Characterization and Pharmacokinetics of Rifampicin Laden

Carboxymethylcellulose Acetate Butyrate Particles

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Abstract

Tuberculosis, caused by *Mycobacterium tuberculosis* (MTB), is a common and potentially lethal infectious human disease. Rifampicin is a front line anti-tuberculosis drug usually prescribed in combination with isoniazid, pyrazinamide and streptomycin for a period of six to seven months. When given orally for the treatment of MTB, rifampicin exhibits low bioavailability. Recent attempts to increase bioavailability and decrease dosage of anti-tuberculosis drugs have focused on creating polymer coated rifampicin nanoparticles. The research effort presented in this thesis evaluates the formation, characterization and relative bioavailability of rifampicin loaded carboxymethylcellulose acetate butyrate (CMCAB) particles using two different formulation techniques. Multi inlet vortex mixer (MIVM) and manual spray drying techniques were used to form the rifampicin containing CMCAB particles. Characterization studies and analyses of particles revealed differences in particle sizes, shapes and drug loading between the different particle formulation techniques. *In vivo* pharmacokinetic studies in BALB/c mice indicate that a single dose of rifampicin laden CMCAB spray dried particle formulations are able to improve pharmacokinetic parameters including relative bioavailability of rifampicin compared to that of the free drug form at the same concentration.
Dedication

I dedicate my thesis to my family. Thank you for being the best part of my life. You all have been my source of encouragement, support and motivation. To my parents thank you for molding and allowing me to become the person that I am. To my sisters and boyfriend thank you for sharing wisdom, encouragement, advice, and laughs. This work is the result of the unrelenting determination and love they have instilled upon me.
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<th>Full Form</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under of the curve</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette – Guérien</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>Maximum concentration</td>
</tr>
<tr>
<td>CMCAB</td>
<td>Carboxymethylcellulose acetate butyrate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetracetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>$K_{el}$</td>
<td>Elimination rate constant</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MIVM</td>
<td>Multi inlet vortex mixer</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MTB</td>
<td><em>Mycobacterium Tuberculosis</em></td>
</tr>
<tr>
<td>MTS</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
</tr>
<tr>
<td>--------------</td>
<td>------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamic</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetic</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>t½</td>
<td>Half-life</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>T(_{\text{max}})</td>
<td>Maximum time</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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Introduction and Objectives

Tuberculosis is the world’s second most common cause of death from an infectious disease, after acquired immunodeficiency syndrome (AIDS) (17). It is spread through the air when people who have an active tuberculosis infection cough, sneeze, or otherwise transmit their infectious droplets through the air. For the treatment of active tuberculosis the World Health Organization (WHO) recommends a 6-month regimen consisting of rifampicin, isoniazid, pyrazinamide and ethambutol given together for the first four months, followed by isoniazid and rifampicin for the next two months (4, 23, 26). Rifampicin is particularly effective in treating tuberculosis because it inhibits RNA synthesis by binding to the β sub unit of bacterial DNA dependent RNA polymerase at very low concentrations (17). It is categorized as one of the first line anti-tuberculosis agent, however various side effects such as hepatotoxicity, allergic rashes, lack of appetite, nausea, or immunological disturbances have been reported in association with the administration of this drug over such long periods (12).

In humans orally administered rifampicin reaches peak plasma concentrations 2 - 4 hours after the dosage is given (5). As rifampicin enters the gastrointestinal tract it is hydrolyzed by the acidic medium found in the stomach to form 3- formyl rifamycin (31). Unaltered rifampicin leaves the gastrointestinal tract and is metabolized by the liver, forming 25- desacetyl rifampicin (3, 25). Both 3-formyl rifamycin and 25-desacetyl rifampicin exhibit high antimicrobial activity in vitro but are inactive in vivo (25). In the treatment of tuberculosis, overcoming low bioavailability of anti-tuberculosis drugs has been a challenge in the treatment process. The reduction in the amount of drug that reaches systemic circulation is responsible for the requirement of daily drug administration. Due to the high incidences of side effects and the need
for daily dosing, the patient compliance of anti-tuberculosis treatment is low, resulting in discontinuation of medication, leading to increased incidence of multi-drug resistant strains (9).

The development and use of nanoparticles as drug delivery systems has recently been used in an attempt to reduce the dose and frequency of administration of anti-tuberculosis drugs while increasing bioavailability (25). Most of these particles contain a unique polymer designed to alter the native release and distribution properties of free drug rifampicin. These formulated particles address increasing bioavailability, the time during which rifampicin remains in systemic circulation and target organs (6, 7, 28). Carboxymethylcellulose esters have been investigated for drug delivery because they possess characteristics that are compatible to the needs of pharmaceutical applications, enabling the formation of drug delivery systems that address patient needs such as consistent extended release (18, 27). These esters provide an excellent matrix for controlling the rate and pH at which the target drug is released into the aqueous environment (27).

Given the documented properties surrounding carboxymethylcellulose esters as possible oral drug delivery excipients and the knowledge of decreased bioavailability of oral rifampicin associated with its degradation in acidic conditions. The presented research presents a novel approach of increasing bioavailability by reducing rifampicin degradation with consistent release and enhanced circulation time when given orally.

The following are specific research objectives of this thesis research:

1) Formulate rifampicin laden CMCAB particles using two methods:

a) Multi Inlet Vortex Mixing and
b) Spray Drying techniques

2) Characterize properties of formulated particle properties using *in vitro* methods

3) Determine pharmacokinetic properties of formulated particles in a mouse model following a single dose high dose administration
Chapter 1: Literature Review

Characterization and Pharmacokinetics of Rifampicin Laden
Carboxymethylcellulose Acetate Butyrate Particles

Pharmacokinetics and Pharmacodynamics

Pharmacokinetics (PK) evaluates the fate of substances administered to a living organism, assessing the absorption, distribution, and elimination of drugs (15, 16). PK factors determine the time course of drug concentration in serum, which ultimately determines the time course of drug concentrations in tissues and body fluids (15). Pharmacodynamics (PD) focuses on the association between serum concentration and the pharmacological and toxicological effects of drugs. PD studies evaluating bactericidal antibiotic drugs are primarily interested in the relationship between concentration and antimicrobial effect (15).

For orally administered drug products a dosage profile is typically constructed by measuring the concentration of active ingredients over time in samples collected from the systemic circulation (1). Bioavailability is an important PK property, as it is defined as the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action (1, 16). Bioavailability data for a given drug formulation provides an estimate of the fraction of the orally administered dose that is absorbed into the systemic circulation. These studies also provide information related to distribution, elimination, and dose proportionality.
Mycobacterium tuberculosis

Tuberculosis is a common, highly contagious and potentially lethal infectious disease caused by Mycobacterium tuberculosis (MTB) (17). M. tuberculosis usually attacks the lungs but can also affect other parts of the body. It is spread through the air when people who have an active MTB infection cough, sneeze, or otherwise transmit their infectious droplets through the air. Tuberculosis is the world’s second most common cause of death from an infectious disease, after acquired immunodeficiency syndrome (AIDS) (17). Though one third of the world’s population is infected with MTB, most infected humans are asymptomatic and are latently infected and the active disease is induced by immunosuppression or immune incompetence (32). When the disease becomes active, its symptoms include a prolonged persistent cough for more than three weeks followed by chest pain, coughing up blood. Systemic symptoms include fever, chills, night sweats, loss of appetite, weight loss and fatigue (2). In 1993 the World Health Organization (WHO) declared tuberculosis a global public health emergency in an attempt to raise public and political awareness. This distinction has never been given to any other disease (26).

When MTB is taken up by alveolar macrophages in the lungs, the bacteria are not killed, as the bacterium prevents the fusion of phagosome with lysozome (34). Specifically, MTB blocks the connection molecule (Early Endosomal Autoantigen 1) necessary for phagocytosis to be complete (35) (34). However this blockage does not restrict fusion of vesicles filled with nutrients. Consequently, the bacteria are able to replicate unchecked within the macrophage. MTB persists in within a small cluster of macrophages, giant cells, T cells, B cells and fibroblasts referred to as a granuloma in the infected lungs (20). The state of the bacteria within the granuloma during latent infections is not well understood. It is speculated that the organism
may be in a dormant non-replicating state, or actively replicating but killed off by the immune response or metabolically altered to produce infrequent replicative cycles (20, 35). When the immune system designed to contain the infection becomes compromised, reactivation and rapid replication of MTB occurs (20).

While mycobacteria do not fit the Gram-positive category from a visual standpoint because they do not retain the crystal violet stain, they are classified as acid-fast Gram-positive bacteria due to their lack of an outer cell membrane (11). First discovered in 1882 by Robert Koch, MTB divides every 15-20 hours. This replication rate is slow compared to other bacteria, which tend to divide in minutes (21). MTB is a small bacillus that can withstand weak disinfectants and can survive in a dry state for weeks (11). Its unusual cell wall, which is rich in lipids such as mycolic acid, is reported to be responsible for this resistance and is a key virulence factor (10, 11).

**Tuberculosis Diagnosis and Treatment**

Tuberculosis is usually diagnosed with a combination of tuberculin purified protein derivative (PPD) skin test, chest x-ray, analysis of sputum, and recent approaches such as polymerase chain reaction (PCR) (13). Chest x-rays can reveal evidence of active tuberculosis, pneumonia, scarring or a hardening (granulomas) in the lungs, which suggest that the infection is contained and inactive (13). Microbiological analysis, relies heavily on microscopic examination of clinical samples for acid-fast bacilli using Ziehl-Neelsen acid faststain, which can detect 60-70% of culture positive samples (30). A positive tuberculin skin test serves as an indicator of MTB exposure. In most people tuberculin reactivity is apparent in 3-6 weeks after
infection, but it can take up to 3 months after initial infection to become skin test positive (22). Due to its slow growth characteristics recently PCR has been used to look for the presence of genetic material of the bacteria, resulting in a faster and more sensitive test than the microbiological analysis which take several weeks to months (13).

Due to the substandard outcomes of tuberculosis control measures, such as vaccination using Bacillus Calmette – Guérin (BCG) and chemoprophylaxis, treatment of tuberculosis with antimicrobials becomes the single most important option (17). The goals of tuberculosis treatment have focused on cure (eliminating the infection without relapse), preventing death, and preventing the emergence of drug resistant bacteria (17). For the treatment of active tuberculosis the World Health Organization (WHO) recommends a 6-month regimen of rifampicin, isoniazid, pyrazinamide and ethambutol given together for the first two months, followed by isoniazid and rifampicin for the next four months (26) (4). Antimicrobials are usually part of therapy for patients with no symptoms. As they are in the inactive state and it will be helpful in preventing the activation of the infection (13). Rifampicin and isoniazid are usually recommended for six to twelve months to prevent future activation of the infection (8).

**Rifampicin**

Rifampicin is a complex semi-synthetic macrocyclic antibiotic derived from *Streptomyces mediterranei* (14). It is an intensely red solid, known for imparting a harmless red-orange color to the urine, sweat and tears of patients, for a few hours after a dose. It is a member of the rifamycin class of antibiotics used for the treatment of tuberculosis, often a component of
the prescribed multi-drug fixed dose combination tablet (24). Rifampicin was developed in the Dow – Lepetit Laboratories in Milan, Italy as a part of a screening program for new antimicrobial agents (29). It was isolated from fermentation cultures of S. mediterranei and found to consist of 5 substances named rifamycin A-E (33). All of the preliminary substances proved to have minimal absorption from the gastrointestinal tract, but rifamycin B was shown to be the most stable, least toxic and most active (29). In 1965, rifampicin, a hydrazone of a rifamycin B, was found to have increased absorption while retaining its parent antimicrobial properties. Rifampicin was approved by the Food and Drug Administration (FDA) in 1971. Subsequent studies began to evaluate its efficacy in tuberculosis treatment (23). Rifampicin inhibits RNA synthesis by binding to the β subunit of bacterial DNA dependent RNA polymerase (17). It is categorized as one of the first line anti-tuberculosis agent, however various side effects such as hepatotoxicity, allergic rashes, lack of appetite, nausea, or immunological disturbances have been reported with the prolonged administration of this drug (12).

**Rifampicin Pharmacokinetics and Pharmacodynamics**

In humans orally administered rifampicin reaches peak plasma concentrations 2 - 4 hours after the dosage is given (5). As rifampicin enters the gastrointestinal tract it is hydrolyzed by the acidic medium found in the stomach to form 3- formyl rifamycin which is an insoluble and poorly absorbed compound (31). Unaltered rifampicin that makes it though the gastrointestinal tract is metabolized by the liver, eliminated in the bile and then reabsorbed from the instine thereby entering enterohepatic circulation. It is during this time that rifampicin is deacylated into
desacetyl rifampicin (3, 25). Both 3-formyl rifamycin and 25-desacetyl rifampicin exhibit high antimicrobial activity *in vitro* but have been shown to inactive *in vivo* (25).

The effectiveness of treatment with rifampicin is questionable due to its variable bioavailability, which was identified over the last four decades (25). Explanations of decreased bioavailability include changes in the crystalline habit of rifampicin, excipients, and degradation in the gastro-intestinal tract, absorption and metabolism (25). Rifampicin is categorized by the Biopharmaceutics Classification System as a class II drug because it has characteristics of low solubility and high permeability (25, 26). Furthermore, rifampicin exhibits pH-dependent solubility affecting its absorption from the gastro-intestinal tract.

Efforts in nanomedicine specifically focused on the encapsulation of rifampicin for oral delivery have been developed to increase sustained release of rifampicin and increase drug encapsulation. Rifampicin encapsulated alginate nanoparticles show therapeutic potential by increasing drug encapsulation efficiency and elevated drug concentrations in plasma after oral dosage for up to 15 days (6, 7). Other use of nanomedicine has lead to the development of microencapsulated rifampicin poly(DL-lactide-co-glycolide). These particles range in size from 1 to 150μm and have demonstrated elimination of MTB from infected mice in a single dose similarly to efficacy obtained with daily free drug dosage necessary to successfully eliminate the infection (19).

**Carboxymethylcellulose acetate butyrate (CMCAB)**

Carboxymethylcellulose acetate butyrate (CMCAB) belongs to cellulose ester family and carries hydroxyl, ester and acid functionalities, CMCAB is insoluble in water and water-
swellable when it becomes partially ionized (27). Carboxymethylcellulose esters have been investigated for drug delivery because they posses characteristics that are compatible to the needs of pharmaceutical applications, enabling the formulation for drug delivery systems that address patient needs such as constant extended release (18, 27). These esters provide an excellent matrix for controlling the rate and pH at which the target drug is released into the aqueous environment (27). For therapeutics that are characterized by fast clearance or issues with instability, the incorporation of CMCAB would be desirable to release the drug slowly at the sites of absorption as needed (27). Cellulose esters have the benefit of low toxicity in humans. Not only are cellulose esters are non toxic and not absorbed from the gastrointestinal tract but they tend to be stable due to the lack of cellulases in the human gut (18)
References


Chapter 2

Formulation Development and Characterization Rifampicin Laden Carboxymethylcellulose Acetate Butyrate Particles

Abstract

Carboxymethylcellulose esters such as Carboxymethylcellulose acetate butyrate (CMCAB) have been commercially evaluated for oral dosage forms because of their low toxicity, and their ability to control the rate and pH at which the drug is released. They are generally used as a major excipient in oral dosage forms serving as a coating or membrane for pharmaceutical drugs. Results from characterization analysis evaluating properties such as dissolution, morphology, and biological effectiveness of tablets coated with CMCAB may not be transferable to formulated particles. Nanoparticles in particular often behave as a whole unit in terms of transport and properties. Their increased surface area and properties can increase the rate of diffusion, leading to enhanced drug absorption and bioavailability. Multi inlet vortex mixers (MIVM) and manual spray drying techniques are common ways of producing drug containing particles. MIVM involves the use of turbulent mixing of inlet streams containing dissolved polymer and drug to form the complex particles suspended in a mostly anti-solvent solution. Manual spray drying is comprised of a one step process beginning with the polymer and drug dissolved in a solvent solution, but resulting in dry formulated particles. Though the properties of rifampicin and CMCAB alone are well documented, particle formulations with the two components have yet to be evaluated. To be able to evaluate the overall use of rifampicin encapsulated CMCAB particles as an oral dosage form, it is important to first characterize their characterization and in vitro properties. Results from these analyses demonstrate a difference in particle size, morphology, and drug loading in the two formulated groups. There is no significant
difference between MIVM and spray dried rifampicin containing particles compared to free drug rifampicin in the killing of intracellular and extracellular bacteria. In summary, rifampicin formulated particles showed difference in physical characteristics; however, both formulations contain biologically active rifampicin necessary for further \textit{in vivo} studies.
Introduction

Particle formation is often categorized in terms of top-down or bottom-up approaches. Top-down approaches consist of milling and homogenization techniques that start with solid particles that are larger than the targeted particles. The larger particles are milled down into smaller sizes for target specific use (12). These processes due to their mechanical nature require excess energy and are usually time consuming (lasting several days). Bottom-up approaches consist of solvent evaporation, and anti-solvent techniques form particles from the molecular level providing more control of particle properties and reduce formulation time (12).

Spray drying is the most commonly used and studied solvent evaporation method (10). In the 1980’s spray drying was investigated as an alternative method of making fine particles (3). In spray drying, a drug solution (usually organic) is atomized to fine droplets which are evaporated by the warm air current to form dry particles (11). This single step drying method from solution to solid, in which the solvent is thermally removed, produces a dry powder with control and consistency of the particle properties (12). The formulation process shown in Figure 1 is accomplished by pumping a feed solution containing the desired drug and polymer though a peristaltic feed tube that connects with the spray nozzle inside the drying chamber. The spray nozzle breaks the solution into a trail of small particles which are mixed with a hot drying gas stream. At this stage of the drying process heat evaporates the solvent from the particles causing the skin to collapse as the solvent evaporates form the core (5). The resulting dry solid particles are typically collected from the gas stream in the product vessel or connected filter.

Particle formation that results in the production of uniform particles by an economical and scalable process has been a significant challenge (6). One of the most common
method for overcoming these challenges in the production of nanoparticles involves flash nano-
precipitation (6). This method has recently been aided by the characterization and design of a
multi-inlet vortex mixer (MIVM) shown in Figure 2. The 4- stream MIVM allows mixing of
various solutions with unequal volumetric flows, providing the ability to control solvent
composition by varying content and flow rates of each individual stream (3). Advantages of the
MIVM system include a resultant final fluid phase that is mostly anti-solvent, and the turbulent
region created by the momentum of the inlet streams provide a mixing environment to create and
precipitate homogeneous nanoparticles (7).

The characterization of formulated particles is necessary to establish understanding
and control of nanoparticle synthesis and possible applications. Characterization is done by using
a variety of different techniques that consider both material and biological science. Material
science often characterizes particles by determining particle size, morphology, poly-dispersity
and thermal analysis. Results from these methods of characterizations are not only useful in
optimizing formulation techniques, but are important in the application process. Characterization
from a biological science perspective frequently involves determination of drug encapsulation,
effectiveness of the encapsulated drug, drug release kinetics and in vivo evaluation.
**Figure 1:** Schematic of the multi-inlet vortex mixer during working state

**Figure 2:** Schematic of BÜCHI B-290 spray dryer during working state
Materials and Methods

Synthesis of Nanoparticles Using MIVM

Carboxymethylcellulose acetate butyrate (CMCAB CAS # 641-0.2 (Lot WG 0077B); approximate molecular weight 22,000 Da, Degree of Substitution of Butyryl group = 1.64, Degree of Substitution Acetyl group = 0.44, Degree of Substitution Carboxymethyl group = 0.33, Eastman Chemical Company) totaling 10mg and 3.33mg of rifampicin were suspended in 100ml of tetrahydrofuran (THF) (Fisher Scientific, Pittsburg, PA) and mixed by magnetic stir bar for 16 - 18 hours at room temperature. Flash nanoprecipitation of CMCAB-rifampicin complexes was carried out in a MVIM that accommodates four solvent streams. The temperature of the streams was adjusted to 25°C prior to injection into the mixer. A New Era 4000 double syringe pump (for the organic stream) and a Harvard Apparatus Remote Infuse/Withdraw PHD 4000 Hpsi Programmable syringe pump (for the three water streams) controlled the inlet flow rates. The New Era pump was controlled by a software syringe pump pro version 1.53. The flow rate for the THF stream containing CMCAB and rifampicin was 12.82ml/min, the water streams were set to a flow rate of 38.47ml/min. The combination of inlet flow rates resulted in a 1:10 THF/water ratio and a Reynolds number 15,000. CMCAB-Rifampicin suspensions were recovered from the mixer and dialyzed to remove THF, free drug, and dissolved polymer molecules. The resulting solution was dialyzed against DI water for two days using cellulose ester dialysis tubing (Spectropore) with a molecular weight cutoff (MWCO) of 25,000. The particle solution in the dialysis tubing was then removed and freeze dried under vacuum conditions.
Formation of Spray Dried Rifampicin Particles

Carboxymethylcellulose acetate butyrate (CMCAB CAS # 641-0.2 (Lot WG 0077B); approximate molecular weight 22000, Degree of Substitution of Butyryl group = 1.64, Degree of Substitution Acetyl group = 0.44, Degree of Substitution Carboxymethyl group = 0.33, Eastman Chemical Company) totaling 1g was suspended in 140ml of tetrahydrofuran (THF) (Fisher Scientific, Pittsburg, PA) and mixed by magnetic stir bar for 16 - 18 hours at room temperature. After mixing, .33g of rifampicin (Sigma – Aldrich, Saint Louis, MO) mixed in 60ml of THF was added to the solution for a total volume of 200ml and allowed to mix for 10 minutes. 3:1 polymer to drug ratio was experimentally determine to be the optimal ratio that provided extensive drug encapsulation and prolonged release of rifampicin over time. The mixture was connected to the inlet valve and pumped though the Buchi B-290 spray dryer (Buchi, Newcastle, Delaware). Spray dryer conditions were programmed with an inlet and outlet temperature of 66°C (to insure solvent evaporation), aspirator chamber at 100% efficiency and pump pressure at 40% for optimal drying and isolation of homogeneous particles. One gram of resulting rifampicin – CMCAB nanoparticles was collected from the spray dryer collection flask.

The collected particles were placed in 250ml of DI water briefly mixed using an automatic wrist action shaker then bath sonicated for 20 minutes. The resulting dispersion of RIF/CMCAB spray dried particles were collected and immediately placed in a 10000 MWCO Millipore centrifugal filter device. The samples were centrifuged at 1,789 ×g for 15 min or until all of the elution had passed though the filter. The filter membranes were rinsed with 10ml of pH 7.4 PBS, redispersing the remaining particles, which were then freeze dried under vacuum.
Dissolution of Rifampicin from CMCAB Particle Formulations

Phosphate buffered saline (PBS) solution was made at pH 7.4 then aliquoted and adjusted with hydrochloric acid (Fisher Scientific) to create PBS solutions at pH 1.2. Each solution was degassed by vacuum filtration through a 0.02µm PES filter. Encapsulated rifampicin totaling 0.1mg drug in MIVM and spray dried formulations were added to 50 ml each of PBS solution containing beakers with a magnetic stir bars. At one hourly intervals 300µl samples were removed from each solution for eight hours and were centrifuged at 1,358 xg for 15 minutes syringe filtered through 0.45µm PES filter and analyzed by high performance liquid chromatography to the determine the concentration of rifampicin released.

HPLC Analysis for Dissolution Studies

The HPLC analysis of rifampicin was performed by a reverse-phase high performance liquid chromatography (HPLC) method using an Agilent 1200 Series HPLC system equipped with an auto sampler and diode array detector. Separation was achieved with C18 column (Zorbax) and data acquisition ChemStation software (Agilent Technologies Inc., Palo Alto, CA). The mobile phase was run with 50% acetonitrile and 50% 1 M ammonium acetate. Analysis was performed on a 5µl injection at a flow rate of 1.0 ml/min through an Agilent Zorbax Eclipse XDB-C18 (4.6×150 mm) column and absorbance recorded at 334 nm.
Cell Viability Assay

The CellTiter 96 AQ<sub>ueous</sub> Non-Radioactive cell proliferation assay is a colorimetric method for determining the number of viable cells. The assay is composed of solutions of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron-coupling reagent (phenazine methosulfate; PMS). MTS is bioreduced by cells into a formazan product that is soluble in culture medium. The absorbance of the formazan at 490nm can be measured directly from 96-well assay plates without additional processing. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture.

J774_A.1 murine macrophage like cell line was obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin-streptomycin solution. Approximately ~2 x 10<sup>4</sup> macrophage cells suspended in 200 µl of DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution were then seeded in 96-well plates. Cells were incubated for 24-36 hours at 37°C in 5% CO<sub>2</sub> atmosphere until ~90% confluency was achieved. After confluency was achieved, the cell culture media was removed and the cells were gently washed with phosphate buffered saline solution (PBS) to ensure complete removal of depleted culture media.

Fresh medium (200µl) containing either free drug or rifampicin - CMCAB nanoparticles (spray dried and MVIM formulations) in at concentrations ranging from 250µg/ml to 62.5µg/ml
were added to the cells and were incubated for 24 hours at 37°C in 5% CO₂ atmosphere. As the control, cells were incubated with only medium. After incubation for 24hrs the culture media was discarded and cells were gently washed with PBS and 100µl of fresh culture media was added to each well sample well along with 20µl of CellTiter 96® Aqueous reagents solution (Promega). Wells containing cells and culture media only served as control for 100% viability. After 3 hours of incubation at 37°C in 5% CO₂ atmosphere, the absorbance of samples in the 96 – well plate were measured at 490 nm using a 96-well ELISA reader (SoftMax Pro Inc., USA). Results were expressed as a percentage mean absorbance by cell upon incubation with various treatments with respect to the untreated control, considering the absorption of control cells as 100% cell viability.

**Determination of Encapsulation Efficiency of Rifampicin Laden CMCAB Particles**

The efficiency of encapsulation was determined by measuring the total amount of rifampicin present in a given sample. A 1mg/ml total weight (rifampicin encapsulated in CMCAB) was dissolved in acetonitrile (Fisher Scientific) by briefly vortexing then filtered with a 0.45µm PES filter. The calibration curve for rifampicin was created by dissolving rifampicin in acetonitrile. Standards used were 10, 20, 30, 40, 60 and 70 µg/ml. Each standard and dissolved particles was analyzed at 475nm using a UV-visible spectrophotometer (Thermo Scientific). The resulting absorbance values from the dissolved particles were compared to the values from the standard to determine encapsulation efficiency.
**Size Determination by Dynamic Light Scattering**

The intensity average hydrodynamic diameters were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd.). The autocorrelation functions of the scattered intensity were fitted using the distribution fit analysis in the ZS 6.12 software to extract the average translational diffusion coefficient, and the hydrodynamic diameters were then calculated with the Stokes-Einstein equation. MIVM particles were prepared at a concentration of 0.01mg/ml in DI water. The solution was sonicated and filtered through a 1μm filter. The Malvern ZS software was set to the manual size measurement standard operating procedure and set to analyze 12 cycles per run for 3 runs at room temperature. Spray dried particles were prepared at a concentration on 0.025mg/ml in PBS, then sonicated and run at the previously stated software conditions un-filtered.

**Scanning Electron Microscope Images**

Morphology of the nanoparticles was examined by using a LEO (Zeisis) 1550 scanning electron microscopy (SEM). MIVM and spray dried particles were sonicated at 0.01mg/ml in DI water. Approximately 100μl of the prepared samples was placed on imaging tape and allowed to dry at room temperature. Samples for SEM were mounted on metal stubs and coated with gold prior to analysis.
Determination of Minimum Inhibitory Concentration (MIC) of rifampicin and rifampicin encapsulated particles

*M. smegmatis* ATCC# 700084 (mc² 155) was routinely grown using Middlebrook 7H10 agar (Difacto, Detroit, MI) containing 0.5% glycerol and 10% OADC enrichment. The bacterial cultures were diluted in 1x PBS (Mediatech, Manassas, VA) the suspension adjusted to equal the McFarland No. 0.5 turbidity standard and 0.02ml aliquots were transferred to each well of the 96-well round bottom plate that contained 0.1ml of Middlebrook 7H9 broth (Difacto, Detroit, MI) and 0.1ml of each particle formulations and free drug at 2-fold serial dilutions in PBS ranging from 500μg/ml to 7.81μg/ml. The inoculated plates were incubated at 37°C for 48hrs and the MIC was defined as the lowest concentration of compound that inhibited visible growth.

Efficacy of Rifampicin and Rifampicin Encapsulated CMCAB Particles against intracellular *Mycobacterium smegmatis* Infected Macrophages

*J774_A.1* murine macrophage cell line was obtained from American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat- inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Approximately 2 x 10⁴ *J774*A.1 cells were suspended in 1 ml of DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution then seeded in 24-well plates. Cells were allowed to incubate for 24 hours at which time new media containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat- inactivated fetal bovine serum (FBS) was added into each well.
Routinely grown *M. smegmatis* ATCC # 700084 (mc² 155) was cultured on Middlebrook 7H10 agar (Difacto, Detroit, MI) containing 0.5% glycerol and 10% OADC enrichment. The bacterial cultures were diluted in 1x PBS (Mediatech, Manassas, VA) to a multiplicity of infection (MOI) of 100 bacteria per cell. Macrophages were incubated with bacteria for four hours then wells were rinsed two times with 1x PBS. Medium containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat- inactivated fetal bovine serum (FBS) placed in each well. Free drug and each particle formulation at 64µg/ml (2x MIC) rifampicin concentration was suspended in PBS and added to each treatment well. At each time point the media was removed and cells were washed with PBS then lysed with 200µl of 0.1% Triton-X. Lysed cell contents were cultured on Middlebrook 7H10 agar (Difacto, Detroit, MI) containing 0.5% glycerol and 10% OADC enrichment in serial dilutions from 10⁻¹-10⁻⁸. The plates were incubated at 37°C for 48 hours then bacterial colony forming units (CFU’s) were determined.

**Statistical Analysis**

All data were analyzed by two-way analysis of variance using Prism software (GraphPad Software, La Jolla, CA). A *P* value of <0.05 was considered significant.
Results

Encapsulation Efficiency

The efficiency of encapsulation of rifampicin was determined by measuring the total amount of rifampicin present in each sample (i.e., drug loading experimental), and comparing this value with the expected amount of rifampicin in each of the samples based on the drug loading during the preparation (i.e., drug loading theoretical). The effect of drug loading was evaluated by comparing the two formulations of particles prepared with the same amount of rifampicin and CMCAB. The efficiency of encapsulation of the MIVM formulation was approximately 10% and that of the spray dried formulation was 21% (Table 1).

Particle Size and Morphology

Polydispersity index is a parameter used to define the particle size distribution of nanoparticles obtained from photon correlation spectroscopic analysis (8). It is a dimensionless number extrapolated from the autocorrelation function and ranges from a value of 0.01 for monodispersed particles and up to values of 0.5–0.7 (8). Results of the particle size analysis using DLS and SEM showed that a change in the formulation technique greatly affected the median particle size and shape morphology. The median particle sizes of the MVIM formulations and spray dried formulations using DLS were approximately 140 and 962 nm respectively (Table 1). SEM morphology illustrated particle aggregation in the MIVM
formulations which was likely measured as an individual particles using DLS (Figure 3). The SEM for the spray dried formulations demonstrated bowl-shaped collapsed poly-dispersed particles ranging from 3 -10μm which exceeds the detectable size measurements of DLS (Figure 4). During spraying and atomization of the liquid some droplets may have been larger than the others accounting for the variation in size of the particles seen in the SEM images (Figure 4).

Physical characteristics of rifampicin/CMCAB MIVM and spray dried particles

<table>
<thead>
<tr>
<th></th>
<th>MIVM</th>
<th>Spray Dried</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encapsulation Efficiency (% rifampicin)</td>
<td>9.94 ± 0.013</td>
<td>21 ± 0.55</td>
</tr>
<tr>
<td>DLS Median Particle Size (nm)</td>
<td>140.8 ± 12.06</td>
<td>962.5 ± 9.26</td>
</tr>
<tr>
<td>DLS Poly-dispersity index (PDI)</td>
<td>0.155 ± 0.005</td>
<td>0.046 ± 0.049</td>
</tr>
<tr>
<td>Morphology</td>
<td>Agglomerates of small particles</td>
<td>Bowl shaped individual particles</td>
</tr>
</tbody>
</table>

Table 1: Physical characteristics of rifampicin/CMCAB MIVM and spray dried particles. Encapsulation efficiency of rifampicin/CMCAB particles determined by UV spectrophotometric analysis. Median particle size and PDI determined by a Malvern Zetasizer Nano ZS. Morphology of the nanoparticles was examined by using a LEO (Zeisis) 1550 scanning electron microscopy (SEM)
Particle Morphology

Figure 3: Scanning electron microscope images for MIVM particles with images. (a) 100x magnification and (b) 200x magnification. The bar in each image corresponds to 200 nm.

Figure 4: Scanning electron microscope images for spray dried particles (a) 2x magnification and (b) 10 x magnifications. The bar in image (a) corresponds to 10 μm and (b) to 3 μm.
Dissolution of Rifampicin from CMCAB Nanoparticle Formulations

The dissolution of rifampicin from both MIVM and spray dried CMCAB formulated particles was pH dependent (Figure 7). Rifampicin release was followed over a period of approximately 24 hours in two different pH buffer solutions (Figure 7). Visual observations of both particle formulations at pH 6.8 revealed a suspension of particles in the aqueous solution. Observations of MIVM and spray dried particle formulations at pH 1.2 demonstrated particle aggregation that remained on the surface of the aqueous solution for approximately 5 hours, at which time the particles remained aggregated and began to collect at the bottom of the dissolution vessel. Analysis of rifampicin contentment revealed approximately 50% rifampicin released from both particle formulations in the first hour of dissolution at pH 6.8. The MIVM particles showed little change in rifampicin released in the first 8 hr after the initial burst release. The spray dried formulated particles showed a gradual release of rifampicin in the first 8 hr after the initial burst release, but at pH 1.2 there was no measurable release of rifampicin from both particle formulations when analyzed using the HPLC method previously described in the materials and methods section (Figure 7). After 24 hr in buffered solutions at acidic pH rifampicin release was unable to be detected. As for the neutral pH after 24 hours both particle formulations released approximately 65% of the encapsulated rifampicin.
Dissolution of Rifampicin from MIVM and Spray Dried CMCAB Particle Formulations

![Graph showing dissolution of rifampicin from MIVM and spray dried CMCAB particle formulations at neutral (pH: 6.8) and acidic (pH: 1.2) buffered solutions over a 24 hr time period.]

**Figure 5:** Dissolution of rifampicin from MIVM and spray dried CMCAB particle formulations at neutral (pH: 6.8) and acidic (pH: 1.2) buffered solutions over a 24 hr time period.

**Cell Viability**

Cell viability assays are vital steps that provide insight to the cellular response to a toxicant. Also, it provides information regarding cell death, survival, and metabolic activities. MTS cell viability assay was performed on all nanoparticle formulations to assess whether the rifampicin
encapsulated CMCAB nanoparticles demonstrated *in vitro* toxicity. In comparing the cell viability of the particle formulations and free drug to the control MIVM particles, spray dried particles and free drug showed no significant reduction of cell viability at the equal rifampicin concentrations and incubation times (Figure 5). Results from this analysis indicate that a rifampicin concentration of 250μg/ml is non-toxic *in vitro* and the formulations containing 250μg/ml of rifampicin were also non toxic *in vitro*. These results are vital given that the MIVM and spray dried formulated particles contain ~10% and ~ 21% rifampicin by weight, meaning that at the 250μg/ml of rifampicin concentration for the MIVM particles there was more than 9 times as much CMCAB present. Microscopic observations of the treated cells after the completion of the incubation period revealed floating and sedimanted particle components in both particle formulations and reduction in the number of attached cells in the MIVM particle treatment group. This observation suggest that the sedimentary CMCAB particle fragments likely attributed to the reduction of attached cells, thus reducing the percentage of viable cells in the MIVM particle treatment group (Figure 5).
Cell Viability of Macrophage Cells after Incubation with Rifampicin Containing Formulations

Figure 6: Data obtained from 96 AQuous Non-Radioactive cell proliferation assay after 24 hour incubation of free drug rifampicin, and RIF/CMCAB formulations (MIVM and spray dried) at 250 μg/ml rifampicin. The y axis represents the percent of reduction in ATP content compared to control. The values represent the mean ± standard deviation of three experiments.

Determination of Minimum Inhibitory Concentration (MIC) of Rifampicin and Rifampicin Laden Particles

The determination of MIC’s for rifampicin and rifampicin laden particles are essential given the various formulation techniques used and the intended application. Particle formulation techniques can alter various factors of the native drug leading to the encapsulation of a less active or inactive complex. Results from the free drug rifampicin and particle formulations indicated that the biological activity of rifampicin was preserved after CMCAB particle formation with MIVM and spray drying techniques (Table 2). The total weight (CMCAB+RIF)
in a particle formulation was used and compared to that of the free rifampicin to more easily distinguish CMCAB particle components from bacterial growth.

Minimum Inhibitory Concentrations of RIF/CMCAB particle formulations for *M. smegmatis*

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Observed MIC (Based on total particle weight)</th>
<th>MIC Range (Determined by calculated % drug loading)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Rifampicin (RIF only)</td>
<td>15.62 – 31.25µg/ml</td>
<td>15.62 – 31.25µg/ml</td>
</tr>
<tr>
<td>MVIM (total weight: CMCAB+RIF)</td>
<td>250 – 500µg/ml</td>
<td>24.75 – 49.5µg/ml</td>
</tr>
<tr>
<td>Spray Dried (total weight: CMCAB+RIF)</td>
<td>125 – 250µg/ml</td>
<td>26.25 – 52.5µg/ml</td>
</tr>
<tr>
<td>Reported MIC*</td>
<td></td>
<td>32µg/ml</td>
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</tbody>
</table>

Table 2: Minimum Inhibitory Concentrations of RIF/CMCAB particle formulations for *M. smegmatis*. MIC values for *M. smegmatis* ATCC # 700084 (mc² 155) total weight formulations (RIF and CMCAB, or RIF alone for free drug) after 48 hr incubation. Reported *M. smegmatis* ATCC # 700084 (mc² 155) MIC values for rifampicin are documented in Alexander. D., et. al., 2003 (1)

Efficacy of Rifampicin and Rifampicin Encapsulated CMCAB Nanoparticles in *M. smegmatis* Infected Macrophages

To be applicable in the treatment of intracellular infections such as MTB, rifampicin containing particles must be capable of either entering the intracellular environment or releasing rifampicin in the extracellular environment to be taken up by infected cells. Colony forming units (CFUs) of macrophage cells infected with *M. smegmatis* then treated with both particle formulations and free drug showed that the particle formulations were as effective in eliminating the intracellular
bacterium as the free drug at 8, 12, and 24 hr time points (Figure 6). However, at the 4 hr time point both particle formulations were not statistically significant when compared to the untreated control (Figure 6). Significant reduction in CFUs were observed at 12 and 24 hr time points for free drug rifampicin and both particles formulations coinciding with a greater than 1 (90%) and 2 log (99%) CFU reduction compared to the untreated control.

**Efficacy of Rifampicin and Rifampicin Laden CMCAB Particles in *M. smegmatis* Infected Macrophages**

![Figure 7: Efficacy of Rifampicin and Rifampicin Laden CMCAB Particles in *M. smegmatis* Infected Macrophages. Significance (*) was determined in all cases by two-way analysis of variance and a \( P \) value of \( \leq 0.05 \) compared to the control](image_url)
Discussion

Advances in particles formation have provided the opportunity to customize particles by selecting for properties that may be an advantage or disadvantage to its potential application. Previous studies have demonstrated the advantages for the possible use of CMCAB as an excipient for orally delivered drugs by conducting classification studies (4, 9). In the current study, the characterization and in vitro analysis of two different formulations of rifampicin encapsulated CMCAB particles were evaluated. Results from these studies not only demonstrate the differences in RIF/CMCAB particle formation using two different techniques and the properties of CMCAB as an excipient, but they provide a foundation from a biological standpoint that communicates and validates the advantages for the possible use of CMCAB as an oral drug delivery system.

Nanotechnology has provided a new avenue in the field of drug delivery with potential applications. In the development of anti-tuberculosis drug delivery systems using particle formulated for oral drug delivery systems two points are important to consider. First the metabolism of M. tuberculosis is slow, resulting in a generation time that is measured in hours. This means that ideal drug therapies will have a low level of toxicity for long term administration and should be bactericidal so that elimination is rapid. Second, M. tuberculosis is a facultative intracellular organism; therefore, drugs should also be able to penetrate host cells. Rifampicin is an effective antimicrobial for eliminating M. tuberculosis through a unique mechanism of action. As an un-altered free drug, rifampicin is able to address the points that should be considered for treatment to an effect; it is important to remember that derivates of the drug formed in vivo are ineffective systemically.
The study presented in this thesis demonstrates for the first time formation, characterization and in vitro evaluation of rifampicin encapsulated CMCAB particles formulated using MIVM and spray drying techniques. Results demonstrated that MIVM and spray dried particles were successfully formulated and contained biologically active rifampicin after the particle formulation process. The differences in shape and encapsulation efficiency are consistent with the formulation technique used and subsequent steps to remove free drug and polymer. MIVM particle formation results in nano-sized particles while spray drying results in micron sized particles.

The use of a 24hr dialysis to remove free rifampicin and polymer may contribute to the reduction in drug encapsulation compared to centrifugal filter method used for the spray dried particles. Results from dynamic light scattering studies used to determine particle size and polydispersity differ from the resulting SEM images. DLS used to determine particle size may not detect aggregates of particles as seen in the MIVM particle images, resulting in a reading of the aggregated small particles. While particle sizes up to 10μm in diameter can be detected by DLS, measurement result from the spray dried particle analysis revealed possible aggregation and large particle sedimentation during the analysis process. The larger particles from the formulation may have aggregated or settled leaving the smaller particles to remain in suspension during analysis.

CMCAB could be an ideal excipient for oral dosage forms in drug delivery of rifampicin. Not only would it provide protection of rifampicin under acidic conditions thus increasing the concentration of unchanged rifampicin that reaches systemic circulation, the use of CMCAB would not add any additional adverse effects as its cellulose ester themselves exude low toxicity in human use (4). Future studies would benefit from the evaluating alternatives to remove free
drug and polymer while enhancing drug encapsulation. The reduction of MVIM particle aggregation and size control of the spray dried particles may prove beneficial to future studies.
References


Chapter 3

Pharmacokinetics of Rifampicin Laden Carboxymethylcellulose Acetate Butyrate Particles

Abstract

Decreased bioavailability of rifampicin has been explained by changes in the crystalline structure of rifampicin, excipients, degradation in the gastro-intestinal tract, absorption and metabolism. Previous studies evaluating rifampicin bioavailability has shown that patient non-compliance and sub-minimal inhibitory concentrations of rifampicin during extended tuberculosis therapy may predispose for the development of resistant strains and ineffective elimination of MTB. Nano and micron size drug encapsulated particles often exhibit different pharmacokinetic parameters than that of the parent free drug. This research thesis examines the pharmacokinetic parameters of rifampicin encapsulated carboxyl methylcellulose acetate butyrate (CMCAB) formulated particles by two different techniques (multi-inlet vortex mixer and manual spray drying) compared to that of free drug rifampicin when given orally in a mouse model. The results indicate that the rifampicin containing CMCAB spray dried particle formulation was able to increase pharmacokinetic parameters of rifampicin compared to that of the free drug form at the same concentration in a mouse model. Therefore, these particles may have the potential to the reduce the rifampicin dose, dosing intervals, treatment time of MTB and rifampicin associated adverse affects, thus increasing patient compliance while maintaining minimal inhibitory concentrations necessary to kill the bacterium.
Introduction

With the advent of nanotechnology, nanomedicine has focused heavily on improving drug delivery, through the development of nano and micron size drug formulations. These particles can be employed to alter factors such as the drug dissolution rate, tissue selectivity, and renal clearance (6). However, the pharmacokinetic (PK) parameters of the parent drug and the drug encapsulated in the formulated particles are often different. Evaluating the PK and pharmacodynamic (PD) properties of these complex particles are essential in understanding and predicting their efficacy as delivery systems. The PK properties of particles are commonly determined by their chemical and physical properties such as size, charge, and surface chemistry (5, 6). PK and PD studies of particles maintain the general principles of analysis associated with that of the parent drugs, measuring drug concentrations in fluids and tissues after drug administration over a period of time until the elimination phase (6).

In the treatment of tuberculosis, overcoming decreased bioavailability of anti-tuberculosis drugs have been a challenge in the treatment process. The reduction in the amount of drug that reaches systemic circulation is responsible for the requirement of daily drug administration. Due to the high incidences of side effects and the need for daily dosing, the patient compliance of anti-tuberculosis drugs is low, resulting in discontinuation of medication, leading to increased incidence of multi-drug resistant strains (3). The development and use of nanoparticles as drug delivery systems has recently been used in an attempt to reduce the dose and frequency of anti-tuberculosis drugs while increasing bioavailability. Past attempts to improve the pharmacokinetic properties of anti-tubercular drugs by applying modified drug delivery systems, such as nanoparticle development have been associated with drawbacks such as the use of toxic polymers and low drug encapsulation (2).
The present studies focus on evaluating pharmacokinetic properties of MIVM and spray dried formulated rifampicin encapsulated CMCAB nanoparticle formulations following a single oral dose in a mouse model.
Materials and Methods

Animals

Approximately 5 week-old BABL/c female mice (27) weighing 25gm were received from Harlan Laboratories (Dublin, VA). All mice were acclimated for two weeks in the animal housing facility of the Infectious Disease Unit in the College of Veterinary Medicine, as per protocol (IACUC #10-048-CVM) approved by Virginia Tech Institutional Animal Care and Use Committee (IACUC).

Experimental Design

Almost 24 hours before experimental study mice were housed (5 mice per cage) in metabolic cages (Techniplast, Philadelphia, PA). Mice were given commercial pellet diet and water ad libitum. Ten hours before experimental study began pellet food was removed from all mice and animals were randomly divided into three groups, receiving treatment as follows: Rifampicin, MIVM formulated rifampicin/CMCAB particles, spray dried formulated rifampicin/CMCAB particles and untreated controls at a single dose of 250mg/kg body weight of rifampicin per os. Mice were fasted to simulate recommended therapeutic dosing conditions of oral rifampicin in humans, in which it has been demonstrated that food has be shown to significantly reduce biodistribution of rifampicin (7),(1). The concentration of rifampicin at 250mg/kg was chosen as safe single dosage concentration that would allow for the detection of rifampicin using HPLC-UV analysis. Material safety data information from Sciencelab Inc. reports an oral LD 50 for rifampicin in mice as 500mg/kg.
Dosing Schedule and Sample Collection

Rifampicin and rifampicin/CMCAB particle formulations suspended in PBS were administered by oral gavage a dose equivalent to 250mg/kg rifampicin. Blood samples were collected retro orbitally into heprinized tubs at 0, 2, 4, 8, 10 hr, and day 1, 2, 3, 4, and 5. Urine and fecal samples were also collected at the previously stated time points and stored -80 °C until analysis. The blood samples were centrifuged at 6708 ×g at 4°C for 10min and plasma was stored at -80 °C until analysis. Mice were euthanized and lung, liver, spleen, kidneys and stomach were obtained at 2hr, 4hr and day 1, 2, 3, 4, and 5. Lung, liver and spleen were homogenized in PBS and stored in -80 °C until analysis. Kidneys and stomach were stored in 10% formalin phosphate buffer (Fishers Scientific) until analysis. Fecal samples were pretreated to form a slurry with the addition of five volumes of water (w/w), the samples were the centrifuged at 6708 ×g at 4°C for 10min. The collected supernatant was collected and stored -80 °C until analysis.

HPLC Analysis for Biological Samples

The HPLC analysis of rifampicin was performed by a reverse-phase high performance liquid chromatography (HPLC) method using an Agilent 1200 Series HPLC system equipped with an auto sampler and diode array detector. Separation was achieved with C18 column (Zorbax) and data acquisition ChemStation software (Agilent Technologies Inc., Palo Alto,CA). The mobile phase was run with 50% acetonitrile and 50% 1 M ammonium acetate. Analysis was
performed on a 5μl injection at a flow rate of 1.0 ml/min through an Agilent Zorbax Eclipse XDB-C18 (4.6×150 mm) column and absorbance recorded at 334 nm.

**Preparation and Analysis of Collected Samples**

For quantitative studies, the sample method involved protein precipitation, and solid phase extraction (SPE) approaches. 200μl of acetonitrile was add to 100μl of the samples e.g., plasma, urine, organ homogenate and feces slurry in a 1.5 ml tube. All samples were the vortexed and centrifuged at 6708 ×g at 4°C for 10min. The collected supernatant was subjected to SPE using HLB® cartridges (Waters, USA). The SPE procedure involved cartridge conditioning with 1ml of methanol followed by 1ml of water, loading approximately 300μl of sample, washing with 1ml of 5% methanol in water and eluting with 1ml of methanol. The recovered samples were dried under a nitrogen stream at room temperature. Samples were reconstituted in 150μl of PBS and duplicate samples were analyzed for rifampicin content under reported HPLC conditions.

**Preparation of working solutions for calibration curve standards in biological matrices**

Blank plasma, urine, lung, liver, spleen, and fecal slurry were spiked with different concentrations of working standard solutions to give respective sets of calibration curve standards. The calibration curves for biological matrices were constructed with standards of rifampicin at 5, 10, 20, 40, 60, 80 and 100μg/ml.
Detection and quantification limits

Limits of detection (LOD) and limit of quantification (LOQ) were determined from the signal to noise ratios. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The limit of quantification was defined as the lowest concentration that produced a peak area with a signal to noise ratio higher than ten times the baseline.

Calculation of Pharmacokinetic Parameters

All the pharmacokinetic parameters were determined by non-compartmental analysis using PK functions for Microsoft Excel. AUC\(_{0-\infty}\) was calculated by linear trapezoidal method. C\(_{\text{max}}\) (the highest drug level measured) and T\(_{\text{max}}\) (the time to reach the highest concentration) were directly analyzed from the concentration time plots. Elimination rate constant (Kel) was estimated from the linear regression line of the elimination phase. Elimination half-life (t1/2) was calculated as a regression of the semi-logarithmic concentration versus time data, and relative bioavailability was determined for each formulation using:

\[
\text{Relative bioavailability} = \frac{\text{AUC}_{0-\infty} \text{ or oral encapsulated drugs}}{\text{AUC}_{0-\infty} \text{ or oral free drugs}} \times \frac{\text{dose of oral free drugs}}{\text{dose of oral encapsulated drug}}
\]
Histopathology of Kidney Tissues

Formalin-fixed paraffin-embedded tissue blocks were prepared by standard protocols (fixed in buffered formalin, processed with xylene and alcohol and embedded in paraffin). The tissue blocks contained kidneys and stomach. A 5 μm section was sliced by microtome from each tissue block, fixed on a glass slide and stained with hematoxylin and eosin for microscopic assessment of necrosis, degenerations, hemorrhage, edema, inflammation or other signs of vascular leakage. Tissues were given a score from 0 to 4 based on the presence of any of these degenerative processes.
Results

Rifampicin Concentrations in Plasma

Free rifampicin and MIVM formulated particles demonstrated a maximum concentration of rifampicin at the first blood collection time point of 2hr after administration (Figure 8) corresponding to a $C_{\text{max}}$ of 2hr (Table 3). Free rifampicin revealed an undetectable drug concentration by 10 and 24 hrs at which time the plasma concentration dropped below MIC. The MIVM formulated rifampicin particles did not reach undetectable limits until 24hr after the initial dose was administered (Figure 8). The calculated PK parameters for the MIVM particles failed to exceed that of the free drug except the elimination rate constant (Figure 8). However, the MIVM particles demonstrated the most linear maintenance of rifampicin concentration above MIC for at least 10hr (Figure 8). The spray dried formulation containing rifampicin demonstrated high onset concentration of rifampicin at the 2hr after dose was administered, resulting in the maintenance of higher levels of rifampicin in plasma even at 24hr after the initial oral dose (Figure 8). This high onset and maintained concentration seen in the plasma from the spray dried particles coincided with increased calculated pharmacokinetic parameters; particularly important was the increase of relative bioavailability when compared to the free drug (Table 3). Surprisingly, the spray dried formulated particles with the highest onset and maintenance concentrations and exhibited the lowest half-life ($t_{\frac{1}{2}}$). All of the rifampicin plasma concentrations for each formulation were undetectable at 48 hr after dose was given.
Rifampicin Concentration in Tissues

Rifampicin concentrations were below quantifiable limit (5μg/ml) at all time points in collected lung, liver and spleen samples using HPLC-UV analysis. Reported rifampicin concentrations in these collected tissues 24hr following a single dose of orally administered rifampicin are 10 fold lower than the limit of quantification established in the reported HPLC-UV methods of this study (2).

Rifampicin plasma drug profile following oral administration of free drug and rifampicin encapsulated formulations

Figure 8: Plasma drug profile following a single 250 mg/kg oral administration of rifampicin and rifampicin containing CMCAB particle formulations. Each formulation treatment group is noted with a different color line and represents n=2. LOQ noted in the figure legend and on the
chart represents the limit of quantification established by the HPLC conditions reported in materials and methods. The MIC noted in the figure legend and on the chart represents the reported minimal inhibitory concentration for *M.tuberculosis* H37Rv (ATCC 27294) as demonstrated in Barrow et.al 1998.

**Pharmacokinetics of rifampicin encapsulated CMACB particles compared to free drug rifampicin**

<table>
<thead>
<tr>
<th>PK Parameter</th>
<th>Free Drug n=2</th>
<th>MIVM n=2</th>
<th>Spray Dried n=2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (µg/ml)</td>
<td>Mouse 1</td>
<td>Mouse 2</td>
<td>Mouse 1</td>
</tr>
<tr>
<td></td>
<td>42.63</td>
<td>95.96</td>
<td>15.82</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>$t\frac{1}{2}$ (h)</td>
<td>2.51</td>
<td>2.06</td>
<td>2.63</td>
</tr>
<tr>
<td>$K_{\text{el}}$</td>
<td>0.27</td>
<td>0.33</td>
<td>0.26</td>
</tr>
<tr>
<td>$\text{AUC}_{0-\infty}$ (µg/ml/h)</td>
<td>193.43</td>
<td>294.76</td>
<td>134.25</td>
</tr>
<tr>
<td>Relative Bioavailability</td>
<td>1</td>
<td>1</td>
<td>0.997</td>
</tr>
<tr>
<td>$\text{AUC}_{0-t}/\text{MIC}$</td>
<td>3448.09</td>
<td>3185.73</td>
<td>489.4</td>
</tr>
</tbody>
</table>

**Table 3:** Pharmacokinetics of rifampicin laden CMACB particles compared to free drug rifampicin. Values are the mean± SD, n=2. $C_{\text{max}}$ = peak plasma concentration; $T_{\text{max}}$= time taken to reach $C_{\text{max}}$; $K_{\text{el}}$= elimination rate constant; $t\frac{1}{2}$= half life; $\text{AUC}_{0-\infty}$= area under plasma drug concentration over time curve.
Rifampicin Concentrations in Urine and Feces

The concentration verses time profile for rifampicin in collected in urine samples is a representation of the elimination profile for all three rifampicin containing formulations. As shown in the plasma concentration data the spray dried formulated CMCAB containing rifampicin particle demonstrated the highest concentrations excreted in urine, followed by the free drug rifampicin and MIVM particles (Figure 9). Unlike plasma samples, rifampicin was detected in urine at 10hr after oral dosing for all formulation groups and 24hr for MIVM and spray dried formulated groups (Figure 9). Rifampicin concentrations of collected urine samples from all rifampicin formulations were undetectable after 24hr. It is also important to note that the concentration of rifampicin could not be detected at various time points due to unavailability of urine samples. Adverse effects were observed in the spray dried particle treatment group approximately 2.5 hrs after administration. Clinical signs included lack of activity, ruffed fur, decreased urination frequency, and lack of water and food consumption. Clinical signs were present for approximately 12 hours after oral administration of the spray dried particles. At 24 hrs after dose mice from the spray dried particle treatment group appeared to be recovered and no clinical signs were observed. Rifampicin concentrations of collected fecal samples from all rifampicin formulations were undetected using HPLC-UV analysis.
Rifampicin Urine Drug Profile Following Oral Administration of Free Drug and Rifampicin Laden Formulations

**Figure 9**: Urine drug profile following a single 250 mg/kg oral administration of rifampicin and rifampicin laden CMCAB particle formulations. Each formulation is noted with a different color line and symbol, points at 2 and 4 hr represent the unavailability of urine.

Potential Rifampicin Degradation Products

Further analysis of HPLC-UV plasma chromatographs showed peaks believed to be rifampicin degradation products as they were not present in blank plasma samples (containing no rifampicin) or rifampicin containing plasma samples used to establish the plasma standard curve (Figure 10). The degradation product peaks appear downstream of the determined rifampicin peak and are more prevalent in the free drug treatment group. In the 4hr plasma samples for each treatment, HPLC-UV chromatographs revealed a degradation peak from the free drug plasma sample that is two times the area percent greater that the spray dried formulation degradation peak (Figure 10). At the same time point the MIVM plasma chromatograph showed a
degradation peak too low to be quantified by the HPLC system, resulting in a zero area percentage.

**HPLC-UV Chromatographs with Potential Rifampicin Degradation Products**

a)

![Free Drug Plasma 4hr](image)

RIF – Potential Degradation Product
Area%: 28.2

b)

![MIVM Plasma 4hr](image)

RIF - Degradation Product
Area%: 0
Figure 10: HPLC-UV chromatograph of plasma samples containing rifampicin and potential rifampicin degradation products. a) Free drug, b) MIVM, c) Spray dried

**Histopathology of Kidney Tissues**

Double blind histopathological analysis was conducted on collected kidneys to microscopically evaluate tissues for the presence of lesions or tissue damage. The collected tissues were evaluated across treatment groups at 4hr, day 2 and day 5 after oral administration of rifampicin (Table 4). Evaluation of kidneys collected from each treatment group at 4 hours after oral dosage demonstrated normal histopathology of the glomeruli. Kidneys from free drug and MIVM treatment group showed minimal signs of damage to the glomeruli receiving a score of 1 (Table 4). The spray dried group at the same time point received scores of 2 to 3 indicating mild to moderate signs of damage to the glomeruli. At day 5 the kidney scores form free drug and MIVM group demonstrated normal histopathology (Table 4). However, the spray dried group at day 5 still showed minimal signs of damage to the glomeruli of the kidneys. The changes seen in evaluation of the glomeruli when compared to the controls include irregularly
thickened basement membranes, hyper cellularity, wire loop capillaries and thickened hypertrophic basement membranes (Figure 12).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Kidney Score 4 hr</th>
<th>Kidney Score Day 2</th>
<th>Kidney Score Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Drug</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>MIVM</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Spray Dried</td>
<td>0</td>
<td>2-3</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 4:** Histopathology scores of kidneys collected at 4hr, day 2 and day 5 after single oral dose administration. Score 0-4: (presence of inflammation, edema, hemorrhage, degeneration, necrosis) 0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

**Histopathology Kidney Images**

**Histopathology Images of Normal Glomeruli**

![Histopathology Images](image)

**Figure 11:** Histopathology images of normal glomeruli imaged at 400x magnification
Histopathology images of abnormal glomeruli

**Figure 12:** Histopathology images of abnormal glomeruli imaged at 400x magnification demonstrating the observed glomerular changes a) wire loop capillaries, and thickened hypertrophic basement membrane b) hyper-cellularity
Discussion

The enhancement of pharmacokinetic parameters such as bioavailability of rifampicin using oral drug delivery system is essential in combating the limitations of the current tuberculosis treatment. Rifampicin has been proven to be an important component in the success of tuberculosis treatment; however its effectiveness comes with the disadvantages of adverse effects and the need for daily dosing. The adverse effects are generally associated with the daily prolonged dosage necessary to maintain above MIC levels of unchanged rifampicin in systemic circulation. Rifampicin encapsulated CMCAB particles represent a drug delivery system that challenges the current treatment particularly by addressing bioavailability.

The differences in pharmacokinetic parameters seen between MIVM and spray dried formulations are likely due to the difference in formulation technique used. Previous studies suggest that drug solubility, drug loading and polymer molecular weight have a great effect on release kinetics depending on the mechanism of release (e.g. erosion, matrix swelling and diffusion). As the same polymer was used for both particle formulations it is unlikely the difference in vivo effect is due to polymer molecular weight. However, the effect of drug loading, solubility and the mechanism of release in vivo are unclear for both formulations.

The improvement seen in the pharmacokinetic parameters from the plasma profile of the spray dried formulated particles compared to free drug is directly attributable to the increased AUC$_{0-\infty}$ for both formulations. Although the increased trend continues for most of the PK parameters determined for spray dried particle formulations, the biologic half life ($t_1/2$) for the spray dried formulation was the lowest of all the formulations despite the high concentration over time. This observation is likely due to the diffusion of the high concentration of rifampicin
moving to region of lower concentration at a faster rate. The rifampicin degradation products was present in all chromatographs in which rifampicin was able to be detected. The degradation product peaks constitutes a smaller peak area percentage for the CMCAB formulations than the free drug. The peaks also do not seem to be concentration dependent, as in the Figure 8 shown the concentration of rifampicin in the spray dried particles is approximately 15 times greater than free drug, yet the degradation peak is significantly less than that present in free drug.

Though urine samples are not evaluated for pharmacokinetic analysis they provide information that can be used to speculate elimination properties of the single oral administered dose. The urine drug profile mimics the plasma drug profile in the differences between drug formulations and their corresponding concentrations. The urine profile demonstrates the elimination of rifampicin of at higher concentrations and for extended times compared to that concentration rifampicin in the plasma profile. This observation of high concentrations of rifampicin has been documented in studies evaluating orally administered rifampicin due to the route of administration (8, 9).

In the kidney, a tubular like structure called the nephron filters blood to form urine. The glomerulus is located at the top of the nephron and is a network of capillaries that perform the first steps of filtering blood. Renal failure is a rare adverse effect associated with the use of rifampicin in the treatment of tuberculosis (4). In humans the mechanism of rifampicin associated renal failure is unknown and varies from acute to chronic failure from case to case (4). Glomerular lesions present in the kidneys of treated mice appear to be reversible and concentration dependent among treatment groups based on histopathology at selected time points. The most observable glomerular changes from the selected time points are seen at 2 days after dosage, and by day 5 the glomeruli appeared normal for the free drug and MIVM groups
(Table 4). The severity of the glomerular changes correlates with the rifampicin plasma concentrations seen for each treatment group. The free drug and MIVM groups that exhibited similar plasma drug profiles and lower rifampicin concentrations also demonstrated the minimal kidney lesions. The spray dried group which demonstrated the highest concentration in plasma resulted in mild to moderate kidney lesions.

In summary an effective drug delivery system for enhancing the relative bioavailability of rifampicin was evaluated. The two formulations for rifampicin CMCAB particles demonstrated variable pharmacokinetics between the two formulation processes used. The MIVM particles did not increase any of the determined PK parameters including bioavailability when compared to the free drug. However, the spray dried rifampicin containing particles showed increased PK parameters including bioavailability compared to the free drug. Future studies related to evaluating PK parameters of rifampicin containing CMCAB particles would benefit from the use of more sensitive methods of analysis as well as conducting studies to evaluate PK parameters of particles given at therapeutic dose.
References


Conclusions

The effects of rifampicin encapsulated CMCAB in formulated nano and micron sized particles *in vivo* have not previously been evaluated. The knowledge available regarding the use of CMCAB consists of *in vitro* characterization analysis for possible use as an oral dosage excipient. The resulting properties have documented the low toxicity of the cellulose ester polymer as well as the controlled and pH dependent release of the contained target drug. The effectiveness and drawbacks of rifampicin in the treatment of MTB has become common knowledge. The goals of tuberculosis treatment have focused on eliminating the infection without relapse, preventing death, and preventing the emergence of drug resistant bacteria. It is categorized as one of the first line anti-tuberculosis drug; however various side effects such as hepatotoxicity, allergic rashes, and lack of appetite, nausea, or immunological disturbances have been reported associated with administration of the drug.

Orally administered rifampicin is hydrolyzed by the acidic medium found in the stomach to form 3- formyl rifamycin which is an insoluble and poorly absorbed compound. Unaltered rifampicin that is able to make it through the gastrointestinal tract is metabolized by the liver and eliminated into the bile and then reabsorbed and enterohepatic circulation proceeds. It is during this time that rifampicin is deacylated into 25- desacetyl rifampicin. Despite these facts, there have not been many efforts that address the degradation of rifampicin while increasing its availability in systemic circulation.

Rifampicin encapsulated CMCAB particles constitute a drug delivery system with the benefits of addressing the two major drawbacks of MTB treatment, toxicity and the need for
daily administration. The enhancement of the pharmacokinetics of rifampicin using drug delivery systems is an important means to overcome the limitations of current tuberculosis treatment.

In the present study, an effective strategy for enhancing the bioavailability of rifampicin oral dosage forms was developed. Rifampicin was successfully incorporated into CMCAB particles by two different particle formulation techniques; manual spray drying and the use of a MIVM. Determination of nanoparticle properties showed that both structures were generally consistent with theoretical assumptions surrounding the formulations techniques. MVIM particles were small particles that aggregated to form clusters of particles approximately 140 nm in size, while the spray dried particles appeared large and poly-dispersed. The demonstrated particle size, morphology and efficiency of drug encapsulation depend on the type of particle formation method, and the resulting free drug/free polymer removal process.

It is common practice in nanoparticle formulation to remove free drug and polymer that remains after the particles are formed. This process however is beneficial in the aspect of quantifying the amount of drug on the encapsulated particles but likely destructive if carried out under conditions that optimal for dissolution of the target drug from the polymer. MIVM formulated rifampicin encapsulated particles were dialyzed for 24 hr to remove remnants of free rifampicin and CMCAB, however this process likely constitutes for the reduction of rifampicin encapsulated since optimal dissolution from CMCAB occurs at neutral pH. Future studies evaluating particle formation, and encapsulation efficiency should consider decreasing the time of dialysis and evaluate alternative methods of removing remnants of free drug and polymer.

Rifampicin particles formulated though MIVM and spray drying methods are not apparent in published literate making comparative analysis to the results presented difficult.
However, Pothayee, N et. al (6) have demonstrated particle sizes of 140-340 nm resulting from the MIVM particle formation process. Though the antimicrobial drug and polymer reported in Pothayee et.al differ from those used in this thesis, the reported drug encapsulation is approximately 80-90%. Friesen et. al (3) have demonstrated bowl shaped polydisperse particles as large as 20μm formed by spray drying techniques.

Dissolution of rifampicin at neutral pH from both particle formulations are similar to reported profiles of CMCAB during characterization analysis. Posey-Dowty et.al (5) have shown a complete and near zero-order release of drugs coated with CMCAB forming compression tablets over 24hr at pH: 6.8 while release was minimal at pH: 1.2. While the release profiles of rifampicin from encapsulated particles presented in this thesis do not mimic zero-order sustained release for 24hrs, the neutral pH at which rifampicin is released coincides with the excipient properties of CMCAB.

Both particle formulations demonstrated in vitro efficiency comparable to free drug rifampicin at the same concentration. The reported results for MIC and intracellular elimination of the particle formulations demonstrate the contained rifampicin remains biologically active after the particle formulation process. More importantly, the pharmacokinetic results showed that spray dried particles improved oral bioavailability of rifampicin in mice. Ahmad et. al (1) demonstrated increase bioavailability of rifampicin using free rifampicin and alginate nanoparticles at 109.2 mg/kg in a single oral dose. The free drug form in this study produced an AUC$_{0-\infty}$ of approximately 6.21 µg ml/h and an AUC$_{0-\infty}$/MIC of 62.1. It is difficult to compare the results presented in this thesis to published results because of the increased dosage used in oral administration and difference in particle properties.
The potential of CMCAB particles to serve as carriers for rifampicin is further supported by the pharmacokinetic parameter AUC\(_{0-\infty}/\text{MIC}\). It has been reported that to achieve maximum efficacy of rifampicin, AUC\(_{0-\infty}/\text{MIC}\) should be \(\geq 75-100\) (4). The decreased \(t^{1/2}\) (biological half-life) seen in the spray dried particles when compared to the MIVM particles and free drug rifampicin is likely attributed to the increase rate of diffusion due to the high concentration of rifampicin released from the spray dried particles.

The glomerulur lesions present in the kidneys of treated mice appear to be reversible and concentration dependent among treatment groups based on histopathology at selected time points. The lesion scores among treatment groups correlates with the rifampicin drug concentration profile of the same groups. Though the highest lesion scores were seen in the spray dried treatment group which also demonstrated the highest rifampicin concentration profile, the onset of glomerulur lesions does not appear to correlate with the concentration profile or observed clinical signs. The plasma drug profile showed the highest rifampicin concentrations between 2 and 4 hours for each treatment group. The onset of clinical signs of urinary retention and lack of physical activity for the spray dried mice were also documented between 2 and 4 hours. However, the analyzed kidney tissues at 4 hours for the spray dried group appeared normal with no visual signs of damage. Renal failure or renal toxicity in humans is an uncommon adverse effect that usually occurs in patients receiving intermittent or interrupted rifampicin related therapy, and very few cases of renal failure have resulted from daily continuous dosages (2, 7). The effect of a single high dose of rifampicin on renal toxicity, gulemerulur lesion formation, and the ability of the golmeruli to reverse any damage caused are unclear. It is presumed that the high rifampicin plasma concentration later concentrated by the kidneys and urinary retention is responsible for the lesions present in the kidneys.
In conclusion, studies presented in this thesis indicated for the first time the formation, characterization, and pharmacokinetic evaluation of rifampicin laden CMCAB particles.
Future Work

Future research should address issues of particle aggregation seen in the MIVM particles, and particle size variation seen in the spray dried particles. Optimizing formulation parameters such as removal of free drug and spray dryer nozzle conditions could result in more consistent particle formation. Methods of confocal microscopy may prove useful in determining how rifampicin and CMCAB are arranged in each particle formation. Fluorescently labeling CMCAB or rifampicin would allow for contrast visualization of the drug/polymer arrangement on the surface of each formulation. Additional particle properties such as structural order in a solid must be evaluated to determine if the formulated particles are crystalline or amorphous using x-ray diffraction and differential scanning calorimetry. These property characteristics could prove useful in explaining in vitro and in vivo results.

Efforts should also evaluate the pharmacokinetic and pharmacodynamic parameters of free drug and particle formulations at therapeutic dosage, and efficacy in an infected animal model. These animal studies should include addition of empty particle formulations for biocompatibility analysis. The analysis of the biological samples would greatly benefit from the use of HPLC with mass spectroscopy (MS) analysis to quantify low amounts of rifampicin, the presence of CMCAB and to track degradation products as well. Similar HPLC analysis would be useful in evaluating mechanisms of drug release during dissolution studies. Developing a HPLC-MS method to analyze CMCAB would allow for further determining mechanisms of release by evaluating the mass of CMCAB components.

In determining the rifampicin content in plasma using HPLC, the presence of what is likely a rifampicin degradation product appears in time points of most samples. The presented
peak was not seen in standards, increase in size with sample time points, and appear less prevalent in some biological samples from CMCAB particle formulations. The degradation products of rifampicin have been evaluated and characterized from their parent compound by simulating optimal degradation conditions, however the commercial availability of these degradation productions are very limited. The determination of these degradation products along with methods of quantification such and mass spectroscopy would be of great benefit in determining the reduction of degradation in the particle formulations compared to free drug.
References


