PULMONARY DELIVERY OF ISOXYL PARTICLES TO TREAT *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

Chenchen Wang: Pulmonary Delivery of Isoxyl Particles to Treat *Mycobacterium tuberculosis*
(Under of the direction of Anthony J Hickey, Ph.D.)

Isoxyl is an effective drug to treat multi-drug resistant tuberculosis but its use was impaired by failure in clinical trials. Due to absence of reliable quantitive analytical methods at that time, the reasons for these poor *in-vivo* outcomes were not determined. In the present work, an HPLC method was developed and validated for determination of isoxyl concentrations in plasma. Pilot studies showed that therapeutic plasma concentrations were not achieved after oral administration of isoxyl to guinea pigs. Pulmonary delivery may rescue the drug by targeting isoxyl particles to macrophages, the host cells of mycobacteria. Isoxyl particles with various size and shape were prepared by antisolvent precipitation. Subsequent spray drying produced microparticles < 5 μm suitable for lung delivery. Isoxyl ≥ 5 μg/ml, independent of particle form, seemed effective in killing intracellular mycobacteria without cytotoxicity. This work is the basis for reviving drugs with undesired physico-chemical properties to treat lung infection.

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LIST OF ABBREVIATIONS AND SYMBOLS

A_s	Surface Area
7AGT	Media Prepared by 0.47% Difco™ Middlebrook 7H9, 0.5% Glycerol, 0.05% Tween 80 and 1X ADS
ACN	Acetonitrile
ADS	Medium consists of 5% BSA, 0.2% dextrose and 0.81% NaCl
ATCC	American Type Culture Collection
AUC	Area Under Plasma Concentration vs. Time Curve
BAL	Bronchoalveolar Lavage
BCG	Bacillus Calmette-Guerin
BD	Becton Dickinson Technologies
BSA	Bovine Serum Albumin
χ	Shape Factor
C	Concentration
C_0	Initial Concentration
C_c	Slip Correction Factor
CFU	Colony Forming Unit
CL	Clearance
Cmax	Maximum Plasma Concentration
CMC	Carboxymethyl Cellulose
CMD	Count Median Diameter
CMI	Cell-mediated Immunity
CML	Count Median Length
CMW	Count Median Width

C_o	Solubility of a Very Large Crystalline Particle
C_s	Solubility of a Small Particle
D	Diffusion Coefficient
d_a	Aerodynamic Diameter
dC/dt	Dissolution Rate
$d_{droplet}$	Droplet Diameter
d_e	Equivalent Volume Diameter (Diameter of a Sphere Having the Same Volume as That of the Irregular Particle)
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DMSO	Dimethyl Sulfoxide
$d_{particle}$	Particle Diameter
DPI	Dry Powder Inhalers
DPPC	Dipalmitoylphosphatidylcholine
DSC	Differential Scanning Calorimetry
DTH	Delayed-type Hypersensitivity
ED	Emitted Dose
ETH	Ethionamide
EthA	Enzyme Required to Activate Ethionamide
F	Bioavailability
FAD	Flavin Adenine Dinucleotide
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
FPD	Fine Particle Dose

FPF	Fine Particle Fraction
γ	Surface Tension of the Particles
GSD	Geometric Standard Deviation
η	Viscosity of Atmosphere
h	Thickness of the Diffusion Layer
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HPMC	Hydroxylpropylmethylcellulose
INH	Isoniazid
IS	Internal Standard
ISO	Isoxyl
k	Boltzmann Constant
LD	Laser Diffraction
LDH	Lactose Dehydrogenase
LOD	Limit of Detection
LOQ	Limit of Quantification
MDM	Human Monocyte-derived Macrophage
MDR	Multi-drug Resistant Strains
MIC	Minimum Inhibitory Concentration
MOI	Multiplicity of Infection
MTB	*Mycobacterium tuberculosis*
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl Tetrazolium Bromide
N.D.	Not Determined

OD_{600nm}	Optical Density at 600 nm
PAS	*p*-aminosalicylic Acid
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PK	Pharmacokinetic
PMA	Phorbol Myristrate Acetate
PSD	Particle Size Distribution
PVA	Polyvinyl Alcohol
PZA	Pyrazinamide
QC	Quality Control
r	Particle Radius
R	Gas Constant
R^2	Regression Coefficient
RD	Recovered Dose
S	Degree of Supersaturation
SEM	Scanning Electron Microscopy
SM	Streptomycin
T	Absolute Temperature
TAC	thioacetazone
TB	Tuberculosis
THF	Tetrahydrofuran
THP-1	Human Acute Monocytic Leukemia Cell Line
Tmax	Time to Reach Cmax
TSC	Thiosemicarbazon

TSLI	Twin-stage Liquid Impinger
UV	Ultraviolet
V	Volume
VM	Viomycin
V_m	Molar Volume
VMD	Volume Median Diameter
VRSA	Volume Ratios of Solvent to Antisolvent
V_β	Volume of Distribution of the Terminal Phase
WHO	World Health Organization
XDR	Extremely-drug Resistance Strains
ΔH	Change in Enthalpy
λ	Elimination Rate Constant
ρ_0	Unit Particle Density (1000 $kg \times m^{-3}$)
ρ_p	Particle Density

Chapter 1

Background and Specific Aims

1.1 INTRODUCTION

1.1.1 Tuberculosis

Tuberculosis (TB) is the leading cause of death by a single infectious micro-organism. One third of the world's population has been infected by TB, with 9.27 million new cases and 1.8 million deaths in 2007 according to World Health Organization (WHO) report.[1] The availability of the vaccine Bacillus Calmette-Guerin (BCG) and curative chemotherapies were responsible for the general decrease in tuberculosis mortality in the past century. Nevertheless, the frequency of occurrence of new cases began to increase in the mid-1980s. Two main factors contributed to the resurgence of TB, the occurrence of immunosuppressive diseases, such as HIV infections, and the emergence of multi-drug resistant (MDR) strains, which are resistant to isoniazid and rifampicin, the two first line anti-tuberculosis drugs.[2-5]

Mycobacterium tuberculosis is mainly transmitted by airborne droplet aerosols containing 1 to 3 tubercle bacilli. After the bacilli are inhaled and deposited into the alveoli, they are phagocytized to endosomes of alveolar macrophages. In healthy immunocompetent humans, this induces cell-mediated immunity (CMI) and formation of granulomas. Early stage granulomas consist of $CD4^+$, $CD8^+$ and other T cells. Late stage granulomas form a fibrotic wall and lymphoid follicular structures to contain the spread of bacilli by depriving them of oxygen and nutrients. Successful CMI forms granulomas containing non-viable bacilli which can persist for decades. [5] However, in the weakened immune system, these bacilli are released from the macrophages. These bacilli are ingested by surrounding poorly activated immunologically compromised monocytes or macrophages and then multiply intracellularly. The development of tissue-damaging delayed-type hypersensitivity (DTH) can kill these bacilli-laden cells, but also expand the caseous necrotic center and cause

damage to lung tissue. This leads to clinical manifestations of the disease. With the progress of the disease, the caseum is liquefied and, thus, provides an excellent intercellular growth environment for the bacilli. The large numbers of intercellular bacilli result in cavity formation as well as resistance even in response to a strong CMI. [5-7] Though the clinical manifestations can occur in almost any organ as bacilli migration via lymphatics to the systemic circulation, about 85% TB infection occurs in lungs in humans without human immunodeficiency virus (HIV) infection. The HIV infection can increase the occurrence of extra-pulmonary infections to 68%.[8]

1.1.2 Anti-tuberculosis Drugs and Isoxyl

The successful modern chemotherapy of TB started in 1944 when streptomycin (SM) was administered to the first patient.[9] The subsequent use of the first-line antituberculosis drugs, such as isoniazid (INH), rifampicin, ethambutol, and pyrazinamide (PZA), lead to a steep decline in TB incidence. The chemotherapy of TB disease requires combination of 3 to 4 drugs over an extended period of 6 months to 2 years. The lengthy therapy results in patient noncompliance, and contributes to poor therapeutic outcomes and emergence of MDR strains.[10] This requires the use of second-line drugs such as capreomycin, ethionamide (ETH), cycloserine and kanamycin which are more toxic and cannot be administered frequently.[11] Therefore, the absence of novel effective TB therapy remains a significant public health threat worldwide due to rapid development of multi-drug resistant strains (MDR) and extremely-drug resistance strains (XDR). Development of potent chemotherapeutic alternatives is crucial to preventing future epidemics of this insidious form of the disease.

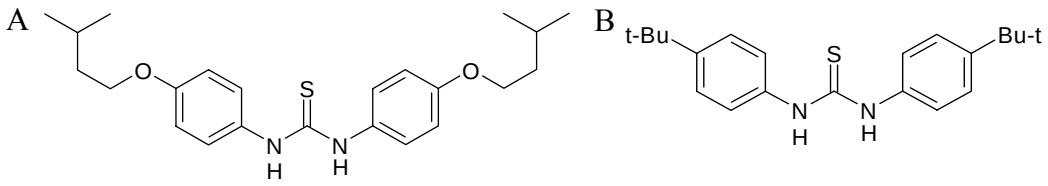

Figure 1. The chemical structures of isoxyl (A) and N,N'-bis[4-(1,1-dimethylethyl)phenyl]-thiourea (B).

Isoxyl (ISO, thiocarlide, 4,4'-diisoamythio-carbanilide) [Figure 1A] is an second-line drug synthesized in 1953 by Buu-Hoi and Xuong [12], and began to be used clinically to treat tuberculosis in the 1960s. Two mechanisms of action of isoxyl have been proposed: 1) It inhibits short-chain fatty acid biosynthesis distinct from the mechanisms of INH and ETH; and 2) Similar to INH and ETH, it inhibits the biosynthesis of mycolic acid which is hallmark of mycobacterial cell wall.[10, 13] Phetsuksiri et al.[14] reported that the antituberculosis effect of isoxyl is primarily due to inhibition of oleic acid biosynthesis other than mycolic acid biosynthesis. Isoxyl inhibits the activities of *des*A3, a fatty acid desaturase in *M. tuberculosis* converting stearoyl-CoA to oleic acid, a monounsaturated fatty acid that is necessary for membrane fluidity to form a lipid bilayer at physiological temperature.[15] Some recent studies proposed that isoxyl is a prodrug that is converted to transient reactive intermediate(s) by the flavin adenine dinucleotide (FAD)-dependent monooxygenase EthA located in *M. tuberculosis*. EthA can activate isoxyl and other thiocarbamide-containing drugs such as ETH and thioacetazone (TAC) to bind to their distinct cellular targets. This explains some cross-resistance between isoxyl, ETH and TAC.[16, 17]

The unique mechanism of action makes isoxyl a potentially efficient antituberculosis drug for treatment of the MDR strains in monotherapy or in a combined regimen. *In-vitro* studies showed that isoxyl have strong antimycobacterial activity with MIC (minimum

inhibitory concentration) of 2.5 μg/ml for *Mycobacterium tuberculosis* H37Rv, MIC of 0.5 μg/ml for *Mycobacterium bovis* BCG, MIC of 2.0 μg/ml for both *Mycobacterium avium* and *Mycobacterium aurum* A+, and MIC of 1-10 μg/ml for various clinical isolates of strains resistant to rifampicin and INH.[13] Clinical studies conducted decades ago, without regulatory controls, revealed that isoxyl was more effective than PZA, thiosemicarbazon (TSC), viomycin (VM) and *p*-aminosalicylic acid (PAS), equivalent to ethionamide and kanamycin, and inferior to INH, SM and cycloserine. It has been reported that isoxyl is effective in treating tuberculosis strains resistant to INH, PAS, ETH, and SM, but not sensitive to the TSC-resistant strains. Isoxyl in combination with SM or ETH showed synergistic effects.[18, 19] In particular, isoxyl can maintain a long-term therapy level in combination therapy. Addition of isoxyl to capreomycin, rifampicin, and ethambutol dramatically increased the survival rate of rabbits infected with *M. bovis* [20][Figure 2]. It was reported that oral administration of isoxyl leaded to no toxicity to liver, kidney and hemopoietic systems but marginal gastro-intestinal symptoms in human. [18, 21-23] Therefore, isoxyl may be a good substitute for the other second-line drugs which are not well tolerated.

However, the effectiveness of isoxyl has been questioned due to failure in some clinical trials.[14, 24] The most plausible explanation for these failures is that isoxyl is almost completely insoluble in water (solubility calculated by advanced chemistry development (ACD/labs) software V8.14 for Solaris: 1.3×10^{-6} mol/L at 25°C) and, consequently, exhibits poor dissolution and bioavailability when it is delivered exclusively by the oral route. Efforts to improve the oral absorption of isoxyl, including use of micropowders or suspension in olive oil, resulted in no improvement of isoxyl absorption and the majority of the administered dose was still eliminated intact via the feces.[12, 25] Development of a more

convenient and effective delivery method is a prerequisite for successful employment of isoxyl for TB chemotherapy.

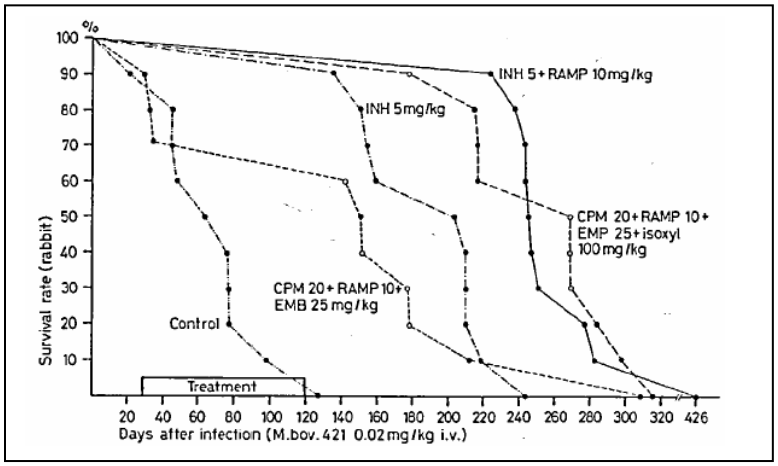

Figure 2. Single or combination therapies increased survival rate of rabbits infected with *Mycobateria bovis.*[20]

1.1.3 Pulmonary Delivery

Pulmonary tuberculosis is a major manifestation of TB implicated in morbidity and systemic dissemination. However, when antitubercular drugs are administered orally, drug concentrations in plasma and the lungs are well below the suggested therapeutic range in some patients.[26-28] Additionally, only a small fraction of an oral dose of drug delivered to the lungs can access granulomas, tubercles or lesions which have very poor blood supply.[29] This suboptimal drug concentration contributes to prolonged duration of treatment, relapse and development of MDR strains.

The previous poor therapeutic outcomes of isoxyl delivered orally may be caused by insufficient and highly variable drug concentrations in the lungs. In contrast, inhalation of isoxyl may achieve higher lung tissue to plasma concentration ratio. Maximizing local

concentration may increase drug efficacy and shorten duration of treatment against pulmonary tuberculosis. Ideally, inhalation therapy will result in higher concentrations of isoxyl in the lungs while maintaining therapeutically effective plasma concentration to treat the systemic infection. However, in some cases, the dose given by pulmonary route may not allow sufficiently high drug levels in systemic circulation. This can be overcome by supplementing with oral therapy using conventional drugs. Compared to parenteral delivery, oral and pulmonary administrations are non-invasive and can be achieved by self-administration. This is important for treatment of TB, because TB is a disease most commonly occurring in the developing world where needles, syringes, water for injection and cold storage conditions may be hard to obtain and the duration of anti-tuberculosis therapy is long.

Since most tubercle bacilli initially reside in the macrophages/monocytes in the lungs, the main targets of isoxyl should be these infected cells. Pulmonary delivery may facilitate efficient dosing of isoxyl to the targets by two main possible pathways: 1) isoxyl particles may dissolve in lung surface lining fluid and the resulting free drug molecules can either diffuse through the thin alveolar epithelium (0.1 to 0.5 μm) via interstitial fluid to the surrounding infected tissue, or pass all the way through the epithelium, interstitium, basement membrane, and vascular endothelium to enter the bloodstream, from which they are then redistributed to the infected tissue; and 2) Isoxyl particles can be taken up by macrophages/monocytes which may be recruited to granulomas. Targeting to alveolar macrophages is known to enhance efficacy due to specific drug action at the main site of infection[30] and through macrophage activation[31]. However, it is still not clear which of the two pathways is more efficient for action of anti-tuberculosis drugs.

The unique features of the lungs offer distinct advantages for pulmonary delivery of drugs, such as isoxyl, that are believed to have low bioavailability following oral administration: 1) Drugs can achieve higher lung tissue to plasma concentration ratio;[32] 2) hydrophobic drugs have higher solubility and more rapid dissolution rates in the surface lining fluid containing large quantities of lung surfactants;[33] 3) the lungs are thought to have fewer metabolizing enzymes and efflux transporters in contrast to the gastro-intestinal tract where orally administered drugs undergo first-pass effects of the gut and liver[34]; and 4) more importantly, drug particles delivered to the lungs have been shown to target to macrophages/monocytes, the host cells for MTB. Theoretically, the respiratory airways in the periphery of the lungs are probably the best place for deposition of isoxyl. These areas are patrolled by most abundant alveolar macrophages with an average of 50 to 100 per alveolus.[35] Mucociliary drug clearance from the deep lung is considerable slower than that from the conducting airways.[34] Deposition in the periphery of the lungs to target macrophage must be balanced against drug dissolution and absorption. The latter may be rapid since there is enormous internal surface area (about 100 m^2)[36, 37], thin alveolar epithelium and tremendous vascular system of the distal lung enable efficient transport of isoxyl to interstitium and bloodstream. An optimal balance between phagocytosis and dissolution would be beneficial for TB therapy. TB is a systemic infection, which might benefit from higher concentrations of isoxyl in the lungs while requiring maintenance of therapeutically effective plasma concentrations.

Deposition of particles in the lungs is mainly determined by five mechanisms, inertial impaction, sedimentation, diffusion, interception and electrostatic deposition. The first three are most important for most therapeutic particles because electrostatic force is only important

in highly charged particles and interception mostly influences long fibers. Inertial impaction is caused by inability of particles to adjust to new directions of airflow before encountering the airway surface. At the average inspiratory flow rate, impaction mostly affects the larger particles (aerodynamic diameter d_a >1 μm). In general, particles with aerodynamic diameter of about 1 to 3 μm deposit in the alveolar region if they are slowly and deeply inhaled.[38] For these particles, diffusion (significant for particles d_a <1 μm) is negligible. Therefore, inertial impaction and gravitational settling are the most important factors for particles depositing in the lower airways. These phenomena occur as a result of aerodynamic properties. The aerodynamic diameter of spherical particles is related to the volume (geometric) diameter according to Stokes' law:

$$V_{TS} = \frac{\rho_p d_e^2 g C_c}{18\eta\chi} = \frac{\rho_0 d_a^2 g C_c}{18\eta} \qquad \textbf{[1]}$$

Where ρ_p and ρ_0 are the particle density and the unit particle density (1000 kg·m^{-3}) respectively, χ is a shape factor, η is the viscosity of atmosphere, d_e is the equivalent volume diameter that is the diameter of a sphere having the same volume as that of the irregular particle, d_a is the aerodynamic diameter which is the diameter of a particle of the unit density having the same settling velocity as that of the irregular particle, and C_c is a slip correction factor necessary for particles with a diameter of less than 1 μm.

1.1.4 Microparticles and Nanoparticles

Particle size and distribution are the most critical factors determining the delivery efficiency of drug to the lungs. Dry powder inhalers (DPI) can be employed to deliver the microparticles or nanoparticle aggregates with aerodynamic diameter between 1 to 5 μm. However, individual isoxyl nanoparticles may be too small to be retained in the lungs, which

should be a rare occurrence due to particle aggregation. Primary sizes of nanoparticles may be achieved by nebulizing nanosuspension to the lungs. Delivery of nanosuspensions may be an attractive means because they may have more homogenous distribution in carrier droplets and based on previous reports produce higher respirable fraction relative to microparticle suspensions.[39-41]

Success of TB therapy is dependent largely on effectiveness of anti-tuberculosis drugs in eliminating mycobacteria residing in phagosomes of alveolar macrophages. It has been reported that particles with diameters of 500 nm to 3μm are phagocytized efficiently when these particles encounter alveolar macrophages.[42-44] Phagocytosis of hydrophobic nanoparticles decreases with decreasing size.[45] Cellular uptake of drug particles results in containment of both drug and bacilli in a cell. This might improve drug efficacy and shorten duration of treatment.[39, 46] Moreover, granulomas are formed by recruiting macrophages/monocytes, it is postulated that accumulation of drug in macrophages will facilitate transportation of drug to granulomas.[47] This may lead to a conclusion that microparticles, or aggregated nanoparticle, might be more effective in drug delivery than separated nanoparticles due to higher macrophage phagocytosis. However, it deserves further investigation. For example, if nanoparticle aggregates are broken down to discrete individual particles or microparticles dissolve quickly outside the macrophages, they may escape phagocytosis. Also, the relation of macrophage phagocytosis and drug efficacy remains controversial. Many studies have showed that accumulation of drugs in phagosomes simultaneously enhanced killing of intracellular mycobacteria[48, 49], while some studies did not [3]. This might be explained by isolation of bacilli and drugs in different intracellular phagosomes.[3] If drugs were trapped in the isolated phagosomes, the effectiveness of drug

against intracellular mycobacteria would be reduced. Therefore, the correlation between macrophage targeting and drug efficacy may be drug-dependent and should be studied on a case by case base.

Besides achieving sufficient drug concentration in macrophages in the lungs, it is also desired to maintain therapeutic drug concentration systemically to teat TB infection. The level of systemic distribution of drug from the lungs is determined by the balance between phagocytosis and dissolution. Particle size is also an important factor for rate of dissolution. The small particle size increases the surface area and intrinsic solubility of the drug that result in a relatively rapid dissolution rate [50]. This is described by the following equations 2 and 3:

$$\ln \frac{C_s}{C_0} = \frac{2\gamma V_m}{2.303\, RTr} \text{ (Kevin equation)} \qquad \textbf{[2]}$$

$$\frac{dC}{dt} = \frac{DA_s}{Vh}(C_s - C) \text{ (Noyes \& Whitney equation)} \qquad \textbf{[3]}$$

Here, C_s/C_o is the saturation ratio that is the solubility of a small particle with radius r over the solubility of a very large crystalline particle, γ is the surface tension of the particles, V_m is the molar volume, R is the gas constant, T is the absolute temperature, dC/dt is the dissolution rate, D is the diffusion coefficient of the drug in solution, A_s is the total surface area of the drug, h is the thickness of the diffusion layer, V is the volume of solution, and C is the concentration of the drug in the solution at time t. Theoretically, nanoparticles exhibit more rapid dissolution rate in alveolar lining fluid and, consequently, a faster absorption rate of the drug through pulmonary epithelium than microparticles. Also, it was reported that particles smaller than 500 nm in diameter were phagocytized by alveolar macrophages to a smaller extent than 1-5 μm microparticles. [43, 44, 51] Dendritic cells may take up these

nanoparticles,[52] but the kinetics of this phenomenon will be influenced by rapid dissolution of the particles in alveolar lining fluid. Therefore, a portion of nanoparticles may escape from monocyte uptake which leads to an increase in systemic drug exposure. However, all of these may not be true due to formation of nanoparticle aggregates which reduce the surface area to the level comparable to microparticles. Moreover, if the solubility of isoxyl in the lung lining fluid is extremely low, dissolution of drug from the nanoparticles may not be fast enough to avoid uptake by macrophages or epithelial cells. In this scenario, the difference of nanoparticles and microparticles may not be obvious. In addition to potential difference in dissolution and absorption, for ultrafine particles (< 100 nm), they may be able to be translocated intact to the blood circulation after inhalation exposure[53-57]. Uptake of nanoparticle by pulmonary epithelial cells will increase with decreasing size.[58] However, there are many conflicting results regarding epithelial cell transport. The amount of drug particles translocated by this mechanism is much smaller than macrophage uptake and molecular drug diffusion. Nemmar et al. [53] reported that significant amount (25-30%) of ^{99m}Tc-albumin nanocolloid particles (<80 nm) will be translocated from the lungs to the systemic circulation rapidly. But Nicolas et al. [59] revealed that 95.7% of the same particles in the lungs 6 hours after inhalation. Particle properties, including size, surface area, charge, number and coating, will influence uptake by macrophages and pulmonary epithelial cells. Therefore, many *in-vitro* studies including dissolution, cellular uptake of particles by macrophages and epithelial cells are necessary to predict *in-vivo* disposition of the drug.

In this study, particles of various size distributions were produced by changing processing conditions. The potentially different disposition of isoxyl nanoparticles and microparticles may result in change in magnitudes or mechanisms of cytotoxicity toward

macrophages and efficacy of the drug in treating the infected cells *in-vitro*. One purpose of the study is to evaluate the effect of isoxyl characteristics on drug cytotoxicity and efficacy. The study will illustrate mechanisms of isoxyl targeting at a cellular level, which may be beneficial for resurrecting the old clinically unproven drug or for designing more novel potent anti-tuberculosis drugs[13, 60].

1.1.5 Cell Culture and Animal Models

Targeting to phagocytic cells, host cells of mycobacteria, is believed to increase antituberculosis drug efficacy. To study the correlation of macrophage targeting and anti-tuberculosis effectiveness of isoxyl, selection of an appropriate macrophage model is a prerequisite. Several primary cell models or continuous cell lines have been used to mimic *in-vivo* macrophages that associate with mycobacteria. Human primary monocytes/macrophages or primary alveolar macrophages were isolated from the blood or lavage of healthy volunteers.[61, 62 63]These cells are the best representatives to *in-vivo* situation, but their application is scarce due to availability of the resources, heterogeneity of the cells and cost consideration. Murine primary alveolar macrophage model are an alternative cell model. These cell are cheaper to obtain and easier to manipulate genetically. However, species differences are always a concern. The human and the mouse have different degrees of susceptibility to mycobacteria, so the killing mechanism of murine macrophages can be different from that of human macrophages.[64] The same potential problem may arise in murine macrophage cell lines such as J774 and NR8383. Human macrophage-like cell line is a good model. Human macrophage-like cells show absence of donor variability, easily proliferate, and are cost-effective. The human acute monocytic leukemia cell line (THP-1)

has been widely used. [65-68] These cells can be differentiated by Phorbol Myristrate Acetate (PMA) to macrophage-like cells. Stokes at el. [69] reported that the differentiated THP-1 cells associate with mycobacteria in the similar way to human monocyte-derived macrophages (MDMs) from blood of healthy adult volunteers. THP-1 cells like MDMs allow intracellular growth of mycobacteria which can be eliminated by incubation of isoniazid.[69] Also, THP-1 cells can model an infection-induced apoptosis response of primary human alveolar macrophages which are believed to be the innate defense to eliminate intracellular bacilli.[70] Therefore, THP-1 cells were selected as a model cell line in the study to predict *in-vivo* disposition and efficacy of isoxyl.

The infected animal model is employed to test drug efficacy *in-vivo*. The mouse model is most commonly used, which is economical, easy to handle, resulting in a highly reproducible infection. The model has been developed thoroughly: there are plenty of immunological agents and genetic manipulation is available to study immune response in mouse. The mouse like the healthy human is more resistant to mycobacteria than guinea pig and rabbit. The mouse is different from the human in response to tuberculosis infection in several ways: 1) The structure of granulomas in the mouse, unlike the human, are not organized (macrophages are surrounded by a ring of lymphocytes);[71] 2) The mouse does not develop measurable delayed-type hypersensitivity after infection, so it does not develop caseous necrosis and cavity commonly seen in human.[72] Therefore, the immune response in mouse can be very different to that in human. The lungs are the natural route of infection following aerosol exposure to mycobacteria. This is achieved in the mouse by a nose-only or whole-body exposure chamber. However, this method of exposure may lead to variation in the number of mycobacteria deposited in the lungs. Alternatively, intravenous injection of mycobacteria

may be used to infect the mouse, which results in much higher loads of bacteria seeded in the spleen and the liver than the lungs (~ 1%) which does not resemble the natural way of disease development.[73] As a good alternative animal model, the guinea pig develops disease and an immune response similar to the human after exposure to low inocula of mycobacteria. The guinea pig develops delayed-type hypersensitivity and caseous necrosis. It also experiences hematogeneous dissemination after primary infections like human. As a result, guinea pigs develop classic human granuloma, primary and second lesions. Unlike the majority of healthy human subjects that have been exposed to mycobacteria without developing disease, guinea pigs are more susceptible to mycobacteria and finally die of the active disease.[71] Also, guinea pigs rarely develop cavities.[74] Therefore, guinea pig is a sensitive model to evaluate effectiveness of chemotherapy and vaccines. The main drawback of the model is that there are a limited number of immunologic reagents commercially available. The rabbit can develop disease by a way closer to that of human than mouse and guinea pig. It can contain mycobacteria, and develop similar granuloma and chronic cavities to human[71]. However, cost and scarcity of immunologic reagents are always concerns. The non-human primate is the closest to human. Moreover, a large number of immunologic reagents for the non-human primates are commercially available. However, practical issues, such as availability of animals, facilities, high cost of labor, and higher variability due to outbreeding, greatly narrow application of the non-human primate model.[73, 75] Consideration of all of these issues, the guinea pig model has been used in our lab to test drug sensitivity.

1.2 HYPOTHESIS AND SPECIFIC AIMS

It is postulated that isoxyl particles will significantly reduce the number of viable mycobacteria in cell culture, indicating potential efficacy of the drug particles after being deposited in the lungs of infected guinea pigs.

Specific aim 1: To develop and validate a quantitative analytical method for determination of isoxyl concentrations in plasma following i.v. and oral administrations.

It was proposed that poor therapeutic outcomes of isoxyl were caused by low *in-vivo* exposure of the drug. However, most studies of isoxyl were performed four decades ago, so a reliable quantification method for determination of this drug concentrationa in biological samples was not available. Consequently, the reports of *in-vivo* behavior of the drug were very scarce and controversial. Development of a sensitive and accurate analytic method to determine isoxyl concentrations in plasma is a prerequisite to understand the mechanism of poor therapeutic outcomes. In this study, a HPLC assay was developed and validated. The method was utilized to determine plasma concentrations of isoxyl at various time points after it was administered by i.v. administration and the oral route. The relationship of concentration-time data was fitted to noncompartment models using WinNonlin analysis program to determine the pharmacokinetic parameters.

Specific aim 2: To optimize particle processing conditions to prepare isoxyl particles suitable for pulmonary delivery.

Particle size affects drug deposition to the lungs and *in-vivo* drug disposition. Theoretically, particles, aggregates or droplets with an aerodynamic diameter between 1 to 5

μm can penetrate deeply to the lungs. The purpose of the study was to prepare microparticle or nanoparticle aggregates in the respiratory size range which can be delivered from a dry powder inhaler (DPI) or to produce nanosuspension that can be delivered by nebulizer. After these particles deposit on the lung surface, microparticles or nanoparticles may exhibit different cytotoxicity and efficacy against intracellular MTB bacilli because of potentially different disposition pathways. The goals were to produce both macroparticles with diameters between 1-5 μm and nanoparticles with diameters less than 500 nm. The effect of processing parameters during antisolvent precipitation and subsequent spray drying were studied using statistical experimental design methods. Batches of isoxyl particles with desired particle sizes were then prepared by optimizing parameters of manufacture. The resulting particles were characterized in terms of their particle size distribution (PSD), morphology, polymorphism and fine particle fraction.

Specific aim 3: To assess cytotoxicity of isoxyl particles on human macrophages, and the efficacy of these particles on intracellular growth of a model mycobacterium, BCG, in these phagocytic cells.

PMA-differentiated human monocytic THP-1 cells were used as a model of human macrophages. Two different isoxyl preparations, spray-dried isoxyl microparticles and isoxyl in 1% DMSO, were tested in terms of cytotoxicity and *in-vitro* efficacy. Preparation of isoxyl in 1% DMSO at higher concentrations produced nanoparticles detected by dynamic light scattering (DLS). The difference in cytotoxicity of these microparticles and nanoparticles/drug molecules was determined by measuring cell viability (Methylthiazol Tetrazolium Assays) and integrity of cell membrane (Lactose Dehydrogenase Assays) after

the cell monolayers were exposed to these drug preparations. The macrophages were infected with *Mycobacterium bovis,* Bacille Calmette-Guerin (BCG). Comparison of the efficacy of these microparticles and nanoparticles/drug molecules was evaluated *in-vitro* by determining viability of the intracellular bacilli after BCG-infected cells have been exposed to various forms of the drug.

Chapter 2

Development and Validation of Quantitative Analytical Method for Determination of Isoxyl Concentrations in Plasma

2.1 INTRODUCTION

Isoxyl was abandoned forty years ago due to failure of early clinical trials. Poor solubility in water appeared to contribute to malabsorption of the drug following oral administration. Due to the absence of a sensitive and accurate quantitative method, the studies of *in-vivo* exposure to the drug were rare and unreliable. The exact mechanism of poor clinical outcomes remains unclear.

Since most studies of isoxyl were conducted in 1960s and 1970s, only two quantitative analytical methods have been reported, microbiological and radiochemical methods. The microbiological activity method was insensitive and the radioactive labeling method could not distinguish between intact drug and metabolites. Consequently, limited *in-vivo* exposure to isoxyl was reported and the data were inconsistent. Published isoxyl blood levels after oral administration are summarized in Table 1. Robinson et al.[76] reported that absorption of isoxyl was rapid, achieving plasma levels significantly higher than MIC (0.4 μg/ml), but highly variable based on the microbiological method. When a single 9 g dose of isoxyl was given orally to 9 human subjects in the fasting state, 5 subjects achieved maximum drug concentrations > 128 μg/ml at 2 h, and the remaining 4 subjects obtained a peak concentration of 8 to 24 μg/ml, after 2 to 6 h. The average isoxyl blood concentrations at specific time points are shown in Figure 3A. In contrast, the other two studies revealed that isoxyl was absorbed to a smaller extent using the same method. Eule et al.[77] gave two doses of 3 g of isoxyl tablets with a 6-hour interval to 31 tuberculosis patients. The average drug concentration in serum reached a peak of 5.5 μg/ml 9 h after the first dose [Figure 3B]. This low level of isoxyl was comparable to another study in 12 healthy volunteers [78, 79], in which drug tablets were administered in two doses of 3 g with 4-hour interval. The maximum isoxyl

concentrations determined by both microbiological activity and radioactive labeling were approximately 10 μg/ml at 10 h [Figure 3C]. Also, as shown in Table 1, the study performed by Eule et al.[77] indicated more rapid elimination and isoxyl plasma levels dropped below the MIC in less than one day, while the other two studies showed that the drug was eliminated slowly and drug concentrations were still higher than MIC after one day. Since these studies were carried out under several different conditions, such as dose, dosing regimen, dosage form, fasting vs. non-fasting, healthy subjects vs. tuberculosis patients, small vs. large population sample sizes, and serum vs. blood samples, comparison is difficult. Further studies are necessary to elucidate the *in-vivo* disposition of isoxyl. In addition, according to a study of isoxyl distribution in rabbits, the drug was poorly absorbed and was eliminated in bile at a fairly high level. After oral administration of drug at a single dose of 200 mg/kg (isoxyl suspension) to rabbits, about 60 % of isoxyl was recovered in feces in 3 days. The highest level of isoxyl accumulated in bile, followed by kidney, spleen and liver respectively. Lungs and blood showed relatively low drug concentrations [80][Figure 4].

To understand the mechanism of poor *in-vivo* efficacy of isoxyl, an HPLC method was developed and validated to determine the isoxyl concentrations in guinea pig plasma and preliminary pharmacokinetics of the drug was reported from a pilot study.

Table 1. Summary of blood levels of isoxyl delivered orally in human subjects.[76-79]

Source	**Robinson et al.[76]**	**Eule et al.[77]**	**Lambelin et al.[79]**
Route (Form)	Oral (Not mentioned)	Oral (Tablets)	Oral (Tablets)
Dose	Single, 9 g	2×3 g (6 h)	2×3 g (4 h)
No. of patients	9	31	6
Subjects	Healthy, fasting	Patients	Healthy, non-fasting
Samples analyzed	Blood	Serum	Blood
Assay Method	Microbiological	Microbiological	Microbiological and Radioactive
MIC	0.4 μg/ml	0.6-0.8 μg/ml	1.2-1.4 μg/ml
Time (C<MIC)	>24 h	< 24 h	> 24 h
C_{max} (T_{max})	>128 μg/ml (2 h) in 5 subjects 8-24 μg/ml (2-6 h) in 4 subjects	5.5 μg/ml (9 h after first dose)	8-11 μg/ml (~ 10 h after first dose)

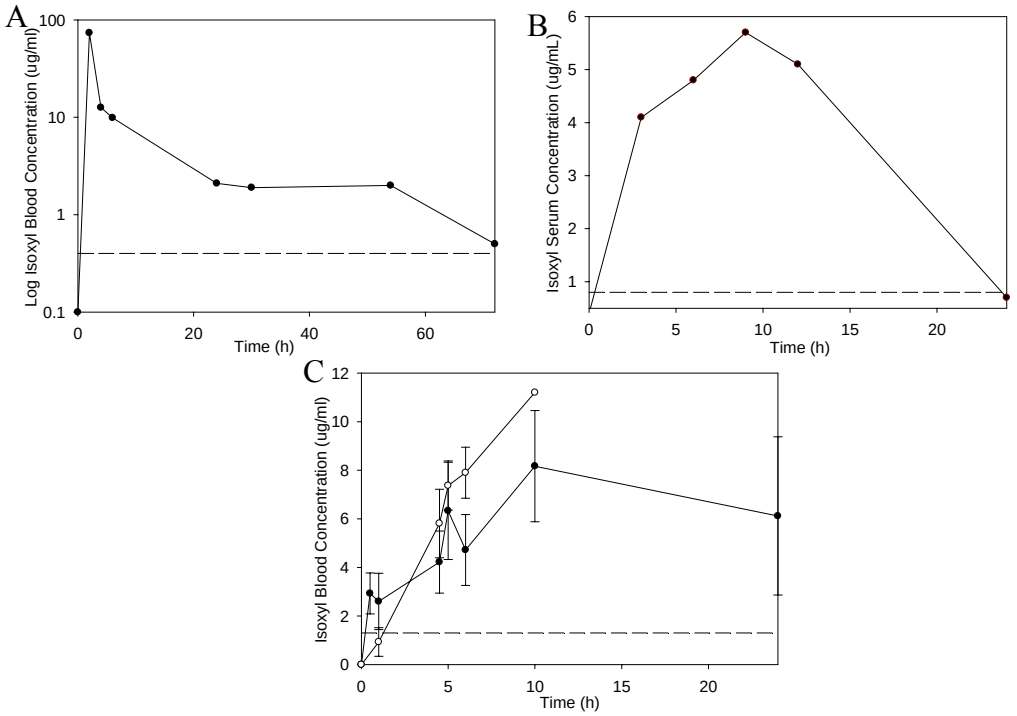

Figure 3. (A) Mean blood concentrations of isoxyl at various periods after a single dose of 9 g to 9 human subjects, 5 of whom exhibited peak levels over 128 μg/ml.[76] (B) Mean serum concentrations of isoxyl at various periods after two doses of 3 g tablets at 6-hour interval to 31 tuberculosis patients.[77] (C) Mean blood concentrations of isoxyl determined by radioactive labeling method (closed circles) and microbiological activity method (open circles) at various periods after two doses of 3 g tablets to 6 patients at 4-hour interval. [78, 79] The dotted lines represent MIC of isoxyl in the studies. (Figures prepared from data in references 76-79)

2.2 RATIONALE AND EXPERIMENTAL DESIGN

The analysis was performed using a Hewlett Packard 1100 series HPLC (Agilent Technologies, Santa Clara, CA) with a binary pump, an autosampler, a Waters Spherisorb 5μm ODS2 (4.6×150 mm) analytic column preceded by a Nova-Pak C18 4μm precolumn cartridge, and a UV detector set at 270 nm (the absorption maximum wavelength of isoxyl in acetonitrile). A narrow-bore column, Spherisorb 5μm ODS2 (2×100 mm), was employed owing to increase detectability, less consumption of solvents, and short run time. N,N'-bis[4-

(1,1-dimethylethyl)phenyl]-thiourea [Figure 1B], having similar structure to isoxyl, was chosen as an internal standard to compensate for variations in sample preparation and injection procedures. The method was validated according to FDA guidance for bioanalytical method validation. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/UCM070107.pdf).

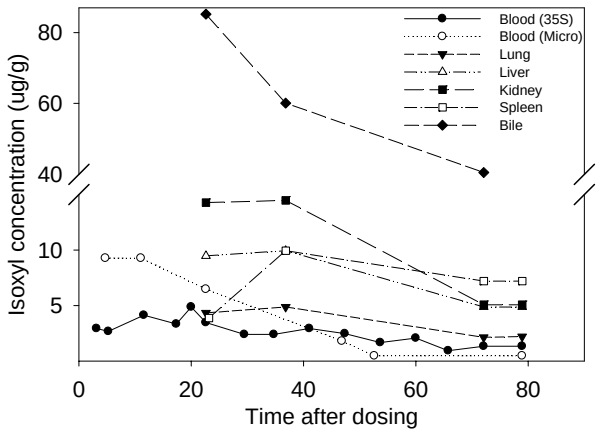

Figure 4. Thiocarlide (isoxyl) concentration determined by ^{35}S-concentration after a single oral dose of 200 mg/kg ^{35}S-thiocarlide to rabbits. Microbiological comparison was measured after the last dose of 100 mg/day thiocarlide for 9 days.[80] (Figures prepared from data in reference 80)

Isoxyl was administered to guinea pigs by i.v. administration. After the treatment, blood samples were collected at various time points and drug concentrations were determined by the HPLC assay. Pharmacokinetic (PK) parameters were determined from plasma concentration vs time data. The disposition of isoxyl following oral delivery was also determined. If the drug plasma concentrations are low, this explains the poor therapeutic outcomes of isoxyl following oral delivery. Further studies will be focused on measuring drug concentrations in the lungs after inhalation of isoxyl.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Isoxyl was purchased from Cayman Chemical Co. (Ann Arbor, Michigan). Acetonitrile (HPLC grade) and ammonium acetate (HPLC) were obtained from Acros Organics and Fisher Scientific respectively. Carboxymethyl cellulose, sodium salt (CMC) was from Sigma. N,N'-bis[4-(1,1-dimethylethyl)phenyl]-thiourea (internal standard, IS) were from Ryan Scientific, Inc (Mt. Pleasant, South Carolina). Dunkin-Hartley guinea pigs were purchased from Hilltop Lab Animals Inc. (Scottsdale, PA). Blood was collected at different time points and plasma was separated, and stored at -80^{o}C until use. Ultrapure water was obtained from Barnstead Nanopure Infinity Ultrapure Water System (Dubuque, IA, USA).

2.3.2 Preparation of the Standards and Quality Control (QC) Samples

A 2 mg quantity of isoxyl was weighed accurately and then transferred to a 2 ml volumetric flask containing ethanol. The stock solutions of isoxyl at nominal concentrations of 0.100, 0.0100, 0.00100 mg/ml were made by a series of 10-fold dilutions using volumetric flasks. These solutions were stored at - 20 ^{o}C until use. From these stock solutions, norminal concentrations of 0.0500, 0.100, 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0, 50.0 μg/ml isoxyl were made freshly using either 50% Acetonitrile (ACN) in 20 mM ammonium acetate buffer or guinea pig plasma to obtain the standards. Three quality controls were made by spiking plasma with isoxyl to prepare solutions at concentrations of low (0.400 μg/ml), medium (6.00 μg/ml) and high (40.0 μg/ml). The standards and quality controls in plasma were stored at -80 ^{o}C until analysis.

2.3.3 Analytical conditions

Mobile phase was screened by varying ratios of acetronitrile to water. Flow rate was 0.5 $ml \cdot min^{-1}$. The optimal analysis condition was determined by running time and separation of isoxyl and internal standard. The standards containing pure isoxyl in 50% ACN and 50% 20 mM ammonium acetate buffer were used to determine linearity of the drug in a range of concentrations. Background levels were measured and the limit of detection (LOD) was measured by signal to noise ratio of 3.

2.3.4 Extraction of Isoxyl from Plasma

Internal standard (0.102 mg/ml) of 5 µl were spiked in 100 µl of the standards, QCs, blank plasma controls and samples. Acetonitrile (400 µl) was then added to precipitate proteins. The samples were vortexed for 2 min and centrifuged at 12,000 rpm for 10 min. The supernatants were pipetted into microcentrifuge tubes and dried in a Savant ISS110 Speedvac concentrator (Thermo Fisher Scientific, Waltham, MA). Each pellet was dissolved in 100 µl of 50% acetonitrile in 20 mM ammonium acetate buffer from which 50 µl was injected.

2.3.5 Method Validation

Briefly, specificity was evaluated by separation of the isoxyl peak from the peaks of impurities from plasma and internal standard. The standard curve was plotted by peak area ratios of isoxyl to internal standard against isoxyl concentrations. The best fit line was obtained by the least regression method and the regression coefficient (R^2) was calculated. Intra-day precision and accuracy of the HPLC method were evaluated by assaying at least four replicates of standard plasma solutions at three concentrations, low (0.400 μg/ml), medium (6.00 μg/ml) and high (40.0 μg/ml) in one day. The inter-day variation was determined by assaying these three concentrations on each of five consecutive days. The limit of quantification (LOQ) was measured by determining precision and accuracy of five replicates of four low concentrations, 0.0500, 0.100, 0.250, and 0.500 μg/ml.

2.3.6 Recovery Evaluation

Extraction efficiency was defined as the amount of the drug extracted from plasma expressed as a percentage of the amount of the drug in the pre-extraction sample (control). Samples of 0.400, 6.00, 40.0 μg/ml in triplicate were extracted and analyzed by HPLC as described in above. For controls, drug free plasma of 100 μl was extracted with 400 μl acetonitrile, as described in sample preparation. Isoxyl and 50% acetonitrile in 20 mM ammonium acetate buffer were added to the extraction pellets to prepare controls of 0.400, 6.00, 40.0 μg/ml. All the controls were assayed in triplicate. An internal standard was introduced into each sample and control. The recovery of isoxyl from plasma was evaluated by comparing peak area ratios of isoxyl to internal standard measured in samples with those of the controls of the respective concentrations.

2.3.7 Stability Studies

The stability of isoxyl was evaluated by determining the drug concentration change in quality control samples (QCs) during storage. Triplicate QCs were stored at room temperature for 5.5 h or underwent three freeze-thaw cycles according to FDA guidance. For long-term stability, the standards were stored at -80 ^{o}C for a month (30 days).

2.3.8 Preliminary Pharmacokinetic Studies

Male Dunkin-Hartley guinea pigs (808-961 g) were employed. All of the animal experiments were consistent with the policies of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. The right external jugular vein of each guinea pig was cannulated under anesthesia (ketamine 50 mg/kg and xylazine 5 mg/kg) using silicone polymer tubing connected to polyethylene PE-50 tubing to facilitate collection of blood samples. Two guinea pigs received a dose of 10 mg/kg or 20 mg/kg by intravenous administration through the cannula implanted in the jugular vein. Blood samples were collected at various time points and drug concentrations were determined by the HPLC assay. The 10-min infusion at a dose of 8.7 mg/kg was performed to prevent isoxyl from precipitation immediately after injection and to obtain accurate pharmacokinetic parameters. For oral delivery, isoxyl was suspended in 1% CMC and ground in a mortar with a pestle. The suspensions were fed orally to guinea pigs in duplicate at a dose of 100 mg/kg.

The isoxyl plasma concentrations vs. time data were analyzed by the WinNolin computer analysis program (Pharsight Corp., Mountain View, CA) using noncompartmental model. Pharmacokinetic parameters of isoxyl were calculated.

2.4 RESULTS

Optimization of chromatographic methods was conducted using samples containing the pure drug solution in 50% ACN and 50% 20 mM ammonium acetate buffer. The optimized mobile phase contained 70% ACN and 30% 20 mM ammonium acetate buffer. The retention times of internal standard and isoxyl were 3.5 min and 4.5 min respectively [Figure 5A]. As the percentage of ACN in mobile phase increased to 75%, isoxyl peak merged with internal standard peak. Decrease of ACN resulted in the longer retention time (T_R). When a volume of 10 µl of the samples was injected onto the HPLC column, a linear calibration plot of isoxyl ranging from 0.1 µg/ml to 50.6 µg/ml was obtained. The regression equation and correlation coefficient were determined:

Peak Area Ratio (Isoxyl/IS) =0.173×Concentration (µg/ml) -0.0044 [R^2=0.9960]

The Limit of detection (LOD) of the method was determined to be 20 ng on the Waters Spherisorb 5µm ODS2 (4.6×150 mm) analytic column.

For plasma samples, isoxyl was extracted with ACN and analyzed by HPLC. Isoxyl peak (4.8 min) was separated well from the peaks of internal standard (3.8 min) and impurities from plasma (1.2 min) [Figure 5B]. The chromatogram of blank guinea pig plasma showed only the peak of impurities from plasma [Figure 5C]. The LOQ was determined to be 0.25 µg/ml based on intra-day variation (precision was $\leq$ 20% and accuracy was between 80% and 120%) and the recovery of isoxyl extracted by ACN was above 68% [Table 2-3]. The standard curve of isoxyl was linear from 0.25 µg/ml to 50 µg/ml:

Peak Area Ratio (Isoxyl/IS)=0.20×isoxyl concentration (µg/ml) -0.023 [R^2=0.9958]

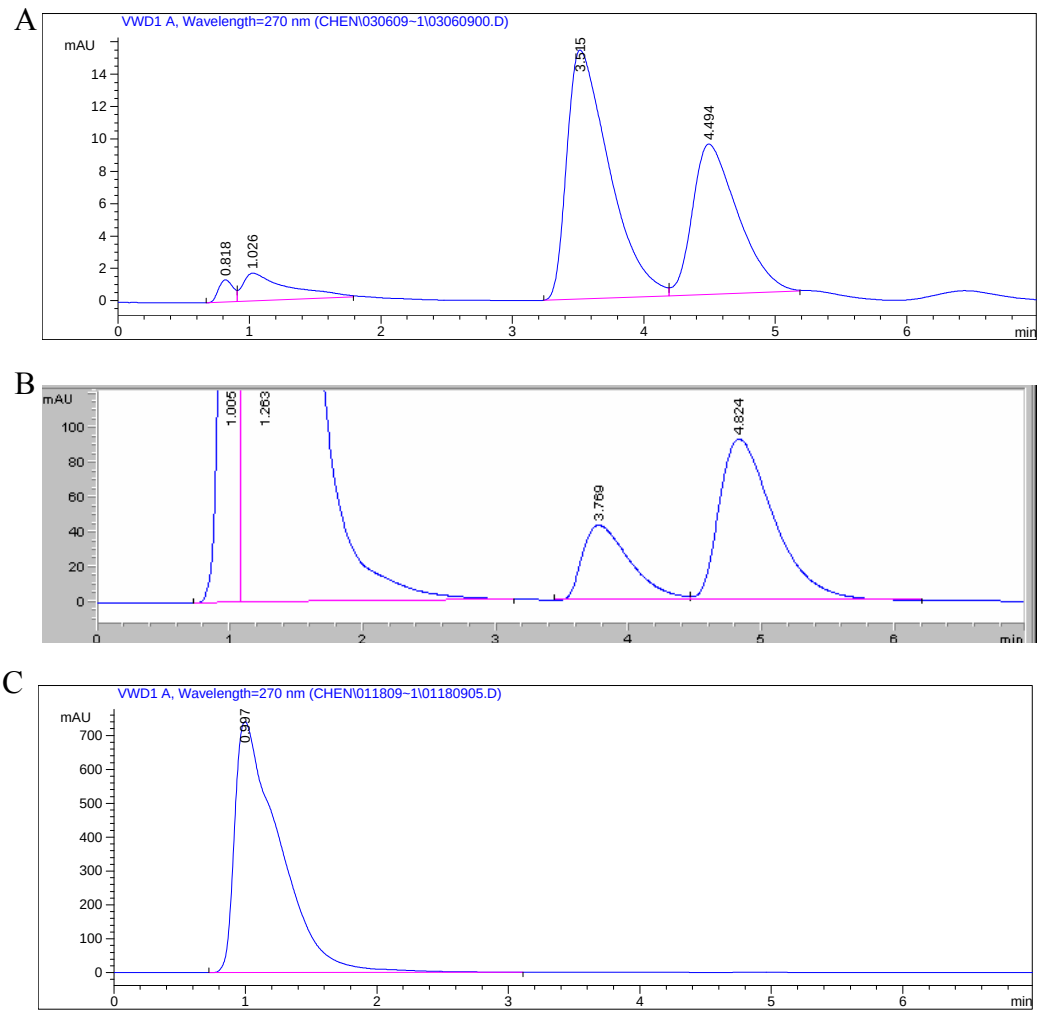

Figure 5. (A) A chromatograph of isoxyl (4.5 min) and internal standard (3.5 min) dissolved in ethanol. (B) A representative chromatogram of isoxyl (4.8 min, 10.3 µg/ml) and internal standard (3.8 min, 5.0 µg/ml) extracted from guinea pig plasma (wavelength=270 nm). (C) A chromatogram of blank guinea pig plasma.

Table 2. Determination of Limit of Quantification (LOQ) (n=5).

Conc. (µg/ml)	Precision (%)	Accuracy (%)
0.0504	43	259
0.101	40	108
0.252	11	87
0.504	12	100

Table 3. Determination of extraction efficiencies in three concentrations (n=3).

Conc. (µg/ml)	Recovery (%)
0.404	77 ± 13
6.05	68 ± 13
40.4	96 ± 5.2

As shown in table 4, the HPLC method was validated for determining isoxyl concentrations in plasma: 1) Intra-day and inter-day precision of the method were between 6.8-14% for 0.400 μg/ml and between 6.0-8.0 % for 6.00 and 40.0 μg/ml; and 2) Intra-day and inter-day accuracy of the method were between 115-119% for 0.400 μg/ml and between 85-99% for 6.00 and 40.0 μg/ml [Table 4]. The HPLC analysis demonstrated that isoxyl was stable under the conditions employed. Isoxyl concentrations in the plasma samples did not change after being frozen and thawed for three cycles or kept at room temperature for 5.5 h [Table 5]. Also, isoxyl was stable after the plasma samples were stored at - 80 ^{o}C for one month [Table 6].

Pharmacokinetic parameters were first determined by intravenous administration of isoxyl to guinea pigs. Two doses of isoxyl (10 and 20 mg/kg) exhibited the same plasma concentration vs. time profiles: the initial concentration of isoxyl in blood circulation was 12-14 μg/ml and the drug was rapidly eliminated to a concentration below MIC (2 μg/ml) in 1 h [Figure 6A and Table 7]. The 10 min infusion did not increase *in-vivo* exposure of isoxyl [Figure 6B]. When isoxyl was administered orally, the drug plasma concentrations at various time points were far below the reported MIC (2 μg/ml). Most of them were also below the limit of quantification (LOQ) [Figure 6C].

Table 4. Intra-day and inter-day variations (n=4 for each concentration).

Spiked Conc. (µg/ml)	Intra-day			Inter-day		
	Measured Conc. (µg/ml)	Precision (%)	Accuracy (%)	Measured Conc. (µg/ml)	Precision (%)	Accuracy (%)
0.412	0.47	6.8	115	0.49	14	119
6.18	5.5	7.5	89	5.22	6.0	85
41.2	39.0	7.3	95	41.0	8.0	99

Table 5. Stability of isoxyl under room temperature (benchtop) and freeze/thaw conditions.

	Time Period	Spiked Conc. (µg/ml)	% of Control
Benchtop (n=3)	5.5 h	0.412	115 ± 21
		6.18	85 ± 1.5
		41.2	104 ± 3.2
Freeze/Thaw (n=3)	3 cycles	0.412	105 ± 12
		6.18	112 ± 12
		41.2	107 ± 10

Table 6. Long-term stability of isoxyl under frozen conditions.

	Time Period	Sample Conc. (µg/ml)	% of Control
Storage at - 80°C	One month	0.510	82
		10.3	119
		25.7	96
		51.5	114

Table 7. Pilot study of Pharmacokinetic parameters after i.v. administration.

Dose (mg/kg)	C_0 (µg/ml)	CL (L/h/kg)	V_β (L/kg)	$\lambda(h^{-1})$	AUC (µg·h/ml)
10	12.83	1.21	2.45	0.49	8.26
20	12.27	2.23	8.28	0.27	8.95

***** C_0: initial concentration, CL: clearance, V_β: volume of distribution of the terminal phase, λ: elimination rate constant, AUC: area under plasma concentration vs. time curve.

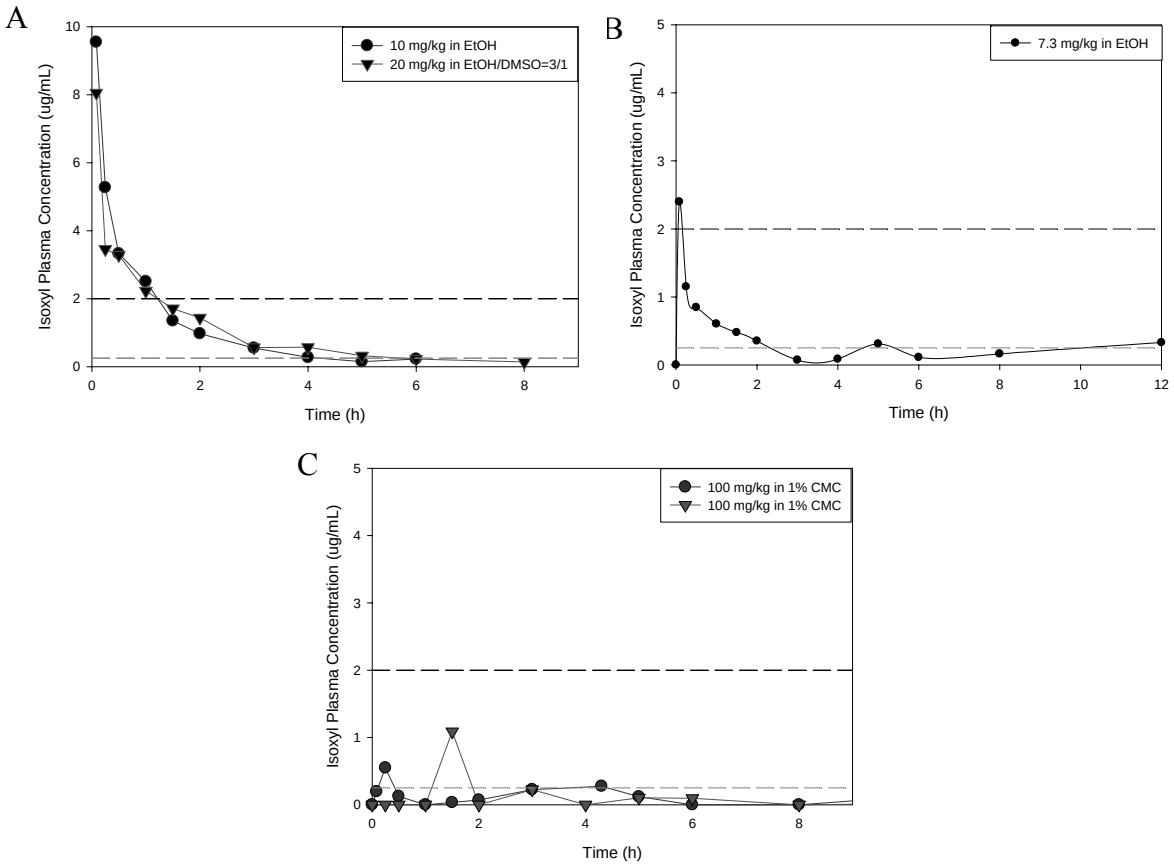

Figure 6. Plasma concentration versus times profiles study of isoxyl after it was given by i.v. bolus administration (A), by a 10-min infusion (B), and oral administration (C). The dotted line in black represents MIC and the lower line in dark gray is limit of quantification (LOQ).

2.5 DISCUSSION

An optimized reverse-phase HPLC method was developed to determine isoxyl concentrations in plasma. The method was a simple procedure with good peak resolution and short run-time. The method was precise, accurate and robust with a sufficiently low limit of quantification as defined by the FDA guidance. Based upon reported MIC, a LOQ of 0.25 μg/ml was adequate since it is about eight-fold lower than the MIC (2 μg/ml) in plasma.

The HPLC method was utilized to determine the preliminary pharmacokinetic profiles of isoxyl after it was given to guinea pigs by intravenous administration. The low and high doses exhibited similar isoxyl plasma concentration vs. time profiles, i.e. the same initial concentration (C_0) and AUC. This suggested that the distribution of isoxyl seemed a solubility-limited process even for direct administration of the drug. Precipitation near the site of injection is likely to have occurred after administration. Consequently, the pharmacokinetic parameters may be inaccurate. To obtain reliable pharmacokinetic parameters of isoxyl, slower infusion was conducted, but it may not have been sufficiently slow to prevent precipitation of the drug at the site of administration. Further investigation of extended infusion times to accommodate the dosing requirements for this sparingly soluble drug are required to evaluate disposition of drug from the circulation.

Isoxyl has been shown to be active in animals and humans at an oral dose of 100 mg/kg. Indeed, some reports suggest that a dose of 50 mg/kg is effective in therapy in guinea pigs.[18] In the present study, a 100 mg/kg dose of isoxyl was delivered orally to guinea pigs. However, this dose of drug did not approach the MIC for *Mycobacterium tuberculosis* in the circulation, as estimated from drug concentrations in plasma. It may be inferred that the equivocal therapeutic outcome observed in some clinical trials may be a result of poor

bioavailability of isoxyl following oral delivery. Surprisingly, the previous reports detected much higher drug concentration than the MIC where the peaks of isoxyl concentration in plasma varied from 5 to 108 μg/ml.[76-79] In these studies, either microbiological or radiochemical methods were employed to determine drug concentrations in blood or serum. The poor bioavailability has several possible explanations. Firstly, isoxyl might be transformed to some active metabolites *in-vivo* which cannot be differentiated from the parent compound by HPLC with UV detection, since they may be hydrophilic and mixed with co-eluting impurities from plasma, but may be detected by both microbiological and radiochemical methods. In this scenario, depending on the activity of the metabolites, individual differences in patient metabolism of isoxyl could result in highly variable therapeutic outcomes. Guinea pig liver microsomes could be used to study metabolism of isoxyl and identify these possible active metabolites. Considering the tedious, costly and high-risk drug discovery process, *in-vitro* metabolism may provide a rapid way to develop new anti-tuberculosis drug alternatives with improved physico-chemical properties and metabolic profiles. An alternative explanation for the differences in bioavailability is species specific factor. It has been reported that isoxyl blood levels in rabbit were significantly lower than those in humans.[78] But this low drug exposure was apparently sufficient to treat tuberculosis in rabbits. Efficacy of isoxyl in the infected guinea pig and human has also been demonstrated.[18, 20, 81, 82] Therefore, concentration difference of isoxyl in different species may not be a critical factor. The formulation of the dosage form for oral delivery of isoxyl might also play a role in drug availability by influencing dissolution rate. However, isoxyl tablets, which require disintegration and deaggregation prior to dissolution, have been shown to achieve plasma concentrations which were far above MIC.[77-79] In the present study, drug was

prepared as a suspension by levigating in 1% carboxymethylcellulose solution. The suspension, with large surface area for dissolution, should exhibit more rapid dissolution than tablets, and, potentially higher bioavailability (rapid uptake, larger peak plasma concentration).

It is anticipated that isoxyl concentrations in the lungs would be low following oral administration. Meissner et al. gave ^{35}S-thiocarlide to rabbits and detected isoxyl concentrations in various organs. The drug concentration in the lungs was close to that in blood.[80][Figure 4] In the present study since isoxyl plasma concentrations following oral administration were below the LOQ (< MIC) in guinea pigs, it is likely that the isoxyl concentrations in the lungs were also less than MIC from 0 to 24 h after drug administration. Also, it is probable that only small quantities of circulating isoxyl can penetrate to granulomas, tubercles or lesions that receive poor blood supply. These barriers to drug delivery would result in extremely low doses of isoxyl arriving at the main site of infection and would contribute to the failure of clinical trials.

There are several possible mechanisms for poor clinical outcomes of isoxyl. Two approaches can be applied to rescue this drug, seeking alternative routes of administration or developing better analogues based on the study of *in-vitro* or *in-vivo* metabolism of the drug. Pulmonary delivery of isoxyl is a promising alternative as: 1) much higher concentrations of isoxyl are delivered to the lungs and are retained for an extended period of time at the main site of infection; and 2) isoxyl paricles, due to their slow dissolution, may be taken up by alveolar macrophages which are host cells for TB bacteria and have local action, or be translocated by these cells to granulomas, lymph nodes or other major sites of infection where drug would be released.

2.6 CONCLUSIONS

A reliable reverse-phase HPLC method was developed for determination of isoxyl concentrations in plasma. The method was used to perform preliminary pharmacokinetic studies of isoxyl after it was administered intravenously and orally. Interpretation of previous reports in the context of the initial pharmacokinetic assessment in these studies, suggests that previous therapeutic failures or conflicting clinical trials outcomes of isoxyl may have been due to either poor absorption of the drug or variation in formation of active drug metabolites. Pulmonary delivery of isoxyl and development of active metabolites may be promising approaches to rescue this abandoned drug and render it an effective agent for treatment of the serious MDR TB disease. This study focused on generating isoxyl aerosols for lung delivery.

Chapter 3

Preparation of Isoxyl Microparticles and Nanoparticles for Pulmonary Delivery

3.1 INTRODUCTION

Low solubility is one primary cause for the previous poor therapeutic outcome of isoxyl following oral administration. Similarly, many active compounds that have been discovered that were subsequently abandoned due to poor solubility. However, improvement of their performance by modifying their chemical structures is laborious and costly. [83, 84] Discovery and marketing of a new drug usually takes billions of dollars and up to 15 years.[85] Therefore, seeking an optimal formulation and desirable dosage forms for these drugs will be the most convenient and cost effective approach. Many methods such as co-solvents, surfactants, emulsions, or polymer complexation can be employed to increase solubility. Though there are some successful cases, the low drug loadings and the toxicities of the excipients are always concerns.[84, 86] Moreover, even if oral absorption can be improved by these methods, the fraction of isoxyl which reaches the granulomas, tubercles or lesions is very limited. Formulation of micrometer or nanometer-sized isoxyl particles would be a simpler and safer method to improve efficacy of the drug. These particles can be delivered to the lungs which increases drug concentration at the main site of infection. A fraction of these drug particles can target macrophages in the lungs, the host cells for mycobacteria. Meanwhile, large surface area of these particles and hydrophobic environment in the lung lining fluid may potentially increase systemic exposure of the drug to eliminate extrapulmonary mycobacteria.

Many approaches have been utilized to prepare microparticles or nanoparticles of therapeutic drugs. A widely used approach is to break up drug particles by mills. However, milling requires long process time, and also may cause contamination with impurities and formation of amorphous drugs. Compared to milling, antisolvent precipitation is simple, and it can control the crystallinity of drugs and produce high drug loading. Preparation of

micrometer or nanometer-sized pure drug particles would also be safer than formulation with a large quantity of excipients to increase solubility. The mechanism of precipitation includes three processes, nucleation, coagulation, and condensation. Addition of antisolvent to the drug solution creates high degree of local supersaturation, which leads to the formation of many small nuclei. The rate of primary nucleation (homogenous nucleation) J is described by the following equation 4:

$$J = A\exp\left[-\frac{16\pi\gamma^3 V_m^2}{3k^3T^3(\ln S)^2}\right] \quad \textbf{[4]}$$

Here, A is a constant, γ is interfacial tension, V_m is molecular volume, k is Boltzmann constant, T is the absolute temperature, and S is degree of supersaturation (S is equal to ratio of the solution concentration to the equilibrium saturation at the given temperature).[87, 88] These nuclei then undergo growth by condensation and coagulation until an equilibrium in which particle sizes are not changed. [86] Therefore, the solid-state properties including particle size distribution (PSD) and morphology of the resulting precipitants can be influenced by the factors that have impact on the rates of these three processes. For example, different solvents can change the crystal habits due to their physiochemical properties; higher temperature may result in larger particle size owing to increasing solubility, diffusion and growth kinetics; increase of antisolvent introduction rates tends to produce more needle-like crystals with smaller particle size and a more narrow distribution; higher degree of miscibility of antisolvent and solvent increases mixing rate and results in smaller crystal sizes; the mixing method with higher energy can also yield smaller particles. [86, 89]

Spray-drying has been used to produce powders for pulmonary delivery due to its ease of operation and ability of controlling particle characteristics. In the following study, this method was applied to dry the particle suspension generated from anti-solvent precipitation.

The suspension is atomized into a spray by a gas under pressure and mixed with hot air stream in the drying chamber. The solvents are evaporated. The resulting solid particles are moved along with air stream and separated from the air in cyclone and collecting vessel. In the spray-drying process, particle size and shape can also be changed by varying feed properties, atomization conditions and drying process.[90] Theoretically, larger particles are produced when nitrogen flow rate is slower, organic strength of the solvent decreases, feed rate is more rapid and drug solution is more concentrated. Particle shape is affected by physicochemical properties of the feed solvent and outlet drying temperature.

Therefore, it is expected that the study of antisolvent precipitation and spray drying conditions of isoxyl would permit production of particles with diverse PSDs and other characteristics. The future efficacy study of these different particles will help to identify the optimal particle characteristics for treating TB disease.

3.2 EXPERIMENTAL DESIGN

The purpose of the study is to produce particles with different sizes for pulmonary delivery. The goal of particle preparation is to produce microparticles with diameters between 1 to 5 μm which is suitable for pulmonary delivery and generate nanoparticles with diameters less than 500 nm. Nanoparticles can be delivered in the forms of suspensions, aggregates or particles in matrix. A modified BUCHI B-191 mini spray-drier containing a 3-fluid nozzle was used to produce isoxyl particles. The nozzle allows water, isoxyl in organic solvent and compressed nitrogen pass independently. The two liquid feeds are mixed and atomized at the tip of the nozzle, where antisolvent precipitation occurs. The precipitants generated by this process are controlled by several variables: inlet temperature, type of

organic solvent, various ratios of organic solvent to water, and mixing energy (nitrogen flow and feed flow).[86, 89] The precipitants were then spray dried. According to the information provided by Buichi Labortechnik AG, particle size resulted from the following spray drying process is influenced by four main variables: spray flow rate and pressure, organic feed rate, organic solvent type, and isoxyl concentration. Consequently, isoxyl particle preparation consists of two processes, antisolvent precipitation and spray drying, which are controlled by totally seven main variables as shown in Table 8. Statistical experimental design (Design Expert®, State-Ease, Inc., Minneapolis, MN) is a quick method for optimizing preparation conditions by studying the effects of several variables and their interactions concurrently.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Isoxyl was purchased from Cayman Chemical Co. (Ann Arbor, Michigan). Acetone and isopropanol were obtained from Fisher Scientific. D-mannitol and ethanol were from Sigma. Tetrahydrofuran (THF) was from EM Science (Darmstadt, Germany). Lactose (Respitose ® ML001) was provided by DMV international. Flexi-Dry™ freeze-drier was from Kinetics Group (Santa Clara, CA). The 3-fluid nozzle was purchased from Buchi Labortechnik AG (Flawil, Switzerland) and fit in a BUCHI B-191 mini spray-drier made by the same manufacturer. Isopore™ Polycarbonate Membrane Filter (Pore size: 0.1 μm, Millipore, Billerica, MA) or a Cyclopore Polycarbonate Membrane Filter (Pore size: 0.1 μm, Whatman, Florham Park, NJ) were used to collect particles in suspensions. Water was deionized by Barnstead Nanopure Infinity Ultrapure Water System (Dubuque, IA, USA). Either SEM (JSM 6300V scanning electron microscopy, JEOL USA, Peabody, NY, USA) or

SEM (Hitachi S-4700 cold cathode field emission scanning electron microscopy) was used to visualize the particles. Laser diffraction (LD, Series 2600C Malvern Instruments, Malvern, UK) and Dynamic Light Scattering (Nicomp particle sizing systems, Autodilute^{PAT} Model 370 Santa Barbara, CA) were utilized to measure microparticle and nanoparticle size distributions, respectively. Thermal properties of isoxyl were measured by Differential Scanning Calorimetry (DSC, Perkin Elmer DSC 6, Waltham, MA).

3.3.2 Particle Manufacture

1) Particles Precipitated from the Injection Method: The preliminary screening of precipitation conditions was performed by injecting isoxyl (1 mg/ml) solution in ethanol into the aqueous phase manually at a speed of approximate 15 ml·min^{-1} during magnetic stirring using a 27 gauge sterile needle with a 10 ml disposable syringe. The tip of the needle was located just above the stir bar to achieve maximum mixing. Three volume ratios of solvent to antisolvent (VRSAs) were assessed, 1:1, 1:2, and 1:5. All the above procedures were conducted at room temperature. The resulting isoxyl particle suspensions were centrifuged (Beckman Model CS-15R) at 8000-9000 rpm for 10 min and then the supernatants were discarded. The pellets were transferred to the freeze-dryer (temperature < -50 ^{o}C and pressure < 0.05 millibar) and dried for 24 to 48 h.

2) Particles Precipitated from the Nozzle Mixing Method: Solvent and antisolvent at a VRSA equal to 1:5 were mixed by a 3-fluid nozzle. Isoxyl concentrations in ethanol, isopropanol, acetone and THF were 11 mg/ml, 7 mg/ml, 100 mg/ml and 100 mg/ml respectively. This was determined by solubility of isoxyl in these solvents. All the other

antisolvent precipitation conditions were kept constant: organic feed rate 0.2 ml/min, water feed rate 1 ml/min, nitrogen flow rate 400 L/min and atomization pressure 3 bar. Atomized fine droplets from the 0.7-mm diameter nozzle were sprayed directly into 50 ml quenching water. Dynamic light scattering was used to measure particle size distribution in the suspensions. The particles in suspension were collected by filtering through a membrane filter. The filters were completely dried in a vacuum desiccator and used immediately for SEM.

3) Spray-dried Particles from the Nozzle Mixing Method: The effects of processing parameters on particle characteristics were evaluated utilizing a 2^{7-4} factorial design. Eight manufacturing runs were conducted and randomized by statistical experimental design [Table 8]. For all the studies, the aspirator rate of the spray drier was kept at 90% of maximum flow (approximately 35 $m^3 \cdot h^{-1}$). Water and isoxyl solution in either isopropanol or ethanol were sprayed by the 3-fluid nozzle into a BUCHI B-191 mini spray-drier. The particles impacted on the bottom of cyclone (<8 cm from the bottom edge) and collecting vessel and were collected, and subsequently characterized.

Table 8. A 2^{7-4} factorial design for isoxyl particle preparations.

	Organic Solvent	Organic Feed (ml/min)	Water Feed (ml/min)	Isoxyl Conc. (mg/ml)	Inlet Temp (^oC)	N_2 Pressure (bar)	N_2 Flow (L/min)
1	Isopropanol	0.3	1.8	2	120	3	600
2	Ethanol	0.3	0.7	5	120	6	600
3	Isopropanol	0.3	0.7	2	100	6	800
4	Ethanol	0.7	1.8	2	100	6	600
5	Ethanol	0.3	1.8	5	100	3	800
6	Ethanol	0.7	0.7	2	120	3	800
7	Isopropanol	0.7	1.8	5	120	6	800
8	Isopropanol	0.7	0.7	5	100	3	600

In addition to alcohol, THF as solvent was also evaluated. Isoxyl was dissolved in THF at a concentration of 100 mg/ml and spray dried using the following condition: organic feed rate 0.5 ml/min, water feed rate 1.8 ml/min, atomization pressure 3 bar, nitrogen flow rate 600 L/min and aspirator rate 90% of maximum flow.

The use of the bulking agent, mannitol, was assessed. The isoxyl solution (7.2 mg/ml) of 4 ml and water were mixed in the 3-fluid nozzle under the following conditions: organic feed rate 0.3 ml/min, water feed rate 1.7 ml/min, nitrogen flow rate 400 L/min and atomization pressure 3 bar. The mixture was directly sprayed into 200 ml of mannitol solution (0.63 mg/ml). This resulted in the ratio of isoxyl to mannitol equal to 1:4.4. The dried particles were resuspended in water to dissolve mannitol. Particle size distributions were measured by dynamic light scattering. To visualize these particles, the suspension was filtered by a membrane filter. The filter was washed with a large quantity of water at least three times to remove residual mannitol and then dried in a vacuum dessicator to be ready for SEM.

3.3.3 Characterization

1) Particle size and morphology: The primary particle size, shape and surface morphology of the isoxyl particles were examined by SEM. The lyophilized particles and commercial isoxyl powders were employed as controls and directly applied to conductive double-sided tapes attached to aluminum stubs. The spray-dried particles are first suspended in water and sonicated for 30 seconds. Small drops of the particle suspensions were placed on the tape and dried in a vacuum desiccator. The filters containing nanoparticles were cut to fit and placed on the tapes. All the samples were sputter-coated using either a Polaron 5200

(Structure Probe Supplies, West Chester, PA) or a Cressington 108 with a Cressington MTM-10/10A high resolution film thickness monitor (Cressington Scientific Instruments, Cranberry, PA) with gold-palladium alloy and micrographs of the particles were taken. The primary particle size distributions and circularities were calculated by Image J program (Image Processing and Analysis in Java, created by NIH image for Macintosh). Here, the circularity indicates degree of particle elongation and a value approaching unity means that the particles are nearly spherical.

LD was used to measure microparticle size distribution, by volume. Briefly, the microparticles were suspended in water and sonicated for 1-2 min. The suspensions were added dropwise in a stirred sample cell containing distilled water until an obscuration level of about 10% to 20% were achieved. Nanosuspensions from precipitation or particle in mannitol matrix were redispersed in water and nanoparticle size distributions were measured by dynamic light scattering.

2) Thermal Properties of Spray-dried Microparticles: Thermal profiles of unprocessed isoxyl powder and a batch of spray-dried isoxyl microparticles (condition 8) were subjected to DSC, in which nitrogen at a flow rate of 30 ml/min was the purge gas. The samples were sealed in standard aluminum pans and heated from 21 ^{o}C to 250 ^{o}C at scanning rates of 40 ^{o}C/min and 5 ^{o}C/min.

3) Fine Particle Fraction and Emitted Dose: Aerosolization of the lyophilized isoxyl particles from the injection method, the spray-dried particles (condition 8), and control from commercial source were determined. Lactose carrier particles were added by mixing

isoxyl microparticles at a concentration of 1% w/w (Fischer Scientific hematology/chemistry mixer, model 346) for 10 min. Isoxyl powders of approximate 1 mg with or without lactose carrier were filled into the gelatin capsule (product size: #3, Capsugel, Reapack, NJ) and then placed in an Inhalator® (Boehringer Ingelheim). A twin-stage liquid impinger (TSLI) was employed to evaluate deposition of the isoxyl particles. The impinger (7 ml and 30 ml of ethanol were placed in the upper and the lower stages) was assembled to be airtight and vertical. After turning on the pump providing an air flow of 60 L/min, isoxyl powder was released from the inhalator. The pump was turned off 10 seconds after emission. After disassembling the apparatus, the capsule shell, the inhaler device, the mouth piece, the throat, and the upper and lower stages were all thoroughly washed with ethanol separately. When isoxyl was dissolved in ethanol, the absorption maximum wavelength was 273.5 nm. The concentration of isoxyl in each sample was determined by UV spectrometry at a wavelength of 273.5 nm based on standard curves (R^2>0.98). Several parameters of the deposition profile of each isoxyl powder were calculated: the emitted dose (ED) is the total drug mass leaving the device (the sum of the amount of isoxyl in the mouthpiece, the throat, and the two stages); the recovered dose (RD) includes ED and the amount in the capsule shells and the inhalator device; the fine particle dose (FPD) is the amount of isoxyl in the lower stage, which contains particles ($\leq$ 6.4 μm aerodynamic diameter); the fine particle fraction (FPF) is the ratio of the FPD to RD; the percent recovery is the ratio of RD to the expected dose; the dispersibility is the ratio of FPD to ED; and the percent emission is the ratio of ED to RD.[91]

3.4 RESULTS

3.4.1 Particle Size Distribution and Morphology

1) Particles Precipitated from the Injection Method: As shown in Figure 1 and Table 2, the commercial isoxyl powder consisted of either large needle-shaped agglomerates (lot # 130365-149120) or extremely long needle-shaped particles (lot # 165311-167669) with heterogeneous distributions. Depending on different lots, these particles may form agglomerates with diameters up to a few hundred microns. When isoxyl was precipitated by the injection method from the mixture of VRSA of 1:1, the particles were needle-shaped with count median diameter (CMD) of 3.3 μm in width and were more loosely aggregated compared to commercial isoxyl. The antisolvent precipitation when VRSA was equal to 1:2 generated both flake-shaped and needle-shaped particles. A VRSA value of 1:5 yielded elongated particles with a shortest dimension of 220 nm [Figure 7 and Table 9].

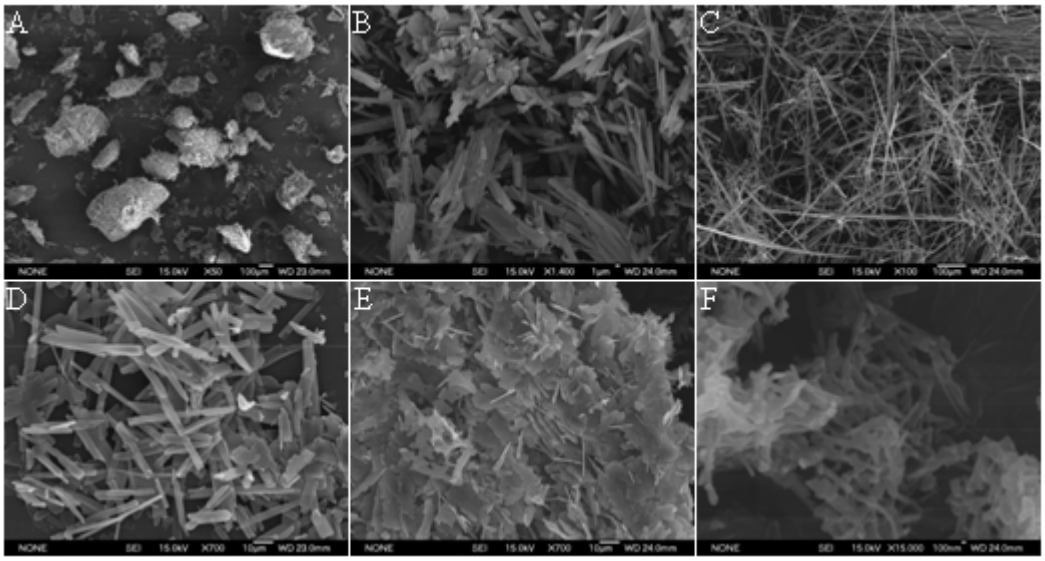

Figure 7. SEM photomicrographs of (A) commercial isoxyl (lot # 130365-149120) at 50× magnification, (B) commercial isoxyl (lot # 130365-149120) at 1400× magnification, (C) commercial isoxyl (lot # 165311-167669) at 100×magnification, and the isoxyl particles from antisolvent precipitation by injection method: (D) VRSA=1:1 at 700× magnification, (E) VRSA=1:2 at 700× magnification, and (F) VRSA=1:5 at 15000× magnification.

Table 9. The count median diameter (CMD) of the shortest dimension and the geometric standard deviation (GSD) of the elongated isoxyl particles in each powder.

		Unprocessed (lot # 130365-149120)	Unprocessed (lot # 165311-167669)	VRSA= 1:1	VRSA= 1:2	VRSA= 1:5
Width	**CMD (µm)**	1.47	3.95	3.30	0.88	0.22
	GSD	1.88	1.57	1.68	2.03	1.55
Length	**CMD (µm)**	8.2	178	17.0	N.D.	0.99
	GSD	1.90	2.48	1.91	N.D.	1.66

2) Particles Precipitated from the Nozzle Mixing Method: Depending on organic solvents, isoxyl particle size and morphology were different: ethanol generated nanofibers (width=385 nm, circularity=0.72); isopropanol and THF produced oblate spherical particles (width=275 nm and circularity=0.89 for isopropanol, width=220 nm and circularity=0.87 for THF); acetone yielded both nanofibers (width=200 nm, circularity=0.4) and oblate spherical nanoparticles (width=285 nm, circularity=0.98) [Figure 8 and Table 10]. Isopropanol was the first choice of solvent for further optimization of nanoparticle preparation because they produced most homogenous nanoparticles with a diameter <500 nm.

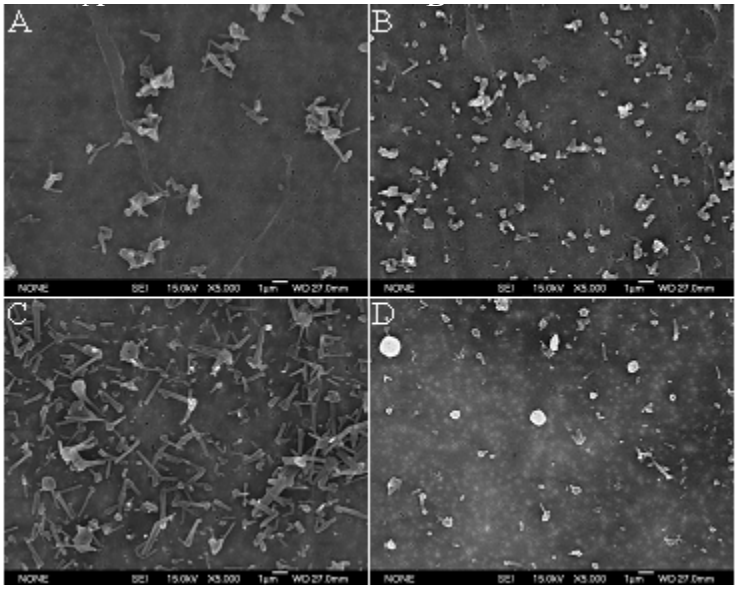

Figure 8. SEM micrographs of isoxyl particles precipitated from ethanol (A), isopropanol (B), acetone (C), and tetrahydrofuran (THF) (D). The micrographs were taken at 5000× magnification.

Table 10. Particle size distributions of isoxyl particles precipitated from various organic solvent.

		Ethanol	Isopro-panol	THF	Acetone	
					Nanofiber	Oblate Sphere
Circularity		0.72	0.89	0.87	0.40	0.98
Width	CMW (nm)	385	275	220	200	285
	GSD	1.5	1.47	1.76	1.44	1.3
Length	CML (nm)	1280	495	450	1190	390
	GSD	1.72	1.61	1.63	1.66	1.5

*PSD: particle size distribution, CMW: count median width, CML: count median length, GSD: geometric standard deviation

3) Spray-dried Particles from the Nozzle Mixing: The modified spray drier produced approximately spherical particles with count median diameters (Feret's diameter[92]) between 1.19- 1.77 μm in these eight conditions. The average circularity of the microparticles in each condition was approximate to unity and these from condition 8 were almost perfect spheres

[Figure 9A-9H and Table 11]. The particle size distributions, by LD, were unimodal for condition 5 to condition 8, bimodal for condition 2 and 4, and multimodal for condition 1 and 3. Condition 5 to condition 8 resulted in 100% of the particles having diameters < 5μm; condition 1 to condition 4 resulted in 70-80% < 5μm [Figure 10]. The yields of the spray drying conditions varied from 11.0 to 29.3% [Table 11]. Statistical experimental design and analysis showed that isoxyl concentration and nitrogen flow rate were two critical factors influencing the yield: an increase in isoxyl concentration and decrease in nitrogen flow rate resulted in greater yield. However, statistical analysis could not identify any key factors influencing particle size distribution and morphology [Figure 11]. When THF was evaluated as a solvent, this produced the undesirable outcome of much larger plate-shaped particles with heterodisperse PSD [Figure 9I]. Condition 8 appeared to achieve the desired high yield, spherical shape and narrow particle size distribution.

The addition of mannitol reduced the particle size. When the drug ratio to mannitol is 1:4.4, the mean count diameter of the isoxyl particles determined by DLS was 455 nm and standard deviation was 299 nm [Figure 12A]. However, SEM micrographs showed that these isoxyl particles were heterogeneous: some particles were almost spherical while the others were elongated [Figure 12B]. Further studies are required to produce nanoparticle aggregates with uniform shape and size.

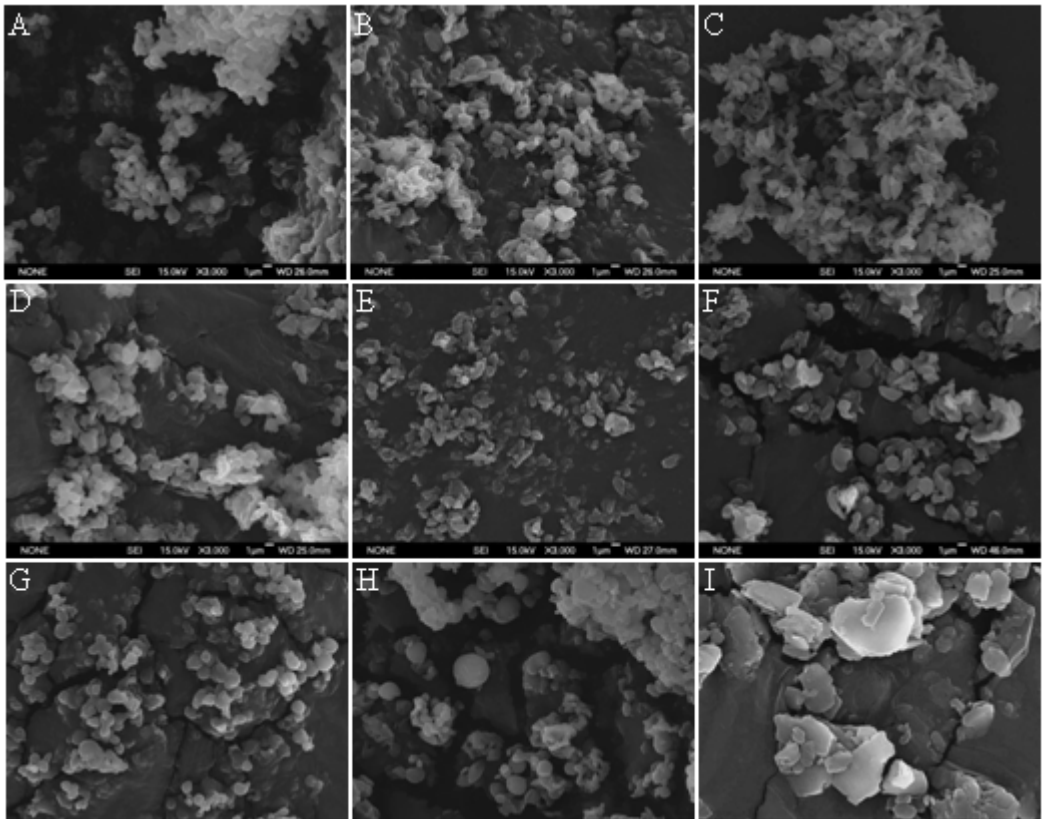

Figure 9. SEM photomicrographs of the spray-dried isoxyl particles produced by nozzle mixing of isoxyl solutions and water. A-H are the particles from conditions 1-8 respectively in Table 1 and I is those from using THF as solvent. The photomicrographs were taken at 3000× magnification.

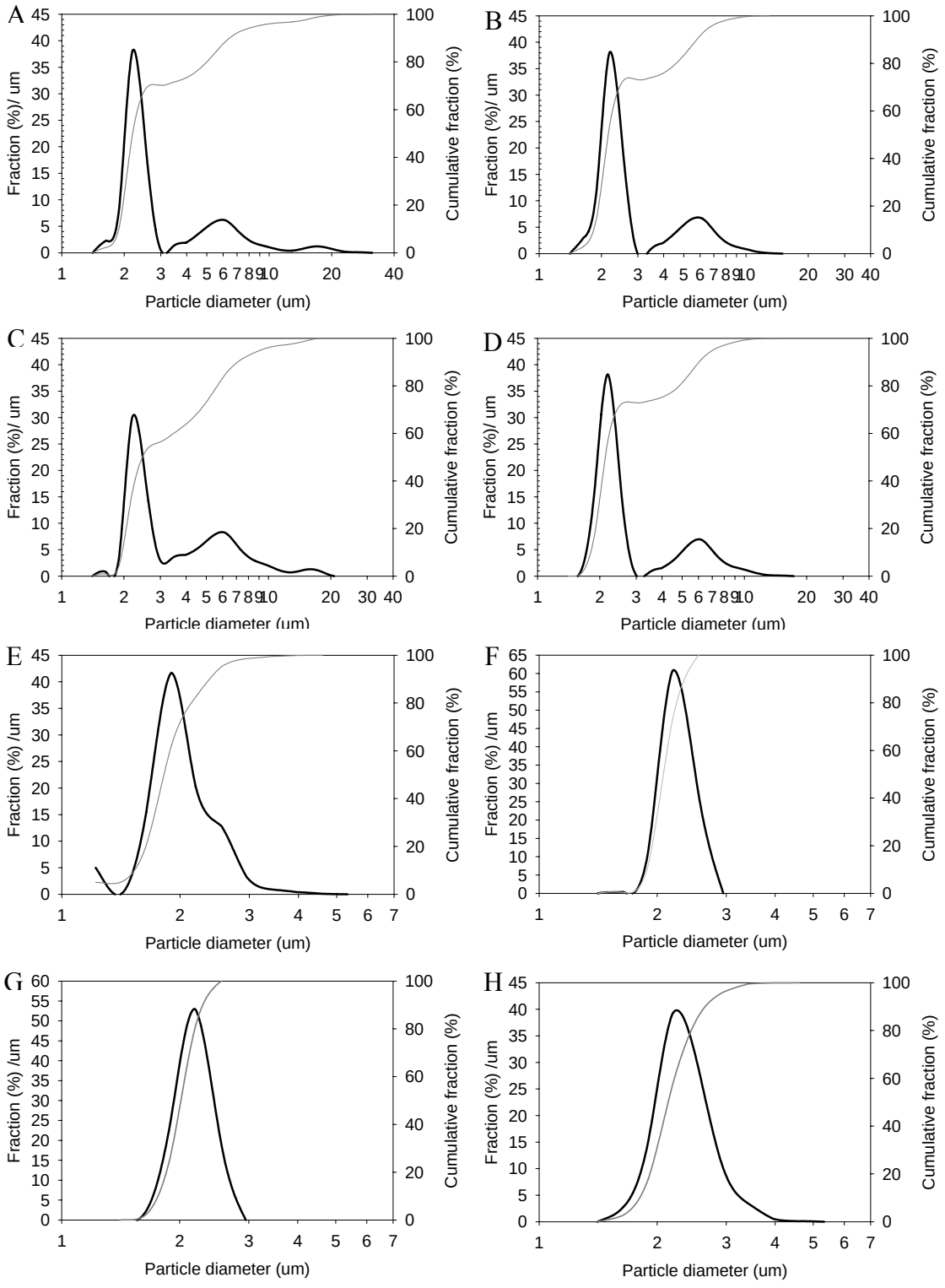

Figure 10. Particle size distributions of the isoxyl particles measured by laser diffraction (LD). A-H represent particles from spray drying condition 1-8.

Table 11. Summary of particle size distributions and circularities produced in the eight conditions.

		1	2	3	4	5	6	7	8
Yield		19.7	29.3	11	25	18.2	11.4	20	28.6
SEM	**CMD (µm)**	1.25	1.24	1.82	1.57	1.33	1.19	1.67	1.77
	GSD	2.01	1.58	1.75	1.34	1.21	1.26	1.47	1.48
	Circularity	0.95	0.93	0.91	0.95	0.91	0.95	0.95	0.98
LD	**$D_{v,10}$ (µm)**	1.88	1.84	1.98	1.81	1.54	1.86	1.77	1.81
	$D_{v,50}$ (µm)	2.20	2.16	2.44	2.13	1.81	2.09	2.02	2.14
	$D_{v,90}$ (µm)	6.43	5.78	7.09	6.05	2.36	2.32	2.28	2.67
	Span	2.06	1.82	2.09	1.98	0.45	0.22	0.26	0.4

*PSD: particle size distribution, CMD: count median diameter, GSD: geometric standard deviation, Circularity: $4\pi(\text{Area}/\text{Perimeter}^2)$, $D_{v,10}$: particle size at 10th percentile of the cumulative distribution, $D_{v,50}$: particle size at 50th percentile of the cumulative distribution, $D_{v,90}$: particle size at 90th percentile of the cumulative distribution, Span: $(D_{v,90} - D_{v,10})/ D_{v,50}$

3.4.2 Thermal Properties

At a scanning rate of 40 ^{o}C /min, the DSC thermogram of unprocessed isoxyl powder showed two peaks at 95.8 ^{o}C and 149.7 ^{o}C (ΔH=98.6 J/g) which corresponded to the boiling point of residual ethanol and melting point of isoxyl crystal form I respectively. Both two batches (lot # 130365-149120 and lot # 165311-167669) of unprocessed isoxyl powders contain the same crystal form of the drug. Isoxyl microparticles from anti-solvent precipitation and consequent spray drying exhibited only one peak at 143.5 ^{o}C (ΔH=76.3 J/g) corresponding to crystal form II [Figure 13A]. This may be explained by the processing conditions removing residual ethanol and converting isoxyl from form I to form II. For the scanning rate of 5 ^{o}C /min, the DSC thermogram revealed the same results except that the peaks of crystal form I, II and ethanol shifted to 140.5 ^{o}C, 136.2 ^{o}C and 80.2 ^{o}C [Figure 13B].

3.4.3 Fine Particle Fraction and Emitted Dose

1)Particles Precipitated from the Injection Method: The isoxyl particles, which were prepared by injecting isoxyl solution into water and sequentially lyophilizing, exhibited good emitted dose performance of 74.9 -76.0% and FPF of 8.8- 12.6% [Table 12].

2) Spray-dried Particles from the Nozzle Mixing: The aerodynamic particle size of the isoxyl microparticles produced in condition 8 was determined. The powder had an emitted dose of 94% and a low FPF of 5.4%. Therefore, cohesive forces of isoxyl microparticles were too high to achieve efficient de-aggregation during aerosol dispersion. Addition of lactose carrier increased FPF of the isoxyl particles to 8.4% [Table 13].

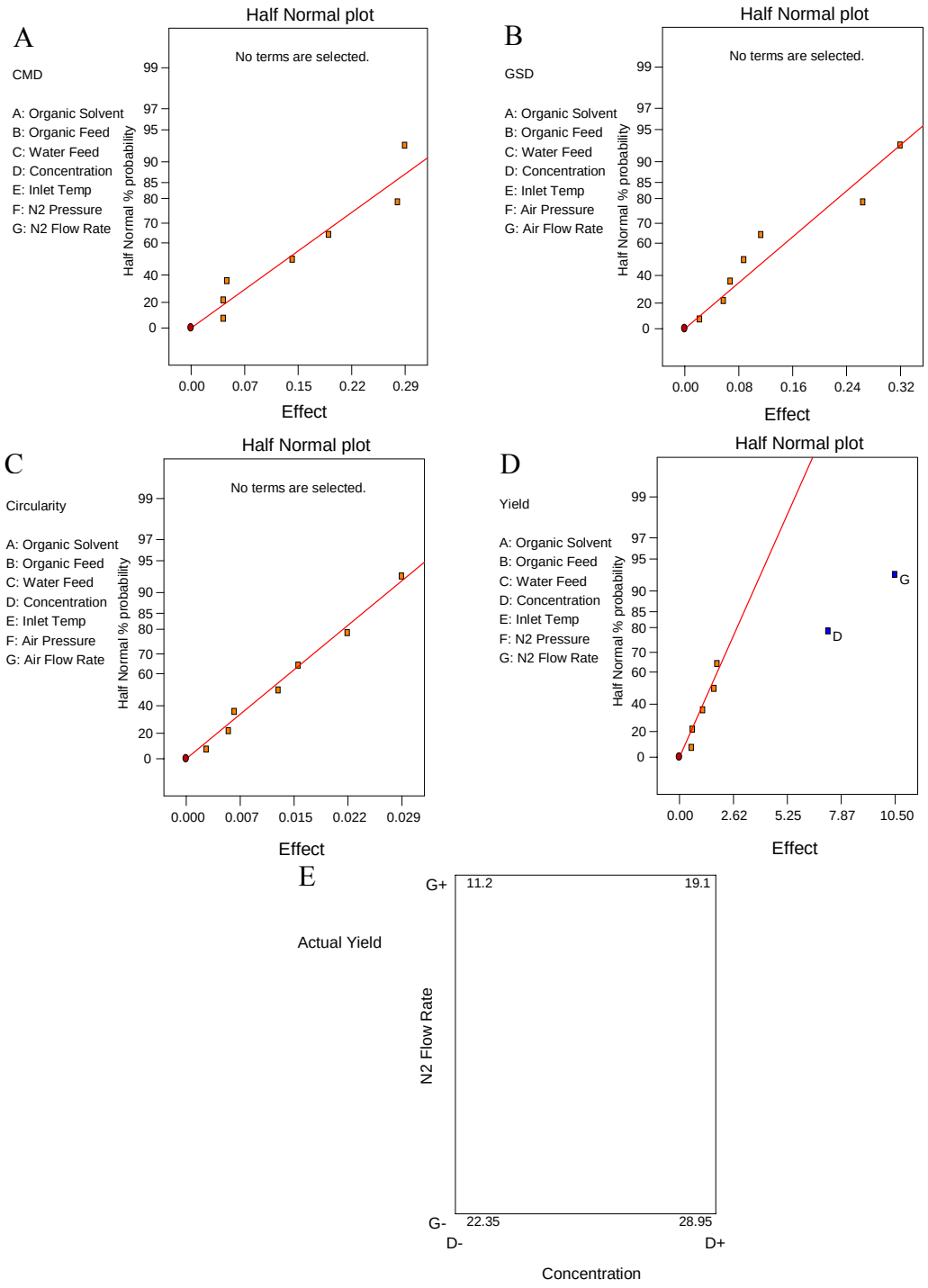

Figure 11. Half normal plot of effects of processing parameters on CMD (A), GSD (B), circularity (C), and yield (D) of spray-dried isoxyl particles. In the selected range, only yield was influenced by the processing parameters, N_2 flow rate (P=0.0025), and isoxyl concentration (P=0.0005). (E) represents square plot of effect of these two parameters on yield.

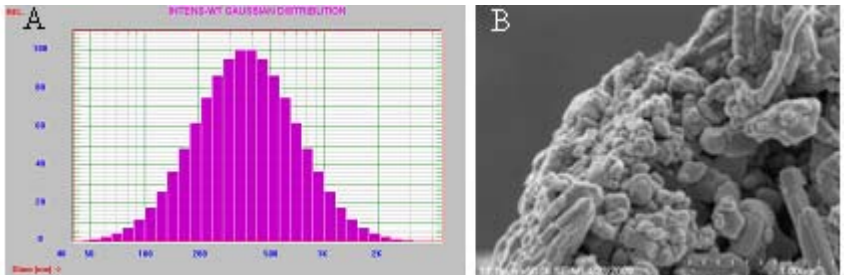

Figure 12. PSD (A) and SEM (B) of the spray dried isoxyl particles produced by addition of mannitol (isoxyl: mannitol=1:4.4).

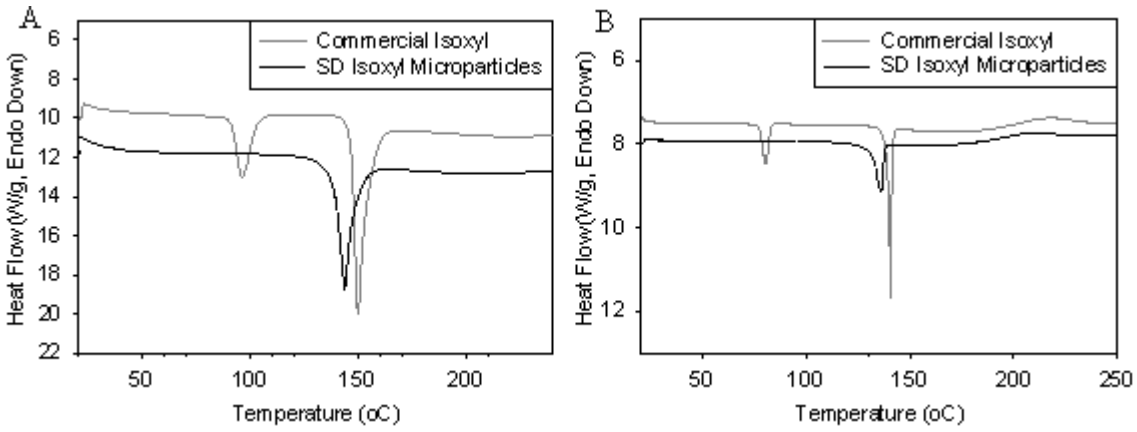

Figure 13. DSC thermograms of commercial isoxyl (gray) and isoxyl microparticles (black) prepared by spray drying using condition 8 (isopropanol feed rate 0.7 ml/min, water feed rate 0.7 ml/min, isoxyl concentration 5 mg/ml, inlet temperature 100 ^{o}C, atomization pressure 3 bar, nitrogen flow rate 600 L/min and aspirator rate 90% of maximum flow). The scanning rates were 40 ^{o}C/min (A) and 5 ^{o}C/min (B).

Table 12. The deposition profile of each commercial isoxyl and isoxyl particles precipitated from the injection method. (n=3)

	Unprocessed	**VRSA=1:1**	**VRSA=1:2**	**VRSA=1:5**
FPF (%)	6.94±2.34	8.76±2.61	12.57±3.35	9.23±1.65
Dispersibility (%)	8.84±3.32	10.52±4.20	16.63±3.97	12.26±2.90
Percent Emission (%)	78.46±2.97	74.90±6.44	75.43±5.74	76.03±5.20
Percent Recovery (%)	103.08±2.43	93.43±0.64	94.52±4.53	101.76±2.76

Table 13. The deposition profile of isoxyl microparticles in condition 8 (isopropanol feed rate 0.7 ml/min, water feed rate 0.7 ml/min, isoxyl concentration 5 mg/ml, inlet temperature 100 ^{o}C, atomization pressure 3 bar, nitrogen flow rate 600 L/min and aspirator rate 90% of maximum flow). (n=3)

	Microparticles w/o Lactose	Microparticles w/ Lactose
FPF (%)	5.4 ± 1.7	8.4 ± 1.6
Dispersibility (%)	5.8 ± 1.9	8.7 ± 1.7
Percent Emission (%)	94.2 ± 2.3	96.6 ± 0.2
Percent Recovery (%)	91.1 ± 3.1	97.4 ± 1.8

3.5 DISCUSSION AND FUTURE STUDIES

The commercial isoxyl consisted of either large fiber agglomerates or long fibers several hundred micrometers in length. Although the sizes of these fibers exceed the respiratory range, they could be inhaled, due to their unique aerodynamic properties, as indicated by FPF of 6.9%. The main reason is that aerodynamic diameters of the fibers are predominantly a function of their cross-section area diameter, or diameter of the smallest dimension. Despite relatively high delivery efficiency, these fibers are not suitable for inhalation therapy: It has been well established that toxicity of certain fibers depends on particle dimension and biopersistence instead of chemical properties. It was reported that occurrence of pleural sarcoma was high in rats when the fibers were more than 4 μm long.[93] One mechanism of fiber carcinogenesis is due to cell transformation. After these long fibers were phagocytized, they persist in the perinuclear region of macrophages and induce chromosomal mutations and consequently cell transformation.[94] Also, persistence of fibers in the lungs can cause cancer and fibrosis. Macrophages cannot take up fibers much longer than their cell dimension. Phagocytosis was incomplete when the rat alveolar macrophages were exposed to fiber of 17 μm in length.[95] The cut-off length of fibers causing frustrated phagocytosis by human alveolar macrophages was expected to be longer than 20 μm,

because alveolar macrophages in human (diameter: ~18 μm) are larger than rats (diameter: ~13 μm). [96] Incomplete phagocytosis leads to persisting of these insoluble fibers in the lungs. As shown in results, commercial isoxyl particles are so long that can be potentially persistent in the lungs and cause toxic effects. In the study, antisolvent precipitation was used to prepare isoxyl particles suitable for the lung delivery.

By simply injecting isoxyl solution into aqueous phase, the drug was precipitated out as needle-shaped particles. It was found that volume ratio of solvent to antisolvent (VRSA) was a critical factor controlling particle size. Keeping other parameters consistent, isoxyl particle size decreased from the micron to the nano range as the VRSAs were changed from 1:1 to 1:5. This is explained by increase in local supersaturation ratio (S) and retardance in particle growth kinetics. [97] Here, the local supersaturation is defined by the ratio of local drug concentration to the equilibrium solubility of drug in the mixture of solvent-antisolvent. After isoxyl ethanol solution was injected to water, solubility of the drug decreased much more in 16% ethanol (VRSA=1:5) than that in 50% ethanol (VRSA=1:1). This led to higher local supersaturation ratio in 16% ethanol and consequentially smaller nuclei. However, isoxyl nuclei were in a more dilute environment in 16% ethanol compared to 50%, which slowed down crystal growth. As a result, when VRSA was equal to 1:5, isoxyl particles were the smallest. In addition, the width of these particles was about 200 nm. This increased surface area dramatically in comparison to microsphere of the same volume, which can, in turn, reduce the residence time of particles in the lungs through rapid dissolution in the lung lining fluid. However, dissolution rate of isoxyl needs to be determined and *in-vivo* exposure study of isoxyl following pulmonary delivery remains to be performed to see whether the rapid dissolution is sufficient to achieve therapeutic isoxyl concentration systematically. If that is

not true, microparticle targeting to the infected cells may be advantageous with respect to the rapid dissolution of nanoparticles and, subsequent, elimination of drug from the lungs before tissue penetration for therapy can occur.

The nozzle mixing method was used to achieve uniform mixing that was hard to obtain by the manual injection method. The appropriate solvent was screened when adopting a VRSA of 1:5. Four organic solvents, ethanol, isopropanol, acetone and tetrahydrofuran (THF), were selected due to their low toxicity in trace quantities. As expected, physico-chemical properties of solvents were a key factor controlling particle size and crystal habit. One explanation for this observation is the difference between these solvents in degree of miscibility with water, a function of supersaturation. Better miscibility resulted in higher supersaturation and smaller particle size. Also, it has been reported that an increase in supersaturation tends to form elongated particles. [98] This may explain the greater production of elongated particles from ethanol or acetone than isopropanol or THF, because isopropanol is more hydrophobic than ethanol and acetone is completely miscible with water but THF is not. However, the effect of solvent can be complicated by other properties such as hydrogen bonding, dipole moment, dielectric constant, which facilitate crystal growth predominately in one dimension.[89] Isopropanol delivered the best product, oblate spherical nanoparticles <500 nm in length with most homogeneous distribution.

The precipitating particles can be simultaneously spray dried. The initial aim was to collect the precipitating nanoparticle in the form of aggregates. The preparation of isoxyl particles in the study consisted of two processes, antisolvent precipitation and simultaneous spray drying, which were controlled by seven main factors, type of solvent, organic feed rate, water feed rate, inlet temperature, drug concentration, nitrogen pressure and flow rate. Effects of these

factors on particle size were evaluated in a statistically designed experiment. However, isoxyl nanoparticle aggregates could not be obtained by simply varying these operating conditions since nano-sized precipitates agglomerate into microparticles in the process. It appeared that primary sizes of the particles were determined by amount of isoxyl in the atomized droplets independent of the precipitate size. Therefore, the median diameter of the spray-dried isoxyl particles can be predicted by the equation 5 [99]:

$$d_{particle} = d_{droplet} \sqrt[3]{\frac{C}{\rho_{particles}}} \qquad \textbf{[5]}$$

Here $d_{particle}$ and $d_{droplet}$ are the spray-dried particle diameter and the atomized droplet diameter. C is the concentration of isoxyl in the mixture of solvent and antisolvent and $\rho_{particle}$ is density of the particle. According to the information from the manufacturer (Buchi Labortechnik AG, Flawil, Switzerland), median volume diameters of the atomized aqueous droplets should be 10.48 μm when nitrogen flow rate is 830 L/h and 30.6 μm when nitrogen flow rate is 440 L/h. Assuming the median volume diameter of the atomized droplets is around 20 μm for 600 L//h and density of isoxyl is close to 1 μg/ml, the median diameter of resulting isoxyl particles is predicted to be between 0.92 to 2.7 μm, which is consistent with the experimental results. This also explains the production of larger microparticles when THF was used as solvent. Isoxyl has a higher solubility in THF than alcohols. Theoretically, during antisolvent precipitation process, smaller particles should precipitate due to an increasing local supersaturation. However, the result showed that final particle size of the spray-dried particles from THF was much larger than those from alcohols because a higher concentration of isoxyl was present in one atomized droplet during spray drying.

The spray drying method failed to produce isoxyl nanoparticle aggregates due to irreversible agglomeration which commonly occurs. The isoxyl nanoparticles can only exist

as separate particles in a dilute suspension. In order to conduct *in-vivo* disposition and efficacy studies, this diluted nanoparticle suspension can be made immediately prior to delivery by nebulizer. The spray-dried nanoparticle aggregates may be prepared by using appropriate surfactants and/or bulking agents. Theoretically, appropriate stabilizers can slow growth of the particles by condensation and coagulation, which reduces particle sizes. Stabilizers and bulking agents can also cover the precipitating particles and prevent them from agglomerating in the drying process and during a long-term storage. The bulking agent, mannitol, was evaluated in the study. Nanoparticle aggregates were produced but these particles were heterodisperse. Therefore, either mannitol itself or the amount of mannitol was not sufficient to prevent nanoparticle agglomeration. Further studies are needed to screen suitable surfactants or bulking agents for isoxyl nanoparticle preparation. The choice of surfactants and bulking agents are very limited for pulmonary delivery. Sodium oleate, sodium stearate, cetyl alchohol, tyloxapol, pluronics, lactose, mannitol, polyvinyl alcohol (PVA), polyethylene glycol (PEG), hydroxylpropylmethylcellulose (HPMC), may be considered, although not all of these are present in approved products. Some of them are present in FDA-approved inhalation products, while some of them have been studied extensively and are generally recognized as safe. Theoretically, 30-40% w/w of these stabilizing agents is required to keep nanoparticles with diameters between 120 to 300 nm separate.[100]

The crystalline/amorphous states influence many physicochemical properties of drugs such as saturation solubility, moisture sorption, particle stability.[83, 101] Isoxyl is known to exist in two different crystal forms. The needle crystals by crystallization from ethanol have higher melting point of 149 ^{o}C (ΔH=98 J/g) and lower aqueous solubility of 0.88 μg/ml than

these from hexane that have melting point of 141 ^{o}C (ΔH=77 J/g) and aqueous solubility of 17.8 μg/mL.[102] During synthesis, isoxyl was purified by crystallization of the drug from ethanol. [103] As expected, DSC profiles showed that the commercial isoxyl powder contains crystal form I and a large quantity of residual ethanol. In comparison, microparticle processing improved properties of isoxyl in the following ways: 1) Spray drying completely removed the residual ethanol; 2) conversion of isoxyl crystal form I to form II occurred. These phenomena contributed to expectations of much higher solubility. However, it remains to be established whether polymorph conversion occurred during precipitation from isopropanol/water or spray drying.

The needle-shaped isoxyl particles generated from the injection method and lyophilization exhibited higher fine particle fraction and lower emitted dose than those spherical particles from the nozzle mixing and spray drying. Theoretically, particle shape greatly impacts on their dispersion and aerodynamic behavior.[92, 104] Inspiratory flow fluidizes and deaggregates particles delivered for the Inhalator®, a passive dry powder inhaler. Dispersion of particles requires several processes: dilation, flow, fluidization and deaggregation. The interparticulate interaction between elongated particles is very unstable. After the elongated particles are dispersed, most of them line up along air streams. As a result, their aerodynamic diameters only depend on their shortest dimension and independent of their length. Particles that are inhaled to the respiratory tract will travel further and achieve better deposition in the deep lung than the spherical particles with the same geometric diameters.[91, 92, 104-106] Consequently, the elongated particles achieved high fine particle fraction due to apparently good dispersion and aerodynamic properties. However, the flow property of needle-shaped particles is worse than spherical particle. This caused that more

particles were adhered to the wall of capsule inside the device and resulted in lower emitted dose. The spray-dried isoxyl microparticles were delivered following blending with carrier lactose, which increased FPF from 5.4 to 8.4%. Further work is required to improve the performance of lactose blends with isoxyl.

3.6 CONCLUSION

Antisolvent precipitation in water was used to prepare isoxyl particles suitable for lung delivery. Nanoparticles with a count median width (CMW) of 275 nm and a count median length (CML) of 495 nm were produced using isopropanol as a solvent and a VRSA equal to 1:5. Spray drying failed to produce nanoparticles or their aggregates in dry form, but generated near spherical microparticles with a count median diameter (CMD) of 1-2 μm. Addition of mannitol in antisolvent at a drug to mannitol ration of 1:4.4 during processing produced isoxyl nanoparticle aggegregates. A range of microparticle and nanoparticle preparations have been evaluated. Further work on their delivery as aerosols is required to assess the potential for inhaled treatment of tuberculosis.

Chapter 4

In-vitro Cytotoxicity and Potency of Isoxyl Particles

4.1 INTRODUCTION

Isoxyl was believed to be a bacteriostatic agent. When tubercle bacilli were exposed to isoxyl at a concentration of 10 μg/ml for 4 days, the viable counts of bacilli decreased but started increasing after removal of the drug.[12] For bacteriostatic agents, they usually kill virulent microorganisms with the help of immune system.

Drug efficacy has been demonstrated in animal studies. Crowle et al. delivered three different doses of isoxyl in corn oil, 62.5 mg/kg, 125 mg/kg or 250 mg/kg, to the infected mouse to test dose-dependent drug effectiveness. A dose > 62.5 mg/kg protected the mouse against tuberculosis which was indicated by suppression of the increase in lung density.[81] As shown in chapter 1, addition of isoxyl to capreomycin, rifampicin, and ethambutol dramatically increased the survival rate of rabbits infected with *M. bovis*.[20] Konopka et al administered isoxyl to the infected guinea pigs and measured extent of disease in the lungs, liver and spleen by histopathologic studies. In comparison to controls, isoxyl demonstrated considerable antituberculosis activity.[82] However, the clinical trials performed in 1960s and 1970s showed that isoxyl had modest effect in monotherapy and combination therapy as shown in Table 14. Some clinical results were even contradictory. Stahle found that isoxyl clearly had good antituberculosis effect which was not significantly inferior to isoniazid (INH).[18] However, Olejnicek et al came to the conclusion that combination therapy of isoxyl and isoniazid was not better than monotherapy of isoniazid in advanced pulmonary tuberculosis based on number of patients who had a conversion of sputum and degree of INH-resistance development.[107]

Isoxyl has been reported to be non-toxic. When the drug was given to rats by the oral route at a daily dose up to 14 g/kg, much higher than therapeutic dose (50-100 mg/kg), for

1.5 years, it showed no effects on food consumption, reproduction potential, growth, renal and hepatic function, and hematopoietic systems, and it is not teratogenic, embryotoxic, and carcinogenic. The high dose only caused a slight increase in the weight of liver, level of alkaline phosphatases, and transitory increase in the weight of the thyroid gland, but no biological dysfunctions were detected.[18, 108]

Due to extremely low bioavailability of isoxyl, the previous *in-vivo* efficacy and toxicity studies of the drug deserve further investigation using *in-vitro* cell models, which allows for higher and more accurate dosing. Especially after a large dose of isoxyl is delivered directly to the lungs, most of the drug may target macrophages in the lungs. Further investigation is needed to assess the cytotoxicity and efficacy of the drug when administered to macrophages alone or coinfected with mycobacteria at higher concentration. This will help to determine the therapeutic window of the drug delivered as an aerosol. However, studies of efficacy and cytotoxicity of isoxyl using macrophages are rare. Phetsuksiri et al reported an *in-vitro* efficacy study of isoxyl using murine bone marrow macrophages and showed a dose-dependent bactericidal activities and killed intracellular bacilli with a 4-log-unit reduction at a concentration of 2.5 μg/ml. The Alamar Blue assay showed that isoxyl was not toxic at up to 2.0 μg/ml in isolated murine bone marrow macrophages. [13] In this study, differentiated THP-1 cells were employed as a macrophage model to evaluate potential of the isoxyl particles with different particle characteristics to be effective agents to eliminate intracellular TB without killing healthy macrophages.

Table 14. Summary of clinical results of isoxyl (table was modified from reference 24 and data from references 20, 109, 115 were added.)

Treatment (Daily Dose)	Time of Therapy (month)	Negative on Culture	X-ray improvement	Develop-ment of Resistance (drug)	Source
Isoxyl (6 g)	1.5-4.5	47% (15/32)	34% (11/32)	N.D.	Heidelbach et al.[109]
Isoxyl (10 g)	> 6	20% (4/20)	15% (3/20)	N.D.	Titscher[110]
Isoxyl (20 g)	3	45% (14/31)	84% (26/31)	N.D.	Dormer[111]
Isoxyl (6 g)	3-5	27% (4/15)	29% (6/21)	N.D.	Boszormenyi [21]
Isoxyl (6 g) plus INH (5 mg/kg)	3	ND	21% (4/19)	N.D.	Boszormenyi [21]
Isoxyl (6 g) plus INH (300 mg)	4	89% (34/38)	69% (22/32)	1	Hekking et al[22]
Isoxyl (6 g), INH (600 mg)	4	89% (32/38)	48% (15/31)	N.D.	Favez et al.[112]
Isoxyl (6 g), INH (10 mg/kg)	6	75% (15/20)	46% (6/13)	15% (INH)	Aksugur et al.[113]
Isoxyl (6 g), INH (300 mg)	6	38% (5/13)		69% (INH)	OLejnicek et al.[107]
Isoxyl (6 g), INH (5 mg/kg)	6	63% (17/27)	60% (16/27)	26% (INH)	Tousek[24]
Isoxyl (4 g), INH (200 mg)	6	40% (16/40)	95% (38/40)	10% (Isoxyl) 50% (INH)	Moodie. et al[114]
Isoxyl (4 g), SM (1 g), INH (200 mg)	6	83% (92/111)	60% (65/109)	8% (INH) 8% (SM)	Kass[115]
Isoxyl (5 g), PAS (12 g)	6	50% (18/36)		8% (Isoxyl) 3% (PAS)	Eule et al[116]
Isoxyl (6 g), INH (5 mg/kg), SM (1 g)	3-4	3 months: 86.5% (16/22) 4 months: 100% (22/22)	4 months: 72.5%	0	Nickling[117]

p-aminosalicylic acid (PAS), Pyrazinamide (PZA), INH: isoniazid, N.D.: not determined

4.2 RATIONALE AND EXPERIMENTAL DESIGN

THP-1 cells can be differentiated by PMA to macrophage-like cells. Factors including particle sizes, shapes, and composition can affect phagocytic activities of macrophages. [118] Spray-dried microparticles may be taken up effectively by macrophages while isoxyl solutions (controls) will transport isoxyl molecules through cell membrane. Since isoxyl is poorly soluble, controls were prepared by diluting concentrated isoxyl solution in dimethyl sulfoxide (DMSO) with cell culture media. It has been shown that DMSO < 2% would not kill mycobacteria. [13] Therefore, the final concentration of DMSO was 1% in the study. Isoxyl precipitates out to form particles during mixing isoxyl solution in DMSO at high concentrations with cell culture media. This will complicate the comparison between isoxyl microparticles and the controls. However, since characteristics of these precipitates from DMSO may be different from the spray-dried microparticles, this can lead to change in disposition of isoxyl in macrophages. Higher uptake of drug into macrophages may simultaneously increase killing of intracellular bacilli and deserves further investigation. Comparison between the spray-dried microparticles and controls of nanoparticles/molecules in 1% DMSO in terms of disposition, toxicity and efficacy may shed a light on this issue. In the present study, BCG, a model mycobacterium, was used to infect PMA-differentiated THP-1 cells. The infected cells were exposed to isoxyl microparticles suspensions and soluble/nanoparticle controls at a series of concentrations from 0.5 to 100 μg/ml, which were determined by the Minimum Inhibitory Concentration (MIC) reported previously. Dose-dependent and preparation-dependent effect on drug efficacy and toxicity were evaluated *in-vitro*.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Difco™ Middlebrook 7H10 agar and Difco™ Middlebrook 7H9 were purchased from BD. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and phorbol myristrate acetate (PMA) were purchased from Sigma. Triton X-100 was obtained from Fluka. Formalin (10%, Certified buffer), anhydrous isopropanol and the eight-well Lab-Tek™ II Chamber Slide™ Systems were purchased from Fisher Scientific. 10X ADS was made from 5% BSA, 0.2% dextrose and 0.81% NaCl. 7AGT media were prepared by 0.47% Difco™ Middlebrook 7H9, 0.5% Glycerol, 0.05% Tween 80 and 1X ADS.

4.3.2 Cell Culture and Bacteria Strains

THP-1 cells were purchased from American Type Culture Collection (ATCC) and grown in complete RPMI 1640 media (Cellgro) supplemented with 10% heat inactivated fetal bovine serum (Atlanta), 10 mM HEPES (cellgro), 1.0 mM sodium pyruvate (Gibco), 0.05 mM 2-mercaptoethanol (Gibco), 1X MEM non-Essential Amino Acids (Gibco).THP-1 cells were seeded on 98-well plate and differentiated into macrophage-like cells by treating with 50 ng/ml of PMA for 48 h, followed by washing with incomplete RPMI media (without FBS) three times to remove PMA. *Mycobacterium bovis,* Bacille Calmette-Guerin (BCG) was from Organon Teknika Corp, Durham, NC. BCG was grown in 7AGT media until OD_{600nm} reached log phase.

4.3.3 Preparation of Isoxyl Suspensions and Solutions

Isoxyl microparticles (condition 8) were prepared by antisolvent precipitation and simultaneous spray drying as described in Chapter 3. Isoxyl microparticles were accurately weighed and then suspended in complete RPMI media to make a concentration of 100 μg/ml. Concentrations of 0.5, 1, 2.5, 5, 10, 50 μg/ml were prepared by a serial dilution with complete RPMI 1640 media. As controls, isoxyl was dissolved in DMSO at concentrations of 0.05, 0.1, 0.25, 0.5, 1, 5, 10 mg/ml and then diluted by 100-fold with RPMI 1640 media. This resulted in the same mass drug concentrations as those of microparticles and DMSO solution with a final concentration of 1%. The spray-dried isoxyl microparticles suspended in 1% DMSO and isoxyl in 0.5 and 2% DMSO were also made as described above to study the effect of DMSO on drug penetration. Particles size of isoxyl precipitates made by mixing DMSO with media in the controls, were measured by DLS (Nicomp particle sizing systems, Autodilute^{PAT} Model 370 Santa Barbara, CA).

4.3.4 Minimum Inhibitory Concentration (MIC) determination

The agar plate dilution method was used to determine MIC of isoxyl, which was defined as the lowest drug concentration that inhibited colony growth on the agar plates. Agar plates were prepared with Difco^{TM} middlebrook 7H10 containing 1X ADS, 0.5% glycerol and a series of concentrations of isoxyl (0, 0.1, 0.5, 1, 2.5, 5, 10 μg/ml). A BCG suspension (2.2×10^5 cells/ml) was prepared. Three 10-fold dilutions of the suspension (10^{-1}, 10^{-2}, 10^{-3}) of 100 μl were plated on isoxyl-containing and isoxyl-free agar plates. The colony-forming units (CFUs) were counted 4 weeks after incubation of agar plates at 37 ^{o}C.

4.3.5 Cell Viability Assays

After the THP-1 cells were exposed to isoxyl microparticle suspensions or isoxyl in 1% DMSO in cell culture medium, the viability of these cells were assessed by their ability to reduce yellow MTT to purple formazan or level of LDH (lactose dehydrogenase) released to the cell culture media. Negative controls consisted of cells without exposure to isoxyl. The cell supernatants were collected and used to measure activities of LDH by using the commercially available kit from Sigma. Fresh media without phenol red and FBS of 100 µl and MTT (5 g/ml) of 10 µl were added to each well and the plates were incubated for 3.5 h. MTT solubilization solution (10% triton X-100, 0.1 N HCl in anhydrous isopropanol) of 100 µl was added to each well. The plates were then incubated overnight at 37 ^{o}C incubator to completely dissolve formazan crystals. Absorbance was measured by using Bio-Rad Model 3550 microplate reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 570 nm and background absorbance at 690 nm.

4.3.6 Preliminary Efficacy Studies

The PMA-differentiated THP-1 cells were cultured in the eight-well Lab-Tek^{TM} II Chamber Slide^{TM} Systems at a concentration of 2×10^{5} cells/well. BCG, which reached mid-exponential growth phase, was diluted in complete RPMI media and then added to the cell monolayer at a Multiplicity of Infection (MOI) of 1. After incubation at 37 ^{o}C for 4 h, the supernatants were discarded and the cells were washed with RPMI media three times to remove the intercellular bacteria. One slide was used to determine the initial infection at Day 0. Three wells of the slide were lysed with buffer (0.05% sodium dodecyl sulfate in PBS). Cell lysates were diluted three times (10^{-1}, 10^{-2}, 10^{-3}) and 111 µl of the dilutions were spread

on drug-free M7H10 agar plates. The supernatants were discarded and the slide was saved in 10% Formalin until analysis. For the rest slides, the BCG-infected THP-1 cells were exposed to a series of concentrations of isoxyl microparticles suspensions or controls of isoxyl in 1% DMSO or 1% DMSO (vehicle control) or blank media (untreated control). After 5-days of incubation at 37 ^{o}C, three wells of each slide were washed once by RPMI media, lysed by buffer (SDS 0.05% in PBS) and then plated. The colony-forming units (CFUs) of BCG were counted 4 weeks after incubation. The slides were saved in 10% Formalin until analysis. Slides stored in Formalin were washed, stained with auramine-rhodamine and visualized by microscopy to count the number of cells and intracellular bacilli.

4.4 RESULTS

4.4.1 Characterization of Isoxyl Particles in the Suspensions

As described in Chapter 3, microparticles produced by antisolvent precipitation and spray drying were approximately spherical and 1.77 μm in count median diameter, determined by microscopy. For the controls, nanoparticles with an intensity-weighted mean diameter of 100-200 nm, from photon correlation spectroscopy, were produced when isoxyl, at concentrations of 5 and 10 mg/ml, was mixed with cell culture media at a ratio of 1:100. The solubility of isoxyl in cell media containing 1% DMSO was ~2 μg/ml [Unpublished data]. Therefore, for isoxyl in 1% DMSO of 5 and 10 μg/ml, nanoparticles may be produced but the concentrations were too low to be detected by DLS. The forms of isoxyl available in 1% DMSO at various concentrations were listed in Table 15.

Table 15. The form of isoxyl available in 1% DMSO at various concentrations.

Conc. (μg/ml)	100	50	10	5	2.5	1	0.5
Dominant Form	NP	NP	N.D.	N.D.	M	M	M

NP: nanoparticles which were detected by DLS, N.D.: not determined, M: molecule

4.4.2 MIC Determination

No visible colony was detected when the isoxyl concentration was $\geq$ 2.5 μg/ml. The CFUs (colony forming units) of the plate containing 1 μg/ml Isoxyl in 1% DMSO were comparable to the control containing 1% DMSO [Table 16].

Table 16. Colony Forming Units (CFUs) on plates inoculated with BCG cells.

Isoxyl Conc. (μg/ml)	0	1.0	2.5	5	10
CFUs	127±38	119±33	0	0	0

4.4.3 Cytotoxicity of Isoxyl Microparticles and Nanoparticles

Isoxyl microparticles did not show any cytotoxic effect on the THP-1 cells one [Figure 14A] or five [Figure 15A] days following exposure. More than 90% of the cells were still viable when $\leq$ 500 μg/ml of isoxyl microparticles was tested. In comparison, the cells exposed to isoxyl in 1% DMSO at high concentrations (10-100 μg/ml) exhibited larger effects on cell viability, as estimated by MTT assay, one day following exposure, which were 50-70% of control [Figure 14A and 15A]. However, the mitochondrial dehydrogenase activity of the THP-1 cells recovered to around 100% of controls two days later [Figure 15A]. Positive controls lost most of their mitochondrial dehydrogenase activity (1.4-2.5% of control). DMSO is a well-known penetration enhancer and can play a role in the drug solution effects at day one. Several controls, isoxyl microparticles in 1% DMSO and isoxyl in 0.5-2% DMSO, were included to assess this possibility. Regardless of DMSO

concentration it appeared similar trends for a series of isoxyl concentrations, from which it was inferred that DMSO has no effect on penetration of the drug into the cells but isoxyl particle characteristics were important for uptake [Figure 14C]. Dipalmitoylphosphatidylcholine (DPPC), a lung surfactant, was employed to mimic the *in-vivo* environment and to aid in microparticle dispersion. As expected, DPPC at 0.05% did not affect viability of THP-1 cells, but seemed to activate the metabolic state of THP-1 cells as indicated by higher mitochondrial dehydrogenase activities than the controls (around 120%) [Figure 14A and 14B].

Levels of LDH leakage were determined as an indication of challenges to the integrity of the cells leading to cytoplasmic enzyme release. All the treatments including 10-100 µg/ml isoxyl in 1% DMSO did not release significantly greater LDH then the controls. However, the positive control resulted in sudden release of LDH [Figure 15B].

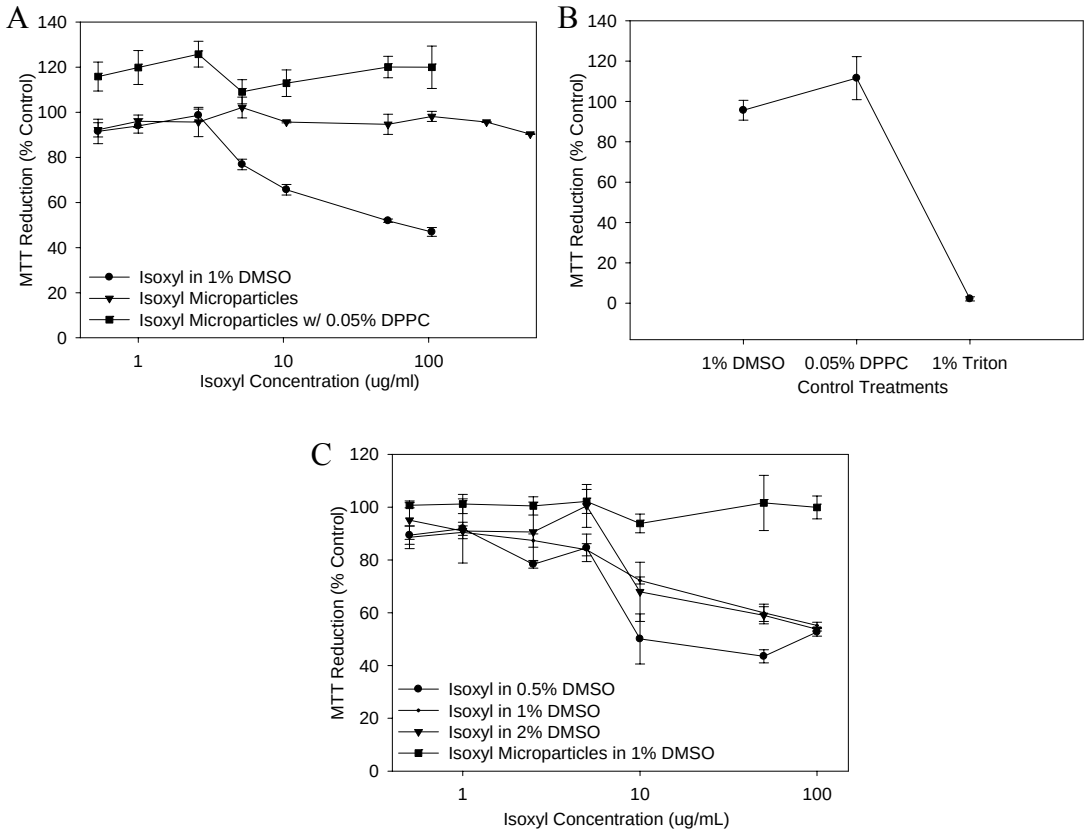

Figure 14. MTT reductions of THP-1 cells after they were exposed to isoxyl for 24 h. (A) Isoxyl in 1% DMSO, isoxyl microparticles, and isoxyl microparticles with 0.05% DPPC. (B) Controls of 1% DMSO, 0.05% DPPC and 1% Triton. (C) Isoxyl in 0.5%, 1%, 2% DMSO and isoxyl microparticles in 1% DMSO.

4.4.4 Preliminary Efficacy Studies

The bacterial burden expressed in colony forming units (CFUs) of the untreated control and 1% DMSO treated cells increased 2.6- and 3.3-fold five days after incubation, but those of isoxyl at concentrations of 0.5 to 2.5 μg/ml in 1% DMSO were unchanged. This may be explained by isoxyl being bacteriostatic at concentration between 0.5 to 2.5 μg/ml. When the drug concentration increased to 5 μg/ml, a 3.9-fold reduction in bacterial count was observed in comparison to Day 0 and a 10.3-fold reduction in comparison to untreated control [Figure 16A]. The microscopy study also revealed antituberculosis effect of isoxyl [Figure 17]. For

isoxyl microparticles suspended in cell culture media, concentrations $\geq$ 5 µg/ml showed bactericidal activity. When isoxyl microparticle concentration was equal to 5 µg/ml, bacterial count reduced by 2.8-fold in comparison to Day 0 [Figure 16B].

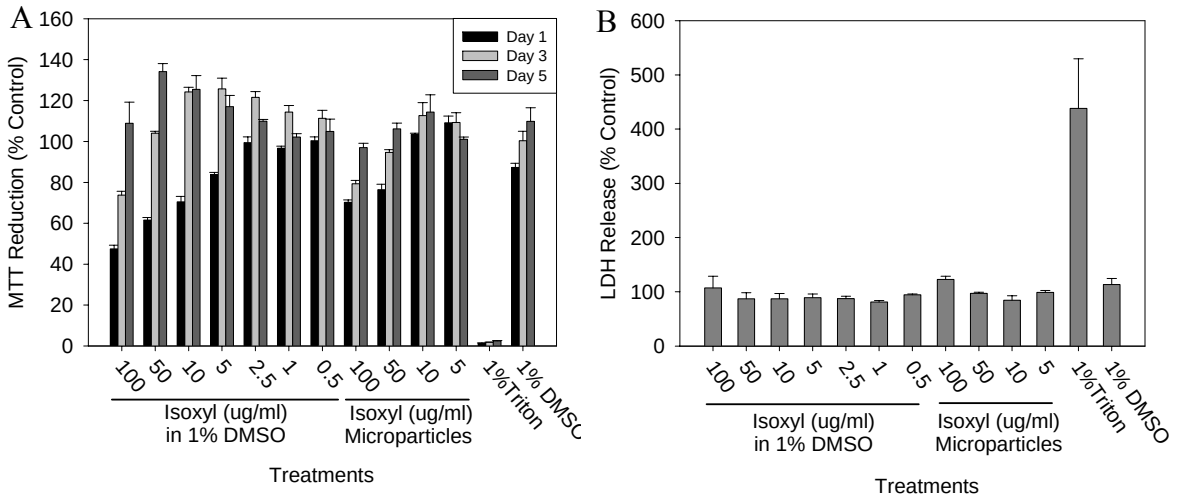

Figure 15. (A) The effect of isoxyl in 1% DMSO or isoxyl microparticles at a series of concentrations on THP-1 cells determined by (A) MTT assays and (B) LDH assays.

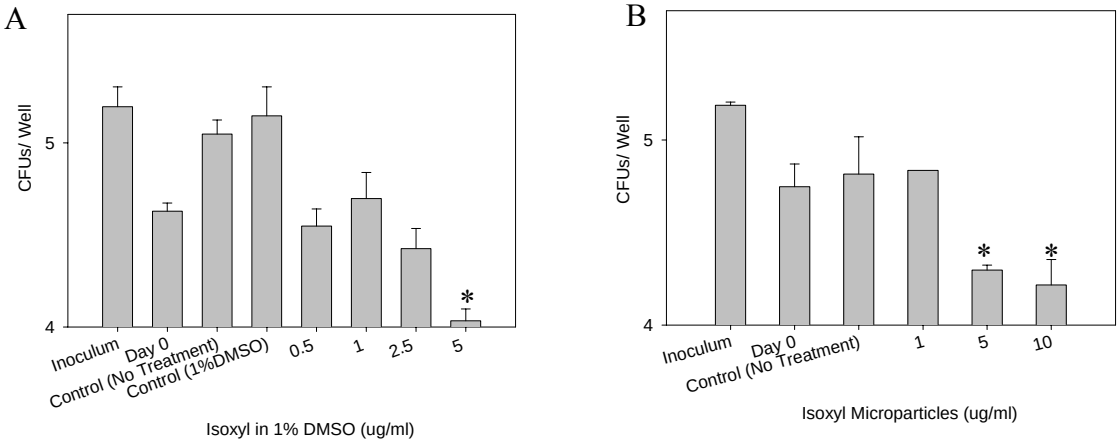

Figure 16. *In-vitro* efficacy of isoxyl in 1% DMSO (A) and isoxyl microparticles (B) determined by CFUs. *Bacterial count of the treatment was statistically different from that of Day 0 ($P<0.05$, Dunnett's t test was performed).

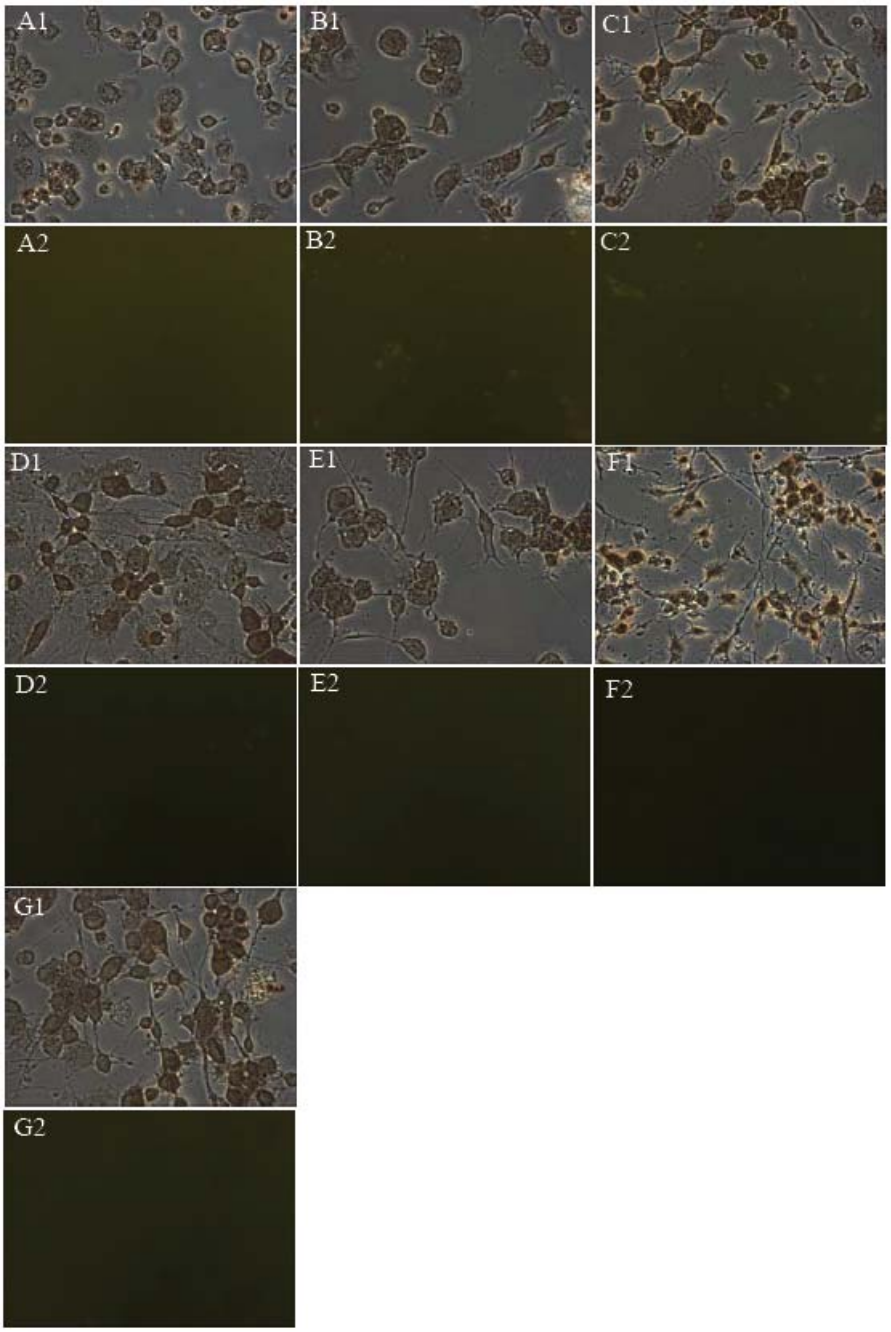

Figure 17. *In-vitro* Efficacy of Isoxyl in 1% DMSO. A1-G1 were taken by phase contrast microscopy to show the infected THP-1 cells and A2-G2 were the same areas examined by Fluroscein-isothiocyanate (FITC) fluorescent microscopy to exhibit intracellular BCG. (A1) and (A2): Day 0; (B1) and (B2): Control (no treatment, day 5); (C1) and (C2): Control (1% DMSO, day 5); (D1) and (D2): Isoxyl (0.5 µg/ml, day 5); (E1) and (E2): Isoxyl (1 µg/ml, day 5); (F1) and (F2): Isoxyl (2.5 µg/ml, day 5); (G1) and (G2): Isoxyl (5 µg/ml, day 5).

4.5 DISCUSSION

The poor solubility of isoxyl limits its use as a therapeutic agent for treatment of MDR strains of tuberculosis following oral route of administration. Isoxyl microparticles, 1-2 μm in median diameter, which may be delivered as aerosols from a dry power inhaler (DPI) directly to the lungs of infected patients, can be prepared by antisolvent precipitation and simultaneous spray drying as described in Chapter 3.[119] Direct delivery of these particles to the lungs will not only result in high local concentrations but may also target alveolar macrophages, the host cells of TB bacilli. It has been reported that particles with diameters of 500 nm to 3μm are efficiently phagocytized when these particles encounter alveolar macrophages.[42-44, 51] Also, phagocytosis of hydrophobic nanoparticles decreases with decreasing size.[45] Therefore, isoxyl microparticles, nanoparticles and solubilized drug (molecules) would be expected to penetrate different sites in the lungs.

Two cytotoxicity studies were performed. MTT assays measured the mitochondrial dehydrogenase activity of viable cells. Interference with the enzyme or cell death decreases the activity of the dehydrogenase as indicated by reduction of methylthiazol tetrazolium (MTT) to coloured formazam. This phenomenon was seen after THP-1 cells were exposed to isoxyl nanoparticles which were produced by mixing the drug solution in DMSO with cell culture media for one day. However, the enzyme activity recovered after the cells exposed to the nanoparticles for an extended period of time. This implied the isoxyl nanoparticles interfered with activity of the mitochondrial dehydrogenase, but the effect was tolerated by the viable cells subsequently. Lactose dehydrogenase (LDH) bioassays also showed that isoxyl, in any form, did not kill the cells. LDH is a stable cytoplasmic enzyme. For a certain culture conditions, the LDH release per cell is constant, but lysis of the cells increases LDH

leakage. When the cells were treated with isoxyl nanoparticles and microparticles at the concentrations $\leq$ 100 μg/ml, LDH level were equivalent to untreated control. In contrast, positive controls had 4-fold increase in LDH leakage in comparison to the untreated controls. The isoxyl in either form did not impair the integrity of the cells as estimated by LDH release. This is further indication that isoxyl microparticles and nanoparticles were not cytotoxic.

The results of the MTT assay suggest that isoxyl interferes with the enzyme to a greater extent when delivered as nanoparticles compared to microparticles but it is not clear that this results in loss of viability. This seems to conflict with the previous reports where microparticles appear to be more effectively phagocytized by macrophages than nanoparticles. However, this is not surprising, because phagocytosis efficiency is associated with not only primary particle size but also other particle characteristics including particle aggregation, shape, charge, crystalline structure, surface and dispersion properties. Isoxyl microparticles are spherical and contain crystal form II.[119] The shape and polymorphism of the isoxyl nanoparticles precipitated from DMSO requires investigation. It is known that isoxyl can occur in crystal form I if it is precipitated from ethanol.[102] Also, dispersion of particles in cell culture media can play an important role. Treatment of 50 and 100 μg/ml isoxyl microparticles provided ratio of particles to cells about 6:1 and 12:1 respectively. However, microscopic examination demonstrated that the isoxyl microparticles formed aggregates in the media. These aggregates can constrain uptake to a limited number of cells. Large aggregates may avoid macrophage uptake altogether. However, the nanoparticles were made freshly in cell culture, so they may attach to macrophages immediately as individual particles or form aggregates in microparticle sizes suitable for rapid uptake. Consequently, the cells were exposed to higher number concentration of the isoxyl nanoparticles than the

microparticles when the mass doses were the same. Therefore, care should be taken when it comes to a conclusion that primary particle size is a key factor for macrophage phagocytosis before studying particle behavior leading to secondary structures in cell culture media.

Higher intracellular drug levels delivered by the nanoparticles compared to microparticles would be expected to lead to improved efficacy, in treating infected cells. However, this was not the case. The preliminary *in-vitro* studies showed that both isoxyl microparticles and isoxyl presented in 1% DMSO had bactericidal activity against intracellular BCG at a concentration of 5 μg/ml. These particles will be employed in future to carry out more *in-vitro* efficacy studies and as possible treatment for tuberculosis in an infected guinea pig model [120].

4.6 CONCLUSIONS

Isoxyl ≥ 2.5 μg/ml inhibited growth of BCG completely on agar plates and ≥ 5 μg/ml showed bactericidal activity to intracellular BCG. Isoxyl nanoparticles precipitated from mixing DMSO with cell culture media may result in the transport of drug more effectively into the THP1 phagocytic cells than micropraticles as indicated, indirectly, by MTT assays, but this did not produce higher cytotoxicity or efficacy in the infected cell model. Further *in-vitro* work is required prior to conducting efficacy studies in animal model of tuberculosis.

Chapter 5

Conclusions and Future Studies

Tuberculosis remains a serious infectious disease resulting in the death of more than two million people annually. Shockingly, the incidence of multiple drug resistance to the first and second line agents employed to treat tuberculosis is increasing globally. New drugs are desperately needed to respond to this major challenge to public health internationally. Assessment of drugs that have previously shown some promise would help in this endeavor.

Isoxyl is an effective drug to treat MDR tuberculosis (MIC: 1-10 μg/ml for various resistant strains). However, the efficacy of the drug has been questioned due to failure of some clinical trials. Due to absence of a reliable method to quantify drug concentrations in the biological samples, the exact mechanism of poor *in-vivo* behavior of the drug was not elucidated. In specific aim 1 a quantitative analytical method was developed and validated for determination of concentrations of isoxyl in plasma following i.v. and oral administrations. The drug was extracted with acetonitrile from guinea pig plasma and quantified by a Hewlett Packard 1100 series HPLC coupled with a Spherisorb 5μm ODS2 (2×100 mm) column and UV detection at 270 nm. The mobile phase was 70% ACN in 20 mM ammonium acetate buffer. Isoxyl peak was eluted at 4.8 min with no interference with the peaks of impurities from plasma and internal standard. According to FDA guidance, the HPLC method was sensitive, reproducible, and accurate for quantification of isoxyl in guinea pig plasma. The preliminary *in-vivo* study of isoxyl showed that two different doses of i.v. administration resulted in the same pharmacokinetic profiles. This implied that drug precipitation might occur near to the site of injection due to poor solubility of the drug. More replicates are required to be conducted to confirm the conclusion and to identify the site of precipitation. Oral administration of therapeutic dose of isoxyl could not approach minimum inhibitory concentration (MIC) in plasma. There are two plausible reasons for this result: 1)

Insolubility of the drug resulted in poor dissolution in the gastrointestinal tracts and malabsorption; and 2) in comparison to the previous reports that detected much higher drug levels in serum using microbiological and radiochemical methods, this suggested that isoxyl may be transformed to active metabolites following oral administration. Therefore, either poor solubility or highly variable drug metabolism may contribute to unsatisfactory clinical outcomes. Delivery of isoxyl to the lungs, a major site of *Mycobacterium tuberculosis* (MTB) infection, may rescue this abandoned drug through increasing local effect.

To further study the drug levels in plasma, slower infusion of isoxyl to guinea pigs will be performed to obtain pharmacokinetic parameters. This experiment may not be necessary if isoxyl plasma concentration do not achieve therapeutic levels even after inhalation of the drug to the lungs. In this scenario, obtaining high local drug concentration would be the primary aim. The drug will be delivered by insufflator or nebulizer to guinea pigs which will then be sacrificed at various time points. Isoxyl concentrations in the lungs of these animals should be determined. These results will be compared to those from the animals who received isoxyl by oral and i.v. administration. If the local drug concentration can not achieve the therapeutic range in the lungs by pulmonary delivery of isoxyl, metabolism of the drug might be a serious problem. Further investigation of isoxyl metabolism using liver and lung microsomes can be carried out to identify the metabolites, and if they are detected, this may permit deducing the role of metabolizing enzymes involved in the process. Also, isoxyl can be incubated with lung and liver miscrosomes and the metabolites can be isolated for the future toxicological and efficacy studies. Study of isoxyl metabolism may allow identification of better anti-tuberculosis drug candidates.

Specific aim 2 was to optimize particle processing conditions to prepare isoxyl particles suitable for pulmonary delivery. Isoxyl particles for pulmonary delivery were prepared by anti-solvent precipitation. Nano-fibers with a width of 200 nm were obtained by injecting isoxyl solution in ethanol to water at a volume ratio of solvent to antisolvent (VRSA) of 1:5. Based on this preliminary result, a well-controlled method, involving nozzle mixing, was employed to prepare isoxyl particles. All the particles were 200 to 400 nm in width but had different lengths depending on properties of the solvents. However, generating these nanoparticles by simultaneous spray drying produced isoxyl microparticles (1.19- 1.77 μm) with no discernible nanoparticle substructure. The processing parameters did not have significant effect on particle size. The primary reason is that nanoparticles agglomerated during the drying process and the final particle size is only dependent on the amount of the drug in the atomized droplet. Addition of the bulking agent, mannitol, helped to prevent these nanoparticles from agglomeration in the process and resulted in nanoparticle aggregates in micron sized superstructures. Future study is necessary to increase homogeneity of the nanoparticles by applying appropriate amount of mannitol or other excipients as discussed in Chapter 3. Aggregation of the spray-dried microparticles resulted in relatively low fine particle fractions. This can be improved by employing different carriers, for example lactose, mannitol or trehalose.[121-123] Also, modifying morphology and surface of these carriers can achieve optimized adhesion forces between drug particles and carriers which allow effective separation of microparticles from aggregates during blending and easy detachment of these microparticles from carriers after aerosolization.[106, 124-132] This may further enhance dry powder inhaler (DPI) performance.

The previous *in-vivo* reports of toxicity and efficacy of isoxyl were not reliable due to poor absorption of the drug. However, the *in-vitro* studies of the drug were very scarce. Specific aim 3 was to assess cytotoxicity of isoxyl particles on human macrophages, and the efficacy of these particles on intracellular growth of a model mycobacterium, BCG, in these phagocytic cells. In the study, THP-1 cells were exposed to a series of concentrations of isoxyl in the forms of either microparticles or nanoparticles/molecules. MTT (methylthiazol tetrazolium) and LDH (lactose dehydrogenase) assays were utilized to test cytotoxicity of the drug. Microparticles and nanoparticles of concentrations up to 100 μg/ml were not toxic to macrophages, but nanoparticles seemed to deliver more drug to the cells because they reduced MTT more than controls and microparticles. Preliminary studies of the *in-vitro* efficacy were performed by incubating the infected THP-1 cells with mycobacteria. The results suggested that different forms of isoxyl seem not have changed in *in-vitro* efficacy to kill tuberculosis bacteria inside the cells. Both isoxyl microparticle suspension and isoxyl in 1% DMSO showed bactericidal activity at a concentration of 5 μg/ml. In the future, the study of *in-vitro* drug efficacy will be repeated and additional drug concentrations will be tested. Also, bronchoalveolar lavage cells (BALs) from guinea pigs will be used to evaluate *in-vitro* cytotoxicity and efficacy of these isoxyl microparticles and nanoparticle/molecule controls again. The results will be compared with those obtained from THP-1 cells and for subsequent comparison with *in-vivo* data. Even though isoxyl microparticles might not be different from nanoparticles/molecules in terms of *in-vitro* efficacy, *in-vivo* studies of both forms of isoxyl are necessary in the future. Particles delivered to the lungs can have very different disposition *in-vivo* due to: 1) The hydrophobic environment of the lungs may increase the rate of dissolution; 2) there are different components of the lungs, such as lung surfactants, in

comparison to cell culture media which may facilitate or prevent phagocytosis; 3) heterogeneous macrophages in the lungs can have variable functions; and 4) availability of other cells such as epithelial cells may eliminate the drug via other pathways.

Modern analytical and drug delivery techniques were employed to resurrect the prospects of isoxyl, an antitubercular drug, as an effective therapeutic agent. Establishing particle manufacturing processes, *in-vitro* effect, in the absence of cytotoxicity, and analytical methods in biological fluids are prerequisites for conducting efficacy experiments in infected animal models as a precursor to regulated clinical studies. The methods described in this thesis form the basis for future work on the treatment of resistant tuberculosis lung infection by isoxyl or other analogs within this class of drugs.

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