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CLOFAZIMINE LOADED SOLID LIPID MICROPARTICLE FOR INHALED TUBERCULOSIS TREATMENT

By
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A THESIS

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ABSTRACT

Tuberculosis (TB), which killed 1.8 million patients in 2015, is still a real threat to public health despite the current therapy. The therapeutic success is limited due to, in part, mycobacterial resistance and side-effects related to the current regimen. Clofazimine (CFM) has activity against several species of mycobacteria and has not been clinically reported to be resisted by *Mycobacterium tuberculosis* which makes it ideal for multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) cases. However, it is classified as third-line agent for TB treatment due to poor pharmacokinetic properties and serious side-effects accompanying its oral delivery. Solid lipid microparticles (SLMs) is a delivery system that contains lipid and surfactant, and it can improve the incorporation and bioavailability of hydrophobic drugs such as clofazimine.

In this project, we believe that inhaled clofazimine delivery in the form of SLMs would be ideal to overcome the challenges of current TB treatment.

A variety of lipids and surfactants were formulated with curcumin as a drug model to estimate how they will behave with clofazimine. Anti-solvent precipitation technique (ASP) followed by spray drying (SD) or freeze drying (FD) was used to produce SLMs. These SLMs were characterized for batch yield, particle size distribution, physical state, and stability. Based in curcumin’s results, it was determined that SD do not change the particle size distribution of the formulations after ASP, while FD may increase the particle size. Also, FD keep high batch yield comparing to SD.

Clofazimine was formulated with eligible lipids and surfactants to produce SLMs. As a drying method, FD was able to keep high batch yield and drug content more than SD especially with formulations containing lipids. Lipids improved drug content more than
surfactants, but they made larger particles with multi-modal distribution. All clofazimine formulations were physically stable, chemically intact, and in crystalline form. Four out of fourteen clofazimine formulations showed excellent aerodynamic properties with mass median aerodynamic diameter (MMAD) < 5 µm in acceptable geometric standard deviation (GSD) and free particle fraction (%FPF). These formulations were FD and SD CFM: Dipalmitoylphosphatidylcholine, FD CFM: Palmitic acid: Lecithin, and SD CFM: Palmitic acid: Choline Chloride.
Dedicated to

My great parents, real brothers and sisters, dear wife, and darling daughter
ACKNOWLEDGMENT

Always and forever, I thank you, God, as how worshipers do for what You gave and prevented and for how You honored and preferred me over much of what You have created with definite preferment.

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<td>Tuberculosis</td>
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<td>WHO</td>
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<td>FDA</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>MDR-TB</td>
<td>Multidrug-resistant tuberculosis</td>
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<td>XDR-TB</td>
<td>Extensively drug-resistant tuberculosis</td>
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<td>SLPs</td>
<td>Solid lipid particles</td>
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<td>SLNs</td>
<td>Solid lipid nanoparticles</td>
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<td>SLMs</td>
<td>Solid lipid microparticles</td>
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<td>Clofazimine</td>
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<td>Curcumin</td>
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<td>Dipalmitoylphosphatidylcholine</td>
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<td>PA</td>
<td>Palmitic acid</td>
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<td>SA</td>
<td>Stearic acid</td>
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<td>Lec</td>
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<td>Sp80</td>
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<td>ASP</td>
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<td>NGI</td>
<td>Next Generation Impactor</td>
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<td>MOC</td>
<td>Micro orifice collector</td>
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<td>UIP</td>
<td>Universal induction port</td>
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<td>MDI</td>
<td>Metered dose inhaler</td>
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<td>DPI</td>
<td>Dry powder inhaler</td>
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<td>FPF</td>
<td>Free particle fraction</td>
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<td>GSD</td>
<td>Geometric standard deviation</td>
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<td>MMAD</td>
<td>Mass median aerodynamic diameter</td>
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<td>Differential scanning calorimetry</td>
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<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<td>UPLC</td>
<td>Ultra-performance liquid chromatography</td>
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<tr>
<td>XRPD</td>
<td>X-ray powder diffraction</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
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Chapter 1: Introduction
1.1 Tuberculosis

Tuberculosis (TB) is a major worldwide threat to public health. Even though it is not a major health concern in developed countries, TB is a leading cause of death in poor counties in Asia and Africa [1]. For example, in 2015 Nigeria reported 99 deaths per 100,000 while Bangladesh reported 45 deaths per 100,000. The United States of America reported 0.18 TB associated deaths per 100,000 while Saudi Arabia has about 2.1 deaths per 100,000. Globally, TB is one of the top 10 causes of death [2]. According to the World Health Organization (WHO), approximately 10.4 million new cases of TB occurred in 2015 while 1.8 million patients died because of the disease in that same year. Approximately 95% of the deaths attributed to TB in 2015 occurred in developing countries. The TB incidence rate has declined from the beginning of the new millennium by an average of 1.5% per year. However, this decline must reach a target reduction of 4-5% yearly to accomplish the WHO’s plan of “End TB Strategy” [2].

Figure 1. TB incidence rate in 2015 [2]
The countries in south Asia and Sub-Saharan Africa bear the brunt of TB incidence rate (Figure 1.1). Consequently, they have the highest mortality rate. For example, six countries from those regions (India, Indonesia, China, Nigeria, Pakistan, and South Africa) account for 60% of TB associated deaths [2]. Moreover, TB is the main lethal disease for people who have acquired the Human Immunodeficiency Virus (HIV) [3]. HIV co-infection is a factor for TB in addition to alcoholism, homelessness, and living in crowded areas [4]. In 2015, 400,000 HIV patients died from TB complications, which represent about 35% of the total number of TB associated deaths [2].

1.2 Pathology of TB

1.2.1 Mycobacterium tuberculosis

TB is an infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). This bacterium is a Gram-positive prototrophic, metabolically flexible bacterium that has high lipid content [5] [6]. *M. tuberculosis* is aerobic and tends to thrive in highly oxygenated environments, such as the lungs. *M. tuberculosis* replicates very slowly with bacterial divisions every 15 to 20 hours, compared to divisions on the order of minutes for several other pathogenic bacteria. Slow growing *M. tuberculosis* cultures may need to six weeks to become visible as colonies that are clustered together due to their hydrophobic nature, and take the form of bacillus, which make them able to withstand in unfavorable conditions for weeks [7].

1.2.2 TB pathogenesis:
In humans, TB is principally a lung infection that is disseminated through aerosolization of *M. tuberculosis* bacilli through the air by coughing, sneezing or inhalation of infected droplets [8]. The pathogeneses process of TB starts when *M. tuberculosis* reaches the healthy person’s lungs coming from infected patient as very small aerosolized droplets. Following inhalation of infecting bacilli, the immune system will mount a response with alveolar macrophages phagocytizing some bacilli. Approximately 95% of the infecting mycobacteria will be killed by this primary response (based on patient factors such as age, underlying immunity, etc.). However, the remaining mycobacteria will multiply within pulmonary macrophages that will eventually spread to lymph nodes. After two to eight weeks, a secondary immune response will be generated against the infecting mycobacteria in an attempt to contain the multiplying infection through the formation of granulomas composed on T-lymphocytes and macrophages. If stable granulomas are formed, then the TB infection will be contained and enter a stable stage, or “latent phase.” However, if unstable granulomas form, for example when the patient is younger than five years old or adult suffering from impaired immunity, the immune system is unable to adequately contain the mycobacteria and the infection will enter an infectious stage, or “active phase” (Figure 1.2). The macrophages themselves will be infected. In addition, the granulomas formed to contain the pathogen will become the source of bloody sputum [9] [10]. There are many symptoms that indicate a patient has active TB and include chronic cough with bloody sputum, fatigue, fiver, night sweats, breathing difficulty and chest pain [9].
1.3 Current treatment of TB

Due to the complexity of the disease pathology, TB infections require a long antibiotic regimen that consists of a combination of anti-TB agents for an extended period of time to eradicate both the slow-growing active phase and stable granulomas in the latent phase. The current clinical recommendations for TB treatment list the most active agents as first line drugs, with second and third line agents reserved for patients who do not respond to first line drugs or who have liver or kidney dysfunction (Table 1.1).
Table 1. Classification of TB drugs

<table>
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<th>Place in Therapy</th>
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<th>Medications or Classifications</th>
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<tr>
<td>1(^{st}) Line</td>
<td>Group 1</td>
<td>Isoniazid, rifampin, pyrazinamide, ethambutol</td>
</tr>
<tr>
<td>2(^{nd}) Line</td>
<td>Group 2</td>
<td>Injectable drugs: kanamycin, amikacin, capreomycin, streptomycin</td>
</tr>
<tr>
<td></td>
<td>Group 3</td>
<td>Fluoroquinolones: levofloxacin, moxifloxacin, ofloxacin</td>
</tr>
<tr>
<td></td>
<td>Group 4</td>
<td>Oral bacteriostatic: para-aminosalicylic acid, cycloserine, terizidone, thionamide, protonamid</td>
</tr>
<tr>
<td>3(^{rd}) Line</td>
<td>Group 5</td>
<td>Clofazimine, linezolid, amoxicillin /clavulanate, thioacetazone, imipenem /cilastatin, clarithromycin</td>
</tr>
</tbody>
</table>

The shortest recommended regimen, based on current treatment guidelines, is six months of active drug treatment divided into two phases. The first phase is called “intensive phase” and occurs during the first two months of therapy. The intensive phase of treatment is designed to stop and kill the replicating *M. tuberculosis* bacilli through the use of isoniazid, rifampin, pyrazinamide, and ethambutol. The second phase of treatment, or “continuation phase” typically lasts for four months and is designed to eradicate the dormant mycobacteria and ensure no “active phase” will develop from latent mycobacteria (Table 1.2). The main drugs in the continuation phase are isoniazid and rifampin. Current treatment guidelines emphasize that no fewer than two drugs used during all the regimen reduce the probability of emergent mycobacterial resistance [12].

<table>
<thead>
<tr>
<th>Phase</th>
<th>Drugs</th>
<th>Duration</th>
<th>Purpose</th>
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<tr>
<td>Intensive phase</td>
<td>Isoniazid Rifampin Pyrazinamide Ethambutol</td>
<td>Two months</td>
<td>To kill replicating mycobacteria (active phase)</td>
</tr>
<tr>
<td>Continuation phase</td>
<td>Isoniazid Rifampin</td>
<td>Four months</td>
<td>To kill dormant mycobacteria (latent phase)</td>
</tr>
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</table>
Although this regimen is effective in many cases, therapeutic success is limited by numerous factors. The development of resistance is the major limitation in TB treatment when mycobacteria respond to selective antimycobacterial pressure. Isoniazid and rifampin resistance are the most common forms of resistance and might be a reason for consideration of second- or third-line agents in treatment [13]. Although there are many causes that provoke *M. tuberculosis* to develop resistance against TB drugs, majority of them arise from mismanagement or misuse of antitubercular drugs, or from infection with a resistant strain. For example, when the drugs are not administrated as they prescribed, a low concentration of treatment will be therapeutically available and consequently, the mycobacteria are likely to develop resistance. Additionally, the six months duration of daily drug administration is a major challenge in TB treatment. So, not all the patients are able to remain compliant with the treatment regimen. As a result, TB resistance may occur [14].

Typically, TB resistance can be classified into either multidrug-resistant (MTD-TB) or extensively drug-resistant (XDR-TB). Mycobacteria resistant to isoniazid or rifampin or both are categorized as MTD-TB. On the other hand, XDR-TB includes mycobacteria with resistance against isoniazid, rifampin, one drug of fluoroquinolone group, and one of the injectable drugs group. Unfortunately, XDR-TB represents 9.5% of resistance cases, and it has been identified in 117 counties as of March 2017 [13]. A consequence of emergent TB resistance is a more complex treatment regimen. For example, the regimen duration for resistant TB infections is prolonged, up to 24 months. In addition, second and third line drugs are incorporated into the treatment regimen with an increase in the severity of side effects [13].
Another limitation of current TB drugs is the route of administration. All current first-line anti-TB drugs are administrated orally and require gastrointestinal absorption into the systemic circulation followed by distribution to the lungs, specifically pulmonary macrophages and granulomas, which are the primary site of the infection. The systemic exposure of absorbed drug then is associated with many side-effects of the medications. Generally, first-line anti-TB agents are associated with serious adverse effects, including hepatitis, dyspepsia, exanthema and arthralgia, that lead to termination of therapy in 23% of patients during the intensive phase of treatment [13]. Specifically, adverse effects of first-line agents include: hepatitis and peripheral nephropathy with isoniazid; hepatitis, thrombocytopenia, and flu-like syndrome with rifampin; gastrointestinal disorder and hepatotoxicity with pyrazinamide; and visual disturbances with ethambutol. Therefore, the basic regimen is not recommended for patients who have hepatic dysfunction [9]. Also, the systemic drug exposure has increased potential for drug-drug interactions, especially in patients taking other medications for co-morbid conditions [16]. For example, coadministration of rifampin and the antiviral zidovudine causes an increased rate of zidovudine clearance. In addition, isoniazid absorption is decreased if it use with aluminum hydroxide, and may enhance phenytoin absorption [9] [17]. Rifampin may diminish the plasma concentrations of many drugs, including methadone, warfarin, oral contraceptives, dapsone, and ketoconazole [9].

Economically, current TB treatment is expensive, especially for the individuals and healthcare systems in developing countries where TB is endemic. The typical price of first-line anti-TB treatment is approximately $2,000. However, the medication cost increases to approximately $25,000 if treatment for resistant TB is required [18]. The economic burden
of treatment can also affect patients and health-care systems in developed countries. For example, the estimated cost of first-line anti-TB treatment in the United States is estimated to be approximately $17,000 in 2005. The price jumped up to $134,000 for treatment of MDR-TB and to $430,000 for XDR-TB [19].

1.4 Inhaled TB treatment as an alternative

1.4.1 The advantages

As discussed above, current first-line anti-TB drugs are systemically administrated and result in many side effects with the potential for drug-drug interactions due to extended treatment. Targeted drug delivery to the lungs at the site of the infection could be lead to improved clinical efficacy and reduce the systemic side effects and potential for drug-drug interactions. Inhaled TB treatment has substantial potential for improved clinical outcomes and can overcome many challenges of current systemic treatment. For example, inhaled TB treatment can overcome the side-effects associated with oral delivery such as hepatitis, dyspepsia, exanthema, and arthralgia by reducing systemic drug exposure [20] [15]. Targeted delivery can also reduce the possibility of resistant *M. tuberculosis* strains developing because inhaled delivery will promote high lung concentrations of the drug [21]. Moreover, inhaled delivery will potentially require lower drug doses and shorter durations of treatment because drug should be available at alveolar macrophages. These advantages should then encourage improved patient adherence to the course therapy [20].
1.4.2 Preliminary studies

Several publications have reported preliminary findings in field of inhaled anti-TB drug delivery. Although no inhaled anti-TB treatment is currently used clinically, the reported results are promising and encouraging that this route could lead to improvements in clinical anti-TB treatment. For example a study in 2015 compared oral, parenteral, and pulmonary administration of rifampicin in healthy guinea pigs. The formulation was prepared by spray drying rifampicin with leucine for inhaled delivery as porous particles. After administration of rifampicin to the animals by these three routes, plasma samples were collected and analyzed for drug concentrations by high performance liquid chromatography (HPLC). They found that the group that received rifampicin by the pulmonary route had faster absorption and higher bioavailability than oral delivery group. Importantly, only the inhaled rifampicin group had drug concentrations in the bronchiole lavage fluid above the minimum inhibitory concentration of *M. tuberculosis*. The authors concluded that pulmonary delivery of rifampicin was potentially more effective in TB treatment than oral delivery [22].

Another study from 2015 prepared two solid lipid nanoparticles (SLNs) of rifabutin in a lipid matrix of glyceryl dibehenate or glyceryl tristearate. Full characterization for chemical and physical stability, morphology, aerodynamic properties, and efficacy was performed on the two formulations. Then, an *in vitro* study was conducted by using THB 1, A549, and Calu-3 cells to measure the cell uptake and viability. They reported that the formulation containing glyceryl dibehenate has drug uptake that was almost twice that of
the formulation containing glyceryl tristearate with low cytotoxicity for both formulations. They concluded that SLNs can be a good preparation method for inhaled delivery [21].

In 2013, clofazimine, a third line drug, was prepared by spray drying in different locations with or without leucine in ethanol, dimethyl sulfoxide or distilled water for inhalation delivery. The formulated powders were suitable for deep lung delivery as they had globular shape with particle size <5 µm. In order to evaluate the therapeutic effect of clofazimine formulations, an animal study was done. Forty-eight Swiss mice were divided into groups of eight; 6 groups were for treatment study, 1 untreated group was for control, and 1 group treated with isoniazid and rifabutin was for comparison. The experiment stated 21 days after the mice were infected with TB by using *M. tuberculosis* H37Rv bacilli and lasted for 4 weeks As a result, Clofazimine could eradicate 99% of the mycobacteria at very low concentration and short period of time [23].

### 1.5 Clofazimine

Clofazimine (CFM) is an antimycobacterial drug used to treat *M. leprae* infections (leprosy) that has good activity against other mycobacterial species, including *M. tuberculosis*. Clofazimine resistance has not been reported in mycobacteria species which makes it ideal for MDR-TB and XDR-TB cases. According to the WHO, clofazimine is classified one of the essential drugs that should be available for general health care [24]. Therefore, clofazimine is of particular interest for further development for inhaled anti-TB treatment.
1.5.1 Physiochemical properties

Clofazimine is a riminophenazine dye (Figure 1.3) that can be red, orange, or even colorless based on the degree of protonation [25]. It has a molecular weight of 473.396 g/mol and is practically water insoluble with an aqueous solubility of 0.225 mg/L. Clofazimine is lipophilic with a partition coefficient value of 7.39 and can be dissolved in non-polar solvents such as chloroform, acetone, and alcohol [26].

![Clofazimine chemical structure](image)

Figure 1. 3 Clofazimine chemical structure

<table>
<thead>
<tr>
<th>Drug</th>
<th>MW g/mol</th>
<th>MP C°</th>
<th>Solubility mg/L</th>
<th>Log P</th>
<th>Density g/cm³</th>
<th>Max λ Nm</th>
<th>Local λ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clofazimine</td>
<td>473.39</td>
<td>210</td>
<td>0.225</td>
<td>7.39</td>
<td>1.29</td>
<td>283</td>
<td>492</td>
</tr>
</tbody>
</table>

1.5.2 Pharmacokinetics of clofazimine

1.5.2.1 Absorption

The absorption of clofazimine is associated with inter- and intra-individual variability due to its aqueous insolubility and large lipophilicity. Maximum drug concentrations occur at
highly variable times and are influenced by the fasted or fed state of the subject. Clofazimine is incompletely absorbed from the gastro-intestinal track following oral administration due to its hydrophobicity. However, there are several factors have effects on bioavailability of clofazimine, including the particle size distribution, dosage form, and co-administration in presence of food. For example, a study showed that administrating clofazimine in presence of food enhanced the AUC up to 60% and the maximum drug concentrations increase up to 30% comparing with administrating it without food [27]. Clofazimine is recommended to be administrated with high fat meal. Additionally, the absorption of clofazimine will not be more than 20% if it is prepared as coarse crystals. However, it may reach 75% if it prepared in microcrystalline suspension in an oil wax base in capsules [28].

It is reported that a 100 mg oral dose has maximal bioavailability (approximately 62%) when compared to higher doses. The oral bioavailability of higher doses is approximately 45% for all other doses [28]. To illustrate, a study stated that patients of leprosy received 100, 300, 400, and 600 mg single oral dose. The patient who received 100 mg dose absorbed 62.2 % of drug while only 42.6 % was absorbed in the patient with 600 mg [29]. High steady state concentrations were observed with increasing the number of clofazimine doses and the dose strength. A study showed that single daily doses of 100 mg of clofazimine achieved steady state concentrations of 0.7 µg/ml and 1 µg/ml with a 300 mg dose. Also, it was 0.5 µg/ml when 100 mg is administrated three times weekly [28].
1.5.2.2 Distribution

Clofazimine is a very hydrophobic drug that preferentially distributes into the organs of reticulo-endothelial system (organs with large numbers of macrophages) and to fatty tissues. This distribution leads to deposition of clofazimine crystals in tissues throughout the body and can lead to discoloration of skin, sclera, fatty organs, and some body fluids. For example, leprosy patients taking systemic clofazimine show clofazimine crystal deposition in their skin in correlation with the size of granule. The mesenteric lymph nodes are the main place of distribution and crystal accumulation [30]. Clofazimine crystals have also been reported in adipose tissue, adrenals, liver, lungs, kidney, spleen, and bone. Clofazimine can cross the placenta and is toxic to a growing fetus. A study reported that three neonates had died in 15 pregnancies that their mothers administered clofazimine. In addition, it can distribute to milk of pregnant women [31]. Therefore, clofazimine use is not recommended during pregnancy and breastfeeding.

Although some studies concluded that clofazimine is not life threatening with presence of massive accumulation in some organs, others concluded the opposite. For example, one study concluded that clofazimine caused a fatal case of pyoderma gangrenosum in a patient taking 400 mg clofazimine every day for five months followed by 300 mg for six months. After these 11 months, the patient was hospitalized for severe abdominal pain laparotomy revealing infarction of the spleen, with violaceous congestion of the small bowel [32]. In a separate report, the peak clofazimine concentration was reached after 4-12 hours following a 200 mg oral dose. However, it takes up to 30 days to achieve peak clofazimine concentrations if clofazimine is administered as multiple doses. High single doses of
clofazimine followed by daily maintenance doses is often recommended to bypass long
term accumulation of clofazimine [27].

Clofazimine has high protein binding. A recent study reported that the percentage of free
clofazimine was less than 15% regardless its initial dose [33]. In general, clofazimine has
the ability to distribute to most parts of body and can accumulate in macrophages, the liver,
and spleen, and it is less concentrated in lungs, small intestine, and heart [34]. A study
mentioned that oral clofazimine did not distribute to the brain and cerebrospinal fluid, but
a recent report in mice reported clofazimine distribution into the brain [35].

1.5.2.3 Elimination

Clofazimine has a very long elimination half-life. The elimination half-life of clofazimine
ranges from 8 days following a single dose up to 70+ days following multiple doses.
Clofazimine has first order elimination and is typically modeled using a two-compartment
pharmacokinetic model consisting of a fast distribution phase and slow elimination phase.
Some studies mentioned that residual clofazimine was detected in patients several years
after discontinuation of therapy. For example, reports indicated it was found in the skin of
patient 2 years after use and in mesenteric lymph nodes of a separate patient 4 years after
use [36].

Clofazimine (N,5-bis(4-chlorophenyl)-3-propan-2-yliminophenazin-2-amine) is excreted
uncharged with presence of two major metabolites. The first metabolite is unconjugated
(phase 1 reaction) and is formed by hydrolytic dehalogenation process: 3-(p-
hydroxyanilino)-10-(p-chlorophenyl)-2,10-dihydro-2-isopropyliminophenazine. The
second metabolite is conjugated (phase 2 reaction) and it is formed by hydrolytic
deamination reaction followed by glucoronidation [27]: 3-(beta-D-glucopyranosiduronicacid)-10-(p-chlorophenyl)-2,10-dihydro-2-isopropyliminophenazin. These metabolites retain the red color and have similar properties to clofazimine with slightly increased polarity than the parent compound [27]. Clofazimine, and its metabolites, are primarily excreted into the bile and eliminated in the feces [37]. This fecal elimination is variable from patient to another with the percentage of excreted clofazimine ranging from 35 to 74% [38]. In addition, this variation in excretion depends on the accumulation of clofazimine in the body.

1.5.3 Mechanism of action

The exact mechanisms of clofazimine’s anti-mycobacterial action is not known. It is hypothesized to kill bacteria by binding to the DNA leading to disruption of the cell cycle. It may also bind to bacterial potassium transporters and increase the levels of cellular phospholipase [39]. It is known to be bactericidal against both *M. leprae* and *M. tuberculosis*. However, it is only has bacteriostatic action against the other mycobacteria.

1.5.4 *In-vitro and in-vivo* studies of clofazimine

Clofazimine’s pharmacological activity prevents its complete elimination from clinical use as an antimycobacterial agent. *In vitro* studies of clofazimine have been reported with several pulmonary derived cell cultures such as human alveolar pneumocyte epithelial cell line, murine macrophage cell line (J774), fresh human peripheral blood-derived macrophages [39], THP-1 [41], U937 [42], NR8383 [43] [44]. Alveolar macrophage cell cultures are suitable for many *in vitro* cell cultures because macrophages play the important
role in TB pathogenesis process [43]. To illustrate, when *M. tuberculosis* reaches the lungs, the immune system will attack the bacteria with macrophages by phagocytosis to kill some mycobacteria. However, some will survive and multiply, leading to that the macrophages themselves being infected and a source of the disease [10]. A study presented variety of clofazimine analogs that have better activity against TB and used J774A.1 (monocyte macrophage cell line) to evaluate the activity of them by using a recovery assay. The authors made a comparison between the extracellular recovery of bacteria following 3 days of intracellular exposure and the analogs at different concentrations. After 7 days of recovery, the bacterial titers for analogs exposed wells were lower than for those of the no compound control. When they designed the macrophage assay, they let J774A.1 grow in 75 cm² cell culture flasks in Dulbecco’s Modified Eagle Medium. Then, these cells separated by cell scraper, centrifuged, re-suspended to final concentration, distributed into well plates, and incubated for TB H37Rv [45]. Several animal models have been reported for *in vivo* TB studies and include mice [46] [30][23][47] [48], rats [46] [49], guinea pigs [46] [50], monkeys [46] [50], cattle [50], and zebrafish [50]. A majority of published animal studies involve efficacy and/or toxicity evaluation in mice [23] [48] and rats [51] [49] with additional reports in hamsters. However, monkey and guinea pig based models were not recommended [52].

One sample mouse model study involved 48 Swiss mice divided into 8 groups of 6 (1 group was a control group, 1 group treated with isoniazid and rifampin, 3 groups treated with oral clofazimine with different concentration, and the last 3 groups treated with inhaled clofazimine). Mice were infected with *M. tuberculosis* by inhaled cultures of the H37Rv strain. They found that clofazimine could eradicate 99% of the mycobacteria at very low
concentration and short period of time [23]. Another study evaluated the toxicity of clofazimine by hematological, hemostatic and biochemical parameters in male Wistar rats. It reported clofazimine caused increases in the plasma level of gamma-glutamyltransferase, increased the numbers of polymorphonuclear cells and degenerating cells, and prolonged the prothrombin time which all suggest that clofazimine can induce hematological, hemostatic and hepatic changes [51].

1.5.5 Side effects

As previously discussed, clofazimine accumulation in tissues as crystalline deposits and cause skin and tissue discoloration, in approximately 75% of patients who received clofazimine, and can develop to ichthyosis in 66% of patients in the first 10 weeks [53]. Because of clofazimine’s long half-life, the skin can require substantial time, from several weeks to years, to eliminate crystal deposit-based discoloration and irritation. The discoloration is not exclusively on the skin, but it can pass to the body secretions, such as sweat, urine, and sputum, and to other organs like conjunctiva and cornea. Consequently, visual disturbance, such as eye dryness and irritation has been reported with clofazimine. Another serious side-effect of clofazimine is gastrointestinal tract disturbance where the dose play important role in the degree of its severity. Approximately 60% of patients report gastrointestinal disturbances, including diarrhea, nausea, and vomiting, and this may lead to other troubles like weight loss [54].
1.5.6 Inhaled clofazimine for TB treatment

As it is discussed previously (Section 1.4 Inhaled TB treatment as an alternative), targeted drug delivery to the lungs could overcome many of the challenges of current TB treatment. Of particular interest is the long retention of clofazimine in tissues and an associated long elimination half-life. Following inhaled delivery, clofazimine should be retained in the lung tissue and pulmonary macrophages for an extended duration which should cause prolonged antimycobacterial to completely eradicate the infection. Inhalation of clofazimine should also minimize the systemic availability of the drug and reduce the side effects associated with clofazimine therapy. Additionally, the long lung retention and poor systemic distribution of inhaled clofazimine could lead to a reduction in the number of doses required to treat TB and a simplification of anti-TB therapy. Nevertheless, the targeted delivery of inhaled clofazimine to the deep lung is non-trivial and requires additional investigation because it has needle shape.

1.5.7 Preliminary studies

Previous work done in this laboratory demonstrated that the formulation of clofazimine with DPPC by organic solvent-based spray drying to prepare an inhalable dry powder can obtain ideal aerodynamic parameter for inhaled TB treatment while retaining antimycobacterial activity [55]. Four formulations were prepared with clofazimine and excipients at a weight-based ratio that ranged from 100% (w/w) clofazimine down to 50% (w/w) clofazimine to 50% (w/w) DPPC. These formulations were characterized for aerodynamic properties, particle size distribution, the patch yield potency, anti-TB activity, physical state and stability, and chemical integrity. All formulations contained crystalline
drug, retained chemical integrity, and had anti-TB activity similar to pure clofazimine. However, the batch yields were less than 50% for all formulations. Of all the prepared formations, the 80:20::clofazimine:DPPC formulation had the best aerodynamic parameters (mass median aerodynamic diameter of 3.2 µm, geometric standard deviation of 2.58, and free particle fraction of 47.43%) and had spherical particles, and was determined to be most compatible for inhalation delivery. Nevertheless, the low batch yield and organic solvent-based spray drying were substantial limitations for further formulation development.

1.6 Solid lipid microparticles as delivery system

One potential formulation improvement for inhaled clofazimine is through the incorporation of solid lipid particles (SLMs). These particles can potentially dissolve clofazimine in a delivery system that contains lipid as a solid core and associated with surfactant molecules. These particles are prepared by dissolving the drug in the lipid phase and then incorporating a surfactant-containing aqueous phase followed by drying (Figure 1.4) [56].
In 1990, solid lipid particles (SLPs) were developed to be an alternative to other colloidal delivery systems that use lipids and surfactants, such as liposomes and emulsions. There are two broad categorizations of SLPs based on the particle size distribution ranges, nanoparticles and microparticles. Solid lipid microparticles (SLM) are potentially better for lung delivery because particles in the low micron range tend to preferentially deposit in the deep lung [57]. The role of the lipids in SLPs is to improve the solubility of the incorporated drug, while the surfactants is to improve the stability by keeping drug dispersion in the lipid [21]. The solid lipid matrix could be used to control drug release for the sensitive pharmaceutical components. To illustrate, the movement of the active components in the solid lipid matrix will be lower than when they are in liquid lipid matrix. Consequently, the chemically sensitive components will be protected from degradation and released slowly from the matrix [58]. There are several relationships that must be considered to determine the drug release process from the solid lipid vehicles. For example, if the drug has high partition coefficient, it will be released slowly from the vehicle. If the drug is homogenously dispersed in the lipid, it will be released slowly. On the other hand,
if the drug has high service area due to small particles, it will be released quickly. Other potential advantages for SLPs are the elimination of organic solvents, compatibility with hydrophilic and hydrophobic drugs, and scale-up capability [59].

SLPs can be prepared by using several techniques. One of them is spray drying which is an easy technique to convert liquid containing the drug to dried particles [60]. Spray drying is a one step process that can be either aqueous based, with environmental air intake and exhaust, or organic based, with a close cycle and solvent trap [60]. Although one major advantage of spray drying is the ability to control several parameters in the same time [60], low product yields can be a concern. Another technique for preparation of SLPs is high shear homogenization which involves drug dissolved in the lipid being melted 5-10 C° above its melting point. Then, the aqueous phase containing surfactant is added with high shear homogenizer [21] [60] [61] [62]. Probe sonication is yet another method to prepare SLPs and can be combined with high shear homogenization or anti-solvent precipitation. Anti-solvent precipitation, by itself, could also be used to prepare SLP [60] [63].

Due to the processing flexibility of SLPs, they have been reported in various applications, including administration via inhalation. For example, salbutamol, a drug used for asthma, has been prepared in SLMs by spray drying with dipalmitoylphosphatidylcoline (DPPC) or lecithin as the lipid phase and leucine as a surfactant. After spray drying, the lipid particles were physically mixed with coarse lactose monohydrate and found that the aerosolization properties has been improved with SLMs [64].

Another study from 2015 prepared two solid lipid nanoparticles (SLNs) of rifabutin in a lipid matrix of glycercyldibehenate or glycercyl tristearate. They reported that the formulation containing glycercyldibehenate has drug uptake that was almost twice that of
the formulation containing glyceryl tristearate with low cytotoxicity for both formulations. They concluded that SLNs can be a good preparation method for inhaled delivery [21].

1.7 Lipids and surfactants for solid lipid particles

The literature clearly identifies that the correct selection of lipids and surfactants is critical factor to prepare SLPs. Additionally, SLP processing and formulation parameters can be adjusted to affect the route of administration, the rate of the drug release, the formulation compatibility and stability with the drug, and the method of preparation [59]. Although there are a variety of lipids and surfactants that can be used in SLPs, only a select few will be discussed. These select excipients include phospholipids (e.g. dipalmitoylphosphatidylcholine [65], lecithin [66]), and choline chloride), sorbitan monopalmitate (span 40), sorbitan monooleate (span 80), steroids fatty acids (e.g. palmitic acid [67], stearic acid [61]).

1.7.1 Dipalmitoylphosphatidylcholine (DPPC)

DPPC is a phospholipid that is composed of two acyl chains of palmitic acid connected to glycerophosphocholine, and because of this structure has surfactant properties based on hydrophobic and hydrophilic regions in the molecule (Figure 1.5). The hydrophobic nature comes from existence of the two palmitate residues that each contains saturated 16 carbon acyl chains, while the hydrophilic nature comes from the glycerophosphocholine group [68]. The molar ratio of palmitate to glycerophosphocholine in DPPC is 2:1. The molecular weight of DPPC is 743.4 g/mol, and has a melting of approximately 200 C° which makes
it ideal for preparation methods that require high temperature like spray drying and probe sonication.

![Chemical structure of DPPC](image)

**Figure 1.5 Chemical structure of DPPC**

In general, phospholipids are the most prominent component of the eukaryotic cell membrane and lung surfactant. They represent 85-90% of the lungs surfactant where 40-50% of these phospholipids are DPPC [68]. DPPC, and other pulmonary surfactants, have an important role in stabilizing alveoli during breathing processes to avoid alveolar collapsing by reducing the service tension at alveolar air-liquid interface [68]. DPPC is also approved by FDA as an excipient for inhalation delivery and is contained with a marketed drug product Survanta® (beractant).

As was discussed in 1.5.7, DPPC has been incorporated with clofazimine in inhaled dry powder to improve aerodynamic and physiochemical properties for inhaled TB therapy, and the best composition was 80% clofazimine to 20% DPPC.

Since DPPC has two natures, and every nature gives pharmaceutical action that is different than the other, we need to know which nature is responsible for DPPC performance.
Therefore, we break down DPPC to lipid part and surfactant part. From each part, we derived different surfactants (lecithin, choline chloride, and sorbitan monopalmitate, sorbitan monooleate) and lipids (palmitic acid and stearic acid).

1.7.2 Soya lecithin (Lec)

Lecithin is a refined natural product that contains a mixture of phospholipids, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylethanolamine, and phosphatidylinositol molecules (Figure 1.6). The source of lecithin determines its composition and structure. For example, egg lecithin and soya lecithin are examples of lecithin with different composition [69]. The molecular weight of soya lecithin is 643.87 g/mol, and it has 9.1 log P value. However, it can behave as surfactant because of choline group. The organic solvents for lecithin are halogenated hydrocarbons and mineral oils [69]. Lecithin is important for healthy nutrition since choline is reinforce the lever function. The FDA has approved lecithin in several dietary and pharmaceutical applications [70].

21% phosphatidylcholine
22% phosphatidylethanolamine
19% phosphatidylinositol

Figure 1.6 Chemical structure of soya lecithin
1.7.3 Choline chloride (ChCl)

Choline chloride is a quaternary ammonium salt that behave as surfactant (Figure 1.7). Endogenously, it is required for cell metabolism and nerve transition. However, choline cannot be synthesized naturally in the body and must be obtained exogenously [71]. The molecular weight of choline chloride is 139.61 g/mol, and it is melted at 302°C which makes it ideal for preparation methods that require high temperature like spray drying and probe sonication.

![Chemical structure of choline chloride](image)

Figure 1. 7 Chemical structure of choline chloride

1.7.4 Sorbitan monopalmitate (Sp40)

Sorbitan monopalmitate or (span 40) is a sorbitan monoester that is partial ester of sorbitol with palmitic acid (C16). It is a surfactant used as dispersing agent, emulsifying agent, nonionic surfactant, solubilizing agent, suspending agent, and wetting agent (Figure 1.8).

![Chemical structure of sorbitan monopalmitate](image)

Figure 1. 8 Chemical structure of sorbitan monopalmitate (span 40)
Sorbitan monopalmitate is a cream solid that has molecular weight of 403 g/mol, and density of 1.0 g/cm³. The hydrophilic lipophilic balance of it is 6.4, which means it is soluble in oil soluble and most organic solvents and desirable in water. Sorbitan monopalmitate can be added for cosmetic, food products, oral and parenteral formulation as it has no toxicity to the human body. Therefore, WHO has estimated its acceptable daily intake at up 25 mg/kg [69].

1.7.5 Sorbitan monooleate (Sp80)

Sorbitan monooleate or (span 80) is a sorbitan monoester that is partial ester of sorbitol with oleic acid (C18). It is a surfactant used as dispersing agent, emulsifying agent, nonionic surfactant, solubilizing agent, suspending agent, and wetting agent (Figure 1.9).

![Figure 1.9 Chemical structure of sorbitan monooleate (span 80)](image-url)
As span 40, sorbitan monopalmitate is a solid that has molecular weight of 693 g/mol, and density of 1.01 g/cm³. The hydrophilic lipophilic balance of it is 4.3, which means it is soluble in oil soluble and most organic solvents and desirable in water [69].

1.7.6 Palmitic acid (PA)

Palmitic acid is a saturated fatty acid containing 16 carbon atoms that has frequently existence in plants and animals, and it represents a major part in human depot fat (Figure 1.8) [72]. Palmitic acid can be extracted naturally from palm oil or olive oil after removal of oleic acid. Also, it can be prepared in the lab by mixing cetyl alcohol and soda lime at 270 C° or by melting oleic acid with potassium hydrate [69]. The molecular weight of palmitic acid is 256.42 g/mol, and it is melted at 63 C°. The log P value of palmitic acid is 7.17 which makes it practically insoluble in water. Ethanol is one of the best solvent for it [69].

![Chemical structure of palmitic acid](image)

Figure 1. 10 Chemical structure of palmitic acid

Palmitic acid has been extensively used in oral and topical formulations. For example, it has been used for controlling the release of insulin for oral administration. They found the blood glucose level in the mice which treated with insulin containing palmitic acid was less than controlled mice. At the end, the concluded that mixing insulin with palmitic acid could be a possible alternative to injected insulin [73].
1.7.7 Stearic acid (SA)

Stearic acid is a saturated fatty acid containing 18 carbon atoms that is frequently used in cosmetics, detergents, and food industries (Figure 1.9). Stearic acid can be prepared in the lab by many methods. One of these methods is hydrolysis of fat by high-temperature water in high pressure chamber. Stearic acid will be collected after purification by vacuum steam distillation and separation by specific solvents [69]. The molecular weight of stearic acid is 284.8 g/mol, and it is melted at 70°C. The log P value of stearic acid is 8.2 which makes it practically insoluble in water. Ethanol is one of the best solvent for it [69].

![Chemical structure of stearic acid](image)

Figure 1.11 Chemical structure of stearic acid

Stearic acid is extensively used in oral formulations as lubricant and binder. Also, it is used in topical preparations as emulsifying and solubilizing agent [69]. In 2015, a study showed that incorporation stearic acid as lipid matrix can increase the cellular uptake of SLNs by epithelial cells [74].
1.8 Hypothesis

As it was discussed in 1.5.7, DPPC has been incorporated with clofazimine in inhaled dry powder to improve aerodynamic and physiochemical properties for inhaled TB therapy, and the best composition was 80% clofazimine to 20% DPPC. In 1.7.1, it was discussed that DPPC has two natures; hydrophobic and hydrophilic. The hydrophobic nature comes from existence of the two chances of palmitic acid, which every chain contains 16 carbon atoms, while the hydrophilic nature comes from glycerophosphocholine group. Since DPPC has two natures, and every nature gives pharmaceutical action that is different than the other, we need to know which nature is responsible for DPPC performance. Therefore, we will investigate the lipophilic and hydrophilic nature of DPPC through the use of lipids and surfactants that are related to DPPC. Specifically, we identified different surfactants (choline chloride, soya lecithin, sorbitan monopalmitate, and sorbitan monooleate) and lipids (palmitic acid and stearic acid) that all relate to components of DPPC (Figure 1.12).
In order to overcome the challenges accompanying the current TB treatment, and the pharmaceutical insufficiency with the previous work of our lab (% yield and using of organic solvent based spray drying), this Master thesis is to formulate and evaluate optimal inhaled formulations for TB therapy with better processing method and parameters. The hypothesis is:

“Solid lipid microparticles can improve aerodynamic and physiochemical properties of aqueous-based methods to prepare clofazimine formulations”

In order to prove the hypothesis, this project has two aims:

1- Screening lipids and surfactant formulations with curcumin as a model drug

These curcumin formulations can be characterized for particle size distribution, batch yield, physical state and stability to predict clofazimine formulations.
2- Preparation and characterization of clofazimine formulations

Based on the results of aim 1, the selected excipients will be formulated with clofazimine and characterized for aerodynamic particle size distribution, physical state, stability, chemical integrity, and particle morphology.
Chapter 2: Screening of lipid and surfactant formulations with a model drug
2.1 Introduction

Clofazimine (CFM) is a drug that principally used for leprosy treatment, and it has a good activity against other species of mycobacteria including *M. tuberculosis*. Clofazimine has not been reported to be resisted by mycobacteria which makes it ideal for MDR-TB and XDR-TB cases. However, it is classified as third-line agent for TB treatment due to poor pharmacokinetic properties and serious side effects accompanying its oral delivery.

As it was discussed in (1.4 Inhaled TB treatment as an alternative), targeted drug delivery to the lungs would be ideal to overcome the challenges of current TB treatment. Clofazimine, by itself, can be a goof alternative for many reasons that were discussed in (1.5.6 Inhaled clofazimine for TB treatment)

The need to create a new delivery system that overcome the physiochemical barriers and keep the activity of the drug is a majorly important for inhaled delivery. Solid lipid particles (SLPs) is an example of this new delivery system which contains lipid as a solid core and covered by surfactant, and drug where the drug is dissolved in the lipid phase and then incorporated to the aqueous phase (water + surfactant).

In order to choose the best excipients that show excellent properties and to estimate how clofazimine will behave when it is formulated with lipids and surfactants, curcumin is chosen as a model for clofazimine. The main reason that curcumin is an acceptable model for clofazimine is the similar hydrophobic nature between the two drugs.

The prepared formulations containing curcumin and excipients can be characterized for the basic properties that can help in estimating how clofazimine will work. The evaluation of particle size distribution is essential for inhaled formulations. The batch yield can be
measured as different excipients and preparation techniques are used. The physical state and stability can also be evaluated as different preparation techniques are used.

2.1.1 Curcumin

Curcumin (Cur) is a polyphenol hydrophobic curcuminoid of the Indian spice turmeric. It is one of three curcuminoids including desmethoxycurcumin and bis-desmethoxycurcumin that grant the characteristic yellow color to turmeric. Thus, it is used as color and flavor agent in food industry. For years, turmeric has been used to treat many illnesses in traditional therapy [75].

![Chemical structure of curcumin](image)

Figure 2.1 Chemical structure of curcumin

The molecular weight of curcumin is 368.38 g/mol. Curcumin has poor aqueous solubility at 3.12 mg/L. Curcumin can be dissolved in non-polar solvents such as acetone and ethanol.

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<thead>
<tr>
<th>Drug</th>
<th>MW g/mol</th>
<th>MP °C</th>
<th>Solubility mg/L</th>
<th>Log P</th>
<th>Density g/cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>468.38</td>
<td>183</td>
<td>3.12</td>
<td>3.2</td>
<td>0.93</td>
</tr>
</tbody>
</table>
The tautomeric forms that curcumin can take are keto and enol. The enol form is the most energetically stable form for curcumin in solid and liquid phases. As it is mentioned above, curcumin is a hydrophobic compound, so it is poorly absorbed when it is administrated orally. As a result, about 75% of it will be unchanged excreted in the fees and urine. The rest absorbed portion (25%) will be metabolized to dehydrocurcumin and tetrahydrocucumin by bio-transformation [75] [76].

2.2 Materials
Curcumin, choline chloride, and palmitic acid were obtained from Acros Organics (NJ). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma-Aldrich (St. Louis, MO). Lecithin was obtained from Alfa Aesar (Ward Hill, MA). Stearic acid was purchased from Amend Dug and Chemical Co. (Irvington, NJ). Acetone was purchased from Fisher Scientific (Pittsburg, PA). Deionized (D.I) water was produced by Purelab Ultra water purification system (ELGA LabWater, Woodridge, IL). Bath sonicator and Isotemp magnetic stir plate manufactured by Fisher Scientific (Pittsburg, PA) were used.

2.3 Methods

2.3.1 Preparation of dried powders
Several formulations were prepared by anti-solvent precipitation technique (ASP) following by either spray drying or freeze drying to obtain dry powders as it is showed in (Figure 2.2).
2.3.1.1 Anti-solvent precipitation

As it was mentioned in (1.6 Solid lipid microparticles as delivery system), anti-solvent precipitation (ASP) is one of the techniques that are conducted to prepare SLPs. It is widely used with poorly soluble drugs as it can alert the particle formation. As is clear from the name, particles are engineered by precipitation after introducing dissolved drug into an anti-solvent that is miscible with the solvent used to dissolve the drug. To illustrate, the poorly soluble drug and the lipid are dissolved in organic solvent that is miscible with water. Then, the formed solution is slowly injected to water (anti-solvent) while it is stirring. Once the solution touch the service of water, the new engineered particles are formed and precipitated.

There are many relationships that should be optimized to obtain good particles. For example, the relationship between the drug concentration and the particle size is inverse.
The relationship of the volume of solvent to anti-solvent ratio to the particle size is inverse. In addition, the increase in stirring speed leads to decrease the particle size [61] [78].

For the experiment, eight formulations were prepared by using ASP method. Seven formulations were composed 80% curcumin and 20% lipid and/or surfactant. One formulation contained 100% curcumin without any excipient. The reason to choose (80:20 % w/w) is that this composition showed best aerodynamic and physiochemical properties of clofazimine formulations in a previous work in our lab as it was mentioned in (1.5.7 Preliminary studies).

The 100% curcumin suspension (Cur:100) was prepared by making a solution of weighted curcumin with acetone in conical tube at a concentration of 40 mg/ml. The solution was sonicated in a water bath for 10 minutes. Then, the curcumin solution was slowly injected to a beaker containing water (anti-solvent) by serological pipette (drop by drop). The volume of the solvent (acetone) to the anti-solvent (water) was (1:10 v/v). The produced suspension was left to be stirred on a magnetic stir plate at 500 rpm for 15 minutes after which the suspension evenly divided for further spray and freeze drying.

The preparation of other suspensions that containing 80% curcumin and 20% lipid (Cur:PA and Cur:SA) were done by dissolving weighted amount of curcumin and lipid in acetone following by bath sonication. Then, the solutions were slowly injected to water (anti-solvent) by serological pipette with keeping the volume of the solvent (acetone) to the anti-solvent (water) at (1:10 v/v). The produced suspensions were left to stir on a magnetic stir plate for 15 minutes after which the suspensions were divided for further spray and freeze drying.
The preparation of the other suspensions that containing 80% curcumin and 20% surfactant (Cur:DPPC, Cur:Lec, Cur:ChCl, Cur:Sp40, and Cur:Sp80 ) were done by dissolving weighted amount of curcumin in acetone following by bath sonication. Then, the solutions were slowly injected to water that is containing the surfactants by serological pipette with keeping the volume of the solvent (acetone) to the anti-solvent (water) at (1:10 v/v). The produced suspensions were stirred on a magnetic stir plate for 15 minutes after which the suspensions were divided for further spray and freeze drying (Figure 2.3 and Table 2.2).

**Curcumin** (w/ or w/ out lipid) in acetone
- 40 mg/ml
- 1 part V

Water (w/ or w/ out surfactant)
- Stirring: **500 rpm** (15 min)
- 10 part V

Figure 2.3 Anti-solvent precipitation method for curcumin formulations
Table 2. 2 Composition of curcumin formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Curcumin</th>
<th>DPPC</th>
<th>Lec</th>
<th>ChCh</th>
<th>PA</th>
<th>SA</th>
<th>Sp40</th>
<th>Sp80</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Cur:100</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Cur:DPPC</td>
<td>80%</td>
<td>20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Cur:Lec</td>
<td>80%</td>
<td></td>
<td>20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Cur:ChCl</td>
<td>80%</td>
<td></td>
<td></td>
<td>20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Cur:SA</td>
<td>80%</td>
<td></td>
<td></td>
<td></td>
<td>20%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Cur:PA</td>
<td>80%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td>7 Cur:Sp40</td>
<td>80%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20%</td>
</tr>
<tr>
<td>8 Cur:Sp80</td>
<td>80%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3.1.2 Spray Drying

Spray drying is a fast, continuous, and direct methods that can be used to produce dry powders. The principle of spray drying is to separate the dry powders from liquid feed by exposing it to hot gas. The solvent will be evaporated, and the dry powder will be collected. The liquid feed can be solution, suspension, or emulsion. Spray drying technique can be explained in three stages; atomization, drying and particle formation, and collection (Figure 2.4). First, the liquid feed is atomized and dispersed to very fine droplets by using nozzle which compress gas to disperse the feed. Therefore, nozzle works as a metric controller. The quality of atomization is pivotal in solvent evaporation and particle formation.
Second, after atomizing the liquid feed, the fine droplets will be dried because of the impact of hot gas that is heated to specific temperature in drying chamber. The inlet temperature is set based on solvent used in the feed, the melting point of both the solvent and ingredients in it. The quality of drying process is achieved by making a balance between inlet temperature, feed rate, and the size of the droplets.

Third, since the solvent starts to evaporate, which starts in seconds, the formed powders will be separated and collected by cyclone chamber. The centrifugal motion that is due to cyclone chamber will help in collecting the dried powder by making the airflow spirally move. Therefore, the dried particles will deposit on the walls of cyclone chamber and in collecting flask, and the exhausted vapor will exit out from the top of the cyclone chamber. The majority of the dried particles will be collected in the cyclone chamber. However,
some of very fine particles will exist with the exhausted vapor, and will be deposited in the
filter [80].

Based on the used solvent, the vapor can be exhausted directly to the atmosphere if it is
water (open cycle). However, if it is organic, it is passed through solvent trap to be condensed
and separated from the drying gas (closed cycle). Spray drying method has many
advantages over other techniques of dry powder production. One of them is the ability to
control several parameters at one time such as inlet temperature, feed rate, and aspiration.
It is fast, continuous, one-step, and combatable with scalability and financial saving. The
dry powders that are produced by spray drying show better flow properties comparing to
other techniques.

However, low yield potency is a real challenge in using spray drying technique. The yield
can be ranged from 20 to 70% because of loss of the formulation on the wall of the
chambers and or with the exhausted vapor if the particle size less than 2 µm. The other
disadvantages is that thermal sensitive compounds can be degenerated because of high
temperature [79].

For the experiment, the purpose of spray drying was not for prepare new particles but only
for evaporate the solvent. It was conducted by laboratory-scale mini spray dryer B-290
manufactured by Buchi Labortechnik AG (Flawil, Switzerland) in open cycle. When the
spray drying was done, the dry powders were collected in scintillation vials and stored for
characterization. The following spray drying parameters were consistently used for all
formulations (Table 2.3).
### Table 2. Spray drying parameters for curcumin formulations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet temperature °C</td>
<td>145</td>
</tr>
<tr>
<td>Outlet temperature °C</td>
<td>84</td>
</tr>
<tr>
<td>Aspiration %</td>
<td>100</td>
</tr>
<tr>
<td>Feed rate (%)</td>
<td>18</td>
</tr>
<tr>
<td>Atomization gas rate (L/hr)</td>
<td>536</td>
</tr>
<tr>
<td>Nuzzle cleaning (pulse)</td>
<td>1</td>
</tr>
</tbody>
</table>

### 2.3.1.3 Freeze drying

Freeze drying, called also lyophilization, is a technique that can be used to remove water from a sample to obtain it dried. The principle of freeze drying is the sublimation of the frozen water (solid state) directly to gaseous state without passing on liquid state. This can be achieved under the triple point where the liquid state is not exist. To illustrate, at normal pressure, water can be existed in three states; solid, liquid, and gaseous states. At certain point, all three states can exists in equilibrium. This point called “triple point”, and it is at 4.58 mm Hg and 0.01 °C for water (Figure 2.5).

![Phase diagram of water](image)

**Figure 2.5 Phase diagram of water**
Freeze drying process can be explained in three steps; freezing, primary drying, and secondary drying. First, the sample is frozen under atmospheric pressure. So, water converts to crystalline ice and the main compound becomes in glassy state. Second, primary drying will take place by sublimation of the formed ice to vapor under high vacuum to reduce the pressure and radiation or conduction to increase the temperature. Finally, secondary drying will take place to remove the remaining water by desorping it from the glass with increasing the temperature under low pressure.

Freeze drying has several advantages over other drying techniques. For example, it is ideal for thermal and oxygen sensitive materials. It keeps high yield potency and low level of contamination. However, it does not help with volatile compounds and its operation is very expensive [78].

For the experiment, suspension of ASP was filled in petri plates and freeze dried by Steller laboratory freeze dryer manufactured by Millrock Technology (Kingston, NY). When freeze drying was done, the dry powders were collected in scintillation vials and stored for characterization. The following freeze drying parameters were consistently used for all formulations (Table 2.4 and Figure 2.6).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time (hr)</th>
<th>Temperature (°C)</th>
<th>Vacuum (mTorr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing</td>
<td>4</td>
<td>From RM to -50</td>
<td>200</td>
</tr>
<tr>
<td>Primary drying</td>
<td>24</td>
<td>From -20 to 20</td>
<td>50</td>
</tr>
<tr>
<td>Secondary drying</td>
<td>6</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>
2.3.2 Particle size characterization

Particle size analysis was conducted for the spray dried and freeze dried formulations beside row curcumin. It was carried out by Malvern Mastersizer Hydro 2000S (Malvern, UK) which is a laser diffraction particle size distribution analyzer. Briefly, laser diffraction mastersizer can measure the particle size according to spatial distribution of the scattered light. To illustrate, a beam of laser crosses through particles and the light will be scattered after the laser hits the particles for specific angels. These angels will be detected on detectors that cover all potential spread of the light. When the particles are small, large scattering angels will be detected. When the particles are large, small scattering angels will be detected [86].

For the experiment, an adequate amount of the sample was dispersed in water in test tube firstly and transferred to the hydro chamber of Malvern mastersizer. The samples left to stir at 3500 rpm and be sonicated at 100% for two minutes before running the measurement. In few seconds, the instrument took three reads for each sample and combined them in an
average read in where \(d (0.1) \mu m, d (0.5) \mu m, \) and \(d (0.9) \mu m\) are reported to describe the particle distribution. To explain, \(d (0.1)\) is the diameter below which 10% of the particles distribution sets. \(D (0.5)\) is the diameter below which 50% of the particles distribution sets (median diameter), and \(d (0.9)\) is the diameter below which 90% of the particles distribution sets.

2.3.3 Batch yield

Batch yield is the percent of the recovered (end) mass of the sample divided by the original (start) mass before processing. Ideally, % batch yield should be 100% as there is no loss of the product. Freeze drying is one of the techniques that keep high % yield. However, yield loss is a major concern in spray drying. It is often to end with very low % yield due to product buildup on the chamber’s wall which is a result of poor drug, poor excipients, or poor drying parameters [87]. For both spray and freeze drying, the percentage of yield potency was calculated by using Equation 2.1:

\[
\text{Yield (\%)} = \frac{\text{Recovered mass}}{\text{Theoretical mass}}
\]

Equation 2.1 Batch yield

2.3.4 Thermal Gravimetric analysis (TGA)

TGA is a thermoanalytical technique that used to check the weight loss of the sample due to some physical and chemical phenomena such as vaporization and dehydration when it is subjected heat. For the experiment, it was conducted using Shimadzu TGA-50 thermogravimetric analyzer (Kyoto, Japan). Approximately 5 mg of the sample was filled
in an aluminum pan and heated up to 300 °C at 10°C / min rate and under nitrogen atmosphere at 20 ml / min flow rate.

### 2.3.5 Differential Scanning Calorimetry (DSC)

DSC is a thermoanalytical technique that is used to evaluate the physical state (crystalline or amorphous forms) of the material when it is heated. Briefly, sample and reference pan are subjected to identical temperature that increases gradually from the room temperature (RT) to 300 °C. Based on the differentiation between the sample and the reference, a thermogram showing the thermal states such as melting, crystallization, glass transition is drawn.

For the experiment, it was conducted using Shimadzu DSC-60 differential scanning calorimeter (Kyoto, Japan). Approximately 5 mg of the sample was filled in an aluminum pan, sealed, and heated up to 300 °C at 10 °C / min rate and under nitrogen atmosphere at 20 ml / min flow rate.

### 2.4 Results and discussion:

#### 2.4.1 Batch yield

For both spray and freeze drying, the batch yield was calculated by using (Equation 2.1)

**Batch yield.** The results were as below:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FD</strong></td>
<td>54.00%</td>
<td>81.50%</td>
<td>73.50%</td>
<td>75.00%</td>
<td>65.00%</td>
<td>57.50%</td>
<td>92.5%</td>
<td>62.50%</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>21.66%</td>
<td>61.66%</td>
<td>29.16%</td>
<td>56.00%</td>
<td>19.16%</td>
<td>27.66%</td>
<td>38.33%</td>
<td>37.66%</td>
</tr>
</tbody>
</table>
According to (Table 2.6 and Figure 2.7), it is clear that freeze drying keeps high batch yield >50%. The freeze dried formulations have % yield ranging from 54.00% to 92.50%. The highest % yield of freeze dried formulation was with (Cur:Span40) while the lowest was with (Cur) and (Cur:SA). On the other hand, spray drying technique reduces % yield in general. Only two spray dried formulations, (Cur:DPPC) and (Cur:Lec) have % yield >50% due to the high melting point, while the other formulations have <50% yield. Excipients help to enhance % yield for both spray and freeze drying. For example, the yield of freeze dried curcumin was 54.00%, and it increased 3% with stearic acid and 27.5% with DPPC. Similarly, % yield of spray dried curcumin increased 5% with stearic acid and 40% with DPPC. In addition, it is concluded that surfactants improve % yield more than lipids. For example, % yield increased with surfactants at least 8.5% and 7.5% for freeze and spray drying respectively. However, the maximum increase % yield with lipids was 11% and 6% for freeze and spray drying, respectively.
2.4.2 Particle size distribution

Particle size distribution was measured to evaluate the effect of anti-solvent precipitation and drying method. The results were as below:

A)
B)  

![Graph showing particle size distribution](image1)

Cur_DPPC before drying  
SD Cur_DPPC  
FD Cur_DPPC

C)  

![Graph showing particle size distribution](image2)

Cur_Lec before drying  
SD Cur_Lec  
FD Cur_Lec
Figure 2. 8 Particle size distribution of curcumin with excipients

In panel A) 100% Cur, ASP produced bimodal particle distribution in micro and sub-micro range. However, FD increased the particle size to be in micro range while SD did not change the particle size. B) Cur:DPPC, C) Cur:Lec, E) Cur:SA, F) Cur:Span40, and G) Cur:Span80 showed similar distribution for ASP and SD while FD increased the particle size. The particle size distribution of D) Cur:PA showed no change in particle size between ASP and SD while FD increased the particle size up to 500 µm.

According to the figures, anti-solvent precipitation (ASP) produced multiple-modal of particle distribution. The main reason for that is inconsistency during the injection of the solvent to the water. For all formulations except F) Cur:Span 40, the majority of the particles are in nano-range. However, the particle size ranged from nano to micro-size. Spray drying worked to obtain dry particles without any changes in their size. Therefore, particle size distribution produced by spray drying matched that produced by ASP. On the other hand, freeze drying made bigger particles than that produced by ASP.

2.4.3 Thermogravimetric analysis (TGA):

TGA is a thermoanalytical technique that used to check the weight loss of the sample due to some physical and chemical phenomena such as vaporization and dehydration when it is subjected heat.
In (Figure 2.9), TGA thermogram shows that all spray dried formulations were stable with no significant weight loss until 183 C° (MP of curcumin). Therefore, it can be understood that all powders are free from water moisture. The weight loss that happened after 183 C° for some formulations was because the degradation of the powders.

2.4.4 Differential Scanning calorimetry (DSC)

DSC is a thermoanalytical technique that used to evaluate the physical state of the material when it is subjected to heat.
In (Figure 2.10), DSC thermograms shows that all the formulations are in crystalline form although they are formulated by ASP and then spray dried. All formulations showed melting peak around 183 C° (MP of curcumin) with slight shifting due to the presence of another component in the formulation. Also, the formulations containing lipids showed melting peaks for the lipids around 60 C°.

**2.5 Key findings of chapter 2 and conclusion**

In order to estimate how clofazimine will work when it is incorporated in SLPs, curcumin was used as a drug model with variety of lipids and surfactants. The formulations were prepared by anti-solvent precipitation method and then divided to be dried by freeze or
spray drying. The formulations were characterized for batch yield, particle size distribution, physical state, and stability.

Based on the curcumin results, it can be concluded that all lipids and surfactants that used are eligible to be formulated with clofazimine except span 40 and span 80. Spray drying will not change the particle size distribution of clofazimine formulations after ASP, while freeze may make them bigger. In addition, freeze drying will keep high batch yield comparing to spray drying. Also, all clofazimine formulations will be physically stable and in crystalline form.
Chapter 3: Preparation and characterization of clofazimine formulations
3.1 Introduction

Inhaled clofazimine, as it was discussed in chapter 1, could be a substantial improvement compared to the current oral anti-TB therapy since it has not been reported to be resisted by *M. tuberculosis*. However, clofazimine needs to be specifically formulated in order to be delivered directly to the lungs since it has challenging physiochemical properties. SLMs, a colloidal pharmaceutical vehicle containing lipids and surfactants, could be a good method for enhanced pulmonary delivery of hydrophobic drugs such as clofazimine because it is aqueous-based system that can improve the drug incorporation and its physiochemical properties.

Choosing proper lipids and surfactants plays important role in obtaining improved SLMs of clofazimine. Thus, variety of lipids and surfactants were suggested on the basis of DPPC, an indigenes lung surfactant, is the baseline for the excipients. As it was discussed in chapter 2, the suggested excipients were formulated with curcumin as a drug model to estimate how they will behave when they formulated with clofazimine. Anti-solvent precipitation technique followed by spray or freeze drying was used to produce SLMs. Then, these SLMs were characterized for batch yield, particle size distribution, physical state, and stability.

Based in curcumin results, it was estimated that all suggested lipids and surfactants are eligible to be formulated with clofazimine except span 40 and 80. In addition, spray drying will not change the particle size distribution of the formulations after ASP, while freeze may make them bigger. Also, freeze drying will keep high batch yield comparing to spray drying. Finally, all clofazimine formulations will be physically stable and in crystalline form. Therefore, SLMs containing clofazimine can be prepared and characterized for
aerodynamic particle size distribution, batch yield, drug content, physical state and stability, chemical integrity and particle morphology.

3.2 Materials

Clofazimine and (DPPC) was purchased from Sigma-Aldrich (St. Louis, MO). Choline chloride and palmitic acid were obtained from Acros Organics (NJ). Lecithin was obtained from Alfa Aesar (Ward Hill, MA). Stearic acid was purchased from Amend Dug and Chemical Co. (Irvington, NJ). Methanol optima grade was purchased from Fisher Scientific (Pittsburg, PA). Ethanol 200 proof non-denatured was obtained from Decon Labs, Inc. (King of Prussia, PA). Deionized (D.I) water was produced by Purelab Ultra water purification system (ELGA LabWater, Woodridge, IL). Bath sonicator and Isotemp magnetic stir plate manufactured by Fisher Scientific (Pittsburg, PA) were used. Probe sonicator 3000 manufactured by Misonix Inc. (Farmingdale, NY) also were used.

3.3 Methods

3.3.1 Preparation of clofazimine formulations

Several formulations were prepared by anti-solvent precipitation technique (ASP) following by either spray drying or freeze drying to obtain dry powders.

3.3.1.1 Anti-solvent precipitation (ASP)

ASP method were used for preparation of clofazimine formulations as it was discussed in (2.3.1.1 Anti-solvent precipitation) with slight changes. For instance, the solvent used for clofazimine formulations was methanol instead of acetone for curcumin formulations.
Also, the stirring speed after injecting the solvent to the aqueous phase was increased to be 1000 rpm instead of 500 rpm. In addition, the formed ASP suspension was probe sonicated in ice bath for 20 minutes at power of 36 to 42 watts before it spray of freeze dried.

Seven formulations were prepared; three formulations were composed 80% clofazimine and 20% surfactant, while four formulations contained lipid and surfactant together were composed of 80% clofazimine and 20% divided between lipid and surfactant.

The reason to choose (%80:20 w/w) is that this composition showed best aerodynamic and physiochemical properties of clofazimine formulations in a previous work in our lab as it was mentioned in (1.5.7 Preliminary studies) The 20% divided between lipid and surfactant was calculated based on that molar ratio as the molar ratio of lipid to surfactant in DPPC is 2:1 as it was mentioned in (1.7.1 Dipalmitoylphosphatidylcholine)

Specifically, the preparation of the suspensions that containing 80% clofazimine and 20% surfactant (CFM:DPPC, CFM:Lec, and CFM:ChCl) were done by dissolving weighted amount of clofazimine in methanol following by bath sonication for 10 mins. Then, the solution was slowly injected to water that is containing the surfactants by serological pipette with keeping the volume of the solvent (methanol) to the anti-solvent (water) at (1:10 v/v). The produced suspension was stirred on a magnetic stir plate for 15 minutes at 1000 rpm. Then, the suspension was probe sonicated in ice bath for 20 mints at 36 to 24 Watts. Finally, the suspension was divided for spray and freeze drying.

The formulations containing lipid and surfactant (CFM:PA:ChCl, CFM:PA:Lec, CFM:SA:ChCl, and CFM:SA:Lec) were done by dissolving weighted amount of clofazimine and lipid in methanol following by bath sonication. Then, the solutions were slowly injected to water containing the surfactant by serological pipette with keeping the
volume of the solvent (methanol) to the anti-solvent (water) at (1:10 v/v). The produced suspensions were left to stir on a magnetic stir plate for 15 minutes and probe sonicated for 20 minutes. Then, the suspensions were divided for spray and freeze drying (Figure 3.1 and Table 3.1).

![ASP method for clofazimine formulations](image)

Figure 3.1 ASP method for clofazimine formulations
Table 3. 1 Composition of clofazimine formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Clofazimine</th>
<th>DPPC</th>
<th>Lec</th>
<th>ChCl</th>
<th>PA</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CFM:DPPC</td>
<td>80%</td>
<td>20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 CFM Lec</td>
<td>80%</td>
<td>20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 CFM:PA:Lec</td>
<td>80%</td>
<td></td>
<td>11.1%</td>
<td>8.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 CFM:SA:Lec</td>
<td>80%</td>
<td></td>
<td>10.6%</td>
<td></td>
<td>9.4%</td>
<td></td>
</tr>
<tr>
<td>5 CFM:ChCl</td>
<td>80%</td>
<td></td>
<td></td>
<td>20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 CFM:PA:ChCl</td>
<td>80%</td>
<td></td>
<td>4.4%</td>
<td>15.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 CFM:SA:ChCl</td>
<td>80%</td>
<td></td>
<td>3.9%</td>
<td>16.1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.1.2 Spray drying

Spray drying was conducted by laboratory-scale mini spray dryer B-290 manufactured by Buchi Labortechnik AG (Flawil, Switzerland) in open cycle to evaporate the solvent. There was no change in spray drying parameters than what used for curcumin formulations in (2.3.1.2 Spray drying). When the spray drying was done, the dry powders were collected in scintillation vials and stored for characterization.

3.3.1.3 Freeze drying

Freeze drying was conducted by Steller laboratory freeze dryer manufactured by Millrock Technology (Kingston, NY). There was no change in freeze drying parameters than what used for curcumin formulations in (2.3.1.3 Freeze drying). When the freeze drying was done, the dry powders were collected in scintillation vials and stored for characterization.
3.3.2 Drug content

Clofazimine content in each formulation was detected and analyzed by Acquity Ultra Performance Liquid Chromatography instrument manufactured by Waters Corp. (Milford, MA). The method parameters used for UPLC were validated by our lab as below (Table 3.2 and Figure 3.2):

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Acquity HSS T3 (2.1 X 100 mm, 1.8μm)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Citrate buffer pH3 : Methanol (26 : 74)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.4 ml/min</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>492 nm</td>
</tr>
<tr>
<td>Retention time</td>
<td>2.26 min</td>
</tr>
<tr>
<td>Run time</td>
<td>3.5 min</td>
</tr>
</tbody>
</table>

Table 3.2 UPLC parameters for clofazimine formulations

\[ y = 28836x - 156.79 \]
\[ R^2 = 0.9999 \]

Figure 3.2 Clofazimine standard curve at 492 nm
For the experiment, 5 ± 1 mg of the sample were dissolved by methanol in 50 ml volumetric flask. Then, the solutions were (2:1) diluted with mobile phase and analyzed by UPLC. The % clofazimine content was calculated by using this equation:

\[
Drug\ content\ (%) = \frac{Calculated\ amount}{Theoretical\ amount}
\]

Equation 3. 1 Drug content

3.3.3 Batch yield
Batch yield was calculated as it described in (2.3.3 Batch yield).

3.3.4 Particle size characterization
3.3.4.1 Hydrodynamic particle size distribution
Particle size analysis was conducted for the spray dried and freeze dried formulations beside row clofazimine. It was carried out by Malvern Mastersizer Hydro 2000S (Malvern, UK) as it was described in (2.3.2 Particle size characterization).

3.3.4.2 Aerodynamic particle size distribution
Aerodynamic particle size analysis is very important for inhaled formulations to describe how particles behave when they are inhaled. It is done by Next Generation Impactor (NGI). NGI (Figure 3.3) is an impactor that classifies particles cascade according to its size [79]. The main object of NGI is to enhance the accuracy of aerodynamic particle size distribution testing by simulating particle deposition in the lung [80].
NGI is composed of seven stages and micro-orifice collector (MOC) [82]. Every stage has a removable impaction cup where the particles are distributed to and collected from. The first stage and the MOC have larger cups than the others. Also, each stage beside MOC has own cut-off aerodynamic diameter (d 0.5, Q) depending on the flow rate [80] (Figure 3.4 and 3.5)). Before the stages, there is a 90 degree metal piece named Universal induction port (UIP). It is also known the throat of the NGI. After MOC, there is a filter holder, where a filter paper is put. Vacuum pump is connected to filter holder and to stages via air flow passageway. From this pump, it can be determined the air flow rate which is measured by flow meter. The air flow rate can be ranged from 15 L/min to 100 L/min. The particle size detected can be ranged from 0.23 µm to 11 µm [82]. NGI can be used with metered dose inhaler (MDI), dry powder inhaler (DPI), nebulizer, nasal spray, and aerosol. All of these devices are known heads of NGI [79].
To make the analysis, the sample is put in the head (like nebulizer, PDI, or MDI). The air pump is ran for a specific time and speed. The particles of the sample will deposit in the stages and MOC according to its aerodynamic properties [82]. After the running is done, the particles are collected from each part of the NGI by washing with suitable solvent and analyzed [80].
The analysis can be done by liquid chromatography or plate reader to determine the concentration in each part [80]. Then, this data is used to calculate the percentage of final particles fraction (%FPF), geometric standard deviation (GSD), and the mass median aerodynamic diameter (MMAD) [80]. MMAD is the diameter where 50% of the particles by mass are above and 50% of them are below [84]. GSD represents the spread of the particle size distribution. MMAD and GSD are calculated by Cumulative Mass % plot [83].
MMAD represents the 50% of the cumulative plot (Figure 3.6). GSD is determined from cumulative plot by this equation [84]:

\[
GSD = \sqrt{\frac{\text{Size X}}{\text{Size Y}}}
\]

Equation 3.2 GSD

FPF % is calculated by this equation:

\[
\%FPF = \frac{R}{\sum A}
\]

Equation 3.3 %FPF
Where $R$ is the total mass of the drug deposited on the impactor stages with the cut-off diameter less than or equal to $5 \, \mu m$. $\Sigma A$ is the total mass of the drug deposited into the impactor [80].

For the experiment, $15 \pm 2 \, mg$ of sample was filled in gelatin capsule size 3 (PCCA, TX). The capsule was loaded into DPI (Handihaler) and attached to the induction port of NGI by silicone mouthpiece adaptor. The NGI was run for 4 seconds at 60 L/min flow rate. Based on their aerodynamic properties, the particles were deposited in the stages. They collected by washing with methanol in $50 \, ml$ volumetric flask. The particle remained in the capsule and induction port were collected by washing with methanol, while the particles remained in DPI were collected by ethanol. Then, the solutions were (2:1) diluted by mobile phase and analyzed by UPLC to calculate MMAD, GSD, %FPF.

3.3.5 Thermal gravimetric analysis (TGA)

TGA is used to check the physical stability by weight loss of the samples. It was done for clofazimine formulations as it was described in (2.3.4 Thermal gravimetric analysis).

3.3.6 Differential scanning calorimetry (DSC)

DSC is a thermoanalytical technique that used to evaluate the physical state (crystalline or amorphous forms) of the material when it is heated. It was done for clofazimine formulations as it was described in (2.3.5 Differential scanning calorimetry).
3.3.7 X-ray powder diffraction (XRPD)

XRPD is a non-destructive technique used to check the crystallinity of the sample when it is exposed to x-ray radiation. When materials exposed to light beam, the crystalline materials diffract the light, while the amorphous materials do not.

For the experiment, the analysis was done by Empyrean X-ray powder diffractometer manufactured by PANalytical (Almelo, Netherlands). The range of analysis used was from 5 to 60 (2 theta).

3.3.8 Infra-red (IR) spectroscopy

IR spectroscopy is a non-destructive technique used to check the chemical integrity of the sample when it is exposed to infra-red radiation. Every material has characteristic fingerprint as its chemical bond rotate of vibrate at specific frequency.

For the experiment, the analysis was conducted by Shimadzu IR Prestige-21 FTIR spectrometer (Tokyo, Japan). The range of analysis used was form 700 to 4000 1/cm.

3.3.9 Particle morphology analysis

As it was mentioned in (1.5.6 Inhaled clofazimine for TB treatment), row clofazimine cannot be inhaled directly to the lungs because it has needle shape morphology, so it needs to be formulated in small particles that has spherical or globular shape to be inhaled.

For the experiment, the particle morphology was checked by Leica Microscope DM2500M (Buffalo Grove, IL).
3.4 Results and Characterization

3.4.1 Batch yield

The batch yield results for clofazimine formulation was as below:

Table 3. 4 Batch yield of clofazimine formulations

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>FD</td>
<td>30.0%</td>
<td>72.5%</td>
<td>48.4%</td>
<td>61.0%</td>
<td>70.2%</td>
<td>63.0%</td>
<td>81.2%</td>
</tr>
<tr>
<td>SD</td>
<td>40.0%</td>
<td>25.5%</td>
<td>33.8%</td>
<td>49.7%</td>
<td>56.4%</td>
<td>18.2%</td>
<td>30.1%</td>
</tr>
</tbody>
</table>

Figure 3. 7 Batch yield trend of clofazimine formulations
According to (Table 3.4 and Figure 3.7), freeze drying was able to keep high batch yield ranging from 48% to 81%. Spray drying was not suitable for formulations containing lipids. For example, % batch yield for (CFM:PA:ChCl) was only 18.2%. The potential reasons are the difficulty of recovering the samples from the glass of spray dryer and because lipids have low MP, and this may explain why there is no literature for spray dried lipids. The only spray dried formulation had batch yield > 50% was (CFM:ChCl) because ChCl has melting point (MP) at 302 C°.

However, the results of spray dried (CFM:DPPC) did not correspond to MP explanation because DPPC has 200 C° MP. It can be explained by losing of DPPC during spray drying or there was an error during ASP method. Freeze dried (CMF:DPPC) reinforces this explanation since its batch yield was <50%.

(CFM:Lec) and (CFM:PA:Lec) showed low batch yield (25% and 33.8%) for spray drying and (72.5% and 48.4%) for freeze drying. That means lecithin was not compatible surfactant for spray drying.

If the effect of drying method was excluded, it is clear that stearic acid increase batch yield more than palmitic acid. The potential reason is that stearic acid has MP above palmitic acid (70 and 63 C° respectively). The same way in surfactants which choline Cl increase batch yield more than lecithin.

### 3.4.2 Clofazimine content

Clofazimine formulations results were as below:
Table 3. 5 Drug content of clofazimine formulations

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FD</strong></td>
<td>147%</td>
<td>67.1%</td>
<td>67.1%</td>
<td>60.0%</td>
<td>57.2%</td>
<td>75.8%</td>
<td>75.0%</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>116%</td>
<td>32.7%</td>
<td>29.5%</td>
<td>65.1%</td>
<td>59.2%</td>
<td>87.1%</td>
<td>71.4%</td>
</tr>
</tbody>
</table>

Based on (Table 3.5 and Figure 3.8), the results of clofazimine content were corresponded to batch yield results. Freeze drying kept high clofazimine content more than spray drying while spray dried lipid formulations had low drug content because of the low MP.
It is clear that there were lost drug during preparation for (CFM:ChCl), (CFM:Lec), (CFM:PA:Lec), and (CFM:SA:Lec) for both spray and freeze drying since the drug content dramatically decreased less than 80%.

On the other hand, freeze and spray dried (CFM:PA:ChCl) and (CFM:SA:ChCl) showed % drug content closes to 80%. That means the incorporation of clofazimine with these excipients was excellent.

If the effect of drying method was excluded, it is clear that lipids improved clofazimine content. For example, the drug content of (CFM:ChCl) was 57%, and it jumped to 75% in presence of stearic acid. Similarly, the drug content of (CFM:Lec) was 32.7%, and it jumped to 75% in presence of stearic acid.

(CFM:DPPC) showed highly concentrated formulations. This may be explained by that there was error in weighting or in ASP since this happened for both spray and freeze drying.

3.4.3 Particle size characterization

3.4.3.1 Hydrodynamic particle size distribution

The results of hydrodynamic particle size distribution was as below (Table 3.6):

<table>
<thead>
<tr>
<th>Formulation</th>
<th>D50 μm (D10 – D90) μm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SD</td>
</tr>
<tr>
<td>CFM:DPPC</td>
<td>4.59 (1.8 - 11.0)</td>
</tr>
<tr>
<td>CFM:Lec</td>
<td>4.91 (1.7 - 12.4)</td>
</tr>
<tr>
<td>CFM:PA:Lec</td>
<td>8.45 (2.5 - 1088.4)</td>
</tr>
<tr>
<td>CFM:SA:Lec</td>
<td>14.32 (5.1 - 31.1)</td>
</tr>
<tr>
<td>CFM:ChCl</td>
<td>4.16 (1.6 - 11.6)</td>
</tr>
<tr>
<td>CFM:PA:ChCl</td>
<td>20.49 (5.4 - 809.5)</td>
</tr>
<tr>
<td>CFM:SA:ChCl</td>
<td>14.69 (5.1 - 41.1)</td>
</tr>
</tbody>
</table>
According to the particle size distribution, A) CFM:DPPC showed unimodal distribution for SD and FD. Similarly, unimodal distribution was in B) CFM:Lec, D) CFM:SA:Lec, and G) CFM:SA:ChCl with slight increase in the particle size of SD. Panels C) CFM:PA:Lec and E) CFM:ChCl showed unimodal distribution for FD and bimodal distribution for SD. This likely happened because of particle aggregation. Panel F) CFM:PA:ChCl showed bimodal distribution for both SD and FD, and this likely happened because of particle aggregation.
The formulations that in acceptable deep inhaled range were A)SD_CFM:DPPC, B)SD_CFM: Lec, C)FD_CFM:PA, lec and E)SD_CFM:ChCl. It is clear that lipids made the particles bigger and in multi-modal distribution because the difference between d (0.1) and (0.9) was big as in C)SD_CFM:PA:Lec, E)FD_CFM:ChCl, F)SD_CFM:PA:ChCl, and F)FD_CFM:PA:ChCl.

Nevertheless, the particle aggregation likely took place because it is a hydrodynamic system, and clofazimine is very hydrophobic. Therefore, aerodynamic particle size distribution was conducted to measure MMAD, GSD, %FPF.

### 3.4.3.2 Aerodynamic particle size distribution

Based on the particle impaction in NGI, a plot of % cumulative particle deposited versus cutoff diameter D 50,Q was drawn and MMAD and GSD were calculated as below (Table 3.7):

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MMAD</th>
<th>GSD</th>
<th>%FPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFM:DPPC</td>
<td>SD</td>
<td>2.48</td>
<td>2.71</td>
</tr>
<tr>
<td></td>
<td>FD</td>
<td>2.49</td>
<td>2.91</td>
</tr>
<tr>
<td>CFM:Lec</td>
<td>SD</td>
<td>6.10</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>FD</td>
<td>6.27</td>
<td>1.47</td>
</tr>
<tr>
<td>CFM:PA:Lec</td>
<td>SD</td>
<td>5.93</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>FD</td>
<td>4.50</td>
<td>2.19</td>
</tr>
<tr>
<td>CFM:SA:Lec</td>
<td>SD</td>
<td>5.99</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td>FD</td>
<td>5.71</td>
<td>1.74</td>
</tr>
<tr>
<td>CFM:ChCl</td>
<td>SD</td>
<td>5.60</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>FD</td>
<td>6.02</td>
<td>1.59</td>
</tr>
<tr>
<td>CFM:PA:ChCl</td>
<td>SD</td>
<td>3.08</td>
<td>3.01</td>
</tr>
<tr>
<td></td>
<td>FD</td>
<td>5.14</td>
<td>2.06</td>
</tr>
<tr>
<td>CFM:SA:ChCl</td>
<td>SD</td>
<td>6.42</td>
<td>1.40</td>
</tr>
<tr>
<td></td>
<td>FD</td>
<td>5.67</td>
<td>1.74</td>
</tr>
</tbody>
</table>
(SD_CFMS:DPPC), (FD_CFMS:DPPC), (SD_CFMS:PA:ChCl), and (FD_CFMS:PA:Lec) were the four formulations that had MMAD in acceptable deep inhaled range <5 µm.

The results of aerodynamic particle size distribution was compatible with hydrodynamic particle size distribution for all clofazimine formulations except (SD_CFMS:PA:ChCl) and (SD_CFMS:SA:ChCl). The potential reason is that the particles were low dense, so the behave as small particles in NGI. Also, it may be the particles aggregated in the hydrodynamic system, so the instrument gave a false read.

GSD value informs about how broad the particle distribution. It means the distribution is unimodal of GSD is low and vice versa. The ideal GSD should be less than 1.7. However, it is still acceptable to be lower than 3. All clofazimine formulations were in acceptable range.

The ideal % FPF should be more than 60% in order for drug to reach the deep lung. Clofazimine formulations that showed MMAD lower than 5 µm had also showed % FPF above 60% which confirms the aerodynamic properties of these formulations.
3.4.4 Thermogravimetric analysis (TGA)

In (Figure 3.10), TGA thermogram shows that all clofazimine formulations were stable with no significant weight loss until 220 °C (MP of clofazimine). Therefore, it can be understood that all powders are free from water moisture. The weight loss that happened after 220 °C for some formulations was because the degradation of the powders.

3.4.5 Differential scanning calorimetry (DSC)
Figure 3.11 DSC thermogram of clofazimine formulations

In (Figure 3.11), DSC thermograms shows that all clofazimine formulations were in crystalline form although they were formulated by ASP and then dried. All formulations showed melting peak around 220 C° (MP of clofazimine) with slight shifting due to the presence of another component in the formulation. Also, the formulations containing lipids showed melting peaks for the lipids around 50 C°.
3.4.6 X-ray powder diffraction (XRPD)

![XRPD Graph of Clofazimine Formulations]

In (Figure 3.12), XRPD graph shows that when clofazimine formulations exposed to light beam of XRPD, they diffracted the light. Therefore, it is concluded that all clofazimine formulations were in crystalline form. These results are compatible with DSC thermograph in showing crystallinity.
3.4.7 IR spectroscopy

In Figure 3.13, all clofazimine formulations were chemically intact. Comparing between crude clofazimine and the formulations, ASP method followed by freeze or spray drying
did not change the chemical structure of clofazimine which its chemical fingerprint was showed in all formulations.

3.4.8 Particle morphology

![Figure 3.14 Microscope image for raw CFM](image)

This image in (Figure 3.14) shows that raw clofazimine has needle shape particles that makes it non compatible with inhalation delivery. Therefore, it needs to be formulated in small particles that has spherical or globular shape to be inhaled.
Figure 3.15 Microscope images for (FD_CFM:DPPC), (SD_CFM:DPPC), (FD_CFM:PA:Lec), and (SD_CFM:PA:ChCl)

(Figure 3.15) shows the four clofazimine formulations that have the best aerodynamic properties. It is clear that there is significant reduction in the particle size to be lower than 5 µm. The particle morphology seems spherical or globular in shape. However, there is particle aggregation in all formulations. This may explain the large hydrodynamic particle size distribution. Nevertheless, optical microscopy is not reliable to confirm the particle morphology. Scanning electron microscopy (SEM) images need to be taken.
3.5 Key findings of chapter 3 and conclusion

Based on process estimation in chapter 2 by using curcumin as a drug model, clofazimine was formulated with variety of lipids and surfactants to produce SLMs. The formulations were prepared by anti-solvent precipitation method and then divided to be dried by freeze or spray drying. Clofazimine formulations were characterized for batch yield, drug content, particle size distribution, aerodynamic properties analysis, physical state, and stability, chemical integrity, and particle morphology.

It can be concluded that freeze drying is able to keep high batch yield and drug content more than spray drying especially with formulations containing lipids. Lipids can improve drug content more than surfactants, but it makes bigger particles with multi-modal distribution. All clofazimine formulations were physically stable, chemically intact, and crystalline form

For aerodynamic performance, 4 clofazimine formulations showed MMAD < 5 \( \mu \text{m} \) in acceptable GSD range and %FPF. These formulations are (FD CFM: DPPC), (SD CFM: DPPC), (FD CFM: PA: Lec), and (SD CFM: PA: ChCl). They were pictured under optical microscope and showed small globular particle size.
Chapter 4: Thesis conclusion and future directions
4.1 Thesis conclusion

In this Master’s thesis, it was hypothesized that SLMs can improve aerodynamic and physiochemical properties of aqueous-based methods to prepare clofazimine formulations. In order to prove the hypothesis, this project had two aims; screening lipids and surfactant formulations with curcumin as a model drug, and preparation and characterization of clofazimine formulations.

In aim 1, curcumin was used as a drug model with variety of lipids and surfactants to estimate how clofazimine will work when it is incorporated in SLPs. The formulations were prepared by ASP method followed by freeze or spray drying. All lipids and surfactants that used were eligible to be formulated with clofazimine except span 40 and 80. Spray drying did not change the particle size distribution of the formulations after ASP, while freeze may make them bigger. Freeze drying kept high batch yield comparing to spray drying.

In aim 2, clofazimine was formulated with eligible lipids and surfactants to produce SLMs by ASP method followed by freeze or spray drying. Four clofazimine formulations showed MMAD < 5 µm in acceptable GSD and %FPF. These formulations were (FD CFM: DPPC), (SD CFM: DPPC), (FD CFM: PA: Lec), and (SD CFM: PA: ChCl). They were pictured under optical microscope and showed small globular particle size.

As a drying method, freeze drying was able to keep high batch yield and drug content more than spray drying especially with formulations containing lipids. Lipids improved drug content more than surfactants, but it made bigger particles with multi-modal distribution. All clofazimine formulations were physically stable, chemically intact, and crystalline form.
The findings of aim 1 and 2 were do not conflict with literature. For example, in literature, no one has published any articles for lipid spray drying. Rationally, we found that spray drying is not suitable for lipids because the low melting point of them. Moreover, many articles have indicated that SLPs can increase the incorporation of hydrophobic drugs. Our results also confirmed this fact. The limitations of the previous work of our lab, which are low batch yield and using of organic solvent-based spray drying system have been resolved by SLMs method for clofazimine formulations. 

To a large extent, the findings of aim 2 were compatible with the estimations of aim.1 Thus, it can be said that the hypothesis is fairly proved. Nevertheless, some confirmative characterizations are required to definitely prove the hypothesis.

4.2 Future directions

As it was stated, some confirmative characterizations need to be conducted to definitely prove the hypothesis. For instance, the particle morphology of clofazimine formulations needs to be analyzed by SEM because the optical microscope is not able to take high resolution images for the samples. SEM is a type of electron microscope that scan the surface of the sample with beam of electrons. The energy will transfer to the sample, and these electrons will drive out the electrons form the sample (primary electron). The dislodged electrons (secondary electrons) will be collected by positive detector and translated to signals. Then, the signals will be analyzed and translated to image.
In addition, minimum inhibitory concentration (MIC) values need to be determined to evaluate TB therapeutic effectiveness of SLMs of clofazimine. MIC is the lowest concentration of the drug that is able to stop growing of bacteria. This test is needed to check if the formulation process increased or decreased clofazimine’s activity. It is determined by preparing solutions of the clofazimine formulations at increasing concentration. Cultured TB mycobacteria are incubated to the solutions. Then, the results are measured by agar dilution or broth microdilution. MIC is the first step in drug discovery process. So, when it is proved, the drug can be analyzed in animal models.

If the particle’s sphericity and therapeutic efficacy of clofazimine formulations have been proved, this project could be extended for animal studies. As it was discussed in (1.5.4 In-vitro and in-vivo studies of clofazimine), there are several animal models that can be used for this study such as mice, rats, hamsters, guinea pigs, monkeys, cattle, and zebrafish. Because used lipids are not suitable to be spray dried, using different lipids with high melting point could help in improve drug content and batch yield for spray drying. The inlet temperature during spray drying should be lower than the melting points of spray dried components to make sure that the components will not be degraded. In our case, the melting points of palmitic acid and stearic acid, (63 Cº and 70 Cº respectively), were considerably lower than inlet temperature used during spray drying (140 Cº).
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adherence to tuberculosis treatment.”


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