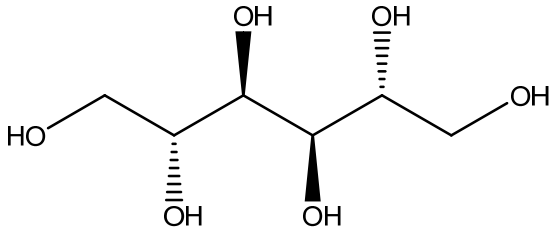


Figure 5.4 FT-IR spectra of (a) porous mannitol, (b-f) PZA-proliposome formulation I to V and (g) PZA

(a)



(b)



(c)

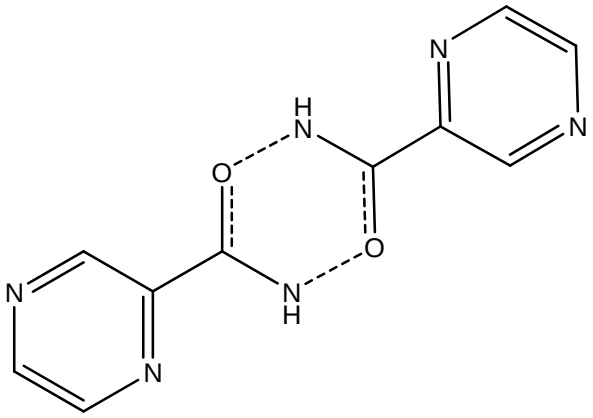


Figure 5.5 Chemical structure of (a) PZA, (b) mannitol and (c) PZA-PZA interaction

From the X-ray diffraction patterns, DSC data and FT-IR spectra, all data indicated that there was some interaction in PZA-proliposomes especially in formulations II to V. It could be explained that there might be an interaction between PZA and mannitol or PZA itself that occurred during the spray drying process. PZA (Figure 5.5a) containing an amide group and mannitol (Figure 5.5b) - an alcoholic sugar, they might interact/form hydrogen bonding between the carbonyl of the amide and the hydroxyl group and PZA can interact with PZA (intermolecular bonding)

through hydrogen bonding of the carbonyl in the amide and hydrogen in primary amine (Figure 5.5c).

5.4.7 Cytotoxicity of PZA-proliposome to cells in the respiratory tract

Human bronchial epithelial cells (Calu-3) were used in this study as an upper airway cell. The human lung adenocarcinoma cells (A549) are human alveolar basal epithelial cell, were representation of lower airway cells. AMs (NR8383) were used as a target site for anti-TB delivery.

The viabilities of Calu-3, A549 and NR8383 were determined after being challenged with different concentrations of PZA and PZA-proliposomes (Formulation I and V). Formulations I and V were the selected because their porous mannitol content varied from the highest to the lowest, aerodynamic properties (MMAD values from lowest to highest) and encapsulation efficacy (highest to lowest). The effect of PZA, present with different amounts of the porous mannitol in the formulations, free drug and reconstituted liposome were established on the respiratory cell lines.

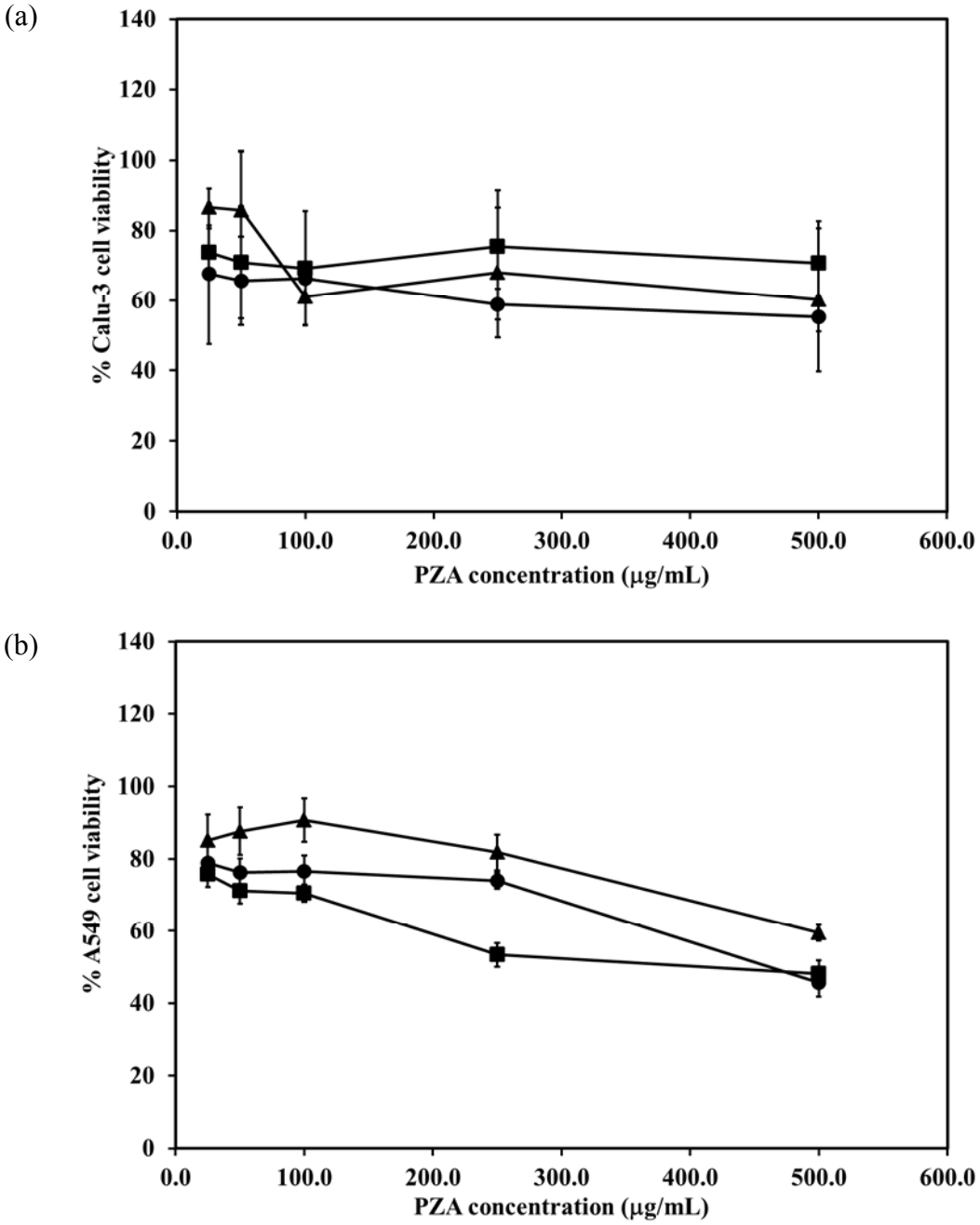


Figure 5.6 Viability of Calu-3 (a), A549 (b) and NR8383 (c) cell lines after exposed with different concentrations of PZA (●), PZA-proliposome formulation I (▲) and PZA-proliposome formulation V (■) (mean ± SD, $n \geq 6$)

(c)

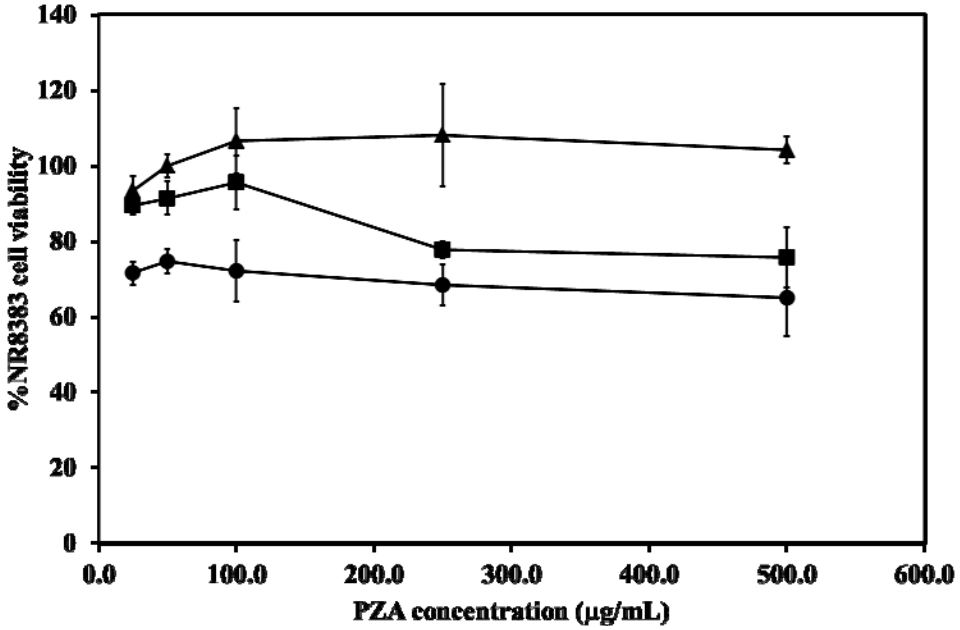


Figure 5.6 Viability of Calu-3 (a), A549 (b) and NR8383 (c) cell lines after exposed with different concentrations of PZA (●), PZA-proliposome formulation I (▲) and PZA-proliposome formulation V (■) (mean ± SD, $n \geq 6$) (Continued)

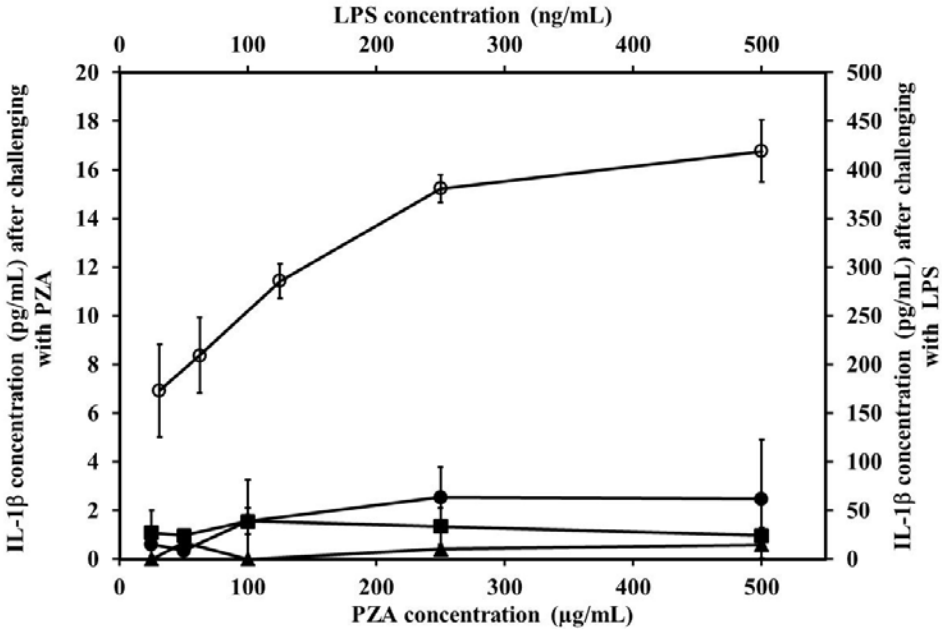
Calu-3 cell viability after being exposed to PZA, PZA-proliposome formulation I and V at various concentrations was less than 80%, while the cell viability was more than 80% only in the case of exposure to PZA-proliposome formulation I concentration of less than 50 µg/mL (Figure 5.6a). At concentrations of 25-500 µg/mL of PZA these were toxic to A549 cells. PZA-proliposome formulation I at less than 250 µg/mL was not toxic to A549 cells (cell viability of more than 80%). For NR8383 cell viability, after being exposed to PZA, was less than 80%. PZA-proliposome formulation I and V were not toxic to NR8383 cells when the concentration was 25-500 µg/mL.

In cytotoxicity studies, cell viability after being exposed to PZA-proliposome formulation I and V were found to be higher than PZA. These results indicated that proliposome could be beneficial to reduce drug toxicity to cells in the respiratory tract. There was slight variation in cytotoxicity among various cell lines in this study as they differ in cell permeability as well as their functions.

5.4.8 Effect of PZA-proliposome on production of IL-1β, TNF-α and NO by NR8383 cell line

The amount of IL-1β generated by NR8383 cells in response to PZA-proliposome and PZA, compared to that to the lipopolysaccharide (LPS) from *E. coli* was minute (figure 5.7a). LPS at 31.25 ng/mL stimulated NR8383 cells to produce 173 ± 47.4 pg/mL IL-1β and with 500 ng/mLof LPS, NR8383 cells produced 419 ± 31.8 pg/mL IL-1β. After challenge with PZA-proliposome and PZA containing PZA at concentrations of between 25-500 μg/mL, IL-1β was produced by NR8383 cells at less than 5 pg/mL. No significant differences in IL-1β concentrations were observed after NR8383 cells were challenged with PZA-proliposome and PZA containing PZA less than 125 μg/mL. After challenge with PZA itself and PZA-proliposome formulation V and formulation I containing 500 μg/mL PZA, NR8383 cells produced 2.5 ± 1.2, 1.0 ± 0.3 and 0.6 ± 0.1 pg/mL IL-1β respectively. These results demonstrated that PZA-proliposome could be used as a delivery system without generating a significant production of IL-1β from alveolar macrophages.

(a)



(b)

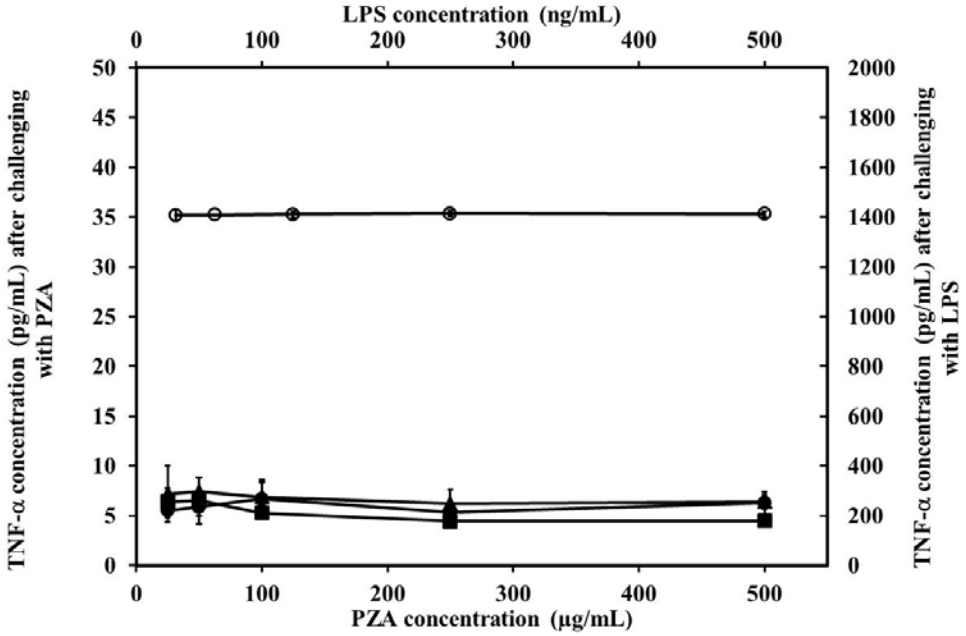


Figure 5.7 The level of inflammatory cytokine (IL-1β (a) and TNF-α (b)) and nitric oxide (c) produced from NR8383 cell lines after exposure with different concentrations of PZA (●), PZA-proliposome formulation I (▲), PZA-proliposome formulation V (■) and LPS from *E. coli* (○) for 24 h (mean ± SD, n ≥ 6)

(c)

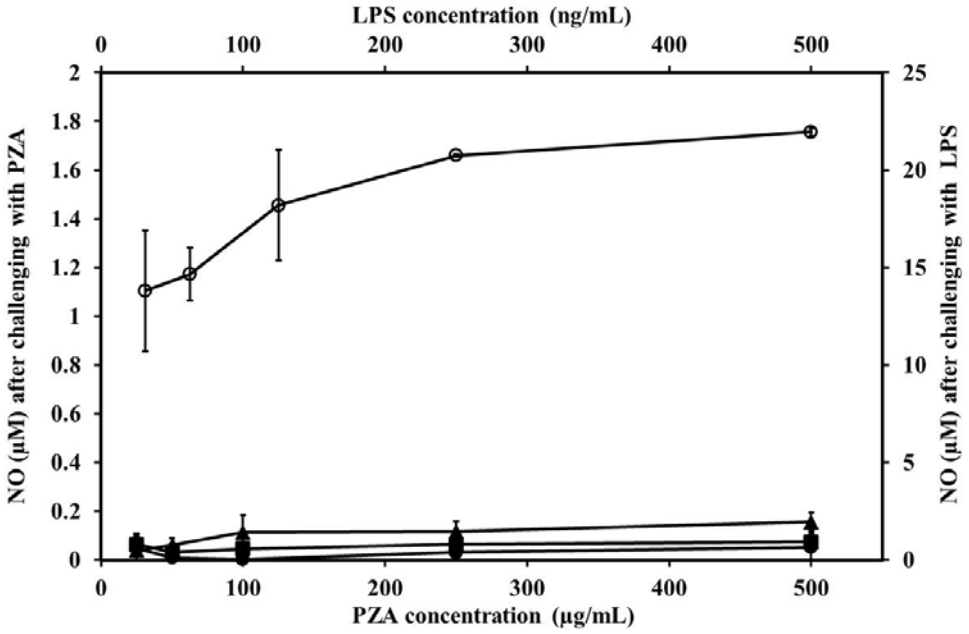


Figure 5.7 The level of inflammatory cytokine (IL-1β (a) and TNF-α (b)) and nitric oxide (c) produced from NR8383 cell lines after exposure with different concentrations of PZA (●), PZA-proliposome formulation I (▲), PZA-proliposome formulation V (■) and LPS from *E. coli* (○) for 24 h (mean ± SD, n ≥ 6) (Continued)

From figure 5.7b, LPS (31.25-500 ng/mL) stimulated NR8383 cells to produce 1385-1425 pg/mL TNF-α while after stimulation by PZA in the range of 125-500 µg/mL present in PZA-proliposome and PZA itself the TNF-α generated from NR8383 was less than 10 pg/mL. Increasing the amount of PZA in the PZA-proliposomes and PZA over the range of 31.25-500 µg/mL had no effect on the amount of TNF-α generated from NR8383 cells (p>0.05).

NO produced from NR8383 cells responding to PZA-proliposome and PZA, compared to the NO response to LPS is shown in figure 5.7c. The response was similar to that observed for IL-1β and TNF-α production. LPS at 31.25 and 500

ng/mL stimulated NR8383 cells to produce NO 13.8 ± 3.1 and 22.0 ± 0.2 μM, respectively. NO generated from NR8383 cells responding to PZA-proliposome and PZA containing between 31.25-500 μg/mL PZA was less than 0.2 μM.

As expected, NR8383 cells produced lower amounts of IL-1β, TNF-α and NO in response to PZA-proliposome and PZA, compared to LPS. The concentration of LPS used to stimulate NR8383 cells to produce an immunological response was 1,000 times less than the concentration of any amount of PZA administered as PZA itself or in PZA-proliposomes. LPS activation of NR8383 cells to produce inflammatory cytokines was significantly greater than when it was challenged with PZA-proliposomes ($p < 0.05$).

5.4.9 Activity of PZA and PZA-proliposome against *M. bovis*

Cell viability can be assessed directly through the presence of cytoplasmic esterases that cleave moieties from a lipid-soluble nonfluorescent probe to yield a fluorescent product. The product is charged and thus is retained within the cell if the membrane function is intact. Hence, viable cells are bright and nonviable cells are dim or nonfluorescent. FDA is a nonpolar, nonfluorescent molecule capable of diffusing across the cell wall and cell membranes of mycobacteria by active transport and passive diffusion (Norden et al., 1995). Once in the cytoplasm, FDA is rapidly (in 5 minutes) hydrolyzed by esterases to fluorescein, whereas nonviable bacteria have decreased quantities of active esterases thus resulting in less production of fluorescein. The viable cells are also detected at a different gate event than nonviable cells.

Only a few particles were detected in the M7H9 medium (Figure 5.8a) and they did not interfere with the analysis. Unstained viable *M. bovis* were present in the M1 region but FDA-stained viable *M. bovis* were present in the M2 zone (Figure 5.8b and 5.8c). The number of *M. bovis* can be calculated from the channel of the event (Sawatdee et al., 2006). Figure 5.8d shows dead cells (in the M1 zone) and viable cells (in the M2 zone) of *M. bovis* incubated with PZA-proliposome formulation I at 200 μg/mL for 6 days and then stained with FDA.

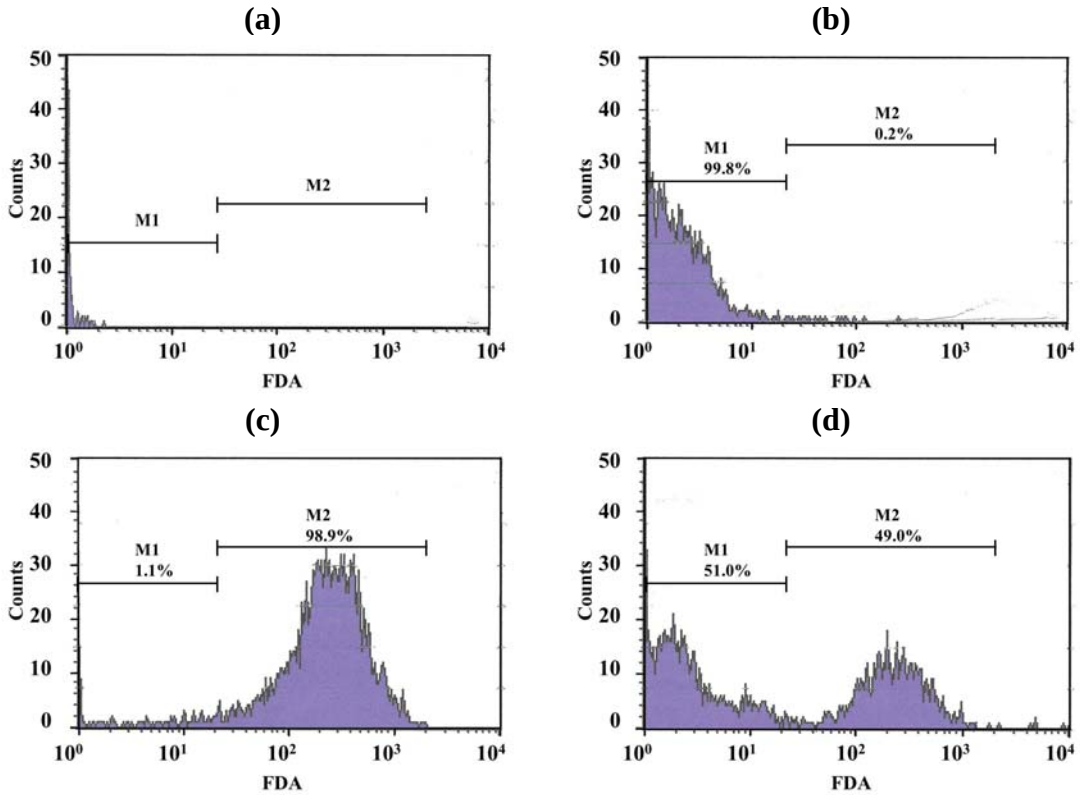


Figure 5.8 Histograms with histogram markers M1 and M2 of (a) M7H9 medium, (b) unstained viable *M. bovis*, (c) viable *M. bovis* stained with fluorescein diacetate and (d) viable *M. bovis* incubated with PZA-proliposome formulation I at 200 μg/mL for 6 days and then stained with fluorescein diacetate

The survival of *M. bovis* after incubation with PZA, PZA-proliposome formulation I or V at various concentrations of PZA. PZA, and PZA-proliposome formulation I and V showed no activity against *M. bovis.* This indicated that PZA could not kill *M. bovis* at any concentrations. Somoskovi and co-workers (2004) have reported the effect of PZA and pyrazinoic acid against *M. bovis*. This mycobacterium is known to have a characteristic single point mutation in its *pncA* gene which is responsible for its natural PZA resistance, but remains susceptible to pyrazinoic acid. PZA at 16 mg/mL gave 16% inhibition of *M. bovis* growth (Somoskovi et al., 2004).

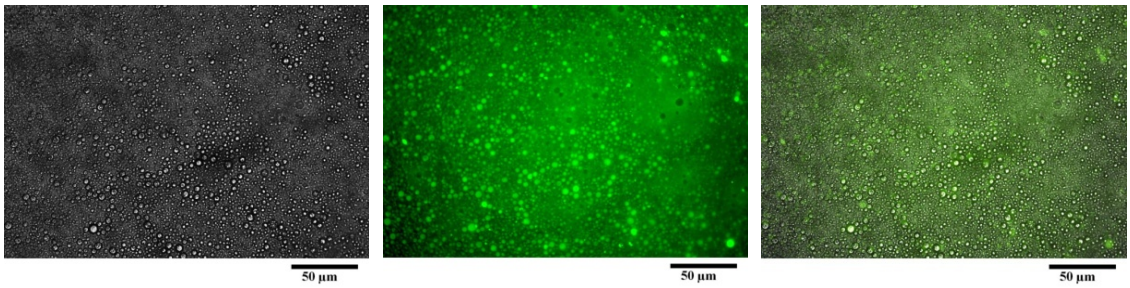
5.4.10 Activity of PZA and PZA-proliposome against *M. tuberculosis*

The MIC values of PZA, PZA-proliposome formulation I and V against *M. tuberculosis* could not be determined. There were no activities because the pH value of the test was 7.0. It might be explained that MIC values of PZA was pH dependent. In previous study, MICs of pyrazinamide for susceptible strains of *M. tuberculosis*, which were determined using radiometric techniques, were 6.2–50 mg/L when tested at a pH of 5.5 (Salfinger et al., 1988). It was recognized early on that pyrazinamide acts only in an acid environment (Rieder, 2001). The antimycobacterial activity of pyrazinamide depends on conversion of the drug to pyrazinoic acid (POA), active metabolite which is preferential accumulated in an acidic pH, by amidase pyrazinamidase (Rieder, 2001).

5.4.11 Phagocytosis of anti-TB dry powder particles by macrophage cells

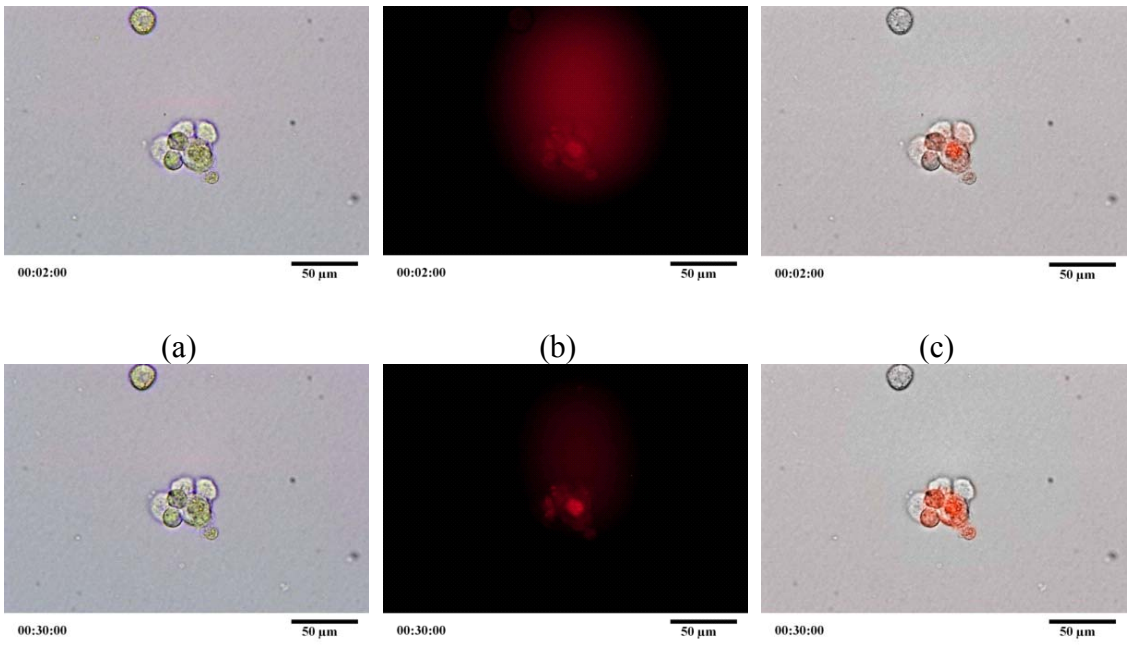
PZA-proliposome formulation I was reconstituted in distilled water and stained with Lumidot® 510. PZA-liposome photographs taken in bright field, fluorescence and overlay mode are shown in Figure 5.9a-5.9c. Their sizes were less than 1 μm. These data were harmonized with the size measurement after the reconstitution study. The liposomes were in spherical shape with narrow size distribution. Uniform nanoparticles gave a high intensity of light with stable images.

NR8383 cells were bright green cells when the cell photographs were taken in bright field mode. To observe the phagocytosis, PZA-proliposome formulation I was reconstituted in distilled water and stained with Lumidot® 640 because Lumidot® 640 could be read in the fluorescence mode. Figure 5.10 shows phagocytosis of NR8383 cells taken in different modes. In the overlay mode, the intensity of the red color increased. The phagocytosis was observed not only by the increase in the intensity of the red, but NR8383 cells were larger and some of them changed their shapes. It indicated that NR8383 could phagocytose the particles stained with Lumidot® 640. These results also correlated with the size measurement after the reconstitution study. Kanchan and Panda (2007) reported that nanoparticles of 200–600 nm in size were efficiently phagocyted (Kanchan and Panda, 2007). These results supported the observation that PZA-liposomes obtained from a PZA-proliposome reconstitution could be phagocyted by NR8383 cells.



(a) (b) (c)

Figure 5.9 Morphology of PZA-proliposome formulation I reconstituted in distilled water and stained with Lumidot® 510 in different mode; (a) bright field, (b) fluorescence and (c) overlay (bar = 50 µm)



(a) (b) (c)

(d) (e) (f)

Figure 5.10 Phagocytosis of reconstituted PZA-proliposome formulation I stained with Lumidot® 640 by NR8383 cells at (a-c) 2 and (d-f) 30 minutes in different mode; (a and d) bright field, (b and e) fluorescence and (c and f) overlay (bar = 50 µm)

5.5 Conclusion

PZA-proliposome powders were successfully produced by spray drying. Dispersion of the powders showed MMADs in a range of 4.26-4.39 μm, with a FPF of 20–30%. From XRD, DSC and FT-IR data, they indicated that there was some interaction between PZA and PZA during the spray drying process. PZA and PZA-proliposomes were shown to be less toxic to respiratory-associated cells, and also did not activate AM to produce inflammatory cytokines and nitric oxide at a toxic level that would cascade to a secondary inflammation. For antimycobacteria activity test, PZA and PZA-proliposomes were inactive against *M. bovis*. The activity of PZA and PZA-proliposomes against *M. tuberculosis* were also inactive due to environmental pH of experiment (pH 7.0).

CHAPTER 6

LEVOFLOXACIN-PROLIPOSOMES: OPPORTUNITIES FOR USE IN LUNG TUBERCULOSIS

6.1 Introduction

Nowadays, antituberculosis drugs are divided into two groups; those used for first and second line medication. First line medication drugs such as isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin have high efficacy. The second line medication drugs are ethionamide, cycloserine, para-amino-salicylic acid (PAS), capreomycin, and fluoroquinolone generally with a lower efficacy, and some have high side effects and are expensive but can be useful in cases of MDR-TB patients (Frieden et al., 2003). Although the first line medication drugs have high efficacy, long term treatment (at least six months) can result in liver and renal toxicity. In addition, it can result in accelerating the production of drug resistant TB especially MDR-TB from non-adherence. Adequate management of MDR-TB is crucial not only for recovery of the individual but also to prevent the acquisition of more resistant mutations, and the spread of these drug-resistant strains between individuals. Extensively drug-resistant TB (XDR-TB) is defined as an MDR-TB with resistance to a fluoroquinolone or at least one second-line injectable agent (amikacin, kanamycin and/or capreomycin). XDR-TB has a high mortality rate, especially among AIDS patients. This is why second line medication drugs have been introduced and the

treatment is of longer duration (Reichman and Hershfield, 2000; Frieden et al., 2003; Katherine et al., 2009).

Levofloxacin (LEV) is a new-generation fluoroquinolone antibiotic. The *in vitro* and *in vivo* activities of LEV against *M. tuberculosis*, that cause TB, is from two to threefold greater than for other quinolone antibiotics (ofloxacin and ciprofloxacin) (Marra et al., 2005). LEV showed the greatest activity against *M. tuberculosis*, with a minimum inhibitory concentration (MIC) of 1 μg/L, while the MIC of ofloxacin and ciprofloxacin was 2 and 4 μg/L, respectively (Rodrı´guez et al., 2001; Rodrı´guez et al., 2002).

LEV inhalation solution (MP-376, Mpex Pharmaceuticals) is a novel formulation for LEV that is currently being evaluated in clinical trials. MP-376 was developed for the management of cystic fibrosis (CF) patients with chronic respiratory infections due to *Pseudomonas aeruginosa* (Traini and Young, 2009; King et al., 2010; Geller et al., 2011). Following administration of MP-376 by nebulizer, high LEV concentrations were achieved in the sputum of CF patients, resulting in a high bactericidal activity (King et al., 2010). Nebulizers are used mostly in hospitals and for management of chronic disease because they are less convenient. Administration takes a long period of time (0.5-1 h) and the dose delivery is unpredictable. Dry powder inhalers, both small and portable forms, are bolus drug delivery devices that contain a solid drug, suspended in a dry powder mix that is fluidized when the patient inhales (Telko and Hickey, 2005).

In this work, LEV-proliposomes used as dry powder inhalers were prepared by a spray drying method using porous mannitol as the proliposome core carrier that has

a low density and high porosity. Their morphologies and aerodynamic properties were evaluated. Toxicity of the LEV-proliposome formulations on human bronchial epithelial cells, human lung adenocarcinoma cells and alveolar macrophages (AMs) were determined. Phagocytosis of LEV-liposomes by AMs was observed to ensure that reconstituted products can be taken up by AMs. The MIC of LEV-proliposome dry powder inhaler formulations on mycobacteria on both extracellular and intracellular within macrophage cells were determined by a flow cytometric method (Sawatdee et al., 2006). *In vivo* repeated dose toxicities of LEV-proliposome were investigated in male Wistar rats administered with the proliposomes by intratracheal instillation.

6.2 Materials

Mannitol, L-α soybean phosphatidylcholine (SPC), and cholesterol from lanolin (CH) were from Fluka (Buchs, Switzerland). Ammonium carbonate was from Ajax Finechem (NSW, Australia). Levofloxacin (LEV) was from Sigma-Aldrich (St. Louis, MO, USA), and dimethyl sulfoxide (DMSO) from Riedel-de Haën (Seelze, Germany). All other reagents were analytical grade.

All solutions (reagent packs) used for maintenance and culture of human bronchial epithelial cells (Calu-3) and human lung adenocarcinoma cell line (A549) were from Gibco (Grand Island, NY, USA). A sample of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and fluorescein diacetate (FDA) was from Sigma-Aldrich. Quantikine® RTA00 and Quantikine® RLB00 kits for rat TNF-α

and IL-1β, respectively, were from R&D Systems Inc. (Minneapolis, MN, USA). The BCG vaccine for *Mycobacterium bovis* (*M. bovis*), Middlebrook 7H9 culture medium, *M. tuberculosis* $H_{37}Ra$ (ATCC 25177) cells and alamar blue solution were supplied by the Queen Saovabha Memorial Institute (Bangkok, Thailand), Becton Dickinson and Co. (Franklin Lakes, NJ, USA), American Type Culture Collection (Rockville, MD, USA) and Alamar Biosciences/Accumed (Westlake, OH, USA), respectively.

6.3 Methods

6.3.1 Production of porous microparticulate mannitol

The method was already described in section 5.3.1.

6.3.2 Production of LEV-proliposome by spray-drying technique

The ingredients of the LEV-proliposome formulations, lipid and powder parts, are shown in Table 6.1. The weighed lipid part and LEV were dissolved in 100 mL of 95% ethanol to obtain the ethanolic lipid solution containing LEV and further sonicated for 15 minutes. Porous mannitol was suspended in the solution, and the suspension was sonicated for 15 minutes in order to deaggregate porous mannitol particles before the spray drying process began. The suspension was continuously stirred to give a homogenous suspension during spray drying. The inlet temperature was set at 90°C and the atomizer pressure was 800 kPa, with a feed rate of 3 mL/minute. The proliposome powder was obtained from the collecting chamber and kept in a desiccator until used.

Table 6.1 Formulation ingredients of LEV-proliposomes

	Lipid part		Powder part	
Formulation	**SPC (mmole)**	**CH (mmole)**	**Porous mannitol (g)**	**LEV (g)**
No. 1	0.06	0.06	0.9	0.1
No. 2	0.06	0.06	0.8	0.2
No. 3	0.06	0.06	0.6	0.4
No. 4	0.06	0.06	0.4	0.6
No. 5	0.06	0.06	0.2	0.8

6.3.3 Morphology of the microparticulate mannitol and LEV-proliposomes

The employed method was previously described in section 4.3.2.

6.3.4 Content uniformity of LEV in the proliposome powder

LEV-proliposome powder (10 mg) was randomly sampled and weighed. The powder was suspended in 10 mL of methanol to dissolve the lipid content. The volume was adjusted to 25 mL with distilled water, followed by sonication to obtain a clear solution. The LEV content from the clear solution was analyzed by high performance liquid chromatography (HPLC) as previously described in section 3.3 and 3.4.

6.3.5 *In vitro* evaluation of aerosol performance of LEV-proliposome dry powder by a cascade impactor

The method was already described in section 4.3.7.

6.3.6 Encapsulation and size measurement after reconstitution of LEV-proliposome into a liposome suspension

The employed method was previously described in section 5.3.6.

6.3.7 X-ray diffraction measurement of LEV-proliposomes

The method was already described in section 5.3.7.

6.3.8 Differential scanning calorimetry of LEV-proliposomes

The employed method was previously described in section 5.3.8

6.3.9 Fourier transform-infrared spectroscopy (FT-IR) of LEV-proliposomes

The method was already described in section 5.3.9.

6.3.10 Cell cultures: Growth of Human bronchial epithelial cells (Calu-3), Human lung adenocarcinoma cell line (A549) and Alveolar macrophage cell line NR8383

Cell culture and maintenance protocol were already described in section 5.3.10.

6.3.11 Determination of cytotoxicity of LEV-proliposome to cells in the respiratory tract

Viabilities of Calu-3, A549 and AMs, after exposed to free LEV, LEV-proliposome formulation No.1 and No.5 in the concentration range of 0.25-5.0 mg/mL were determined by using MTT assay as described in section 5.3.11.

6.3.12 Determination of the alveolar macrophage response to LEV-proliposome

a) Production of inflammatory cytokines

Evaluation of the inflammatory cytokine levels (TNF-α and IL-1β) generated from NR8383 cells responding to LEV, LEV-proliposomes or lipopolysaccharide (LPS) from *E.coli,* (positive control) was determined as described in section 5.3.12.a.

b) Nitric oxide assay by the Griess reaction

Nitric oxide (NO) generated from NR8383 cells after being challenged with LEV, LEV-proliposomes or LPS in a concentration range of 0.25-5 μg/mL for LEV and LEV-proliposomes and 25-500 ng/mL for LPS was detected as described in section 5.3.12.b.

6.3.13 Assessments of the antimycobacterial activity of LEV and LEV-proliposome

a) Culture of *M. bovis* from BCG vaccine

The method was already described in section 5.3.13.a.

b) Determination of minimum inhibitory concentration against *M. bovis*

The minimum inhibitory concentrations (MIC) of LEV and LEV-proliposome (Formulation No.1 and No.5) in the LEV concentration range of 0.25-8 µg/mL were evaluated as described in section 5.3.13.b.

c) Determination of minimum inhibitory concentration against *M. bovis* intracellular macrophage cells

The MIC of LEV and LEV-proliposome (Formulation No.1 and No.5) against intracellular growth of *M. bovis* in NR8383 cells was examined (Rojanarat et al., 2011). Prior to infection, NR838 cells were plated at a density of 10^5 cells/well in 24 well tissue cultureplates and incubated overnight in the CO_2 incubator to allow the cells to adhere on the well surface. Fresh medium containing 1% fetal bovine serum was replaced in order to reduce cell proliferation and penicillin-streptomycin mixture was excluded to avoid any interference by antibiotics on the following day. *M. bovis* was suspended in F 12 Kaighn's medium containing 1% FBS and the suspension was dispersed into individual wells at a density of five mycobacterium per macrophage. Infected NR8383 cells were then incubated at 37 $^{\circ}$C, in a 5% CO_2 incubator for 4 h.

Following incubation, the supernatant was aspirated and the wells were washed by 3 x 1000 μL with PBS to remove unphagocytosed mycobacteria. Fresh media with and without sample was added into each well. The final concentrations of LEV in the wells were 10, 20 and 40 μg/mL for LEV and LEV-proliposome formulation No.5 and 5, 10 and 20 μg/mL for LEV-proliposome formulation No.1. The infected NR8383 cells were incubated in the 37 °C incubator. Every day for one week, the media was discarded and the wells were carefully washed 3 times with PBS to remove excess sample. Determination of *M. bovis* CFU was conducted by lysing NR8383 cells with 0.125% sodium dodecyl sulfate (SDS) in PBS (500 μL) (w/v) and incubated at 37 °C for 15 minutes. The sample was taken from each well and placed in a sterile screw-cap micro-tube containing 500 μL of FDA (500 ng/mL in PBS at pH 7.4). Samples were then incubated at 37°C for 30 minutes before being analyzed using a flow cytometer and CellQuestTM software for data acquisition and analysis.

d) Determination of the minimum inhibitory concentration against *M. tuberculosis*

The antimycobacterial activity of LEV and LEV-proliposomes (Formulation No.1 and No.5) against *M. tuberculosis* H37Ra (ATCC 25177) was determined as described in section 5.3.13.c.

6.3.14 Phagocytosis of anti-TB dry powder particles by macrophage cells

Phagocytosis of anti-TB dry powder particles by macrophage cells was evaluated as described in section 5.3.14.

6.3.15 Statistical analysis

Data, when applicable, were presented as a mean ± standard deviation (SD) from at least three samples unless indicated. The data were compared using analysis of variance (ANOVA) followed by a One-Way ANOVA to determine the difference between data sets. All statistical comparisons were calculated using SPSS software version 17 (SPSS Inc., Chicago, IL). A p-value < 0.05 was considered statistically significant.

6.4 Results and discussion

6.4.1 Morphology of the microparticles

Mannitol and ammonium carbonate as pore forming agents were spray dried together. In the spray drying process, mannitol solution passed through the nozzle as droplets. These droplets dried up rapidly and the pore forming agent was volatilized. Spray dried mannitol was prepared for comparison with porous mannitol. Obtained non-porous and porous mannitol microparticles were spheres of around 3 μm (Figure 6.1a and 6.1b). Porous mannitol had a lower density than the non-porous one because of its porosity (data not shown), and this could improve the aerodynamic properties of the mannitol particles. In this study porous mannitol was used as a core carrier for proliposome, so the proliposomes were expected to have improved aerodynamic properties (Daniher and Zhu, 2008; Calvert et al., 2009).

LEV-proliposomes containing SPC, CH, porous mannitol and LEV were successfully produced using the spray drying technique. The morphology of the LEV-

proliposome formulations No.1 to No.5 are shown in Figure 6.1c-6.1g. Spherical microparticles were observed in the formulation No.1 to No.3 (Figure 6.1c-6.1e) with a high (40-90%) content of porous mannitol, while formulation No.4-5 (Figure 6.1f and 6.1g) containing porous mannitol produced an irregular shape with some tiny particles (less than 1 μm) or elongated particles (Figure 6.1f and 6.1g) adhering to large aggregated particles. The morphology of the LEV raw material is shown in Figure 6.1h. To prepare the LEV-proliposomes, the lipid part and LEV were dissolved in 95% alcohol. Porous mannitol was then dispersed in that solution. Porous mannitol acted as a proliposome core carrier, LEV could be adsorbed on the carrier and coated with lipid. In this study, LEV-proliposome formulations No.1 to No.3 were spherical in shape. This might result from the formulations having sufficient porous mannitol. However formulation No.4 and No.5, had irregular particle shapes with some spheres. It could be that the amount of porous mannitol in formulation No.4 and No.5 was insufficient to produce spherical LEV-proliposome particles. Porous mannitol is an important ingredient for producing spherical particles using the spray drying method. LEV-proliposome formulations were different in shape from pure LEV (Figure 6.1h). The spray drying process modified the particle morphology. The ideal LEV-proliposome formulation should contain not less than 40% porous mannitol to obtain perfect spherical particles.

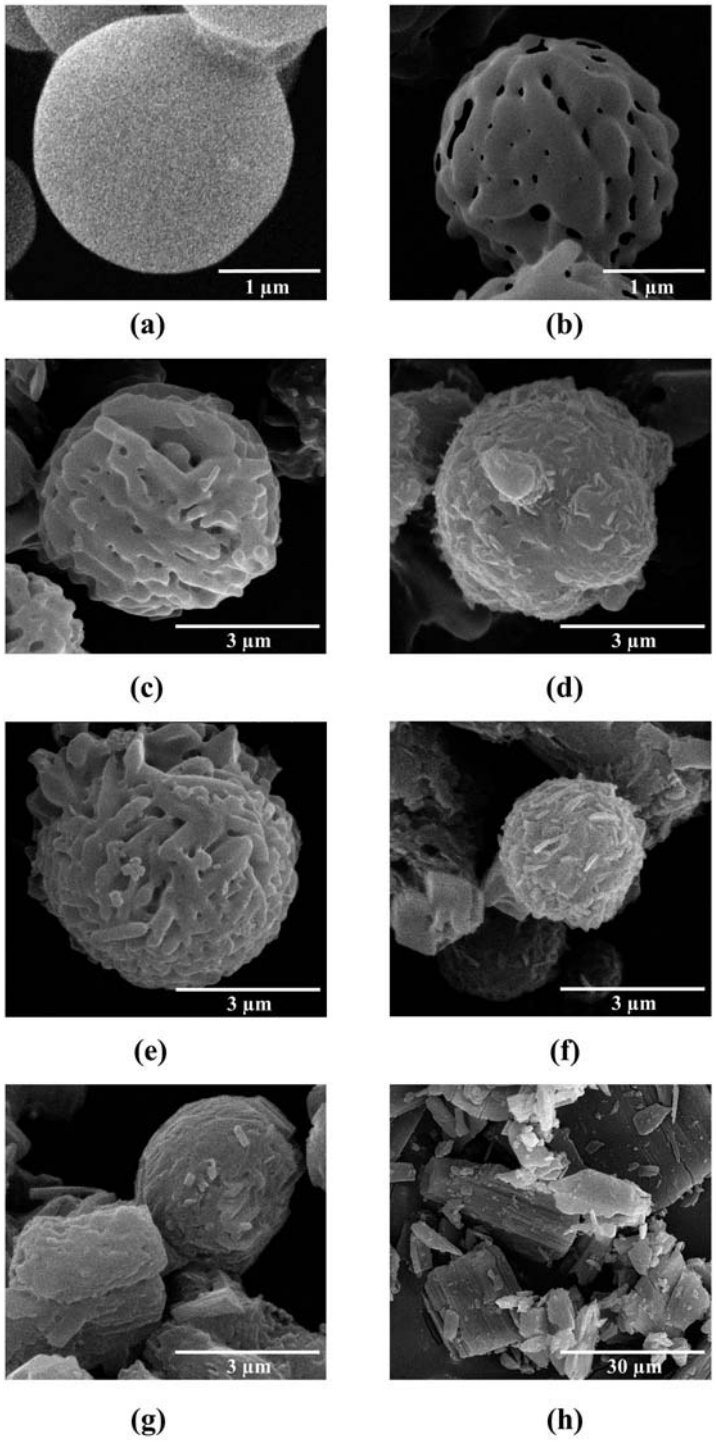


Figure 6.1 The SEM images of (a) spray dried mannitol (bar =1 µm), (b) porous mannitol (bar =1 µm), (c-g) LEV-proliposome formulation No.1 to No.5 (bar =3 µm) and (h) LEV (bar =30 µm)

6.4.2 *In vitro* evaluation of aerosol performance of LEV-proliposome dry powder by a cascade impactor

From Table 6.2, yields of LEV-proliposomes were 63-90%. Formulation No.1 had the highest yield. Higher content of LEV, lower yield of proliposomes were obtained. And the % RSD of the uniformity of the drug content was less than 6. This uniformity was acceptable (USP 30 and NF 25; USP-NF, 2007). It indicated that there was a uniform distribution of the active ingredient throughout the LEV-proliposomes produced using the spray drying process. All formulations showed their contents in the range of 96-110% of the labelled amount.

LEV-proliposome formulations showed no difference in MMAD (4.15-4.44 μm, $p > 0.05$). MMAD values were correlated with the FPF (the fraction of particles smaller than 4.4 μm) in the range of 13-38% and the ED value was in the range of 76-91%. The FPF increased when the MMAD value decreased. LEV-proliposome formulation No.4 gave a lower MMAD, and a higher FPF, with the highest ED value of 91% among all the formulations. The particles that could reach to the lower part of the respiratory tract should be larger than 1 μm and smaller than 5 μm when traveling in the air (Daniher and Zhu, 2008). From the results, all prepared LEV-proliposome formulations met this requirement.

LEV-proliposome formulations in this study were prepared using porous mannitol as the core proliposome carrier in different contents (20, 40, 60, 80 and 90%). From the aerosolization properties of all formulations, the LEV-proliposome formulation No.4 containing only 40% porous mannitol had the most suitable aerosolization properties and the particle morphology was spherical in shape. To

deliver dry powder particles to the lower part of the respiratory tract, both the formulation and device factors were considered.

6.4.3 Encapsulation and size measurement after reconstitution of LEV-proliposome into a liposome suspension

Proliposomes immediately formed a liposomal dispersion on contact with water (Wagner and Vorauer-Uhl, 2011). Liposome can be used for the encapsulation of hydrophillic and hydrophobic substances (Wagner and Vorauer-Uhl, 2011). The LEV-proliposome was reconstituted into an LEV-liposome, the vesicle sizes of the LEV-liposomes are shown in Table 6.2. The sizes of the LEV-liposome had a normal distribution. The mean size ranges were 466-670 nm from all formulations except Formulation No.5 had sizes around 1000 nm. All polydispersity index was about 0.3. Formulation No.4 gave the lowest vesicle size (466 nm). Macrophage cells can take up particles that were 200-600 nm in size (Kanchan and Panda, 2007). This indicated that many of the LEV-liposomes might be phagocytised.

Table 6.2 Aerosolization properties of LEV-proliposome formulations and properties of LEV-liposomes (mean ± SD, n=3)

Formulation (LEV: porous mannitol)	% Yield	Content uniformity (%)	MMAD (µm)	FPF (%)	Emitted dose (%)	Encapsulation (%)	Size measurement (nm)	Polydispersity index
I (1:9)	90.1 ± 4.5	103.5 ± 5.3	4.44 ± 0.26	13.5 ± 1.8	76.3 ± 3.5	23.9 ± 1.5	563 ± 18	0.290
II (2:8)	84.7 ± 1.3	96.6 ± 2.8	4.33 ± 0.23	22.5 ± 6.0	80.3 ± 6.2	21.9 ± 1.7	672 ± 25	0.337
III (4:6)	70.6 ± 1.7	109.9 ± 3.8	4.20 ± 0.01	27.2 ± 1.2	81.6 ± 5.1	18.7 ± 1.3	617 ± 14	0.296
IV (6:4)	62.9 ± 3.4	97.4 ± 1.4	4.15 ± 0.03	38.1 ± 4.6	91.3 ± 6.3	16.9 ± 1.5	466 ± 9	0.221
V (8:2)	66.9 ± 2.6	104.1 ± 3.7	4.17 ± 0.02	28.6 ± 5.0	87.2 ± 6.9	14.9 ± 1.9	1005 ± 63	0.389

For the encapsulation study, the indirect method was used to determine the LEV encapsuled in the liposome. The encapsulation efficiency of LEV was in the range of 14.9-23.9% (Table 6.2). After the LEV-proliposome was reconstituted with distilled water, free LEV would dissolve instantly. Ultracentrifugation was used for separation of the free LEV and LEV-encapsulated liposomes, the liposome was packed into a pellet at the bottom of centrifuge tube. Free LEV from the supernatant was measured. The encapsulation efficiency was calculated from the difference between the LEV content and the free LEV values. Formulation No.1 had the highest LEV encapsulation, while formulation No.5 had the lowest. Increasing the drug loading produced a lower encapsulation. Perhaps the lipid amount was not sufficient to encapsulate the drug, so a low LEV loading amount in the formulation gave a high encapsulation efficiency. In the spray-drying process, LEV could be incorporated into the lipid part before the lipid coated onto the porous mannitol particles. An LEV:porous mannitol ratio of 1:9 gave the highest encapsulation efficiency. It might be explained that hydrophobic drugs can be encapsulated in lipid layers, while hydrophilic drugs are encapsulated inside the aqueous phase (Thompson et al., 2009). LEV is a hydrophobic drug, so it can be encapsulated only in the lipid layers resulting in low encapsulation. To enhance the encapsulation of a hydrophobic drug in liposomes, increasing the water-solubility of hydrophobic drug could be improved such as by using liposomes-encapsulated with cyclodextrin inclusion complexes (Chen et al., 2007).

6.4.4 X-ray diffraction measurement of LEV-proliposome

Figure 6.2 and Table 6.3 show the XRD pattern and XRD data of the porous mannitol, LEV-proliposome formulations No.1 to No.5 and LEV. The porous mannitol diffractogram exhibited sharp reflections at 2θ values of 14.58°, 16.76°, 18.70°, 20.38°, 21.06°, 21.67°, 23.35° and 29.43° (Figure 6.2a). Whereas the diffractogram of LEV showed reflections at 2θ values of 6.62°, 9.68°, 13.06°, 15.76°, 18.88°, 19.44°, 26.31° and 26.67° (Figure 6.2g, Table 6.3). The sharp reflections of the diffractogram of the porous mannitol and LEV confirmed the crystallinity of the ingredients.

LEV-proliposome formulations No.1 and No.2 showed intense peaks of crystallinity (Figure 6.2b and 6.2c), similar to that of porous mannitol. For the formulations No.3, No.4 and No.5, new distinct reflections were presented at 2θ value (calculated $d_{spacing}$) of : 5.99° (14.75) and 10.91° (8.11) for formulation No.3, 5.98° (14.77), 10.90° (8.12) and 25.23° (3.53) for formulation No.4 and 6.02° (14.69), 10.93° (8.09), 13.96° (6.34), 20.74° (4.28) and 25.96° (3.43) for formulation No.5 (Figure 6.2d-6.2f, Table 6.3).

The X-ray diffraction patterns of the LEV-proliposome formulation No.3, No.4 and No.5 were different from that of porous mannitol and LEV. It may indicate that new crystals or cocrystal had been produced.

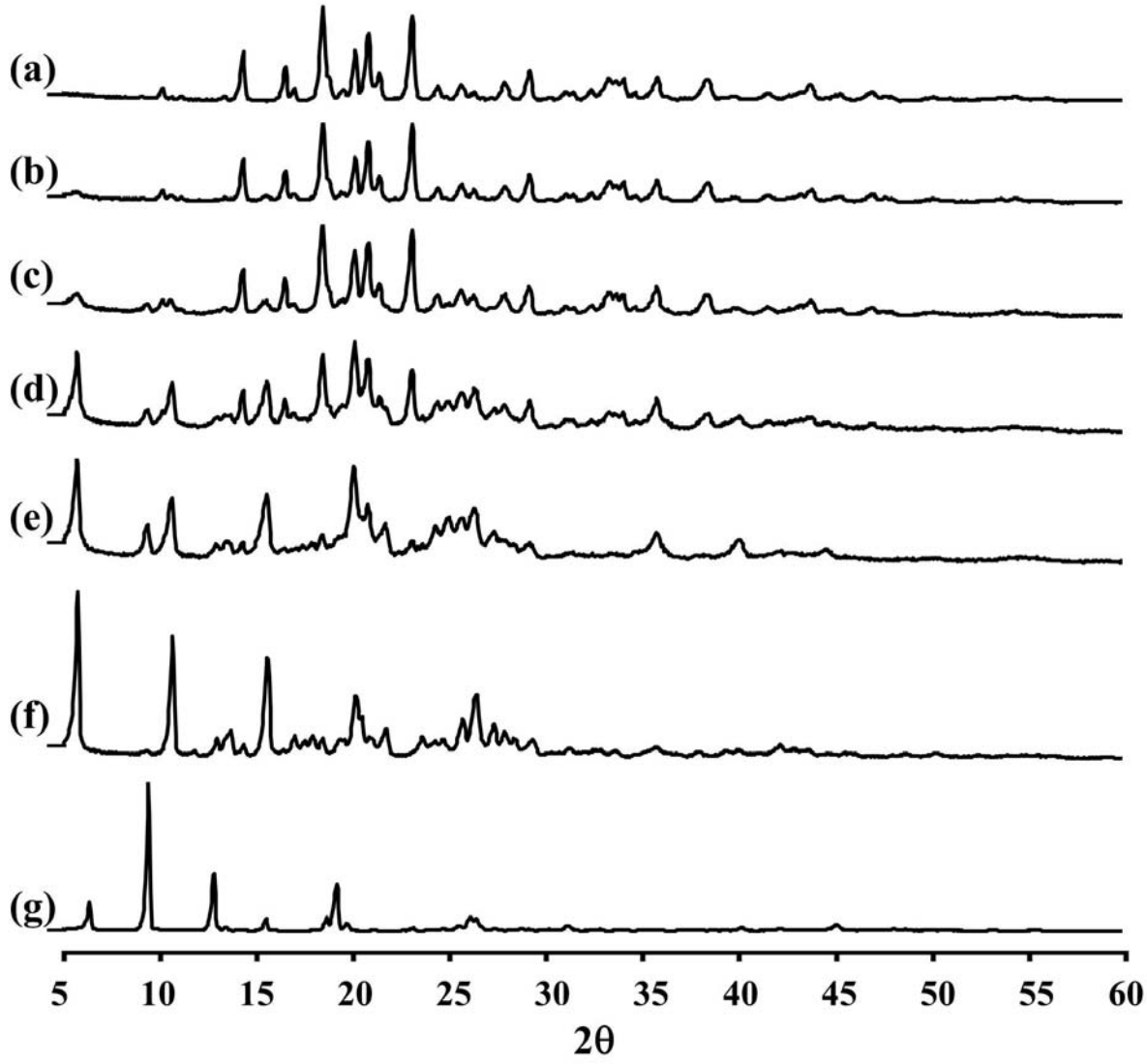


Figure 6.2 The X-ray diffraction patterns of (a) porous mannitol, (b-f) LEV-proliposome formulation No.1 to No.5 and (g) LEV

Table 6.3 X-ray diffraction peaks (2θ) with d-spacing in the bracket (°A) of porous mannitol, LEV-proliposomes and LEV

Material or formulation						
Porous mannitol	No. 1	No. 2	No. 3	No. 4	No. 5	LEV
14.58 (6.08)	14.57 (6.08)	14.57 (6.08)	5.99 (14.75)	5.98 (14.77)	6.02 (14.69)	6.62 (13.34)
16.76 (5.29)	16.76 (5.29)	16.74 (5.30)	10.91 (8.11)	9.62 (9.19)	10.93 (8.09)	9.68 (9.14)
18.70 (4.74)	18.71 (4.74)	18.70 (4.75)	15.85 (5.59)	10.90 (8.12)	13.96 (6.34)	13.06 (6.78)
20.38 (4.36)	20.39 (4.36)	20.36 (4.36)	18.70 (4.75)	15.84 (5.59)	15.87 (5.58)	15.76 (5.62)
21.06 (4.22)	21.06 (4.22)	21.06 (4.22)	20.36 (4.36)	20.32 (4.37)	20.39 (4.35)	18.88 (4.70)
21.67 (4.10)	21.66 (4.10)	21.66 (4.10)	21.04 (4.22)	21.04 (4.22)	20.74 (4.28)	19.44 (4.57)
23.35 (3.81)	23.36 (3.81)	23.34 (3.81)	23.34 (3.81)	25.23 (3.53)	25.96 (3.43)	26.31 (3.39)
29.43 (3.04)	29.43 (3.04)	29.43 (3.04)	26.59 (3.35)	26.66 (3.34)		26.67 (3.39)

6.4.5 Differential scanning calorimetry of LEV-proliposome

The thermal behavior of the LEV-proliposomes in relation to the individual ingredients was evaluated using DSC experiments. Figure 6.3 and Table 6.4 show the DSC thermogram and thermal data for porous mannitol, LEV-proliposome formulation No.1 to No.5 and LEV. Porous mannitol showed an endothermic peak at 163.5°C and a melting point at 151.8°C. Its enthalpy ($\triangle H_f$) was 266.5 J/g (Figure 6.3a and Table 6.4). LEV displayed an endothermic peak at 236.4°C, and a melting point at 231.9°C and $\triangle H_f$ -195.6 J/g (Figure 6.3g).

The endothermic peak, melting point and $\triangle H_f$ of LEV-proliposome formulations No.1 to No.5 are shown in Figure 6.3b-6.3f and Table 6.4. For the LEV-proliposome formulations the No.1, DSC curve was similar to porous mannitol. There were two peaks in the DSC curves of formulations No.2 to No.5; the first peak was an endothermic peak and the second was an exothermic peak. They were from the porous mannitol and LEV peaks, respectively. The endothermic peak tended to decrease when the porous mannitol ratio decreased, while the exothermic peak tended to decrease when the LEV decreased. Both peaks of the LEV-proliposome formulations No.2 to No.5 showed a broadening peak with a shift to a lower temperature. This indicated that there was change in crystallinity of LEV. These results also correlated with the X-ray diffraction patterns of the LEV-proliposome formulation No.2 to No.5. There was no doubt that cocrystallisation or crystallinity changes had occurred.

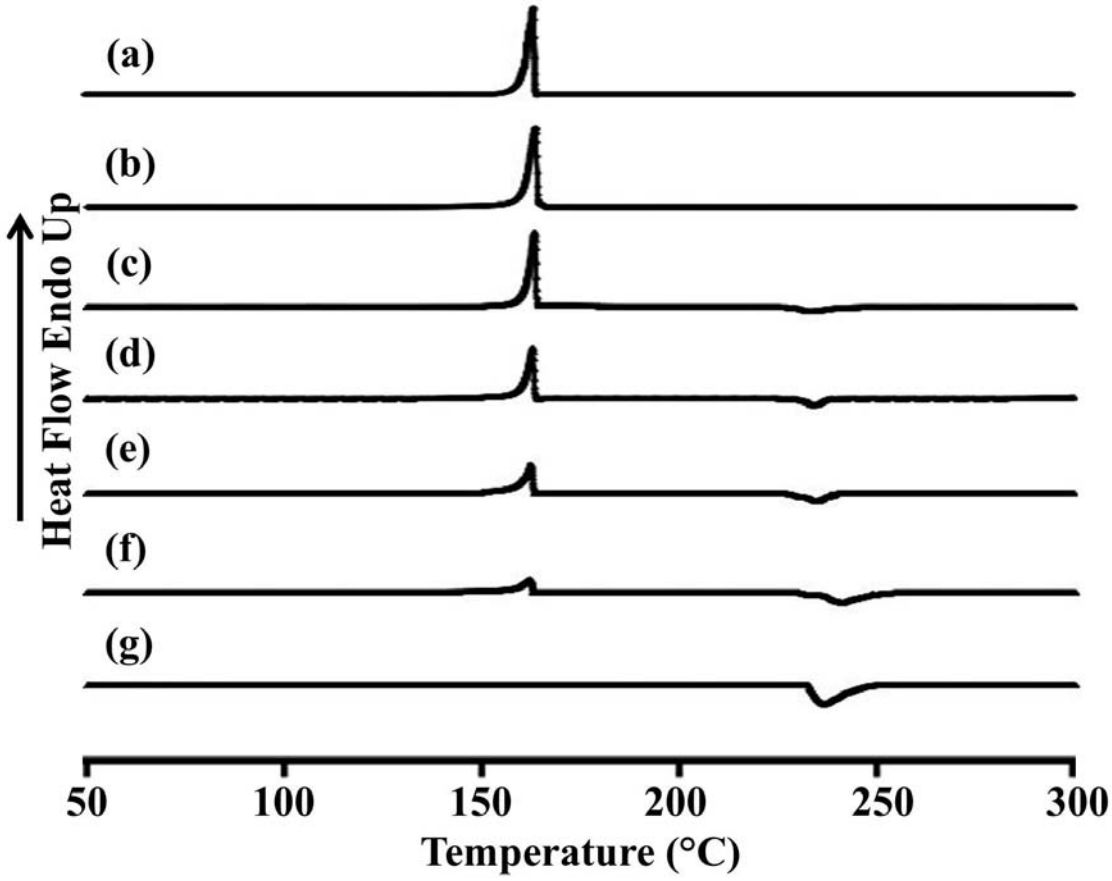


Figure 6.3 Differential scanning calorimeter thermogram of (a) porous mannitol, (b-f) LEV-proliposome formulation No.1 to No.5 and (g) LEV

Table 6.4 Differential scanning calorimeter data of porous mannitol, LEV-proliposome formulations and LEV

Material or formulation	Peak (°C)	Onset (°C)	End (°C)	Peak area (J/g)
Porous mannitol	163.5	151.8	166.5	266.5
No. 1	163.5	150.3	166.0	245.1
No. 2	163.3	151.0	166.4	196.4
	233.3	227.2	240.5	-25.2
No. 3	162.8	145.2	165.4	139.8
	234.0	223.9	241.8	-57.1
No. 4	162.3	146.4	164.9	97.4
	235.4	222.8	242.4	-79.9
No. 5	161.8	150.6	165.1	151.8
	241.2	233.2	250.3	-52.3
Levofloxacin	236.4	231.9	249.5	-195.6

6.4.6 FT-IR of LEV-proliposome

FT-IR studies were conducted to investigate the relationships between the components of the proliposomes. The mannitol spectrum (Figure 6.4a) exhibited characteristic bands of O-H in the plane vibration (1419 cm^{-1}) and O-H stretching vibration (1080 and 1019 cm^{-1}). The characteristic peaks of LEV (Figure 6.4g) were seen at 1725 cm^{-1}, 1621 cm^{-1} and 1241 cm^{-1} that corresponded to C=O stretching (vibration), C=O stretching and C-F stretching (vibration), respectively. The O-H in the plane vibration peak was absent in the formulations No.4 and No.5. The C-F

stretching vibration disappeared in the formulation No.1, No.2 and No.3. In the formulation No.1 to No.5 spectrum (Figure 6.4b-6.4f), the C=O stretching vibration peak shifted about 10 cm^{-1}(Table 6.5).

From the X-ray diffraction patterns and FT-IR spectra, all data showed that some changes of crystallinity did occur in the LEV-proliposome formulations No.2 to No.5. This is interesting point to be further investigated whether LEV new polymorph was formed.

Table 6.5 Specific characteristics of LEV-proliposomes as compared with porous mannitol and LEV from the IR spectra

Material or formulation	**Assignments**			
	C=O stretching vibration	**O-H in plane vibration**	**C-F stretching vibration**	**O-H stretching vibration**
Porous mannitol	-	1419	-	1080, 1020
No. 1	1624	1419	-	1080, 1020
No. 2	1715, 1624	1419	-	1080, 1020
No. 3	1715, 1623	1407	-	1081, 1020
No. 4	1715, 1622	-	1242	1085, 1022
No. 5	1714, 1622	-	1241	-
LEV	1725, 1621	-	1241	-

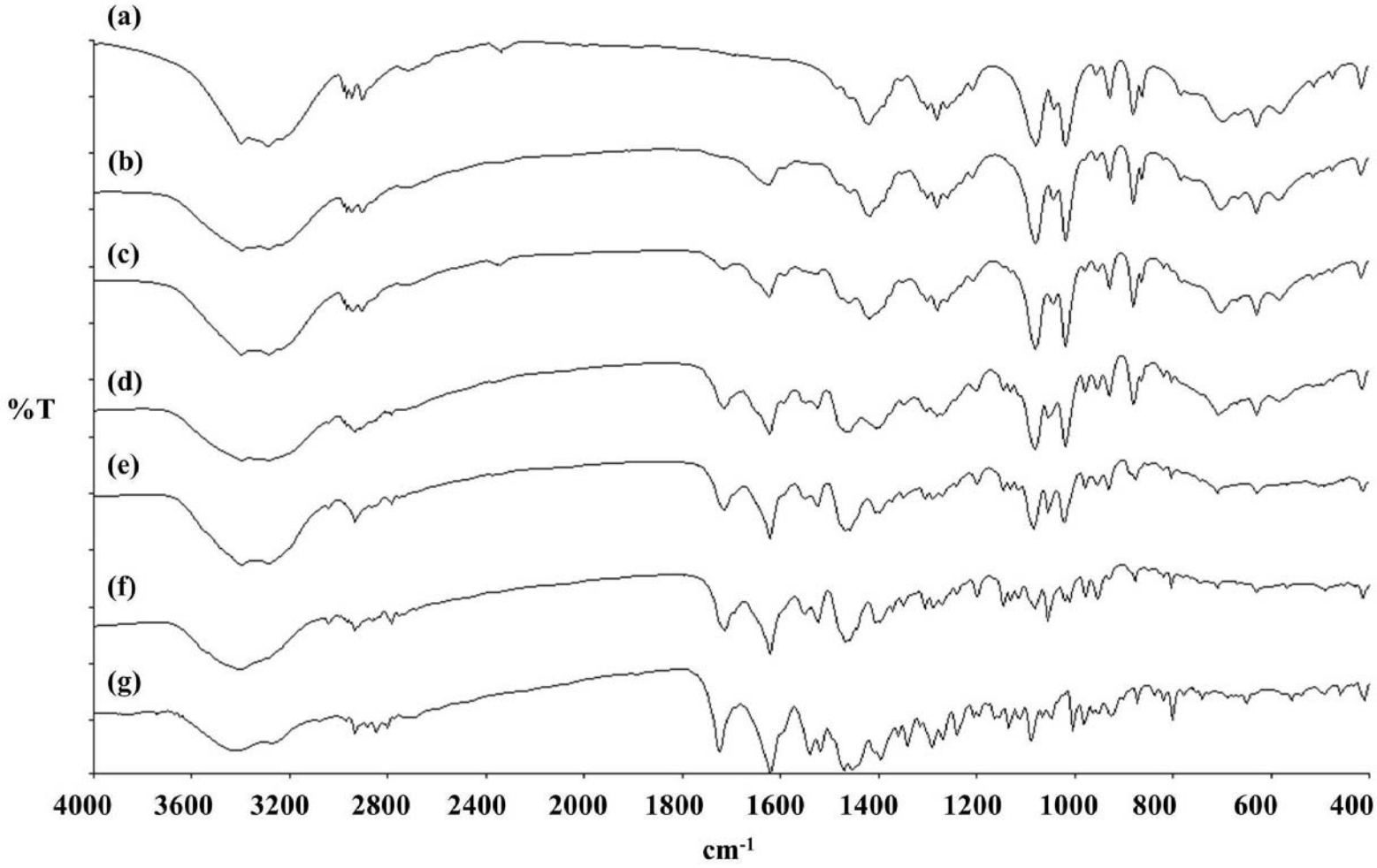


Figure 6.4 FT-IR spectra of (a) porous mannitol, (b-f) LEV-proliposome formulation No.1 to No.5 and (g) LEV

6.4.7 Cytotoxicity of LEV-proliposome to cells in the respiratory tract

The viabilities of respiratory associated cells including bronchial epithelial cells, alveolar basal epithelial cells and AMs were evaluated after being challenged with different concentrations of LEV and LEV-proliposomes (Formulation No.1 and No.5). In the cytotoxicity study, formulations No.1 and 5 were chosen because of their porous mannitol content, aerodynamic properties and encapsulation efficacy. LEV-proliposome formulation No.1 and 5 contained the highest and lowest amounts of porous mannitol and encapsulation efficacy. Not only was an effect of the amount of LEV, there was also effects of the amount of porous mannitol, free drug and LEV-liposome in the formulation on the respiratory cell lines were determined.

Figure 6.5a shows the Calu-3 cell viability after being exposed to LEV, and LEV-proliposome formulations No.1 and No.5 at various concentrations (0.25-5 μg/mL LEV contents). Calu-3 cell viability was more than 80% after being exposed to both LEV-proliposome formulations, while the cell viability was less than 80% only in the case of being exposed to LEV alone. The concentrations of the 0.25-5 μg/mL LEV content of LEV and LEV-proliposome formulation No.5 was toxic to A549 cells (cell viability less than 80%), while the LEV-proliposome formulation No.1 was nontoxic to A549 cells (Figure 6.5b). For NR8383 cells (Figure 6.5c) the viability after being exposed to LEV, LEV-proliposome formulation No.1 and No.5 at all LEV concentrations (0.25-5 μg/mL LEV content) was not affected.

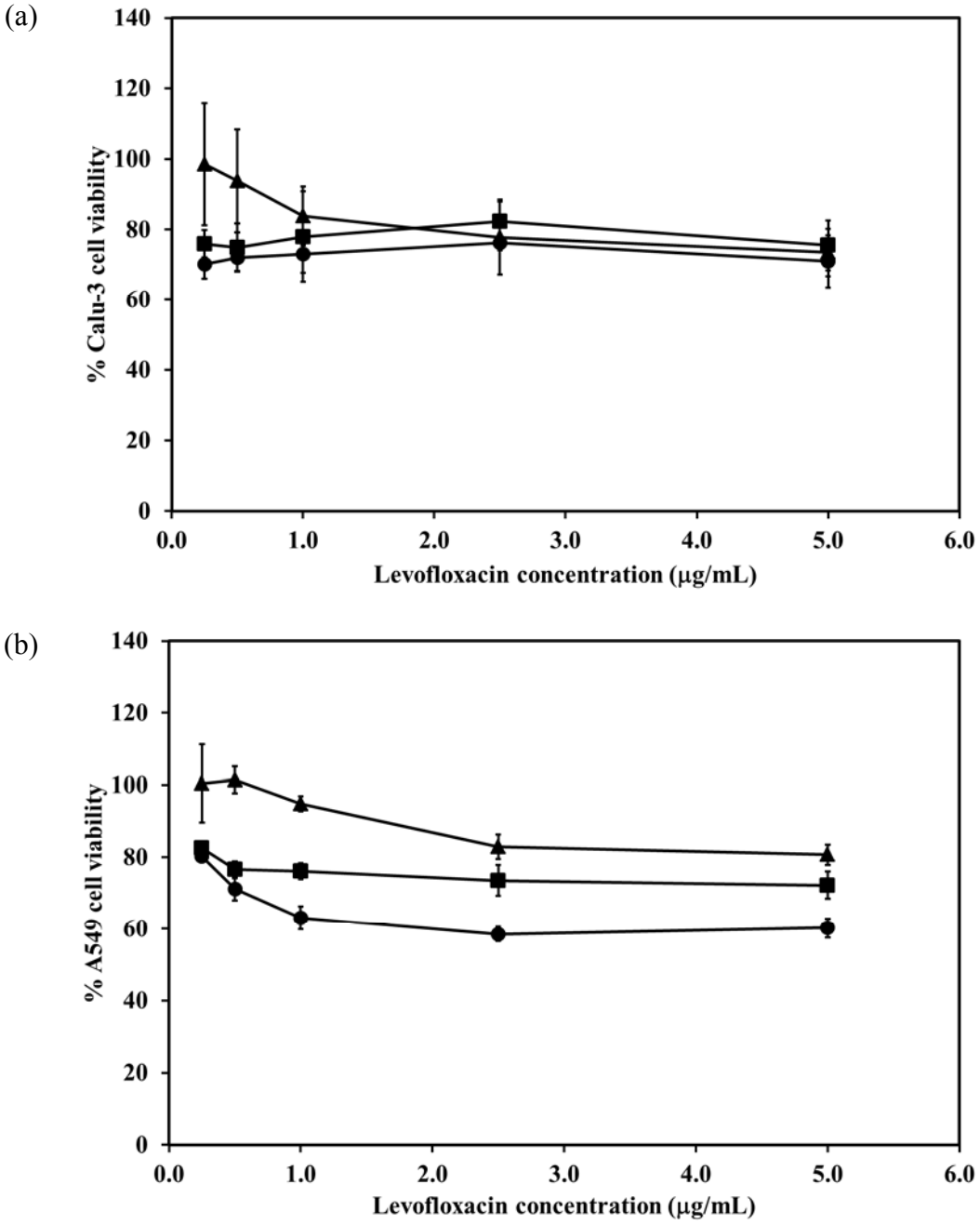


Figure 6.5 Viability of Calu-3 (a), A549 (b) and NR8383 (c) cell lines after exposed with different concentrations of LEV (●), LEV-proliposome formulation No.1 (▲) and LEV-proliposome formulation No.5 (■) (mean ± SD, $n \geq 6$)

(c)

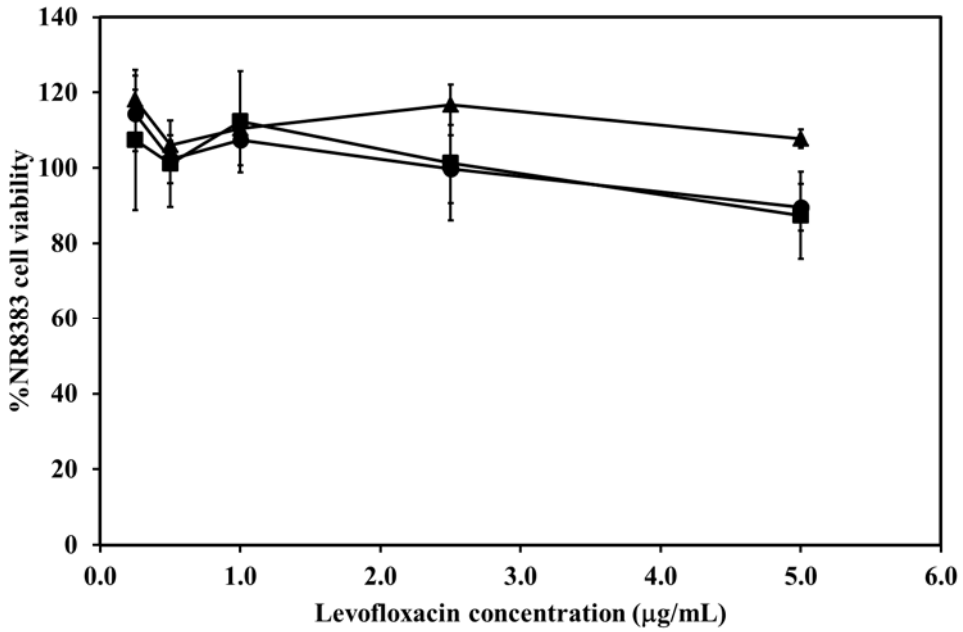


Figure 6.5 Viability of Calu-3 (a), A549 (b) and NR8383 (c) cell lines after exposed with different concentrations of LEV (●), LEV-proliposome formulation No.1 (▲) and LEV-proliposome formulation No.5 (■) (mean ± SD, $n \geq 6$) (Continued)

This cell viability study showed that all 3 cultured cell lines were more resistant to the toxic effects of LEV in the form of LEV-proliposome formulation No.1 and No.5 than pure LEV. Hence proliposome could be used to reduce the drug toxicity to cells in the respiratory tract (Chang et al., 2008).

6.4.8 Effect of LEV-proliposome on production of IL-1β, TNF-α and NO by NR8383 cell line

Alveolar macrophage NR8383 cells, produce inflammatory mediators including IL-1β, TNF-α and NO after being stimulated. To evaluate the effect of LEV, LEV-proliposome formulation No.1 and No.5 on the production of

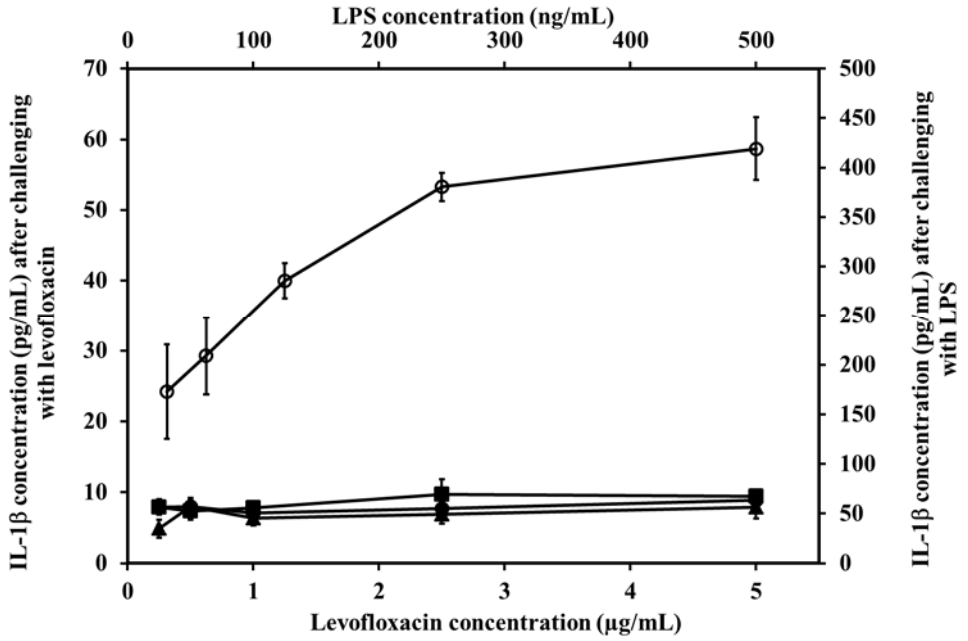
inflammatory mediator by NR8383 cells, LPS (lipopolysaccharide) from *E. coli* was used as positive control and cell culture media was used as negative control.

The IL-1β levels produced from NR8383 cells responding to LPS, IL-1β increased from 173±47.4 pg/mL after being challenged with 31.25 ng/mL of LPS and 419±31.8 pg/mL after challenging with 500 ng/mL LPS. In contrast after stimulation by LEV, LEV-proliposome formulation No.1 and No.5 (0.25-5 μg/mL LEV content) NR8383 cells produced less than 10 pg/mL IL-1β and there were no significant differences among the preparations (p>0.05) (Figure 6.6a).

A similar situation was obtained for the generation of TNF-α. Stimulation by 31.25 and 500 ng/mL LPS induced production of 1538 and 1833 pg/mL TNF-α respectively by NR8383 cells whereas the response to LEV, LEV-proliposome formulation No.1 and No.5 in the range of 0.25-5 μg/mL as LEV content, cytokine was always less than 10 pg/mL (Figure 6.6b). Again TNF-α production from NR8383 cells responding to LEV, LEV-proliposome formulation No.1 and No.5 in range of 0.25-500 μg/mL as LEV content did not differ (p>0.05).

Nitric oxide producted by NR8383 cells also responded in a similar way. Stimulation by LPS at 31.25 and 500 ng/mL stimulated NR8383 cells to produce NO at 13.8±3.1 and 22.0±0.2 μM, respectively. NO generated from NR8383 cells responding to LEV, LEV-proliposome formulation No.1 and No.5 in range of 0.25-5 μg/mL as LEV content was less than 0.2 μM (Figure 6.6c).

(a)



(b)

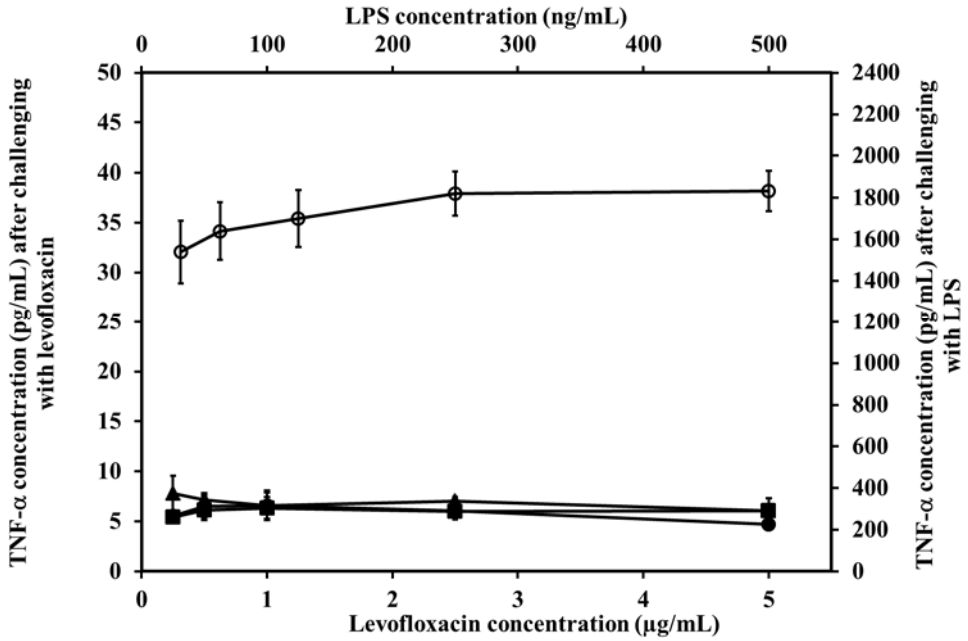


Figure 6.6 The level of inflammatory cytokine (IL-1β (a) and TNF-α (b)) and nitric oxide (c) produced from NR8383 cell lines after exposure with different concentrations of LEV (●), LEV-proliposome formulation No.1 (▲), LEV-proliposome formulation No.5 (■) and LPS from *E. coli* (○) for 24 h (mean ± SD, n ≥ 6)

(c)

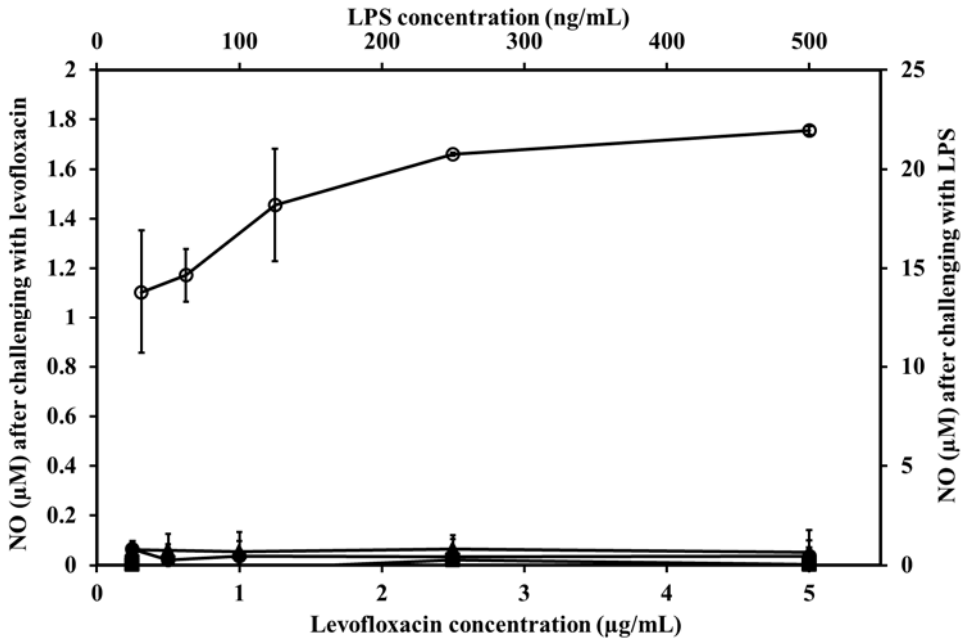


Figure 6.6 The level of inflammatory cytokine (IL-1β (a) and TNF-α (b)) and nitric oxide (c) produced from NR8383 cell lines after exposure with different concentrations of LEV (●), LEV-proliposome formulation No.1 (▲), LEV-proliposome formulation No.5 (■) and LPS from *E. coli* (○) for 24 h (mean ± SD, n ≥ 6). (Continued)

As expected, NR8383 cells produced negligible amounts of IL-1β, TNF-α and NO in response to LEV, LEV-proliposome formulation No.1 and No.5 compared to the LPS. The concentration of LPS used to stimulate NR8383 cells to produce the immunological response was 10 times less than the concentration of any LEV-proliposome. LPS activation of NR8383 cells to produce inflammatory cytokines was significantly greater than when it was challenged with LEV, LEV-proliposome

formulation No.1 and No.5 ($p < 0.05$). LEV did not affect cytokine production by AMs (Tsivkovskii et al., 2011).

6.4.9 Activity of LEV and LEV-proliposome against *M. bovis*

Cell viability can be assessed directly through the presence of cytoplasmic esterases that cleave moieties from a lipid-soluble nonfluorescent probe to yield a fluorescent product. The product is charged and thus is retained within the cell if the membrane function is intact. Hence, viable cells are bright and nonviable cells are dim or nonfluorescent. Fluorescein diacetate (FDA) is a nonpolar, nonfluorescent molecule capable of diffusing across the cell wall and cell membranes of mycobacteria by active transport and passive diffusion (Norden et al., 1995). Once in the cytoplasm, FDA is rapidly (in 5 minutes) hydrolyzed by esterases to fluorescein, whereas nonviable bacteria have decreased quantities of active esterases thus resulting in less production of fluorescein. The viable cells are also detected at a different gate event than nonviable cells.

Figure 6.7 shows the survival of *M. bovis* after incubation with LEV, LEV-proliposome formulation No.1 and No.5 at various concentrations. LEV and LEV-proliposome formulation No.5 showed similar activity against *M. bovis*, while LEV-proliposome formulation No.1 showed different activity at day 6. At day 1-4, all samples showed no activity at all concentrations. At day 6, viable bacilli were only 10% in most samples treated with LEV at >1 μg/mL either by itself or in liposomes. It indicated that LEV could kill *M. bovis*. The MIC values of levofloxacin against *M. bovis* in this study were 1 μg/mL for LEV and 1 μg/mL for LEV-proliposome

formulation No.5 and 0.5 μg/mL for LEV-proliposome formulation No.1. It meant that formulation No.1 had better activity against *M. bovis* than other 4 formulations. These results are similar to those reported by Rastogi and co-workers (1996).

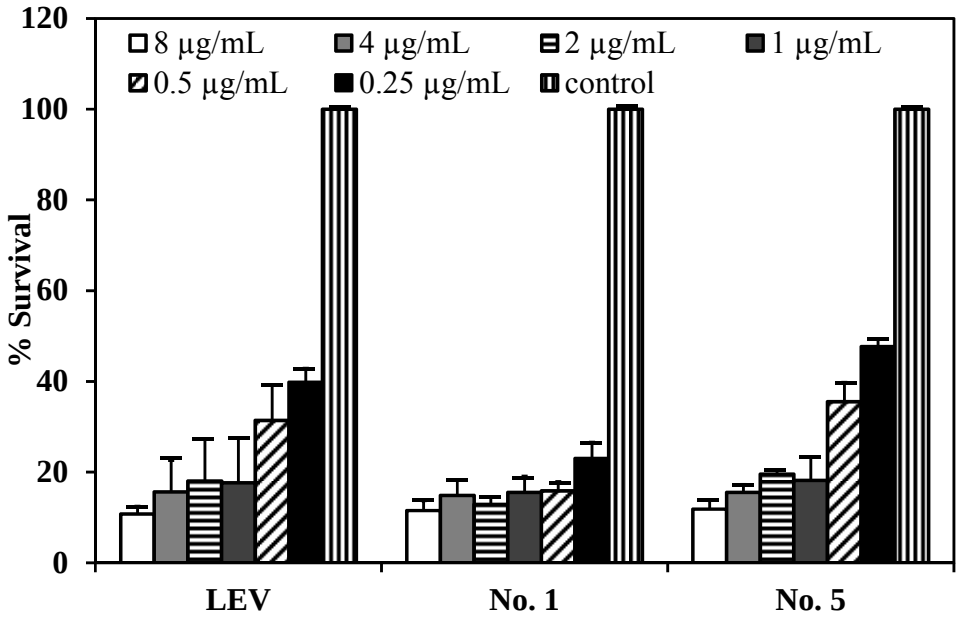


Figure 6.7 The reduction of *M. bovis* (% Survival) after incubated with (a) LEV, (b) LEV-proliposome formulation No.1 and (c) LEV-proliposome formulation No.5 at different concentrations for 6 days (Mean ± SD, n ≥ 3)

6.4.10 Activity of LEV and LEV-proliposome against intracellular *M. bovis* in macrophage cells

The number of *M. bovis* present in intracellular macrophage cells was determined using a dot plot analysis after infected macrophage cells were lysed then stained with FDA. Lysed NR8383 cells showed few particles in selected regions (Figure 6.8a) but viable *M. bovis* intracellular macrophage cells displayed large

numbers of particles in the selected region (Figure 6.8b). The particle counts in the selected region decreased when *M. bovis* infected macrophage cells were incubated with LEV-proliposome formulation No.1 (5μg/mL) for 5 days and the numbers decreased further after 7 days (Figure 6.8c and 6.8d).

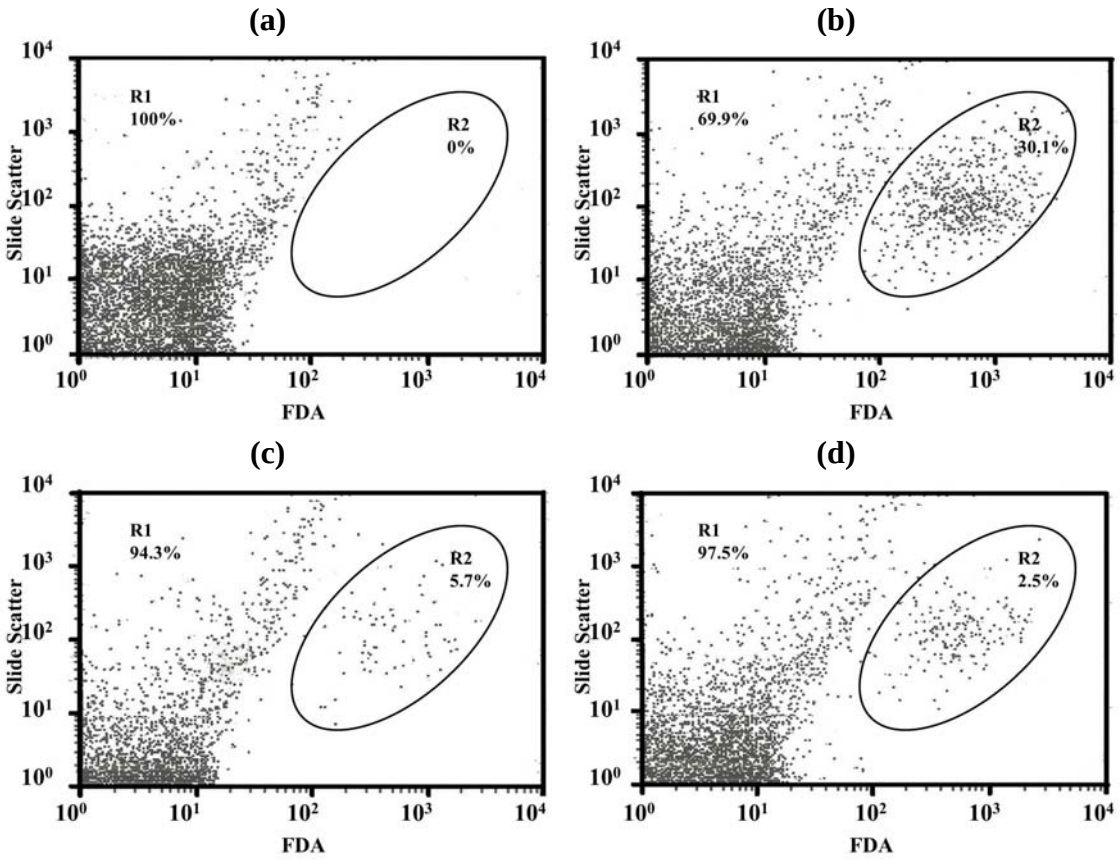


Figure 6.8 Dot plot with region of (a) un-infected macrophage cells, (b) viable *M. bovis* intracellular macrophage cells (control) and viable *M. bovis* intracellular macrophage cells incubated with LEV-proliposome formulation No.1 at 5 μg/mL for (c) 5 and (d) 7 days and then stained with fluorescein diacetate

The survival of intracellular *M. bovis* in macrophage cells after incubation with LEV, LEV-proliposome formulation No.1 or No.5 at various concentrations is shown in Figure 6.9. LEV-proliposome formulation No.1 and No.5 showed a similar curve of inhibition against *M. bovis* present in NR8383 cells, while LEV itself showed a different curve. From the 1st day to the 3th day, all samples showed no loss of viability at any concentrations. At day 4, the numbers of viable bacilli decreased after incubation with LEV alone then decreased steadily to < 10% at day 7. For the LEV-proliposome formulation No.1 from day 3 to day 4 there was no change and for the LEV-proliposome formulation No.5 had a slight loss of viability of < 10% but from day 4 to 5 the numbers decreased dramatically by < 10% for both proliposome formulations. This is due to the slow growth rate of mycobacteria and the drug did not affect on mycobacteria during first three days as micro-organisms were in dormant stage and thereafter there was dramatic reduction in micro-organisms as they were in log phase and drug could kill in this phase. This indicated that LEV-proliposome was more effective than LEV in killing *M. bovis* in infected NR8383 cells.

6.4.11 Activity of LEV and LEV-proliposome against *M. tuberculosis*

The MIC values of LEV, LEV-proliposome formulation No.1 and No.5 against *M. tuberculosis* were 0.195 μg/mL, it was lower than found in a previous study (Rastogi et al., 1996). Rastogi and co-workers (1996) reported that MIC of LEV against *M. tuberculosis* was 1 μg/mL. It showed that LEV-proliposomes had antimicrobial activity against *M. tuberculosis*. The spray drying method could therefore be used to produce effective LEV-proliposomes.

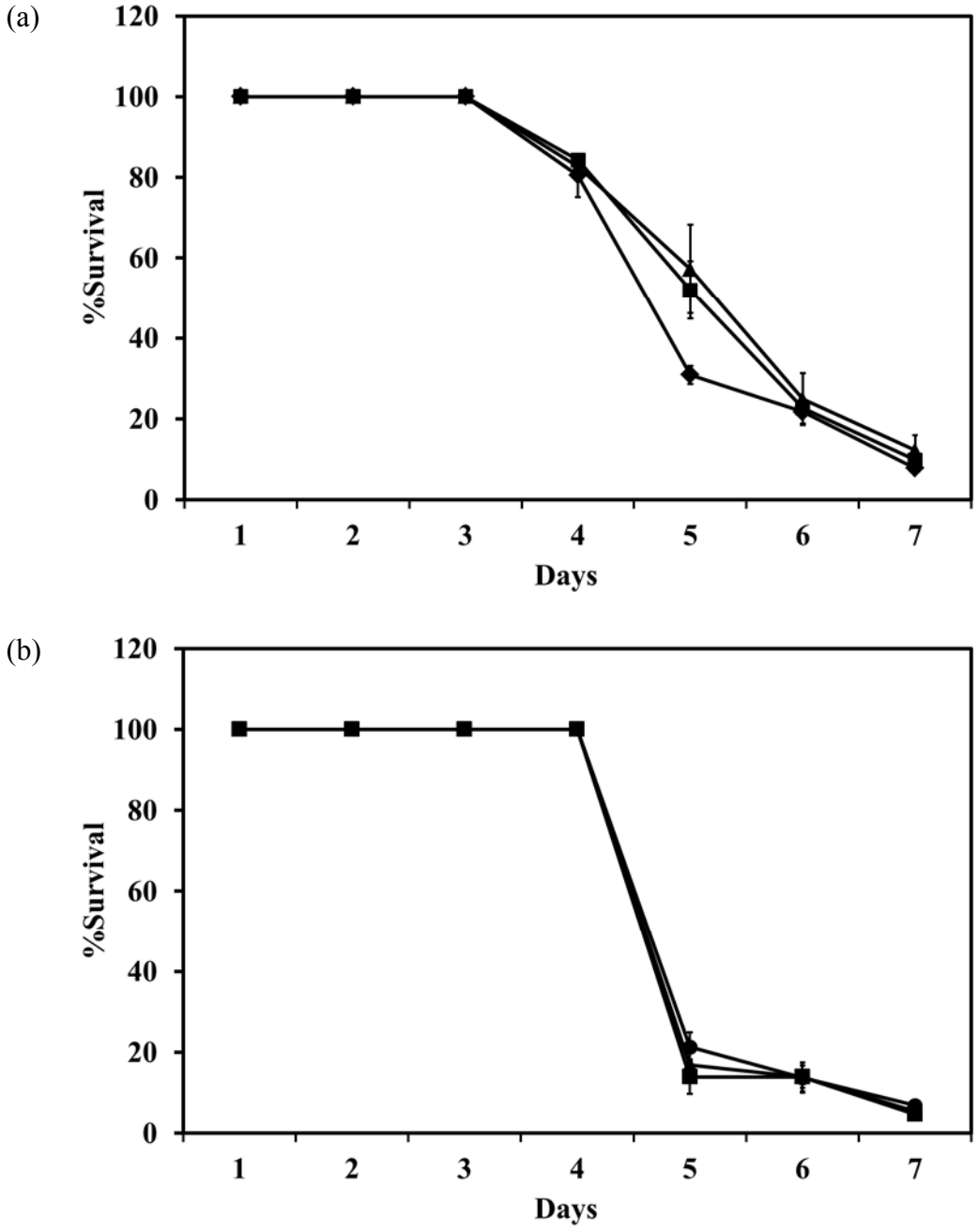


Figure 6.9 The reduction of *M. bovis* (% Survival) intracellular macrophage cells after incubated with (a) LEV, (b) LEV-proliposome formulation No.1 and (c) LEV-proliposome formulation No.5 at different concentrations; 5 (●), 10 (▲), 20 (■) and 40 (◆) µg/mL (Mean ± SD, n ≥ 3)

(c)

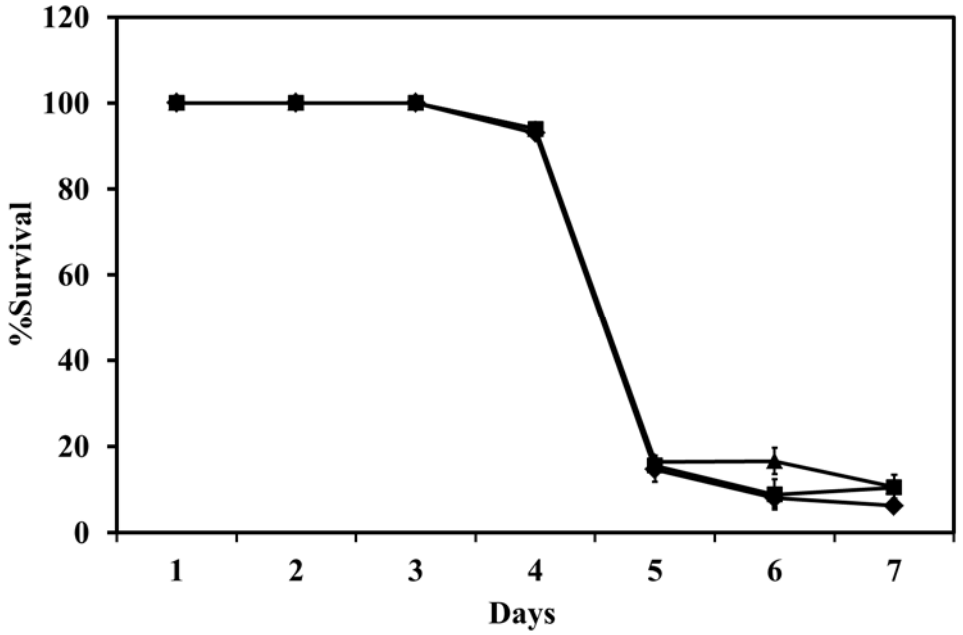
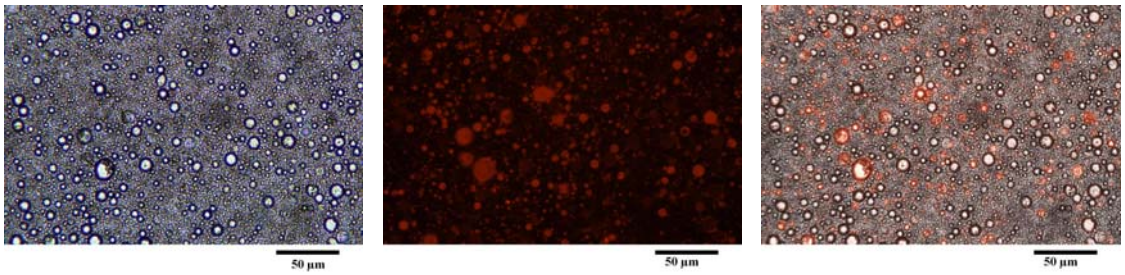


Figure 6.9 The reduction of *M. bovis* (% Survival) intracellular macrophage cells after incubated with (a) LEV, (b) LEV-proliposome formulation No.1 and (c) LEV-proliposome formulation No.5 at different concentrations; 5 (●), 10 (▲), 20 (■) and 40 (◆) μg/mL (Mean ± SD, n ≥ 3) (Continued)

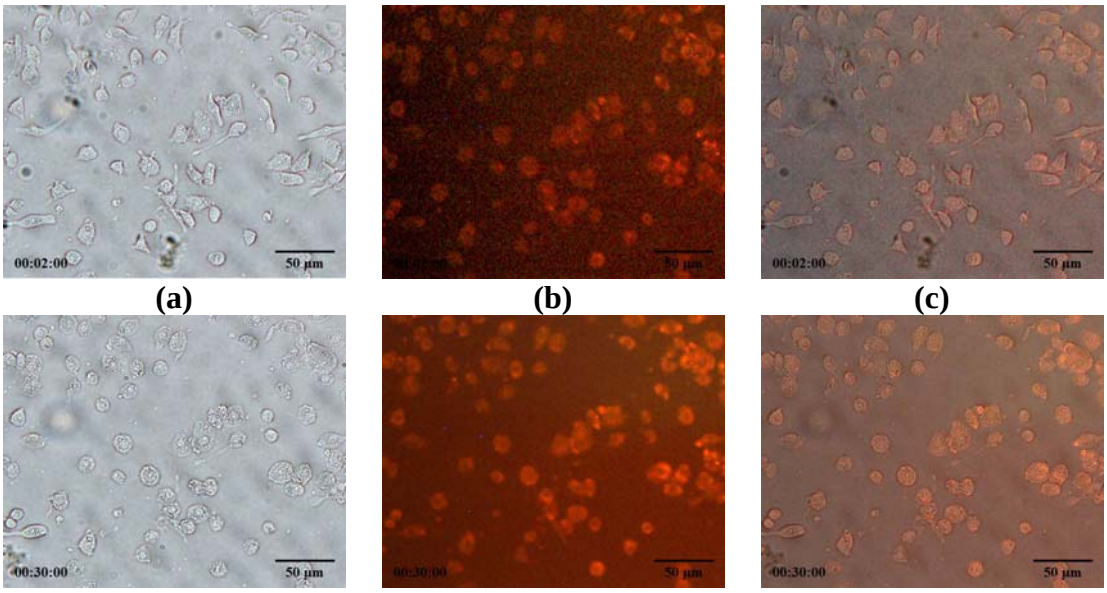
6.4.12 Phagocytosis of anti-TB dry powder particles by macrophage cells

LEV-proliposome formulation No.1 reconstituted in distilled water was stained with Lumidot® 640. Figure 6.10 shows the morphology of LEV-liposome stained with Lumidot® 640, the picture was taken in bright field (Figure 6.10a), fluorescence (Figure 6.10b) and overlay mode (Figure 6.10c). Vesicle sizes were less than 1 μm. They remained un-changed after reconstitution within 24 hours.



(a) **(b)** **(c)**

Figure 6.10 Morphology of LEV-proliposome formulation No.1 reconstituted in distilled water and stained with Lumidot® 640 in different mode; (a) bright field, (b) fluorescence and (c) overlay (bar = 50 µm)



(a) **(b)** **(c)**

(d) **(e)** **(f)**

Figure 6.11 Phagocytosis of reconstituted LEV-proliposome formulation No.1 stained with Lumidot® 640 by NR8383 cells at (a-c) 2 and (d-f) 30 minutes in different mode; (a and d) bright field, (b and e) fluorescence and (c and f) overlay (bar = 50 µm)

LEV-proliposome formulation No.1 was reconstituted in distilled water and stained with Lumidot® 640 to observe phagocytosis. Phagocytosis by NR8383 cells was taken in different modes (Figure 6.11). At 30 minutes after incubation, NR8383 cells had increased in size and shape and after staining for liposome, the intensity of red color in the overlay mode had significantly increased. This indicated that NR8383 could take up reconstituted LEV-liposomes stained with Lumidot® 640. The optimum size for efficient phagocytosis was in the range of 200-600 nm (Kanchan and Panda, 2007).

6.5 Conclusion

LEV-proliposome powders for inhalation were successfully produced using spray dry method. Their shapes were spherical, except formulation No.5 that contained only 10% porous mannitol. The MMAD values of all formulation were in a range of 4.15–4.44 μm, with a FPF of 13–38%. LEV-proliposome powders reflected the different crystallinity from LEV. LEV new polymorph may occur in the formulation. LEV and LEV-proliposomes were shown to be less toxic to respiratory-associated cells, and also did not activate AMs to produce inflammatory cytokines and nitric oxide at a level that would cascade to a secondary inflammation. LEV-proliposomes showed good antimycobacterial activity against *M. bovis*, *M. tuberculosis* and intracellular *M. bovis* in macrophage cells.

CHAPTER 7

SINGLE AND REPEATED DOSE TOXICITY OF PYRAZINAMIDE AND LEVOFLOXACIN INHALED PROLIPOSOME IN MALE WISTAR RATS

7.1 Introduction

Anti-TB drugs can be formulated in dry microparticles for pulmonary delivery. This includes capreomycin and para-aminosalicylic acid. The results from such administrations have shown that direct delivery to the lungs resulted in high local concentrations and reduced bacterial burden compared to the same treatments delivered *via* other routes, thus offering the possibility of reduced doses and systemic side effects (Misra et al., 2011). An alveolar delivery system is an attractive approach to deliver drugs to TB infected lung especially for attacking infected macrophage (AM) (Ahsan et al., 2002). A recent review of the most effective way to deliver drugs to infected animals has been published (Misra et al., 2011). In most cases a significant benefit was noted in terms of the reduction in bacterial bioburden in the lungs. These observations have led to the prospect of the use of aerosols to supplement therapy by other routes and the possibility of a rapid reduction in the lung bacterial bioburden. A recent patent indicated that complete sterilization (no visible count from lungs or spleens) was achieved in mice following pulmonary delivery of an anti-TB drug (Misra et al., 2011).

Pyrazinamide (PZA) is an important first-line drug for the treatment of TB. PZA plays a unique role in shortening the therapy from previously 9–12 months to 6 months because it kills a population of semi-dormant tubercle bacilli in an acidic pH environment that are not killed by other anti-TB drugs. PZA is only active against *M. tuberculosis* at acid pH, an environment that is produced during active inflammation (Zhang et al., 2003). It is not active against *M. tuberculosis* under normal culture conditions near neutral pH. The second-generation quinolone antibiotics (ofloxacin and ciprofloxacin) have moderate *in vitro* activity against *M. tuberculosis* but the activity (both *in vitro* and *in vivo*) of the newer-generation fluoroquinolones, such as levofloxacin (LEV), is two to threefold greater than that of the second-generation quinolone antibiotics. LEV is one of the new fluoroquinolones with a high bactericidal activity against *M. tuberculosis* (0.195 μg/mL) (Marra et al., 2005). PZA and LEV are interesting anti-TB drugs. Both drugs were incorporated into proliposomes and administered by a dry powder inhaler.

PZA-proliposomes and LEV-proliposomes were prepared by the spray drying method with porous mannitol as a core carrier. Drug delivery by inhalations can be from either a single or repeated administration. For this reason, single and repeated dose toxicity experiments become important. In animal experiments, especially in studies on TB therapy, pulmonary delivery is often accomplished by intratracheal delivery (Forbes et al., 2011). Although *in vitro* methods such as cell culture test are being developed and have the potential to reduce costs of animal experimentation as well as providing mechanistic insights. There are currently no validated assays with regulatory acceptance that may be substituted for the single and repeated dose toxicity

in vivo studies (Forbes et al., 2011). Establishing non-clinical toxicology including, mutagenicity and safety pharmacology of inhalation (Forbes et al., 2011) is required before commencing single dose human clinical trials of inhaled medicines or repeated dose (repeated dosing of 2 weeks to 1 month duration). In the present study, both single and repeated dose toxicity in male Wistar rats of PZA-proliposomes and LEV-proliposomes by a dry powder inhaler was investigated.

7.2 Materials and Methods

7.2.1 Experimental animals and housing conditions

Male Wistar rats (230-290 g) used in this study were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. They were allowed to acclimatize to the conditions of the animal house for one week before the experiments and kept in a room maintained under standard environmentally controlled conditions of 22 ± 2 °C and 12 h light–12 h dark cycle. They were supplied with standard rodent diets and water *ad libitum*. All procedures were reviewed and approved by the Animal Ethics Committees, Prince of Songkla University, Thailand (No. Ref 11/51).

7.2.2 Production of PZA- proliposomes or LEV-proliposomes by spray-drying technique

Lipids were composed of a mixture of L-α soybean phosphatidylcholine and cholesterol from lanolin in a mole ratio of 1:1. These reagents were weighed and

dissolved in 500 mL of 95% ethanol to obtain an ethanolic lipid solution. PZA or LEV (500 mg) was added to the ethanolic lipid solution and sonicated until a clear solution was obtained. Porous mannitol (4.5 g) was dispersed in the solution, and the suspension was sonicated for 15 minutes in order to deaggregate mannitol particles before the spray drying process began. The suspension was continuously stirred to provide homogeneity of the suspension during spray drying. It was sprayed through a 1.5 mm nozzle using a mini spray dryer B-290 (Büchi, Flawil, Switzerland) at an inlet temperature of 110°C, an atomizing pressure of 800 kPa, and a feeding rate of 3 mL/minute. Proliposome powder was collected from the collecting chamber and kept in a desiccator until used.

7.2.3 Single dose toxicity study design

Wistar rats were randomly divided into a control and two treatment groups including those that inhaled PZA-proliposomes and LEV-proliposomes (n=5 for each group). Before the samples were administered, blood samples were collected as for pre-treatment samples using the retro-orbital plexus sampling technique. The proliposome formulations (4 mg/kg body weight) were administered by intratracheal instillation. Briefly, intratracheal instillation with operation for single dose toxicity study was performed after anesthetized by Zoletil®100 at 40 mg/kg body weight. Polyethylene tube No. 240 (Intramedic®, New Jersey, USA) 5 cm in length was inserted through the tracheal. The proliposome powder was loaded into the tube, the proliposome traveled into the rat airways when the rat inhaled. One hour after drug

administration, the blood samples were collected as post-treatment samples using the same technique.

7.2.4 Repeated dose toxicity study design

Twenty-one Wistar rats were randomly divided into a control and two treatment groups including those that inhaled PZA-proliposomes and LEV-proliposomes (n=7 for each group). Before the samples were administered, a blood sample was collected for the pre-treatment samples using the tail vein sampling technique. The proliposome formulation I (4 mg/kg body weight) was administered by intratracheal instillation at day 1st followed by intratracheal inhalation from the 2nd day to the 28th day every day. Intratracheal instillation and inhalation were modified from previous study (Brain et al., 1976). Briefly, intratracheal instillation was performed after anesthetized by Zoletil®100 at 40 mg/kg body weight. Polyethylene tube (No. 240) 10 cm in length was inserted through the mouth to insert into tracheal and about 5 cm was left outside the oral cavity for drug administration. Proliposomes were loaded into the tube. When the rat inhaled, the proliposome powder traveled automatically into the rat airways. For intratracheal inhalation, the rats were allowed to breath through plastic tube (diameter = 1 cm, length = 8 cm) for at least 2 minutes, while proliposomes were blown from the other end. The blood samples were collected after being first administered for 7, 14, 21and 28 days. Clinical signs for example food intake, feces, hair and behavior were observed once daily and body weights were recorded every week during the experiments.

7.2.5 Serum biochemical parameter assay

The collected blood was centrifuged at 2000 rpm for 10 minutes to obtain serum in separated tubes. Blood urea nitrogen (BUN) and creatinine (Cr) were determined for acute toxicity. Alanine aminotransferase (ALT), asparatate aminotransferase (AST), BUN and Cr were evaluated for the repeated dose toxicity study. All Serum biochemical parameters were measured with a Cobas Mira Plus Chemistry Analyzer (Roche Diagnostics, USA).

7.2.6 Statistical analysis

Data are presented as a mean ± standard deviation (SD) from at least five animals unless indicated. The data were analyzed by one-way analysis of variance (ANOVA) and the Student's *t*-test was used to determine the level of significance of the differences in population means. A significant difference was accepted with $p<0.05$ between data sets. All statistical comparisons were calculated using the SPSS software version 16 (SPSS Inc., Chicago, IL).

7.3 Results and discussion

From the MIC of PZA for susceptible of *M. tuberculosis*, which were determined using radiometric techniques, are 6.2-50 µg/mL when tested at pH of 5.5 (Salfinger and Heifets, 1988). The expected PZA amount in lung was at least 20 µg/kg body weight. Therefore PZA-proliposome formulation I was inhaled at least 200 µg/kg body weight. PZA-proliposome formulation I amount was calculated to be

4 mg/kg body weight for inhalation (20 times of expected PZA amount in lung). In this study, the MIC of LEV against *M. tuberculosis* was 0.195 µg/mL, so the expected LEV amount in lung was at least 0.2 µg/kg body weight. Therefore LEV-proliposome formulation No.1 was inhaled at least 2 µg/kg body weight. LEV-proliposome formulation I amount was calculated to be 4 mg/kg body weight for inhalation (2,000 times of expected LEV amount in lung). The toxicity studies were carried out at these extremely high doses for practical reasons associated with real life application because only a fraction of dose delivery reached alveoli.

Wistar rats were administered by intratracheal instillation for the single dose toxicity study. After administered for 1 hour, the blood samples were collected. Serum biochemical parameters, including BUN and Cr, present in Figure 7.1. Significant differences in BUN of pre-treatment groups were observed ($p<0.05$). Although BUN values were significantly different, these data were in the normal biochemical ranges for rats (11-25 mg/dL) (Sharp and LaRegina, 1998). There were no significant differences in BUN and Cr between the pre-treatment and post-treatment data when compared within the same group ($p > 0.05$). The tendency of post-treatment BUN value was slightly higher than that of the pre-treatment (Figure 7.1a), while Cr values of post treatment were slightly lower than those of the pre-treatment (Figure 7.1b). There were no adverse acute renal effects of PZA-proliposomes and LEV-proliposomes on rats after administration of both proliposome formulations within 1 hour.

For the repeated dose toxicity experiment, the samples were administered by intratracheal instillation at 1st day and administered daily by intratracheal inhalation

until day 28. During this experiment period, no significant abnormalities in food intake, feces, hair and behaviour in any group of animals were observed. Animals in all groups gained weight for the duration of the study and no statistically significant changes were observed when compared with the control group ($p > 0.05$). For serum biochemical parameters, a significant increase in AST was observed in all groups after administration for 7 days. Apart from that, there were no significant differences in BUN, Cr and ALT (Figure 7.2a-7.2d). The ALT data showed a tendency to increase. The increase in AST might be due to the animals readjusting themselves to the new environment. Although AST values increased slightly, they were still within the normal biochemical ranges for rats (52-224 U/L) (Sharp and LaRegina, 1998). Serum levels for both ALT and AST become elevated whenever disease processes affect liver cells, with ALT being the more liver-specific enzyme. Elevations of ALT activity persisted longer than those of the AST activity (Johnston, 1999). Renal and liver toxicity in the repeated dose toxicity study were not observed.

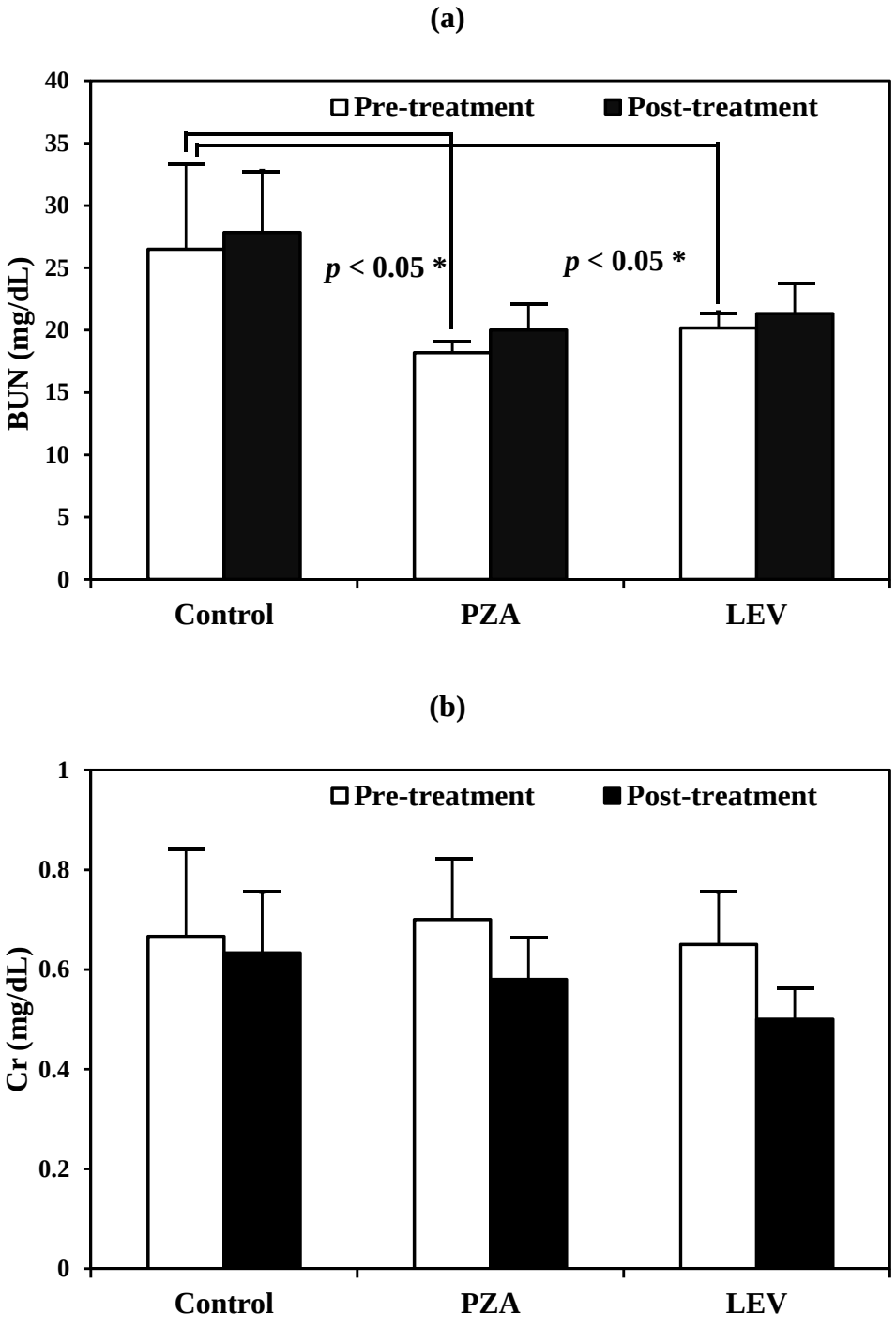


Figure 7.1 Serum biochemical parameters of (a) BUN and (b) Cr of male Wistar rats in single dose toxicity study

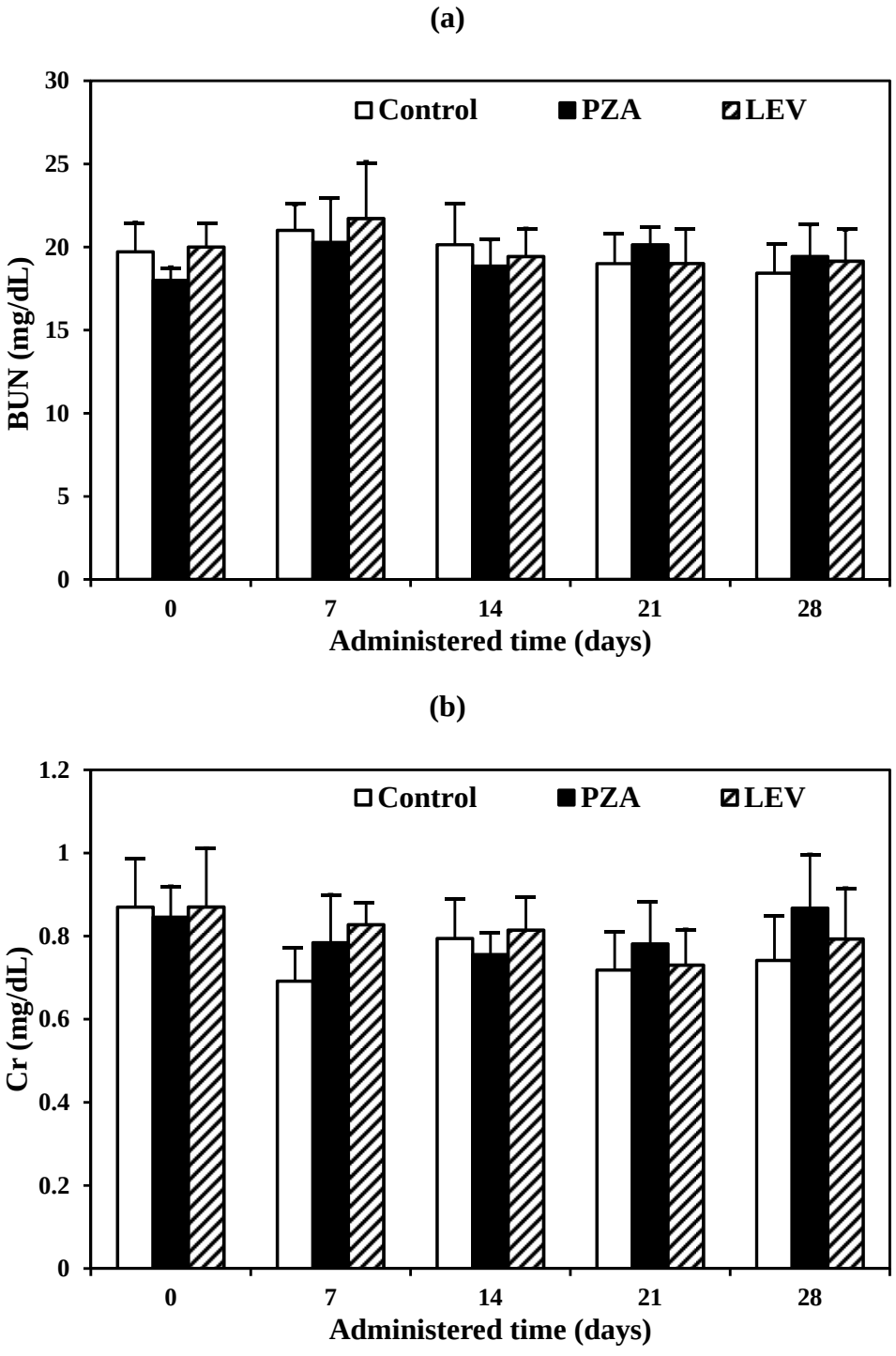


Figure 7.2 Serum biochemical parameters ((a) BUN, (b) Cr, (c) ALT and (d) AST) of male Wistar rats in repeated dose toxicity study for 28 days

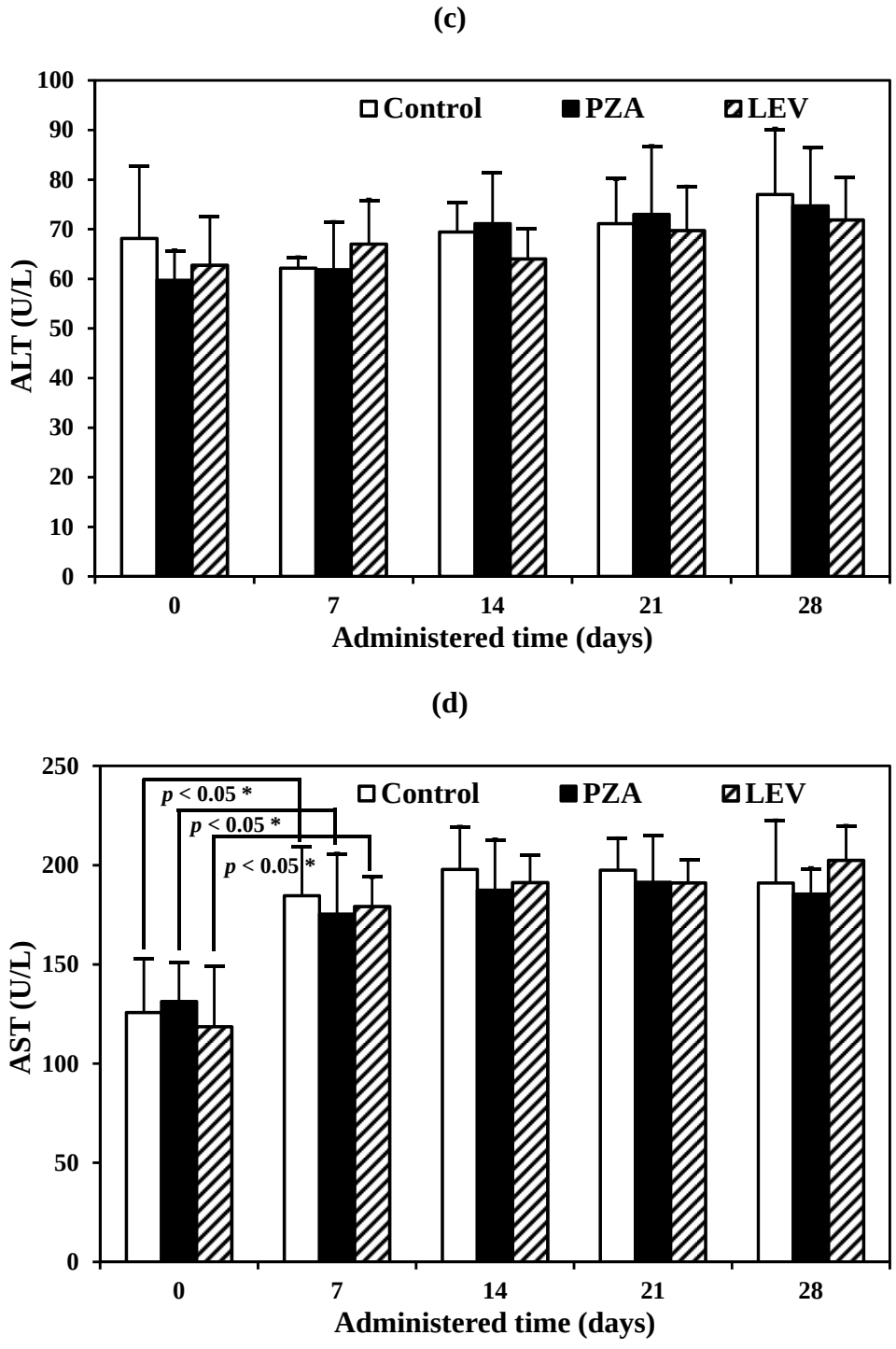


Figure 7.2 Serum biochemical parameters ((a) BUN, (b) Cr, (c) ALT and (d) AST) of male Wistar rats in repeated dose toxicity study for 28 days (Continued)

7.4 Conclusion

The renal functions after PZA or LEV inhaled proliposome administered were determined in the single dose toxicity study, while renal and liver functions were evaluated in the repeated dose toxicity experiment. From the single and repeated dose toxicity data, no significant toxicological changes were observed when the rats were treated by intratracheal instillation with 4 mg/kg body weight of the drug proliposomes. The inhaled proliposome with drug is an optional formulation that delivers the drug to the lung for local action. This may reduce systemic toxicity. Anti-TB drug inhaled proliposome therefore could be a safe formulation for TB treatment. The efficacy of anti-TB drug inhaled proliposome in infected animals or human will be determined.

CHAPTER 8

GENERAL DISCUSSION

An approach for the preparation of porous particles using a spray drying method is to use volatile salts as pore forming agents to create voids (Straub et al., 2002). The volatile pore forming agents can be added in various ways to the droplets, as either a micronized solid in an organic solvent (Narayan et al., 2001), as an aqueous solution (Gervelas et al., 2007), or via the dispersed phase of a water-in-oil emulsion (Straub et al., 2005). Adding the volatilizable salt before the spray drying process has been used in this thesis to prepare porous mannitol . Ammonium carbonate as a pore forming agent was necessary to prepare porous particles in water (Nolan et al., 2009). Spherical particles of porous mannitol as a proliposome core carrier were successfully developed. The determination of porosity was evaluated using the liquid displacement method by employing absolute ethanol as an antisolvent. Ethanol penetrated easily into the pores and at the same time produced no shrinkage or swelling. Porous mannitol displayed a low bulk density with a high porosity. It indicated that increasing the formation of pores resulted from the addition of the pore forming agent. The pore forming agent and mannitol were then spray dried at 110°C when water and volatile substances evaporated. The pore forming agent was decomposed by volatilization. The volatilization enhanced the formation of pores in the particles. All ammonium salts decomposed into ammonia and the acid component when heated. Proliposomes with non-porous mannitol gave a much higher mass

median aerodynamic diameter (MMAD), while the MMAD of the formulation containing porous mannitol was the lowest. This resulted from the lower density of porous mannitol. The aerodynamic particle diameter was directly proportional to the square root of the particle density hence the particle density was in this case an important parameter. As the particle density decreased, the particle's aerodynamic diameter decreased. The aerosolization properties of the proliposome could be improved using porous mannitol as the proliposome core carrier.

In this study, hydrophilic and hydrophobic antituberculosis (anti-TB) drugs were prepared in proliposomes using the porous mannitol as a proliposome core carrier. Pyrazinamide (PZA), the first line medication drug, was chosen as a hydrophilic model drug and levofloxacin (LEV), as a second line medication drug, was selected as the hydrophobic model drug.

Proliposomes can be prepared by many methods including : the crystal-film method, a carrier method by deposition ona film, a fluidized-bed method, a powder bed grinding method, a freeze-drying method and a spray drying method (Gupta et al., 2008; Zhao et al., 2011; Janga et al., 2012). Spray drying has been considered to be a one-step process for the production of small particles (<5 μm) for pulmonary administration. This allows for better control for forming particle, and hence can be easily scaled up. Spray drying is not only limited to aqueous solutions, but can also be used for non-aqueous systems to prepare particles suitable for aerosol delivery (Alves and Santana, 2004; Chougule et al., 2007). Therefore in this study the anti-TB-proliposomes were prepared by the spray drying method. The lipid component containing soybean phosphatidylcholine, cholesterol and the drug were dissolved in

ethanol to obtain a clear solution. Porous mannitol as a proliposome core carrier was added to the solution to form a suspension. The suspension was spray dried to obtain proliposomes. Spherical proliposome microparticles were produced from the formulation that contained more than 60% porous mannitol for the PZA-proliposomes and more than 40% for the LEV-proliposomes. This was to provide sufficient amounts of porous mannitol in the formulation to produce spherical particles.

The PZA-proliposome and LEV-proliposome formulations had MMAD values that were correlated to a fine particle fraction (FPF) (the fraction of particles smaller than 4.4 μm). The particles that can reach the lower part of the respiratory tract should be in the range of 1-5 μm when traveling in the air (Daniher and Zhu, 2008). From the results, all prepared PZA-proliposomes and LEV-proliposomes formulations met this requirement.

The aims of the dry powder inhaler (DPI) formulation were to deliver a uniform pulmonary distribution of the drug, with very small dose variations, a good flowability, adequate physical stability in the device before use and good performance in terms of the emitted dose and FPF. The performance of DPI formulations was enhanced through engineering particles by lowering the aerodynamic diameters of the particles using particles with small geometric diameter, a lower of particle density (by increasing the porosity of particles), altering the shape (pollen-shaped particles) (Hassan and Lau, 2009) and by creating a rough surface (to increase the air drag force) (Maas et al., 2010). The performance was also improved by blending and the use of ternary mixtures (by using fine carriers and ternary components) (Jones et al., 2010), by lowering the bulk density (loose particle packing to reduce particle

contacts), lowering the inter particulate forces in between the particles by creating a rough surface (to reduce particle interaction) and by lowering the surface energy by modifying the surface composition (Chan, 2006). On the basis of improving aerosol performance, blending and use of ternary mixtures, reducing aerodynamic diameters through use of low density particle, preparing less cohesive and adhesive particles were considered for producing novel DPIs.

After the proliposomes were reconstituted with distilled water, vesicle sizes showed a normal distribution. Reconstituted PZA-proliposomes and LEV-proliposomes were contained in nanosized vesicles. The vesicle sizes in the range of 200–600 nm were efficiently taken up by macrophages. It is expected that the reconstituted PZA-proliposomes and LEV-proliposomes in this study were suitable for targeting AMs.

The proliposomes immediately formed a liposomal dispersion on contact with water (Song et al., 2002; Wagner and Vorauer-Uhl, 2011). Hydrophillic and hydrophobic substances could be encapsulated in reconstituted proliposmes (Wagner and Vorauer-Uhl, 2011). All proliposome formulations were reconstituted with distilled water, whereas the free drug dissolved instantly. After ultracentrifugation, drug-encapsulated liposomes were packed into pellets at the bottom of a centrifuge tube. The supernatant was analyzed to determine the amount of free drug. The encapsulation efficiency was calculated from the difference between the drug content and the free drug values. The advantages of this method when compared with the membrane dialysis method were that it was rapid and easy. A low drug loading amount in the formulation provided a high encapsulation efficiency. That might result

from the lipid amount not being sufficient to encapsulate the drug, so a low amount of drug loading in the formulation gave a high encapsulation efficiency. The encapsulation efficiency of the LEV-proliposomes was less than for the PZA-proliposomes. One explaination was that the hydrophobic drugs were encapsulated in a phospholipid layer, while hydrophilic drugs were encapsulated inside the core. LEV is a hydrophobic drug, so it can be encapsulated only in the phospholipid layers resulting in a low encapsulation efficiency. While PZA is a hydrophilic drug, it was encapsulated inside the core. To improve the encapsulation efficiency for a hydrophobic drug in the liposomes, a method to enhance the water-solubility property of the hydrophobic drug should be considered (Chen et al., 2007). An alternative method would be to increase the amount of phospholipid in the formulation but that would have a negative effect in allowing for particle aggregation and resulting in a low FPF.

Interactions of the proliposome powder and its physical properties were evaluated using X-ray diffraction (XRD), differential scanning calorimeter (DSC) and fourier transformed infrared (FT-IR). From the XRD patterns, DSC data and FT-IR spectra, all data indicated that some interactions occurred in the PZA-proliposomes and LEV-proliposomes. One explanation could be that there was an interaction between the drug and mannitol or the drug-drug itself that occurred during the spray drying process. This could be confirmed by using solid-state nuclear magnetic resonance spectrometry (SS-NMR).

In order to target the alveolar macrophages (AMs), the inhaled particles have to travel through the upper airways and reach the lower airways. Finally, the particles

in the lower airway are phagocytosed by the AMs. The toxicity of proliposomes to respiratory-associated cell lines and its potential to provoke immunological responses from AMs were examined. The proliposome formulations containing drug contents that varied from a low loading (%) to a high loading capacity were compared. The effect of the amount of drug, and the amount of porous mannitol in the formulation was also established on respiratory cell lines. In the Calu-3, A549 and NR8383 cell viability study, the cytotoxicity results were similar. Cell viabilities after being exposed to PZA-proliposomes or LEV-proliposomes were higher than for the pure drug. One explanation was that the proliposome could be used to reduce drug toxicity to cells in the respiratory tract. For the immunological responses from the AMs, NR8383 cells produced a lower amount of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and nitric oxide (NO) in response to the drug, and drug-proliposome formulations compared to the effect of lipopolysaccharide (LPS). The concentration of LPS used to stimulate NR8383 cells to produce the immunological response was 1,000 and 10 times less than the concentration of any PZA-proliposome and LEV-proliposome. LPS activation of NR8383 cells to produce inflammatory cytokines was significantly greater than when it was challenged with the drug, drug-proliposome formulations ($p < 0.05$). PZA or LEV did not affect the alveolar macrophage cytokine.

We employed a flow cytometry method to evaluate the PZA-proliposome and LEV-proliposome activities against mycobacterium as previously described (Norden et al., 1995; Bownds et al., 1996; Kirk et al., 1998; Moore et al., 1999; DeCoster et al., 2005; Pina-Vas et al., 2005). Results from the flow cytometry were available

within 24 h. Fluorescein diacetate (FDA) is a nonpolar, nonfluorescent molecule capable of diffusing across the cell wall and cell membranes of the mycobacteria by active transport and passive diffusion (Norden et al., 1995). Once in the cytoplasm, FDA is rapidly (in 5 minutes) hydrolyzed by esterases to fluorescein, whereas nonviable bacteria have decreased quantities of active esterases thus resulting in less production of fluorescein. The viable cells are also detected at a different gate event than for nonviable cells. From the results, PZA and PZA-proliposomes inhibited the growth of *M. bovis* at concentrations of PZA of more than 25 µg/mL. PZA-proliposome formulations containing 80% PZA showed that it was slightly more effective than the proliposme formulations containing 10% PZA and PZA itself at the same concentration. *M. bovis* is known to have a characteristic single point mutation in its *pncA* gene which is responsible for its natural PZA resistance, but remains susceptible to pyrazinoic acid. PZA at 16 µg/mL that gave a 16% inhibition of *M. bovis* growth after adjusting the pH to be 5.5 (Somoskovi et al., 2004). On other hand, viable *M. bovis* were around 10% in the LEV and LEV-proliposome samples at day 6. This indicated that LEV could kill *M. bovis*. The minimum inhibitory concentrations (MIC) values of levofloxacin against *M. bovis* in this study were 1 µg/mL for LEV and for the LEV-proliposome formulation containing 80% LEV and 0.5 µg/mL for the LEV-proliposome formulation containing 10% LEV.

The activity of the drug and drug-proliposomes against *M. bovis* intracellular macrophage cells was performed only with LEV because PZA could not kill *M. bovis*. LEV-proliposomes showed a dramatic decrease in bacilli at day 5 and the remaining live bacilli were not more than 10% at day 7. This proved that LEV-proliposome

could be taken up by AMs easier than LEV, so LEV-proliposome could kill *M. bovis* present in macrophage cells at day 6.

The MIC values of PZA, PZA-proliposomes against *M. tuberculosis* could not be determined in this study because the pH value of the test was 7.0. It might be that the MIC value of PZA was pH dependent. It has been recognized for some time that PZA acts only in an acid environment (Rieder, 2002). The antimycobacterial activity of pyrazinamide depends on a conversion of the drug to pyrazinoic acid (POA), an active metabolite that preferentially accumulates in an acidic pH, after the reaction with amidase pyrazinamidase (AHFS, 1999; Rieder, 2002). The MICs of pyrazinamide against *M. tuberculosis* using radiometric techniques were 6.2–50 mg/L at a pH of 5.5 (Salfinger et al., 1988). The MIC values of LEV and LEV-proliposomes against *M. tuberculosis* reported in this study were 0.195 µg/mL similar to those reported by Marra and co-workers (2005) but it was lower than the 1 µg/mL as reported by Rastogi and co-workers (1996).

To observe the phagocytosis, PZA-proliposomes and LEV-proliposomes were reconstituted in distilled water and stained with Lumidot® 640. Lumidot® is to produce quantum dots that are used in modern biological imagry. They have quickly filled this role, being found to be superior to traditional organic dyes on several counts, one of the most immediately obvious was the increased brightness (owing to the high extinction co-efficient combined with a comparable quantum yield to fluorescent dyes) as well as to their stability (allowing much less photo-bleaching). It has been estimated that quantum dots are 20 times brighter and 100 times more stable than traditional fluorescent dots (Michalet et al., 2005; Walling et al., 2009). The

phagocytosis was observed by the increase in the intensity of Lumidot® 640, larger macrophage cells and changes to macrophage cell shapes. Reconstitued PZA-proliposomes and LEV-proliposomes could be phagocytosed by AMs. The vesicles that were 200–600 nm in size were efficiently phagocytosed.

In vivo single and repeated dose toxicity studies were carried out at extremely high doses for practical reasons associated with real life applications because only a fraction of the delivery dose reached the lower airways. Zoletil® 100 at 40 mg/kg body (i.p.) was used to produce anesthesia because it showed no hepatic or renal toxicity (Ferrari et al., 2005). Wistar rats were administered by intratracheal instillation during anesthaesia for the single toxicity study. Blood urea nitrogen (BUN) and creatinine (Cr) were in the normal biochemical ranges for rats (Sharp and LaRegina, 1998) and there were no significant differences in BUN and Cr between the pre-treatment and post-treatment data. These results indicated that there was no adverse acute renal effect observed for at least 1 h after the administration of the PZA-proliposomes and LEV-proliposomes to rats.

For the repeated dose toxicity experiment, the samples were administered by intratracheal instillation during anesthaesia at day 0 then administered daily by intratracheal inhalation until day 28. Alanine aminotransferase (ALT), asparatate aminotransferase (AST), BUN and Cr were within the normal biochemical ranges for rats (Sharp and LaRegina, 1998), so no renal or liver toxicity was observed in the repeated dose toxicity study.

CHAPTER 9

CONCLUSIONS

Pyrazinamide and levofloxacin were successfully prepared in proliposome formulation forms. Aerosolization properties were improved by using porous mannitol as a proliposome core carrier. From X-ray diffraction patterns, DSC thermograms and FT-IR spectra, all techniques indicated that there were some interactions probably cocrystallization occurred in pyrazinamide-proliposomes and levofloxacin-proliposomes. Both pyrazinamide-proliposomes and levofloxacin-proliposomes were evidently to be non-toxic to respiratory-associated cell lines. In determination of the alveolar macrophage response to proliposomes, alveolar macrophage cells produced lower amounts of TNF-α, IL-1β and NO in response to pyrazinamide-proliposomes and levofloxacin-proliposomes, compared to lipopolysaccharide. Levofloxacin-proliposomes showed good activity against *M. tuberculosis* and *M. bovis* including *M. bovis* intracellular alveolar macrophage cells, while pyrazinamide-proliposomes were inactive against *M. tuberculosis* and *M. bovis*. The phagocytosis was observed after incubation alveolar macrophage cells with Lumidot® 640-stained reconstituted proliposome. Furthermore, single dose toxicity and repeated dose toxicity experiments showed that no significant toxicological changes were observed when male Wistar rats were treated by intratracheal instillation with 4 mg/kg body weight of the drug proliposomes. Proliposome dry powder inhalers prepared in this study is a formulation that is able to deliver the drug

to lower airways. Batch production, scale up, stability study and the efficacy of proliposome dry powder inhalers containing antituberculosis drug in infected animals or human will be the next step of future studies.

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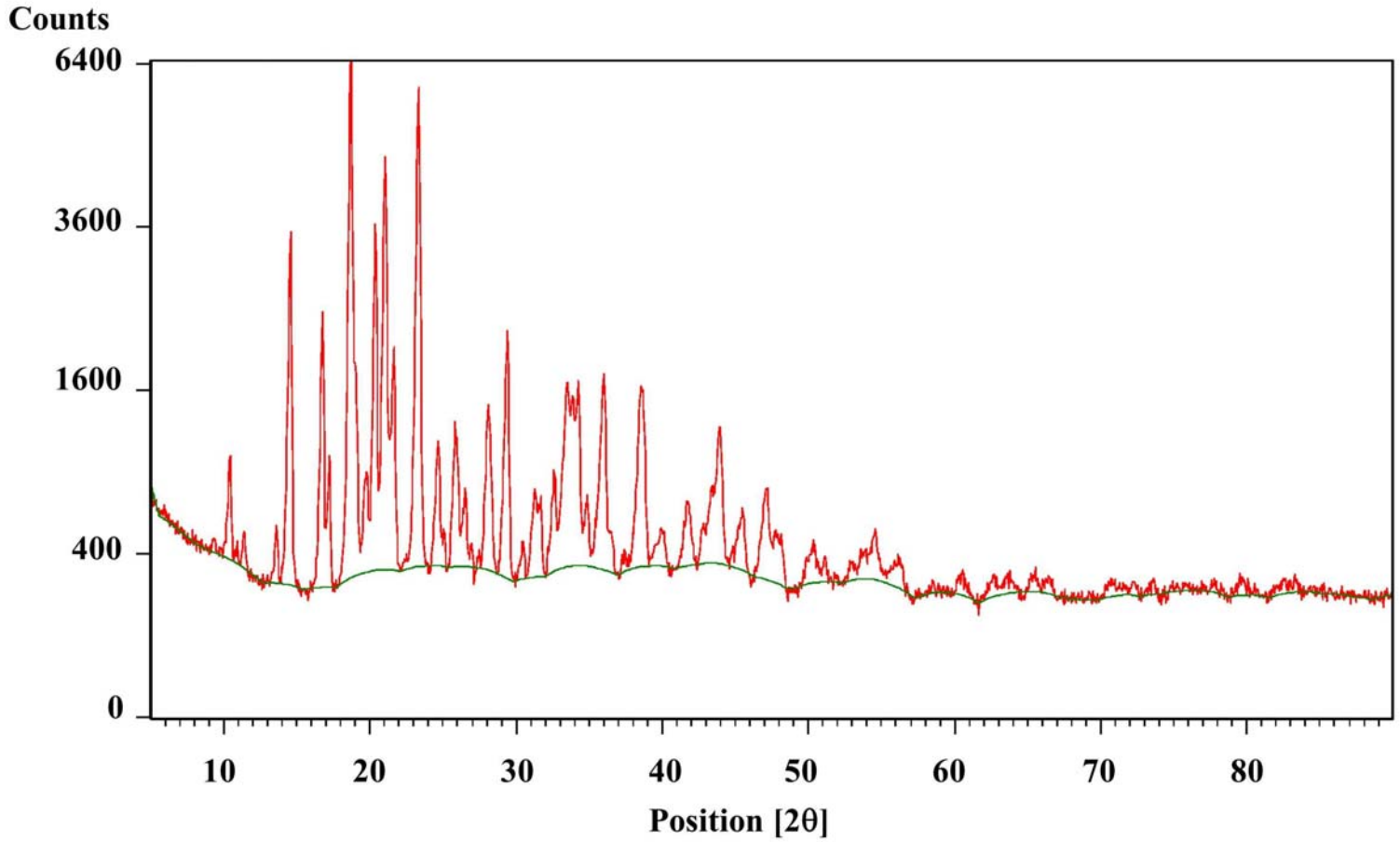
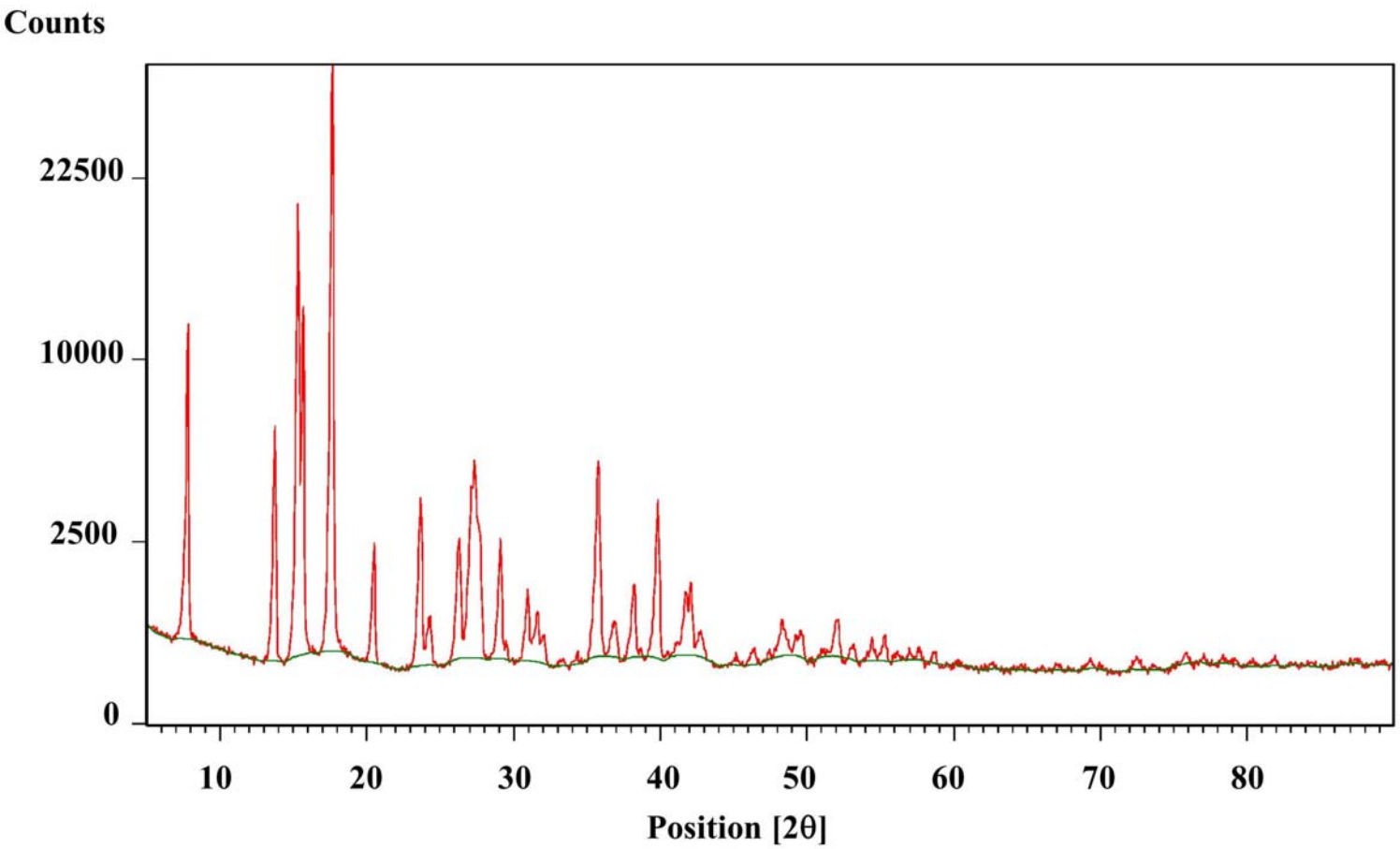
APPENDICES



The X-ray diffraction pattern of porous mannitol (AC2)



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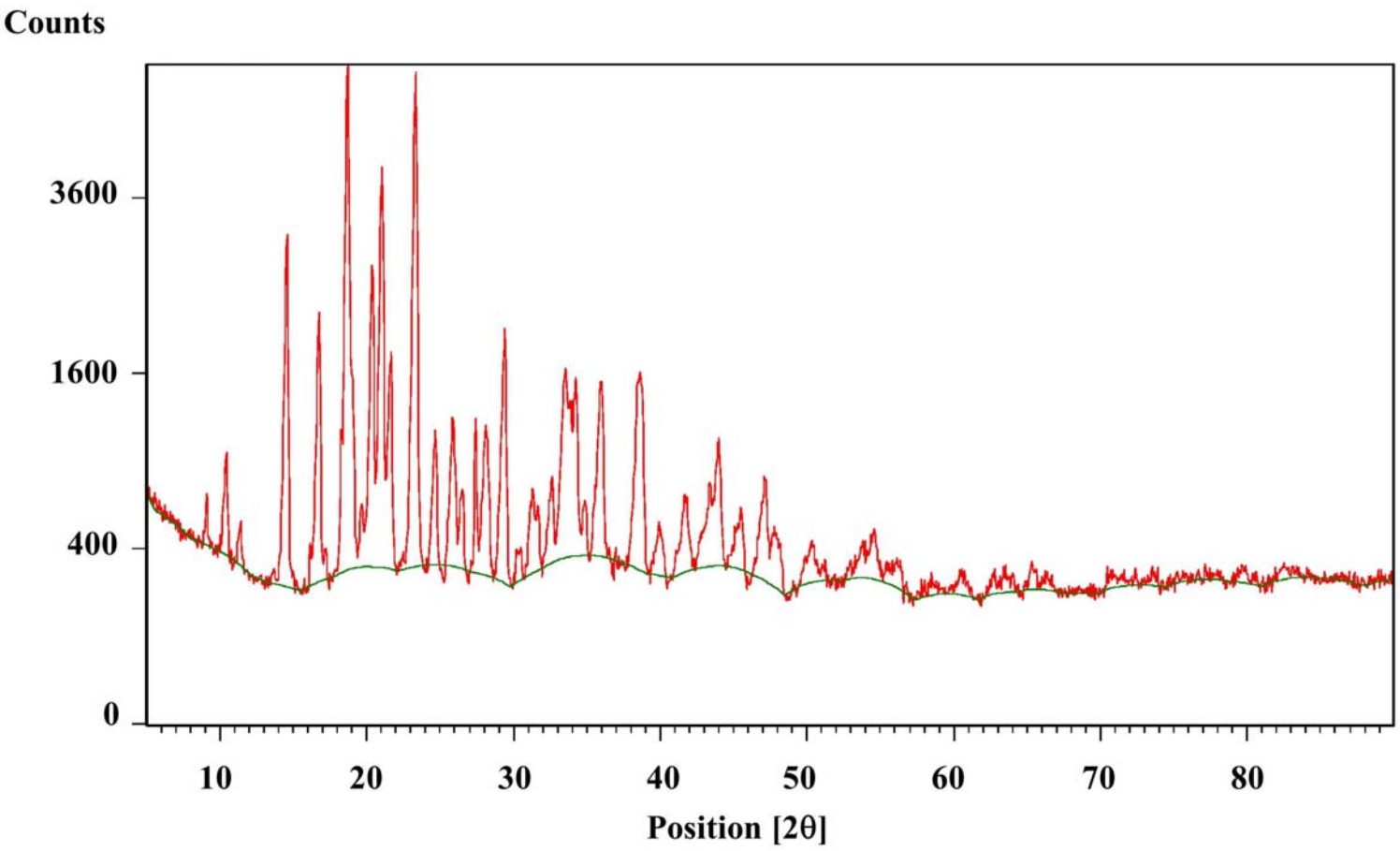
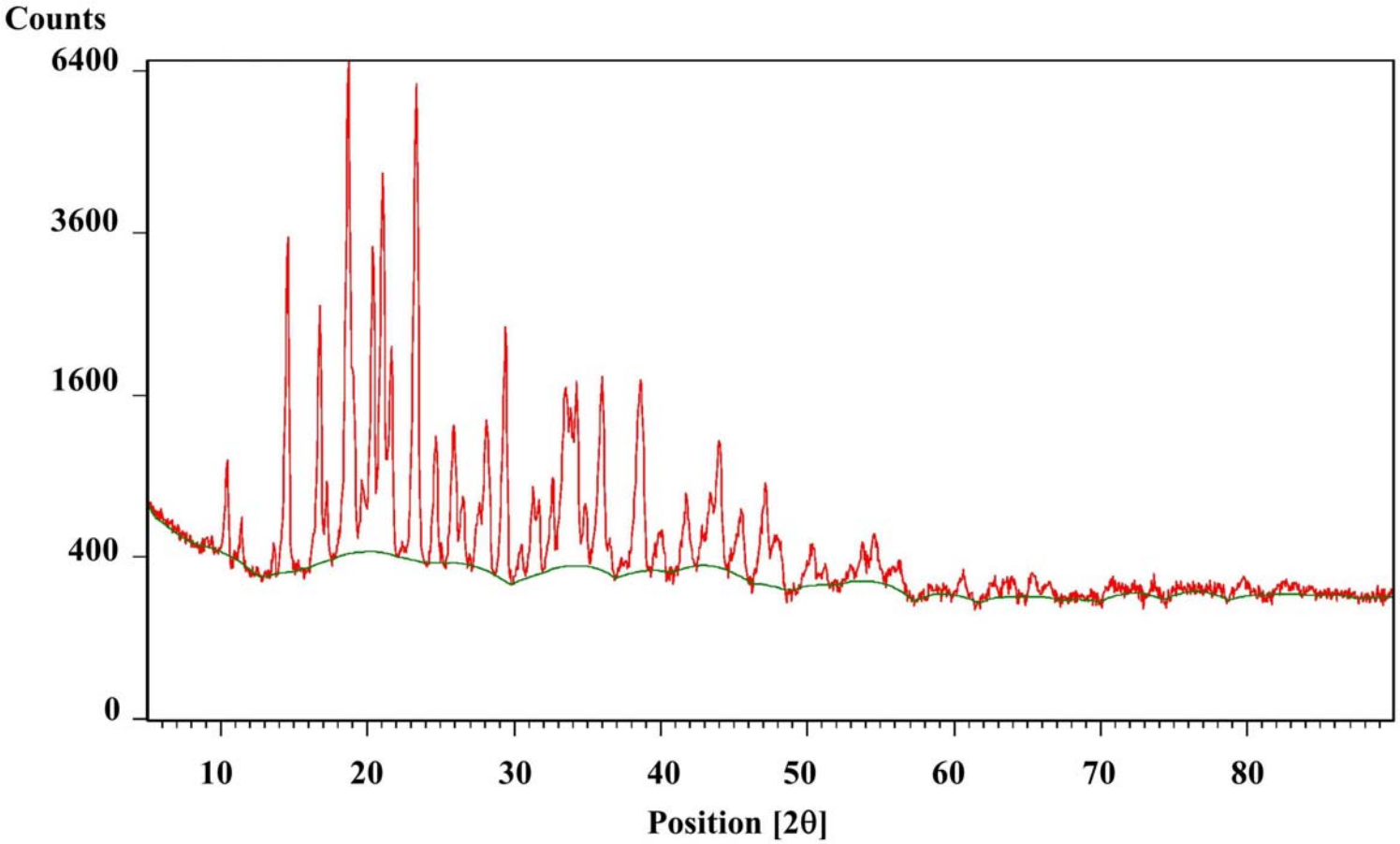


The X-ray diffraction pattern of PZA-proliposome formulation I



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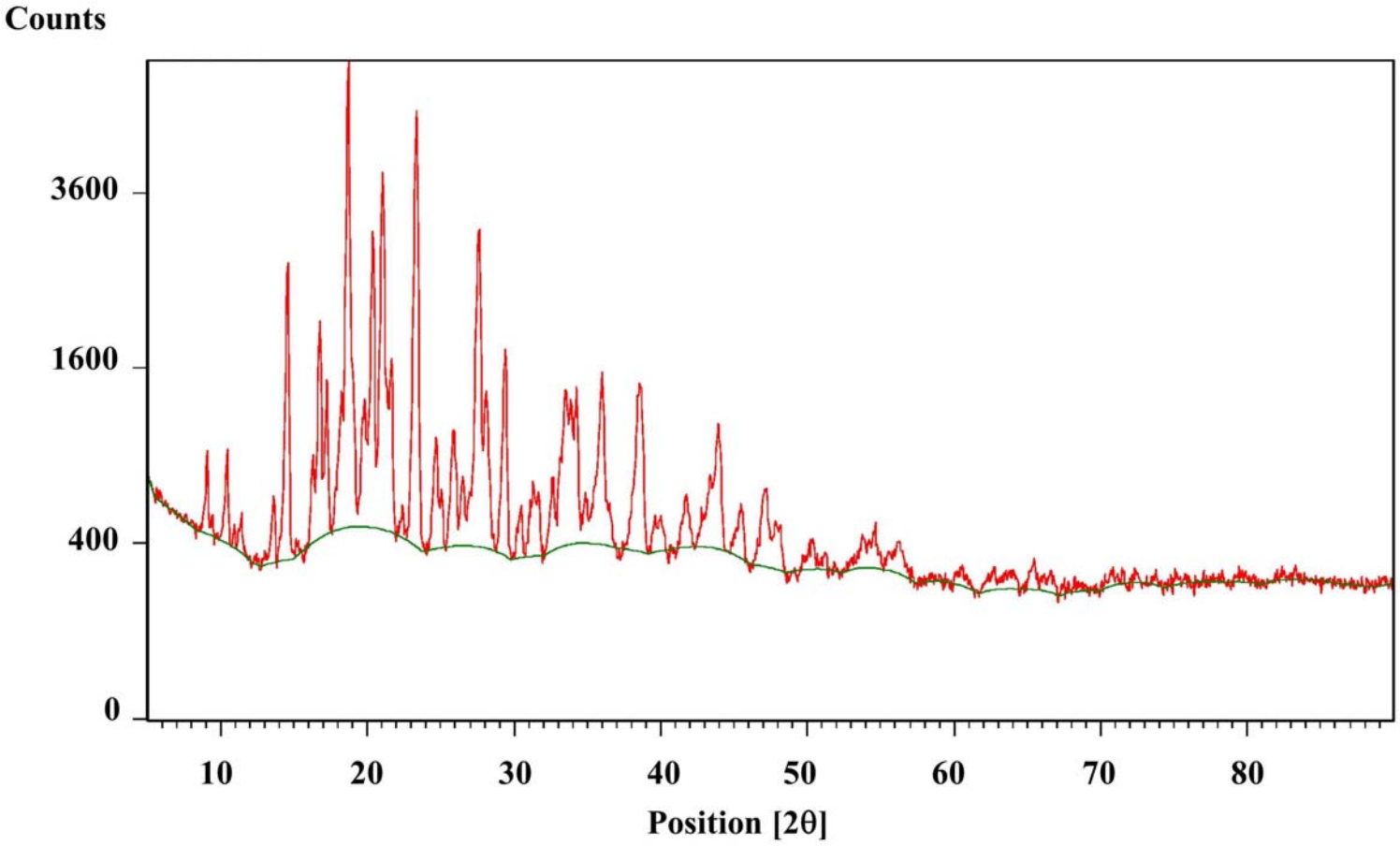

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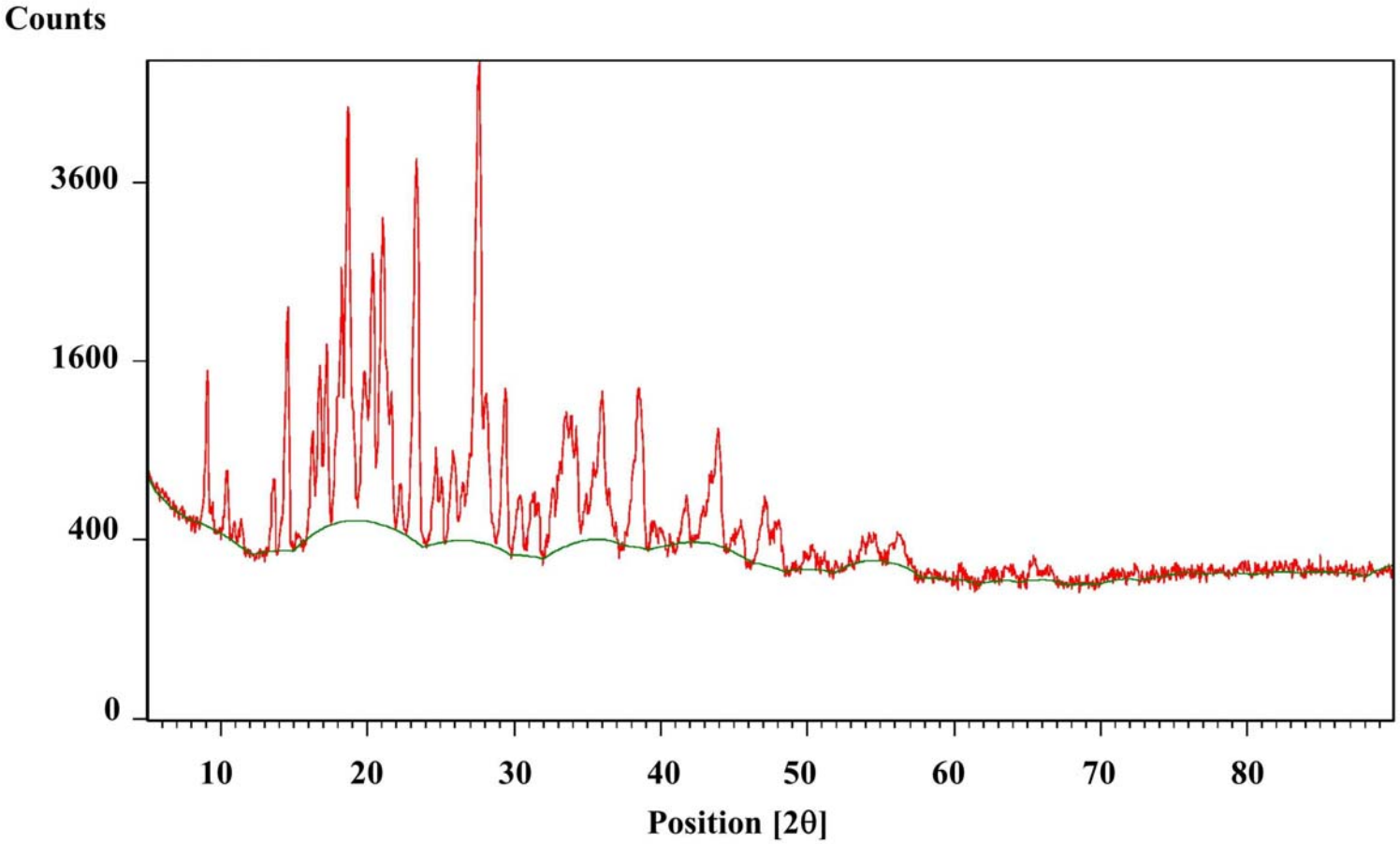

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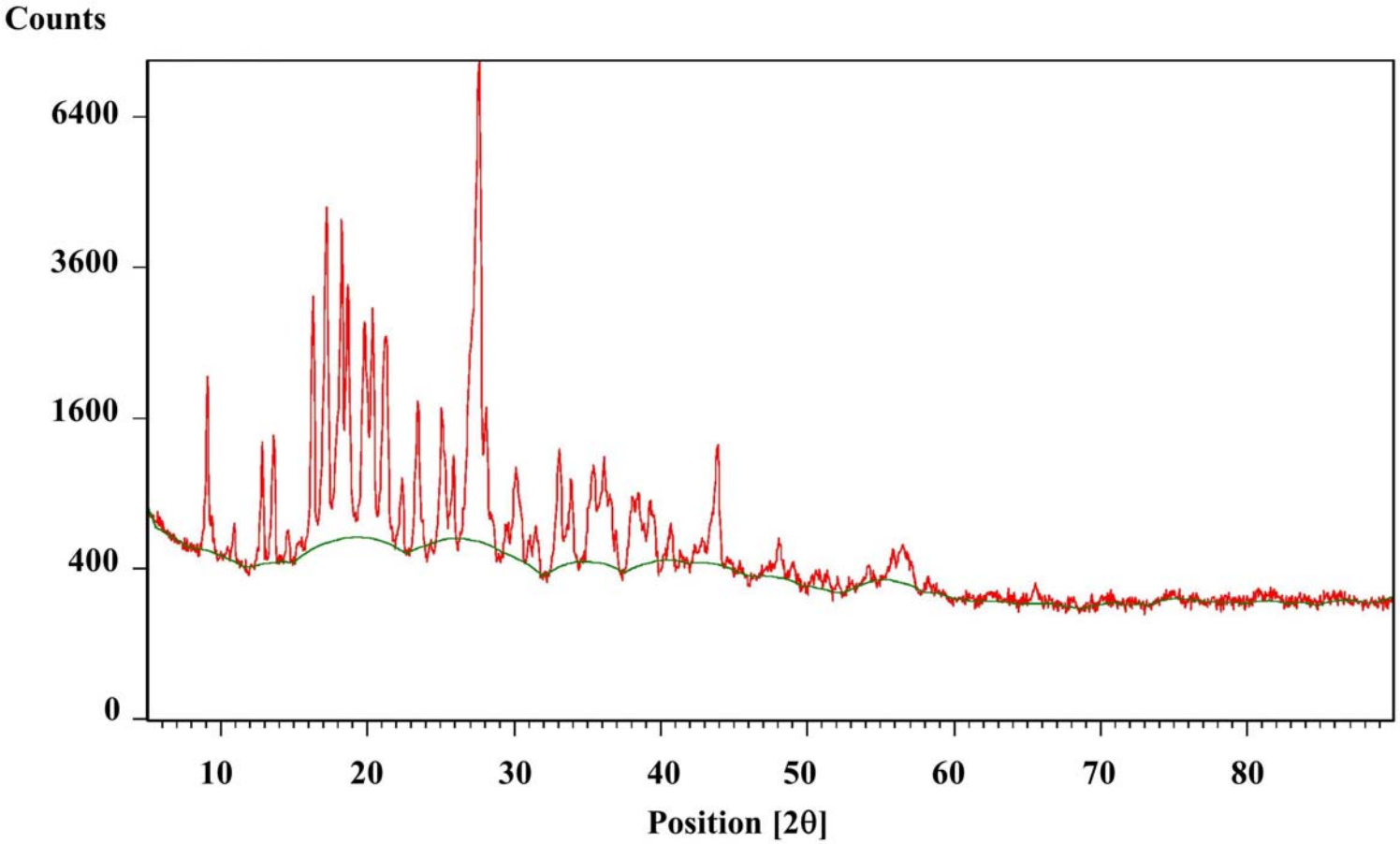

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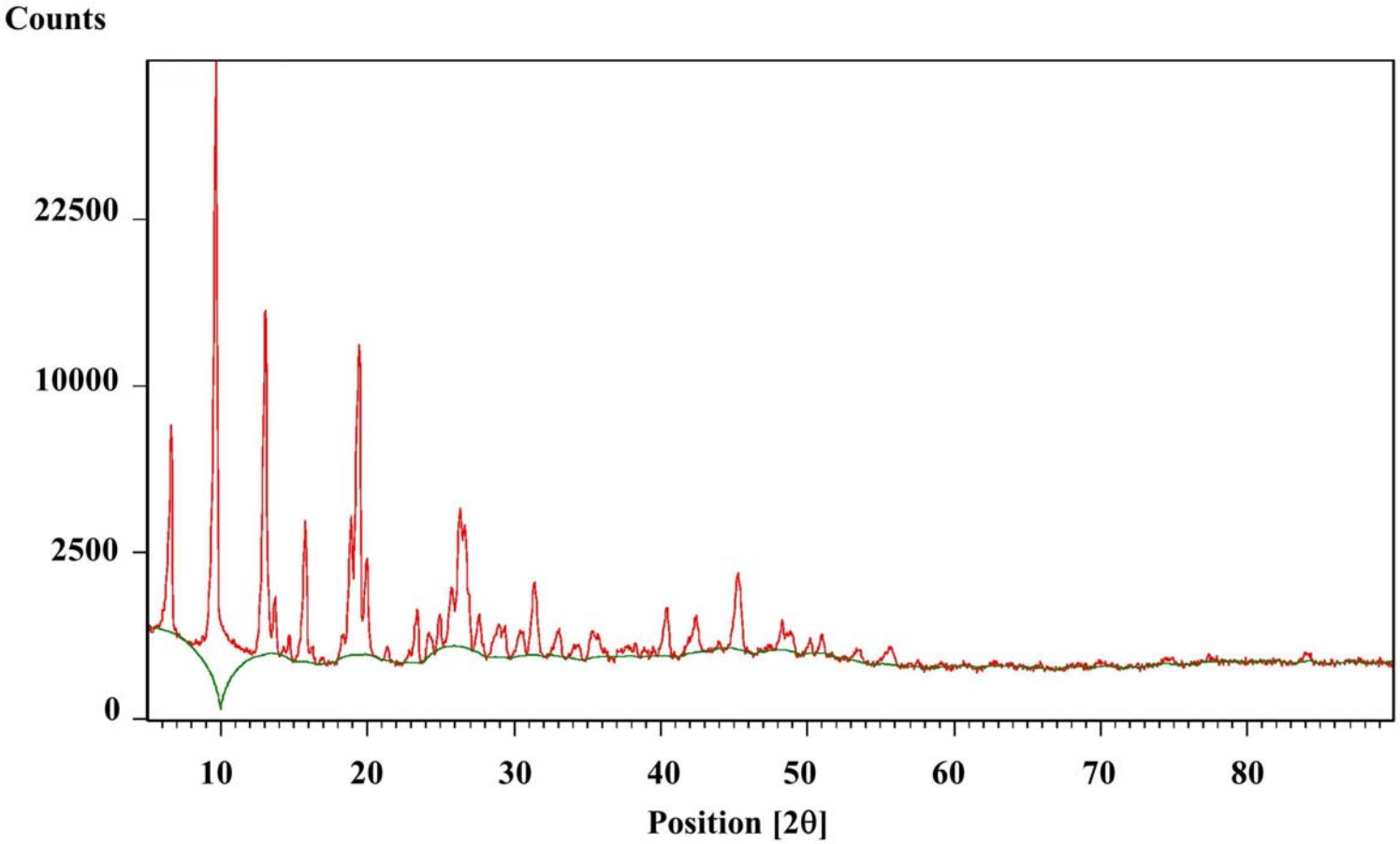

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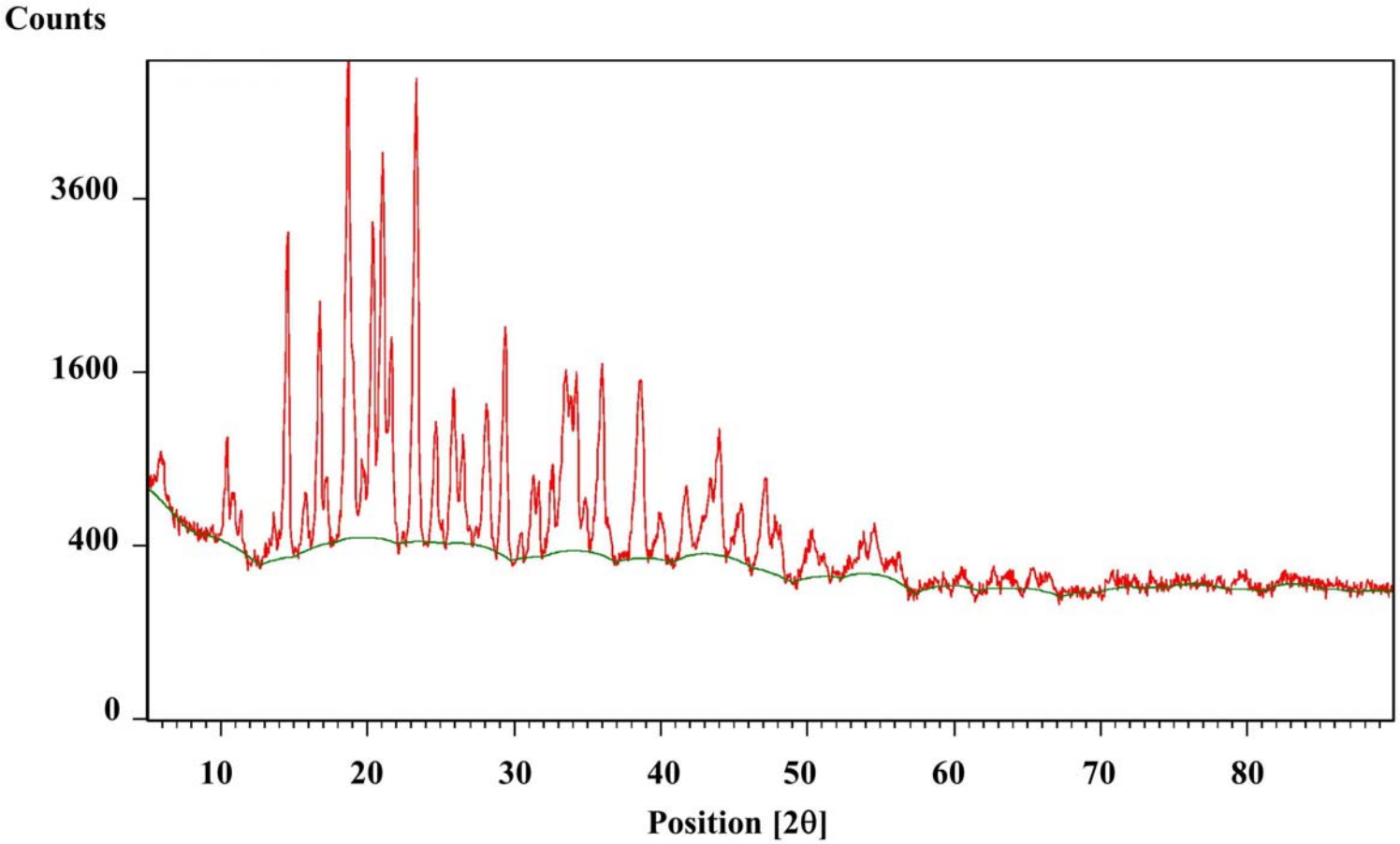

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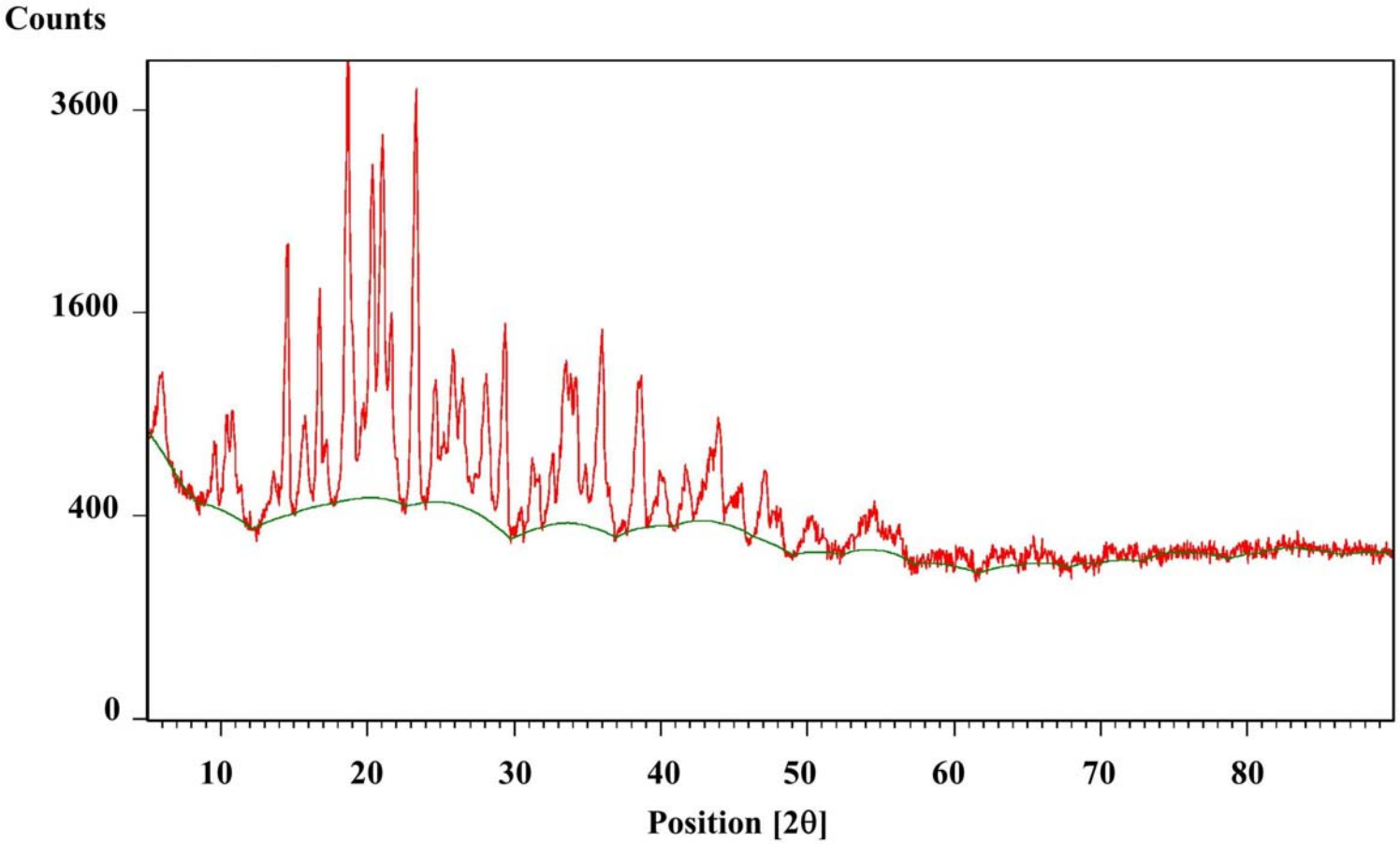

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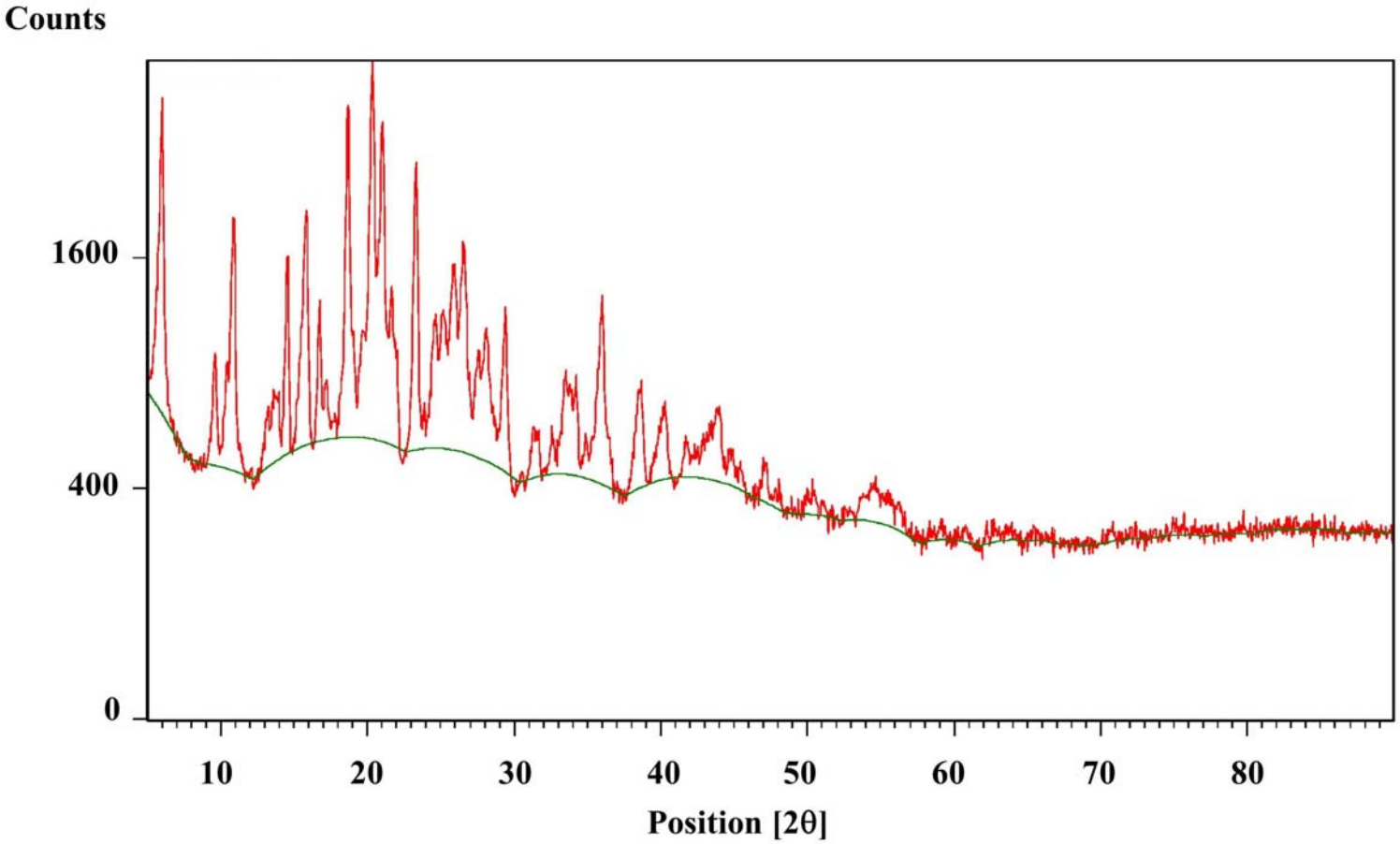

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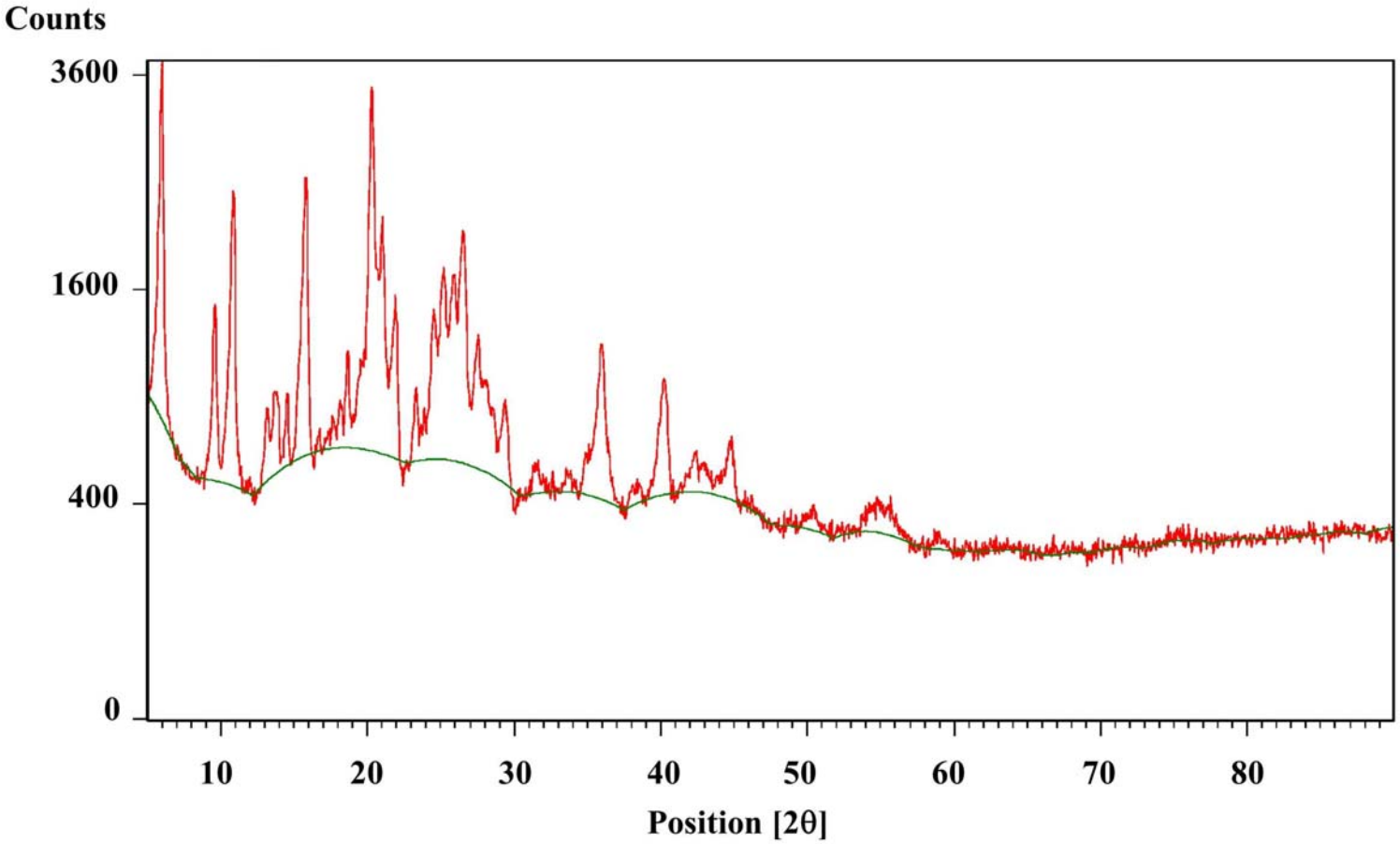

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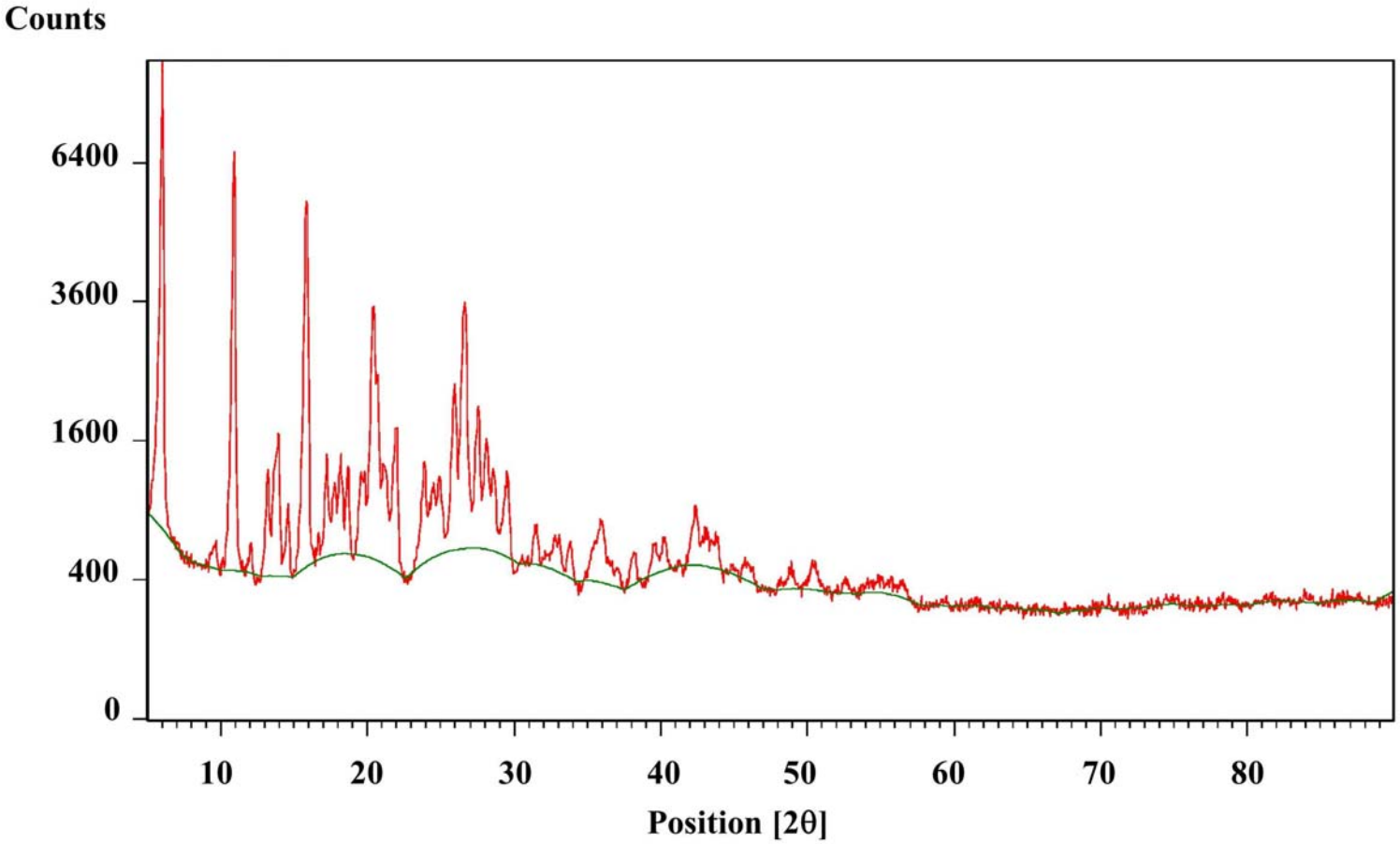

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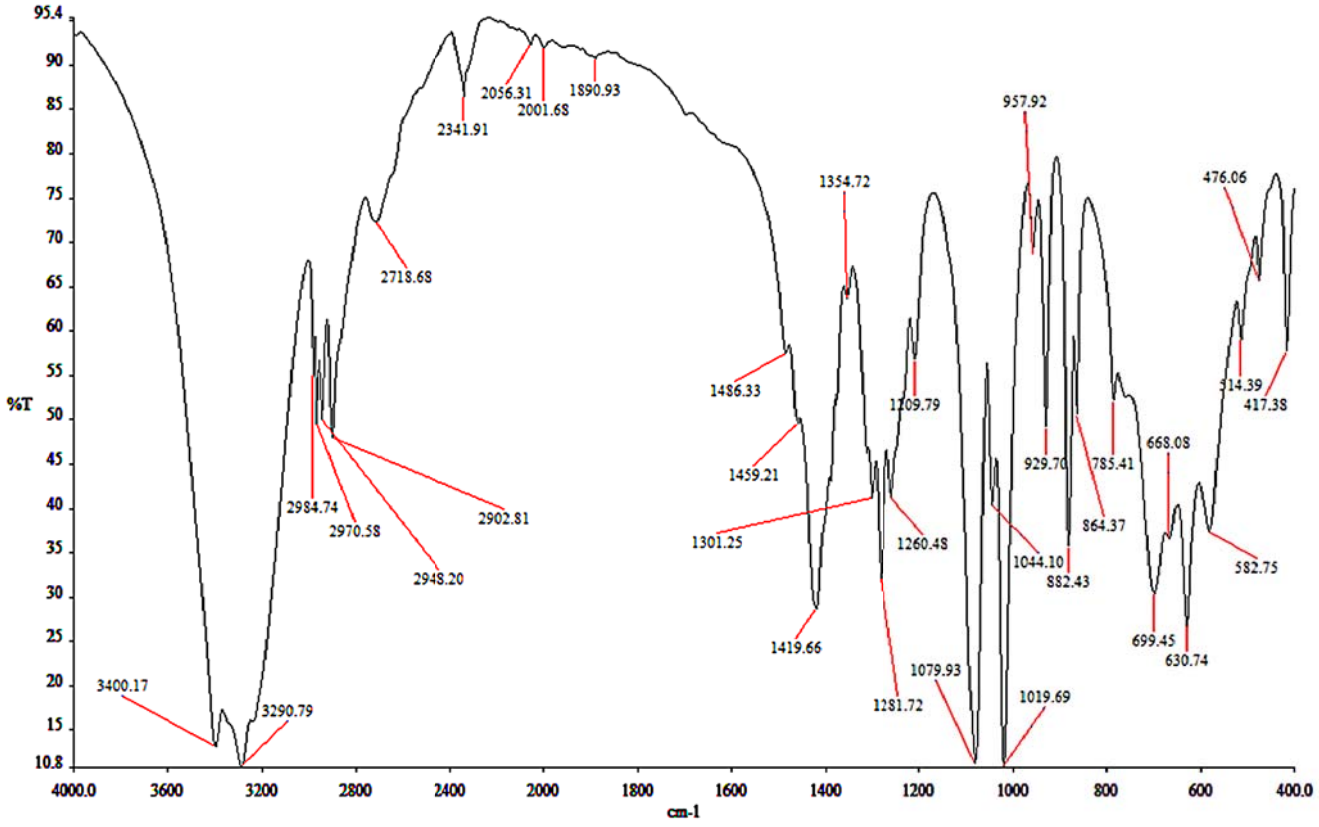
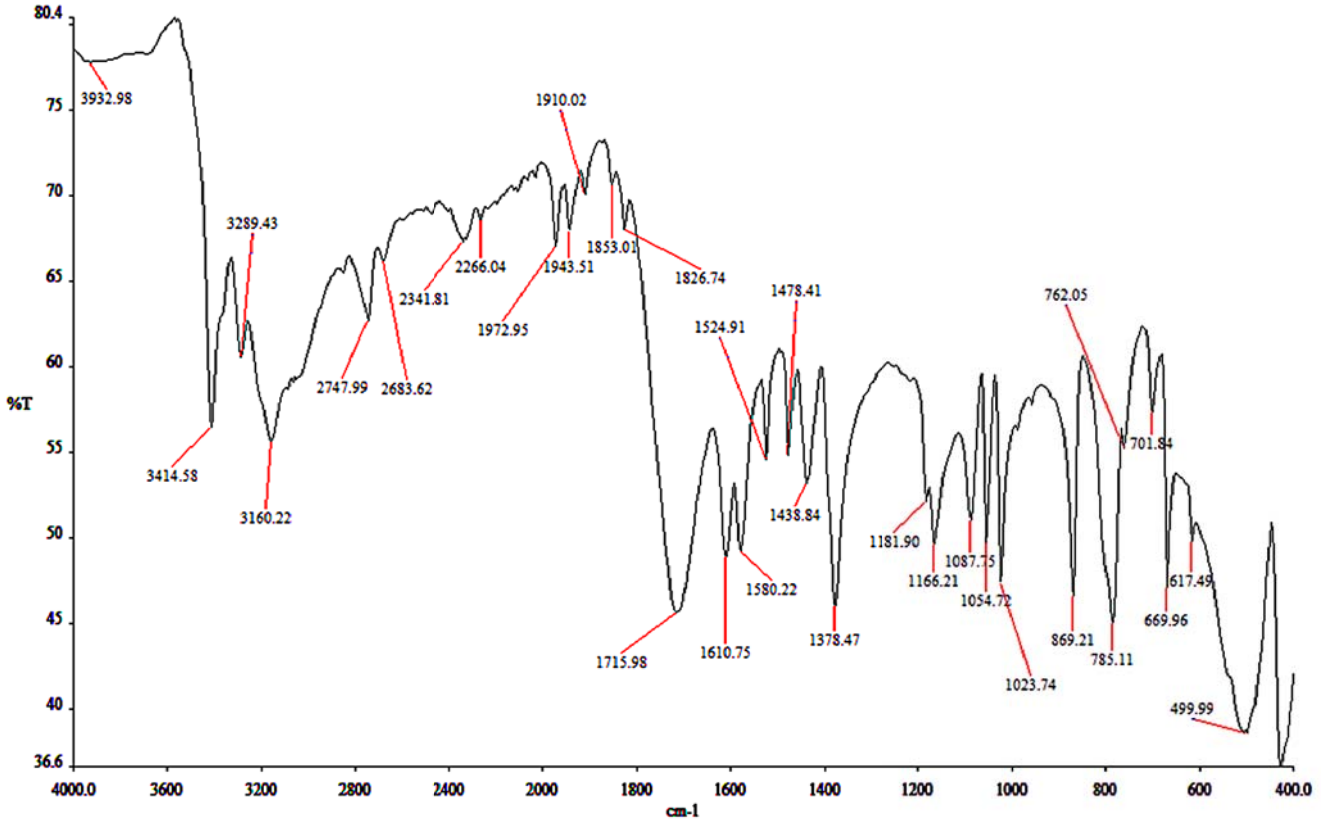


FT-IR pattern of porous mannitol (AC2)



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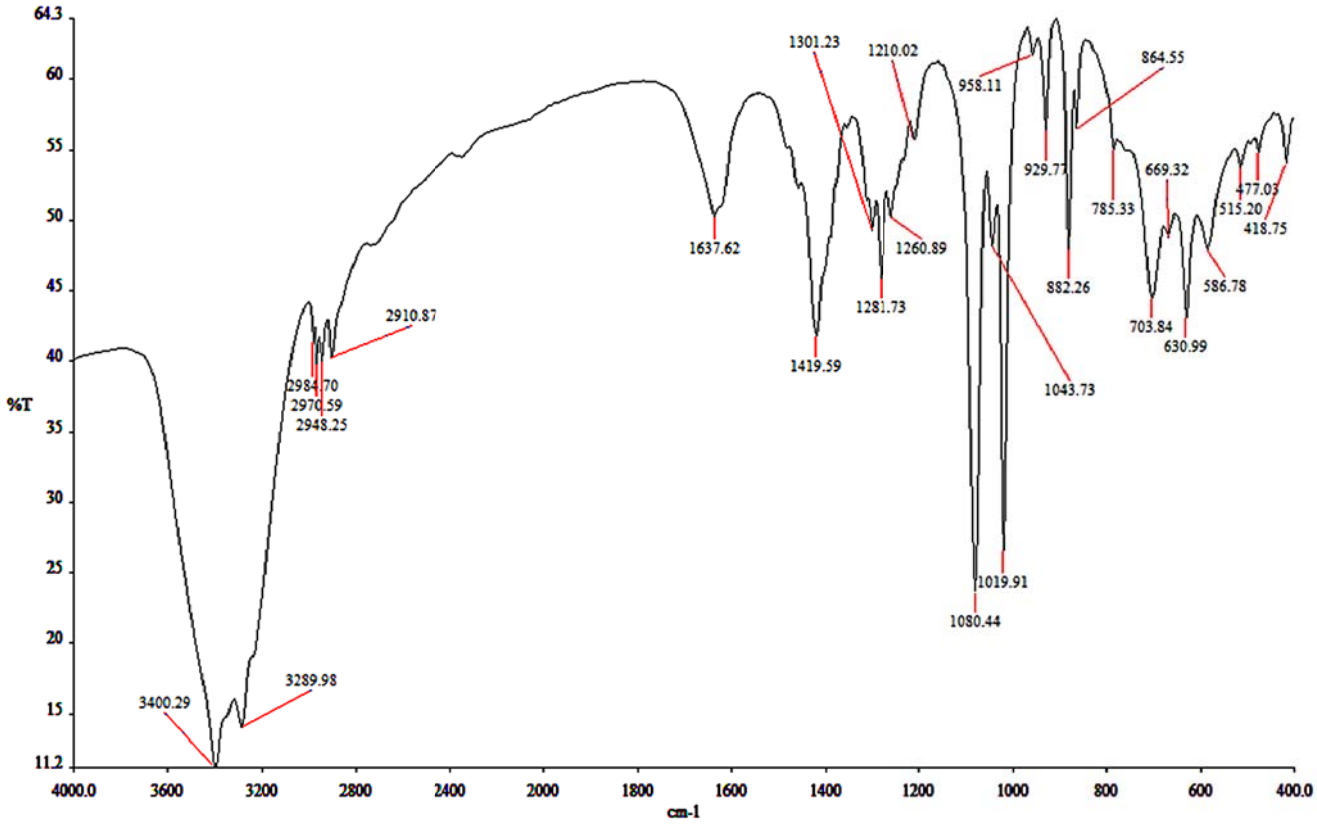

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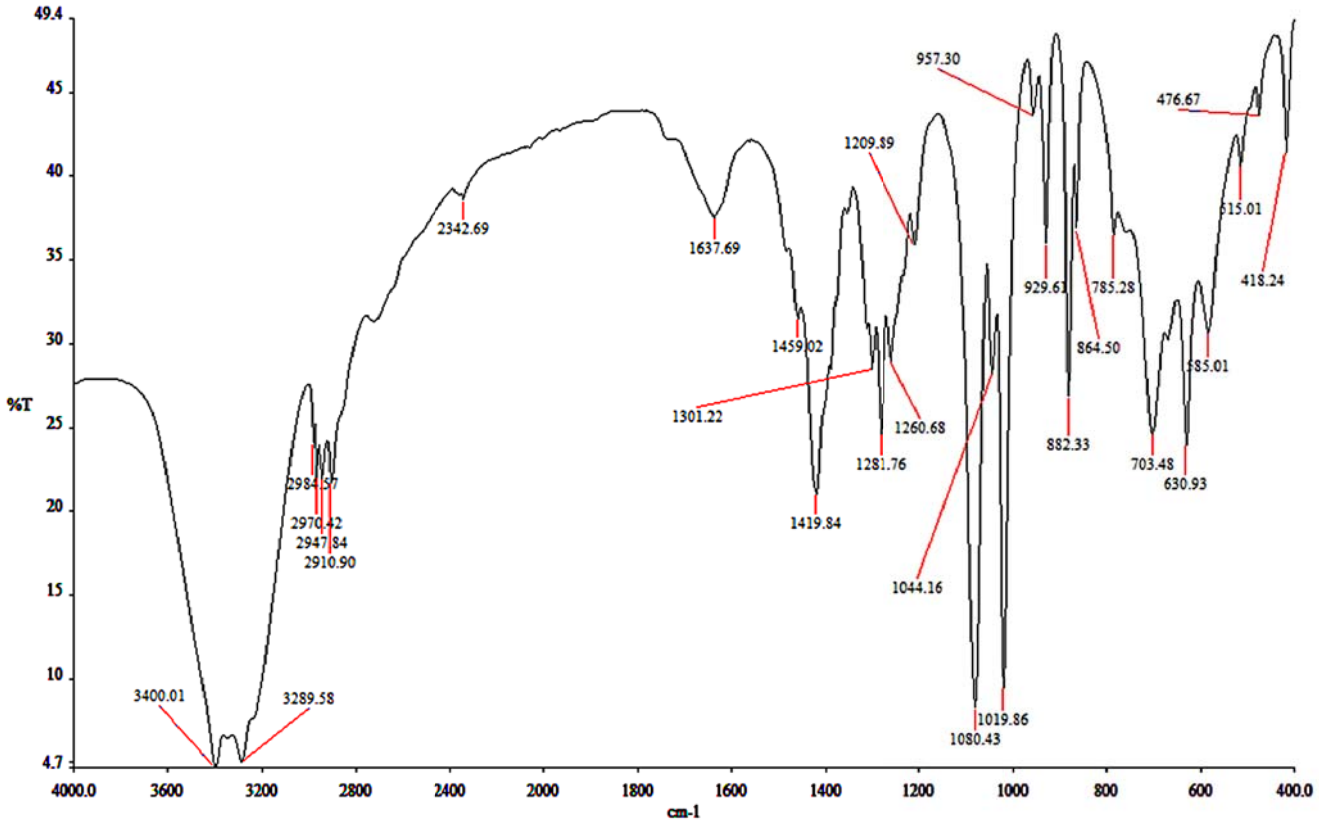

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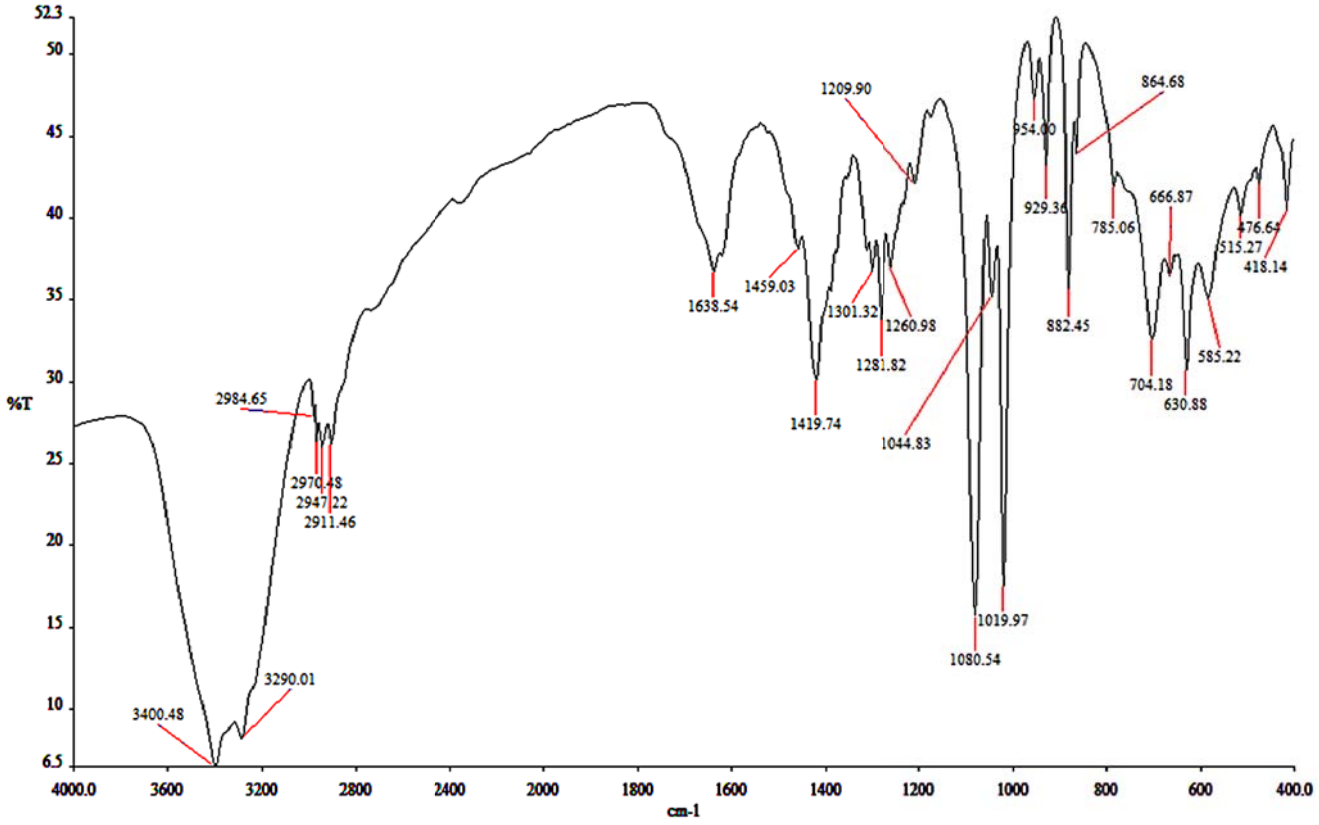

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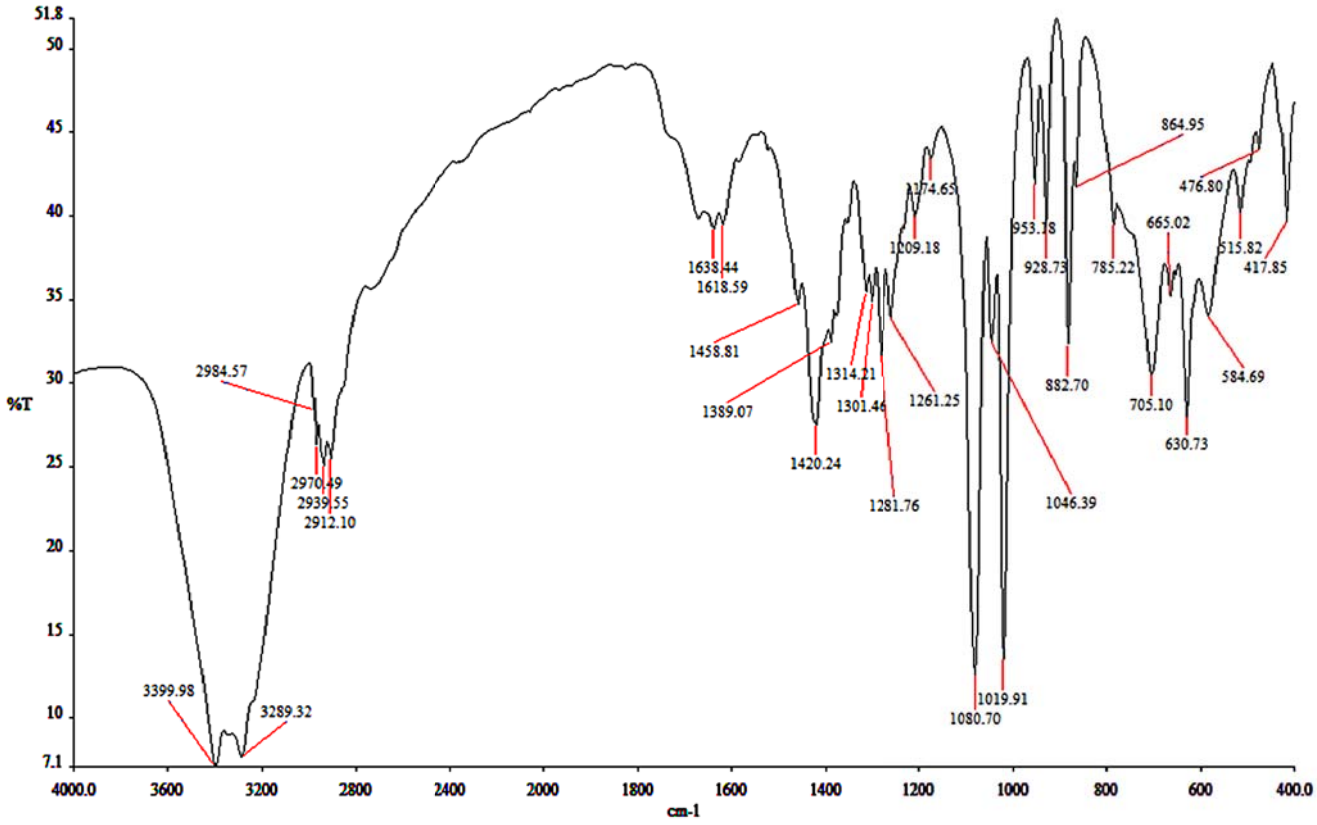
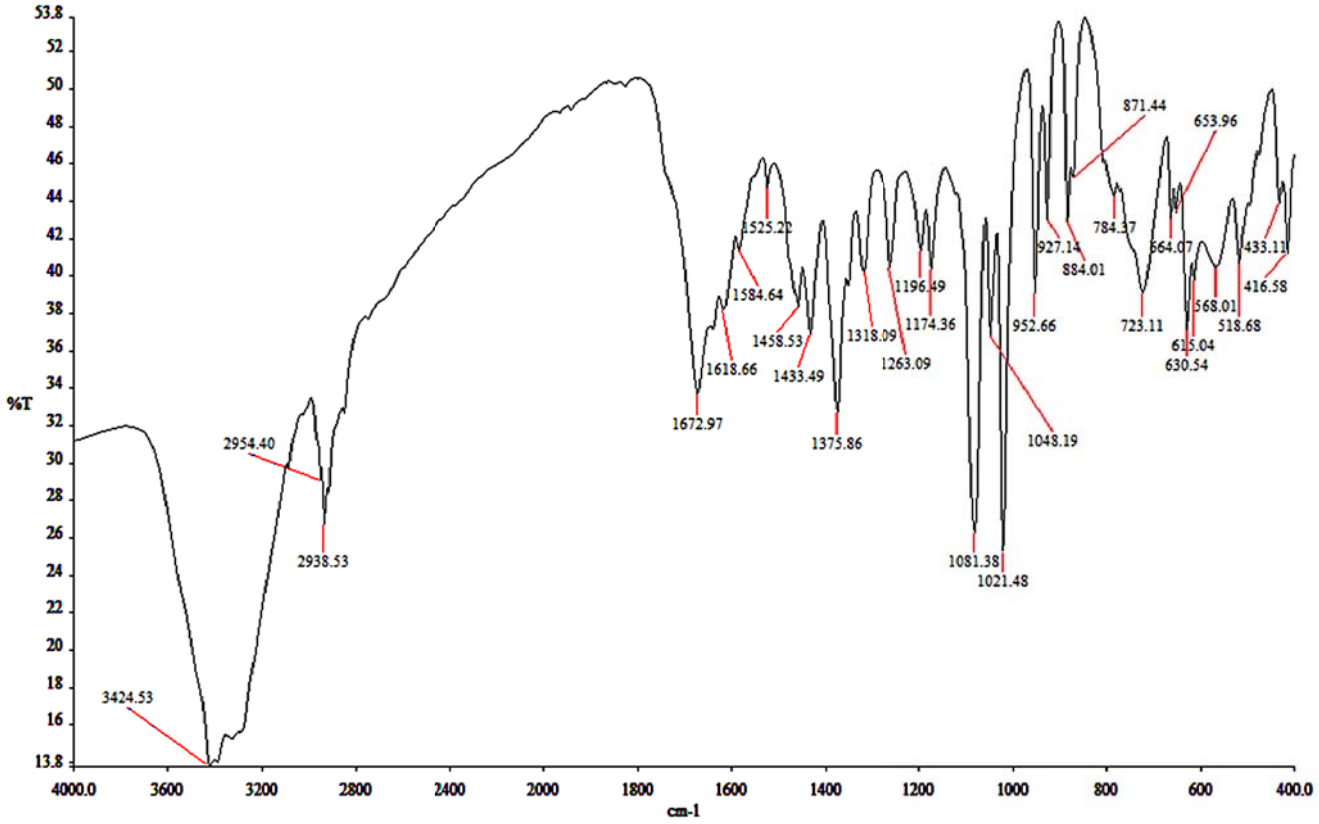
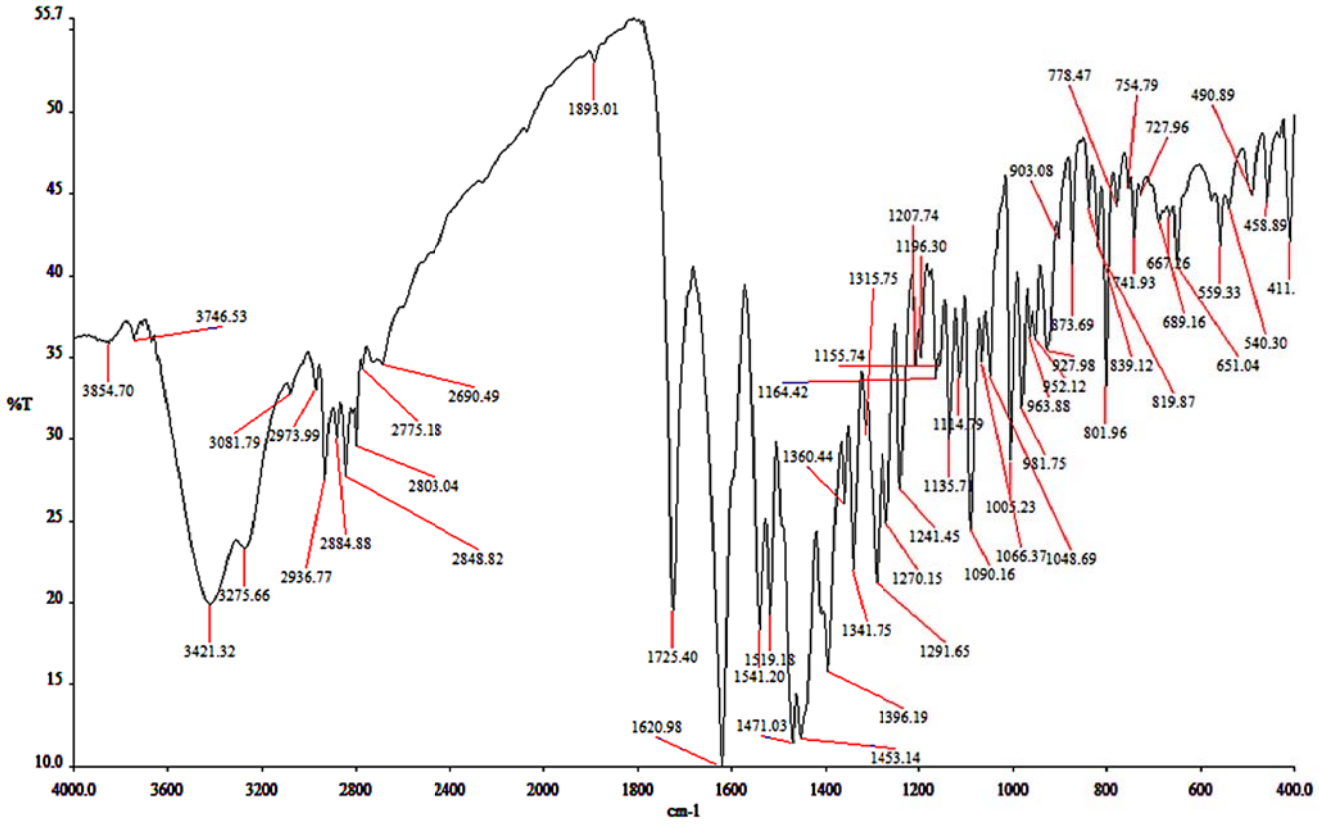


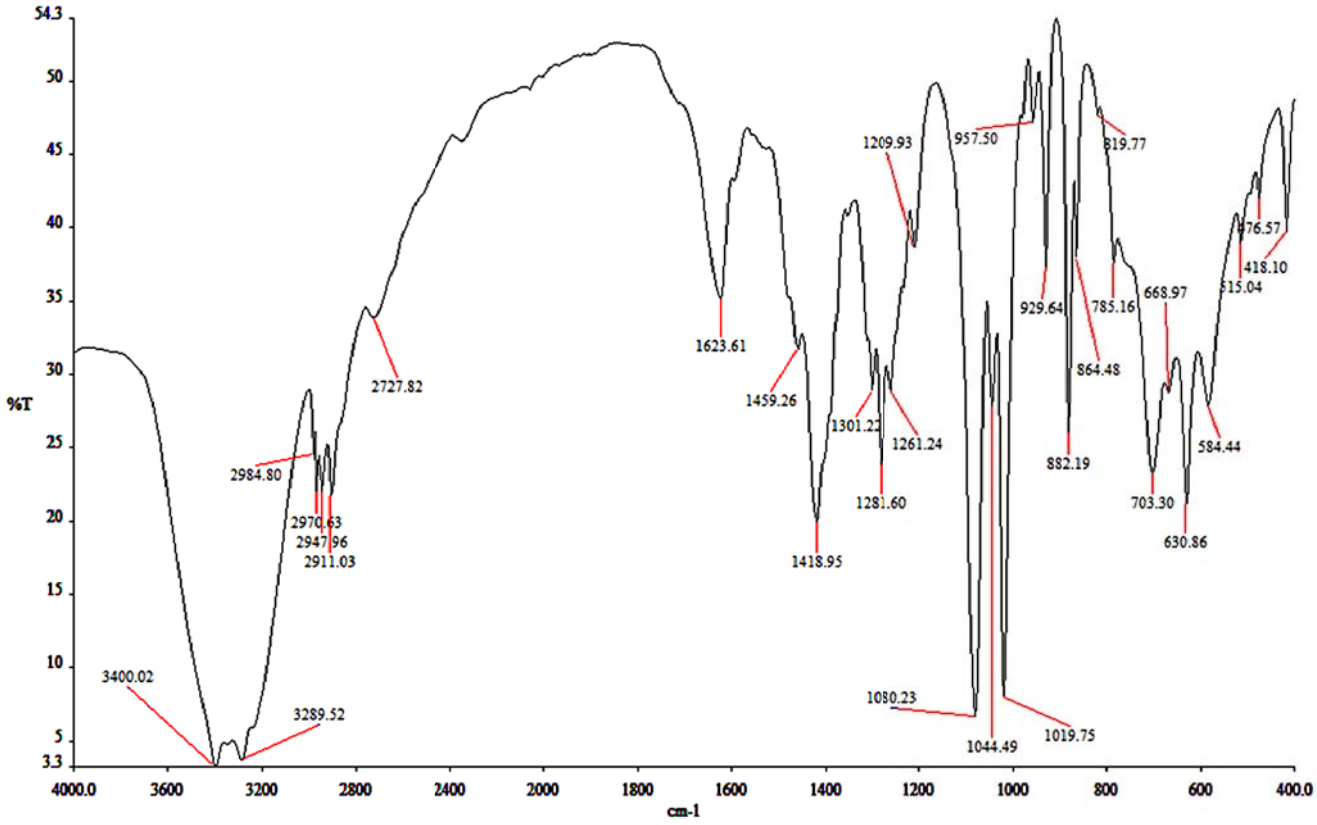
FT-IR pattern of PZA-proliposome formulation IV



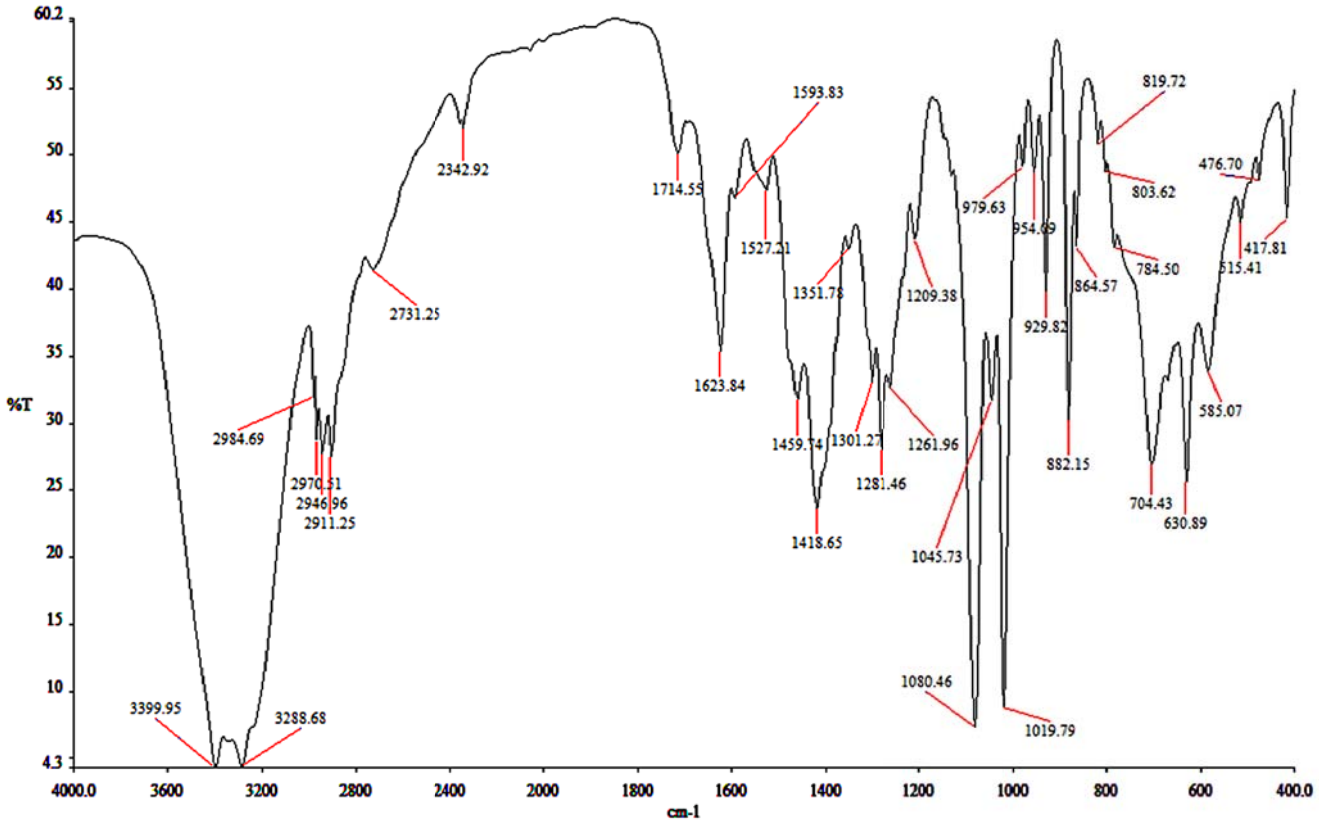
FT-IR pattern of PZA-proliposome formulation V



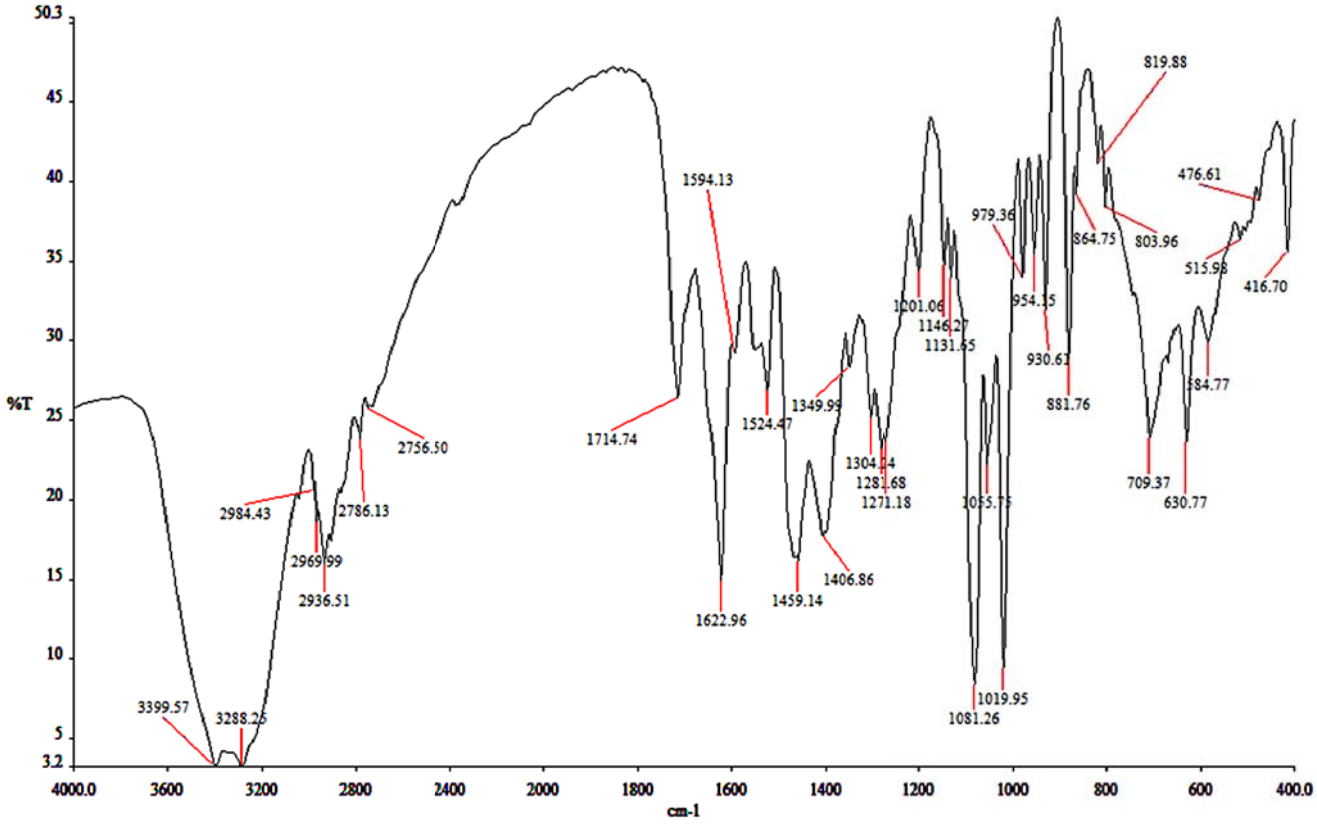
FT-IR pattern of LEV



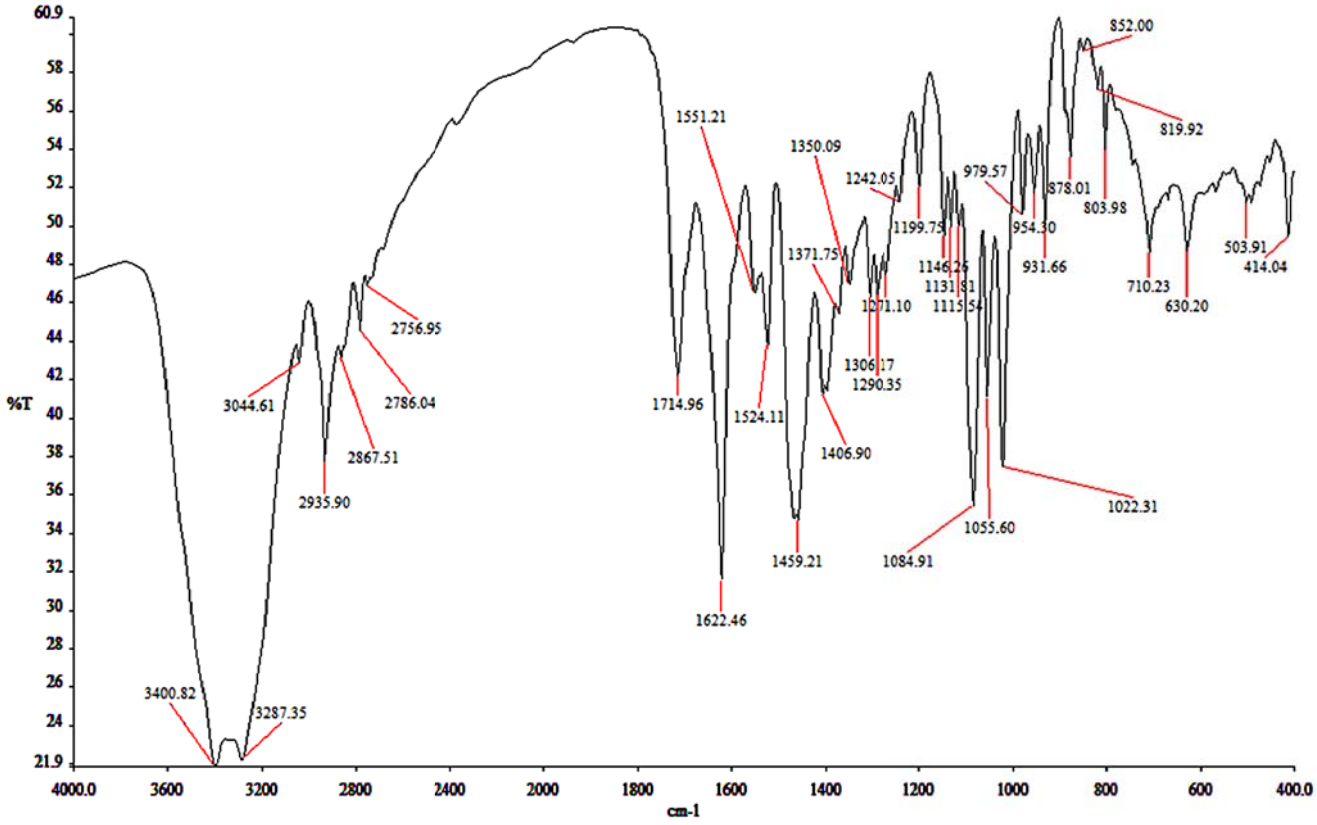
FT-IR pattern of LEV-proliposome formulation No.1



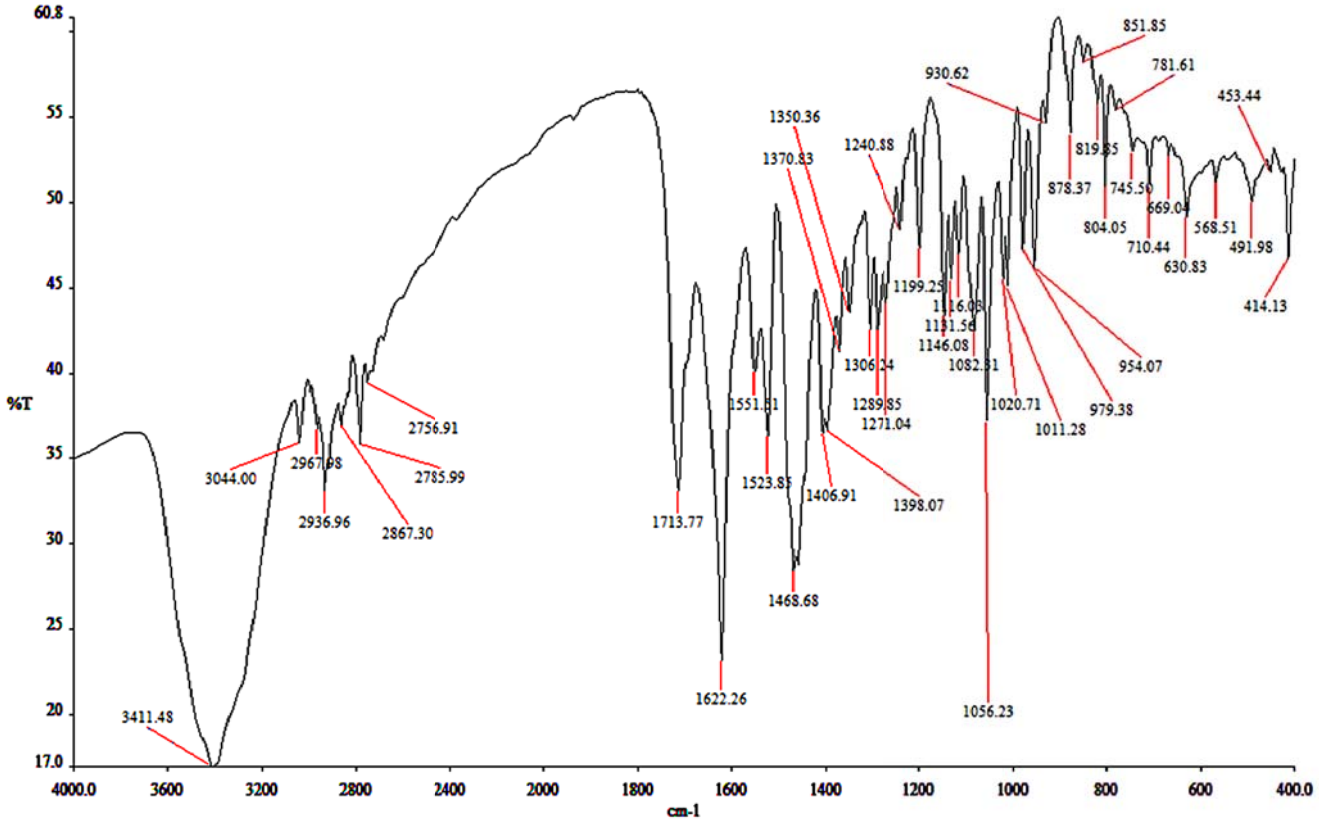
FT-IR pattern of LEV-proliposome formulation No.2



FT-IR pattern of LEV-proliposome formulation No.3



FT-IR pattern of LEV-proliposome formulation No.4



FT-IR pattern of LEV-proliposome formulation No.5

APPENDIX C: The acceptance for animal experiments from local Ethics Committee, Prince of Songkla University, Thailand (No. Ref 11/50)

ที่ ศธ 0521.11/๒๔๑

สำนักวิจัยและพัฒนา
มหาวิทยาลัยสงขลานครินทร์
อ.หาดใหญ่ จ.สงขลา 90110

Ref.11/51

หนังสือรับรอง

โครงการวิจัย เรื่อง การเตรียมยาต้านวัณโรคบรรจุในถุงขนาดอนุภาคนาโนและความเป็นไปได้ในการรักษาวัณโรคปอด

หัวหน้าโครงการวิจัย รศ.ดร.ธีระพล ศรีชนะ

ได้ผ่านการพิจารณาและเห็นชอบจาก คณะกรรมการจรรยาบรรณการใช้สัตว์ทดลอง มหาวิทยาลัยสงขลานครินทร์

ให้ไว้ ณ วันที่ ๓๐ มิถุนายน 2551

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MS. Pharm. (Pharmaceutical Sciences)	Prince of Songkla University, Thailand	2005
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List of Publications and Proceedings

Rojanarat, W., Yanyium, N. and Srichana, T. Single and repeated dose toxicity of pyrazinamide and levofloxacin inhaled proliposome in male Wistar rats. The 2nd current drug development international conference, Phuket, Thailand, 2-4 May, 2012.

Rojanarat, W., Changsan, N., Tawithong, E., Pinsuwan, S., Chan, H.J. and Srichana, T. Isoniazid proliposome powders for inhalation-preparation, characterization and cell culture studies. Int. J. Mol. Sci., 12: 4414-4434, 2011.

Rojanarat, W., Thawithong, E., Nakpheng, T. and Srichana, T. Levofloxacin proliposome as dry powder inhaler. 7th Joint seminar on biomedical sciences, Faculty of Medicine conference center, Prince of Songkla University, Thailand, 13-15 October, 2011.

Rojanarat, W., Thawithong, E. and Srichana, T. Porous microparticles of pyrazinamide-proliposome. RGJ seminar series LXXXIII, Faculty of Science, Prince of Songkla University, Thailand, 31 August, 2011.

Rojanarat, W., Thawithong, E. and Srichana, T. Microparticles of porous mannitol as dry powder inhaler carrier. The 1st current drug development international conference, Phuket, Thailand, 6-8 May, 2010.