

CHAPTER 5- Pharmacokinetic/Pharmacodynamic analysis of novel PLGA nanoparticles encapsulating four first-line anti-tuberculosis drugs

Abbreviations

6-ANA - 6-aminonicotinic acid

DF- dilution factor

ETB - ethambutol

INH - isoniazid

LCMS - liquid chromatography mass spectrometry

LLOD- lower limits of detection

LLOQ- lower limits of quantification

MeOH - methanol

MBC - minimum bactericidal concentration

MIC - minimum inhibitory concentration

MRM - multiple reaction monitoring

M.tb - *Mycobacterium tuberculosis*

OADC - oleic acid-albumin-dextrose-catalase

OD₆₀₀ - optical density at 600 nm

PDI - polydispersity index

PD - pharmacodynamics

PK - pharmacokinetics

PLGA - poly-(DL)-lactic-co-glycolic acid

PZA - pyrazinamide

RIF- rifampicin

Rb- rifabutin

TB- tuberculosis

5.1 Introduction

The challenges with current tuberculosis (TB) therapy and the potential for nanoparticulate drug delivery to address these challenges have been discussed previously (Chapter 1). It has been hypothesised that the size and physicochemical properties of nanoparticles may improve bioavailability of the drugs by improving drug absorption (Ahmad *et al.* 2006:415) facilitate transport through biological barriers (Lockman *et al.* 2002:1) as well as enhance uptake of poorly soluble drugs (Kipp 2004:110). The largest knowledge contribution towards understanding the potential of nanoparticle-based drug delivery systems in TB chemotherapy has been the research group of Prof G.K. Khuller (Pandey *et al.* 2003a; Pandey *et al.* 2003b; Sharma *et al.* 2004a; Sharma *et al.* 2004b; Pandey & Khuller 2005; Pandey *et al.* 2005a; Pandey *et al.* 2005b; Ahmad *et al.* 2006; Pandey & Khuller 2006; Ahmad *et al.* 2007; Pandey & Khuller 2007; Ahmad *et al.* 2008). These are summarised in Table 5.1. The very important consideration that stemmed from these studies and others (Langer *et al.* 1996; Lamprecht *et al.* 1999; Kisich *et al.* 2007; du Toit *et al.* 2008; Jin *et al.* 2008) was that each nanoparticle formulation was unique and often demonstrated unique results.

The data presented in Chapters 3 and 4 demonstrated the advantages of PLGA nanoparticles in terms of cellular internalization at the specific size and physical chemical properties without an acute immune reaction. Furthermore, it was also confirmed that these nanoparticles are able to traverse biological barriers in the biodistribution data generated. However, the PK/PD of the nanoparticles encapsulating drugs has not been evaluated *in vivo*. Therefore, a detailed PK/PD study was warranted to try to elucidate the drug delivery kinetics of these nanoparticles.

As discussed previously in Chapter 2, various formulation parameters such as molecular weight, crystallinity, zeta potential, surface hydrophobicity, particle size, porosity, surface area and excipient characteristics all contribute to the drug delivery kinetics of nanoparticles. The extensive results summarised in Table 5.1 all supports the hypothesised promise of nanotechnology for drug delivery, especially in the field of TB chemotherapy. However, the one common denominator in the formulations listed below is the method of manufacture, namely solvent evaporation by means of freeze drying. This is a time consuming process which takes a number of days to produce a single batch thus presenting possible scaling up limitations in attaining similar nanoparticle characteristics. The method of solvent

evaporation by means of spray-drying is much more efficient, requires a few hours to produce a batch, produces spherical particles with a low polydispersity index and are generally smaller in size compared to freeze-dried nanoparticles. This has been previously described in Chapter 2.

Table 5.1 Studies conducted to show potential efficacy of nanoparticles in tuberculosis chemotherapy. EE-encapsulation efficiency; []- concentration; cfu- colony forming unit; SGF-simulated gastric fluid; SIF- simulated intestinal fluid; IM- intramuscular; SLN- solid lipid nanoparticles

Route of administration	Chemotherapeutic efficacy	Results	Conclusions/assumptions	Ref.
Oral PLG nanoparticles encapsulating with INH, RIF and PZA in murine tuberculosis	Administration once over 10 days showed no <i>M.tb</i> growth for 25 days. No evidence of biochemical hepatotoxicity	<u>Characterization</u> : Size:186-290nm, EE=56.99-68.02% <u>In vitro studies</u> : <1% drug for up to 42 days <u>In vivo studies</u> : Plasma [] from hrs. onwards for up to 9 days. Free drugs only detected for up to 24hrs. Drug detected in the tissues from day 7 to day 11	The first to report on an extensive <i>in vivo</i> study and concludes the therapeutic potential of oral dosing every 10 days	(Pandey, Zahoor, Sharma, & Khuller 2003b)
Inhalable PLG nanoparticles encapsulating INH, RIF and PZA. <i>In vivo</i> in guinea pigs against experimental tuberculosis	Administration once over 10 days showed no detectable cfu counts in the lungs of guinea pigs. No evidence of biochemical hepatotoxicity	<u>Characterization</u> : Size:186-290nm, EE=56.99-68.02% <u>Aerodynamic characterization</u> : approx. 96% ≤ 6µm <u>In vivo studies</u> : Plasma [] from 6hrs. onwards for up to 8 days	The first report detailing the evaluation of nano-encapsulated TB drugs for pulmonary administration every 10 days. From the results it is evident that either the same batch of nanoparticles was used for this analysis or the method is 100% reproducible	(Pandey, Sharma, Zahoor, Sharma, Khuller, & Prasad 2003a)
Oral PLG nanoparticles encapsulating INH, RIF and PZA. <i>In vivo</i> in guinea pigs against experimental tuberculosis	Administration once over 10 days for 6 weeks showed no detectable cfu counts in the organs of guinea pigs	<u>Characterization</u> : Size:186-290nm, EE=55-73% <u>In vivo studies</u> : Plasma [] from 6hrs. onwards for up to 12 days. Comparative study with only 2/3 of the therapeutic dose yielding the same results	The validity of this data is based on the pathophysiology of guinea pigs, which is similar to humans. The most significant finding of this study is the improved efficacy even at only 2/3 of the therapeutic dose	(Sharma, Pandey, Sharma, & Khuller 2004a)
Inhalable alginate nanoparticles encapsulating INH, RIF and PZA. <i>In vivo</i> in guinea pigs against experimental tuberculosis	3 nebulized doses spaced 15 days apart resulted in undetectable cfu in the lungs and spleen of infected guinea pigs. No evidence of hepatotoxicity	<u>Characterization</u> : Average size: 235.5nm, EE=80-90% <u>Aerodynamic characterization</u> : Size range: 0.4-2.1µm <u>In vivo studies</u> : Plasma [] from 3hrs. onwards for up to 14 days. Drug was present in the tissues for up to 15 days	Results showed increased bioavailability in the lungs and tissues. Retention of drug [] above the MIC for up to 15 days raised the conclusion of a possible fortnightly aerosol administration of these inhalable alginate	(Zahoor <i>et al.</i> 2005)
Oral solid lipid nanoparticles. <i>In vivo</i> in mice against murine tuberculosis	No detectable cfu in the organs of <i>M.tb</i> H ₃₇ Rv infected mice after 5 doses. No evidence of biochemical hepatotoxicity	<u>Characterization</u> : Size:186-290nm, EE=41-51% <u>In vitro studies</u> : <9-15% in first 6 hours and 11-15% for up to 72hrs. <u>In vivo studies</u> : Plasma [] were maintained from 3 hours onwards up to day 8 above the MIC	The findings of this study were comparable to previous studies using SLNs for pulmonary administration	(Pandey, Sharma, & Khuller 2005b)
Oral nanoparticle-based encapsulating INH, RIF, PZA and ETB for delivery to the brain. <i>In vivo</i> in mice in an experimental model	6.8 log cfu in the brain post infection. 6 weeks of treatment every 10 days resulted in undetectable bacilli in the brain. No inflammation cells detected	<u>Characterization</u> : Size:186-290nm, EE=43.11-68.32% <u>In vivo studies</u> : Plasma levels were maintained for up to 8 days. Bioavailability were enhanced 15- to 30 fold	Results show drug concentrations in the brain for up to 3 days. The data indicate the potential use of this drug delivery system in extrapulmonary tuberculosis	(Pandey & Khuller 2006)
Oral administration at 2 dose levels with alginate nanoparticles encapsulating INH, RIF, PZA and ETB. <i>In vivo</i> in mice	This was a PK/PD analysis showing improved bioavailability	<u>Characterization</u> : Average Size:235.5nm, EE=70-90% <u>In vivo studies</u> : Plasma levels were detected from 3 hours onwards and were observed for up to 11 days	This study evaluated the PK/PD parameters of these nanoparticles and different drug doses. Both doses achieved significantly higher values for AUC and T _{MIC} than required for substantial killing	(Ahmad, Pandey, Sharma, & Khuller 2006)
Oral PLG Nanoparticles encapsulating streptomycin. <i>In vivo</i> in mice	Eight weeks of therapy resulted in a significant decrease in cfu in combination therapy with INH and PZA. No significant hepatotoxicity	<u>Characterization</u> : Size:11-250nm, EE=32.12% <u>In vitro studies</u> : 3% in SGF and 16% in SIF for up to 12hrs. only. <u>In vivo studies</u> : Plasma [] were maintained for up to 4 hours following IM, but none detected following oral administration	Although the drug was detected in organs following oral administration, none was detected in the blood or intestines	(Pandey & Khuller 2007)
Oral alginate nanoparticles encapsulating azole and antituberculosis drugs. <i>In vivo</i> in mice	ATD administered fortnightly and econazole once weekly. 90% clearance of bacilli from lungs and spleen. No hepatotoxicity detected	<u>Characterization</u> : Size:229nm, EE=92-97.5% (azole) 70-95% (other) <u>In vivo studies</u> : Plasma levels were detected from 3hrs. onwards for up to 11 days	These results show the superior efficacy of alginate nanoparticles over other methods for the delivery of azole drugs. The potential of this system is improved bioavailability as well as retention of full chemotherapeutic potential	(Ahmad, Sharma, & Khuller 2007)
Lectin-functionalized PLG nanoparticles for oral/aerosolized delivery. <i>In vivo</i> in guinea pigs	Undetectable cfu in infected animal after fortnightly doses for 45 days. No hepatotoxicity detected	<u>Characterization</u> : Size:350-400nm, EE=54-66.8% <u>In vitro studies</u> : Minimal release in SGF and SIF within the first 7 days <u>In vivo studies</u> : Plasma levels were observed for up to 13 days	The data shows that the lectin conjugation provides an improved formulation showing similar chemotherapeutic efficacy to the same dose as previous reports every two week	(Sharma, Sharma, & Khuller 2004b)
Econazole and MXF loaded PLG nanoparticles. <i>In vivo</i> in mice	Total bacterial clearance within 8 weeks in combination with RIF following once weekly administration	<u>Characterization</u> : Size:217nm, EE=33.69-52.27% <u>In vivo studies</u> : Therapeutic drug concentration maintained for up to 5 days	This is the first report demonstrating the encapsulation of this combination of drugs. Although efficacy in murine TB is shown, further studies will need to show efficacy in drug resistance	(Ahmad, Pandey, Sharma, & Khuller 2008)

5.2 Objectives of this study

The aim of this study was to assess the PK/PD of the PLGA nanoparticulate drug delivery system encapsulating the four first-line anti-TB drugs (RIF, INH, PZA and ETB) encapsulated in individual formulations, for the following parameters to determine:

- *In vitro* MIC and minimum bactericidal concentration (MBC) of drug released from the nanoparticles;
- Whether *in vivo* drug plasma levels from nanoparticles are sufficiently bioavailable to reach MIC and facilitate growth inhibition of *M.tb in vitro*;
- The drug release plasma concentration versus time profile of the four anti-TB drugs in the nanoparticles over 10 days; and
- The subsequent biodistribution of these drugs following release from the nanoparticles since biodistribution of the drug-free nanoparticles has already been established.

These studies were conducted as part of a PhD exchange program at the Colorado State University in Fort Collins, CO, USA under the supervision of Prof. Anne Lenaerts.

5.3 Materials and Methods

5.3.1 Materials

The reagents used in the *in vivo* PK and *in vitro* PD studies are summarised in Table 5.2.

Table 5.2 Summary of reagents for *in vitro* PD *in vivo* PK/PD study

Reagent	Supplier
7H9 Broth	Becton Dickinson, Sparks, MD
oleic acid-albumin-dextrose-catalase	Becton Dickinson, Sparks, MD
Tween 80	Fisher Scientific, Fair Lawn, NJ
Rifampicin	Sigma Chemical Co. St. Louis, MO, USA
Isoniazid	Sigma Chemical Co. St. Louis, MO, USA
Pyrazinamide	Sigma Chemical Co. St. Louis, MO, USA
Ethambutol	Sigma Chemical Co. St. Louis, MO, USA
6-aminonicotinic acid	Sigma Chemical Co. St. Louis, MO, USA
Rifabutin	Sigma Chemical Co. St. Louis, MO, USA
Methanol (HPLC grade)	Sigma Chemical Co. St. Louis, MO, USA
Formic acid (98% pure)	Sigma Chemical Co. St. Louis, MO, USA

5.3.2 Preparation of PLGA particles

PLGA nanoparticles and drug free nanoparticles were prepared as described in Chapter 3. The nanoparticles encapsulating anti-tuberculosis drugs were prepared as described in Chapter 4 where the hydrophobic drug, RIF, was included in the oil phase of the w-o-w emulsion and the hydrophilic drugs, PZA, ETB and INH was included in the water phase. The drugs were incorporated individually in separate formulations.

5.3.3 Particle characterization

Particles were characterized as described in Chapter 3.

5.3.4 Animals used in assays

For the *in vivo* experiments specific pathogen-free, immunocompetent female Balb/C mice six-eight weeks old were acquired from Charles River Laboratories, Wilmington, MA. The mice weighed 18-23 g and were housed under standard environment conditions at ambient temperature of 25 °C, and supplied with food and water *ad libitum*. Ethics approval was obtained from this study from the Colorado State University's Institutional Animal Care and Use Committee (IACUC), Fort Collins, CA (See Appendix A).

5.3.5 Culture of *Mycobacterium tuberculosis* (*M.tb*)

M.tb (strain H37Rv, Trudeau Institute, Saranac Lake, NY) was grown in 50ml of 7H9 broth (Difco) containing oleic acid-albumin-dextrose-catalase (OADC) enrichment (7H9-OADC) and 0.05% Tween 80. The cultures were incubated at 37 °C with rotary agitation, grown to mid-exponential phase (optical density at 600 nm [OD₆₀₀] of approximately 0.6 to 0.8, at 14 to 21 days), and harvested by centrifugation. The cell pellets were resuspended in a small amount of the enriched 7H9-OADC medium containing 10% sterile glycerol, transferred to cryogenic vials, and stored at -70 °C as starter stocks for further use. To prepare stock for an experiment, starter stock was added to 50 ml 7H9-OADC containing 0.1% Tween 80 and incubated at 37 °C with agitation. The starter culture was grown to an OD₆₀₀ of 0.3 to 0.5 and then diluted to an OD₆₀₀ of approximately 0.1 by using 7H9-OADC containing 0.1% Tween 80 (resulting in $\sim 3 \times 10^5$ CFU per well). OD₆₀₀ readings were measured spectrophotometrically (BioRad Benchmark Plus).

5.3.6 MIC/MBC of nanoencapsulated RIF, INH and ETB

The purpose of the MIC and MBC studies was to determine whether nanoparticle drug release over time was sufficient to inhibit and/or result in significant killing of H₃₇Rv *M.tb*. PZA was not included in these assays due to assay incompatibility demonstrated by previous experiments conducted (personal communication, Prof. Anne Lenaerts).

The microdilution method was employed to determine the MIC of nanoencapsulated drugs over time. Briefly, washed nanoparticles, i.e. nanoparticle formulations in which the free drug was removed was suspended in deionized water and ten serial dilutions (1:2) were prepared and added to 96-well microtitre plates. Nanoparticle dilution range was 32 µg/ml to 0.125 µg/ml. Standard dilutions of free drug was also included based on the known MIC of each drug (ETB 16-0.0625 µg/ml, RIF 0.96-0.00375 µg/ml and INH 0.48-0.001875 µg/ml). The difference in the concentration ranges of the free- and nanoencapsulated drugs was because preliminary results showed that no inhibition was observed for nanoencapsulated drugs at similar ranges as free-drug. This suggested that insufficient drug release occurred to reach MIC concentrations. The concentrations were increased and MIC profiles were compared to the free-drug MIC profiles. The negative controls contained no drug. In addition, blank (drug-free) nanoparticles were added as a control to determine whether the PLGA nanoparticles had an inhibitory effect on *M.tb*. *M.tb* was added as described in section 5.3.5 at 5×10^4 per well and incubated. OD₆₀₀ readings were measured every two to three days until day 18.

MBC studies were conducted for PLGA nanoparticles encapsulating RIF and INH. The MBC assay was conducted similarly to the MIC assays with the major variant being the concentration of the inoculants. For the MBC assay a concentration of 1×10^6 CFU per well was used. OD₆₀₀ readings were measured every one to two days up to day 18 and confirmed by visual inspection.

5.3.7 *In vivo* bioavailability studies for PLGA nanoparticles encapsulating RIF and INH

The purpose of this assay was to evaluate the bioavailability of nanoencapsulated drugs versus free (unencapsulated) drug using *M.tb* is indicator strain. A published method for the rapid assessment of the oral bioavailability of experimental compounds against *M.tb* was used for this assay (Gruppo *et al.* 2006:1245). ETB was excluded because of incompatibility

with the assay method (personal communication from author of publication). PZA was excluded as described in section 5.3.6.

The animals were dosed at 60 mg/kg for RIF (free and encapsulated) and INH 150mg/kg (free and encapsulated) via oral gavage. Blood samples were collected via cardiac puncture per time point per drug, at 2 hr., 8 hr., 1 day, 2 days, 3 days, and 7 days for nanoparticle-encapsulated drug and at 2 hr., 8 hr., 1 day and 2 days, for free drugs. Blood was collected aseptically in serum separator tubes and centrifuged to collect serum. Serum was stored at -70 °C and used within one week.

For the assay, serum samples were prepared as two-fold dilutions using the sera of naïve (untreated) mice as diluents. The dilutions ranged from 10% to 0.312%. The serum was then added in 10 µl aliquots to 96-well plates with 10% serum of drug-treated mice as starting point in the top well. The reason for the maximum concentration of 10% was due to the fact that higher serum concentrations demonstrated growth inhibition of *M.tb*. Free-drug standards were included on the same 96-well microtitre plate in threefold dilutions ranging from 30 µg/ml to 0.51 ng/ml, in the presence of- and without serum. The inclusion of wells with and without serum for drug standards was to determine whether serum protein-binding to drugs had any effect on the assay results. The free drugs in the standard lanes were diluted with 100% DMSO to avoid possible solubility problems with a resultant 2% DMSO as final concentration. The *M.tb* stock described in section 5.3.5 was added to the 96-well plates at 2×10^5 CFU per well in a volume of 50 µl 7H9 medium. The final volume was adjusted to 100 µl. The plates were subsequently sealed in plastic bags for containment and incubated at 35 °C. OD₆₀₀ readings were measured every three to four days until day 14. Results were confirmed with visual inspection. Inhibition of bacterial growth in this bioassay would indicate whether there are sufficiently high concentrations of bioactive product in the bloodstream. Wells were scored as positive (drug containing) when the OD₆₀₀ values were less than 50% of the OD₆₀₀ value of the untreated control wells. An estimation of serum drug levels (in µg per ml serum) was obtained by using the MIC data from the standard drug lanes.

5.3.8 LCMS-MS method development

To analyse plasma drug levels of nanoencapsulated anti-TB drugs (RIF, INH, PZA and ETB) a published method (Song *et al.* 2007:1331) was optimized for use on the Liquid Chromatography Mass Spectrophotometer (LCMS-MS) Triple Quadrupole, API 3200

(Applied Biosystems, ABSciex, Germany). The internal standards used were 6-aminonicotinic acid (6-ANA) and Rifabutin (Rb). Rb served as internal standard for RIF and 6-ANA for INH, PZA and ETB.

5.3.8.1 Drug optimisation

Stock solutions of 10mg/ml of PZA, INH and ETB in 50% methanol (MeOH) in water and 10 mg/ml of RIF in 100% MeOH were prepared. Serial dilutions of each stock were prepared to reach a concentration of 5µg/ml for tuning and quantitative optimisation. The 5 µg/ml was diluted further for three concentrations of 5, 50 and 500 ng/ml to determine detection limit and linearity of analysis.

5.3.8.2 LCMS-MS conditions

Positive ion electrospray ionization (ESI) mass spectra were obtained with a MDS Sciex 3200 Q-Trap triple quadrupole mass spectrometer (Applied Biosystems). A turbo ion spray source was interfaced with a Shimadzu high performance liquid chromatographer (HPLC). Chromatography was facilitated by a C18 Reverse-phase column and protect by a C18 guard cartridge (4.0 x 2.0 mm). The LC gradient was employed with mobile phases A (0.3% formic acid in deionized water) and mobile phase B (0.3% formic acid in methanol). Chromatographic resolution was achieved by increasing mobile phase A linearly 25% to 95% from zero to five minutes for RIF, INH and PZA and 0% to 95% from zero for ETB. The LC flow rate was 0.75 ml/min for INH, PZA, ETB and RIF and the sample injection volume was 10 µl. MS/MS conditions were determined per compound. Quantification was achieved by multiple reaction monitoring (MRM).

Figure 5.1 illustrates the chemical structures of the compounds of interest. The arrow indicates the expected transition point of the compound following ionization and fragmentation (Song *et al.* 2007:1334).

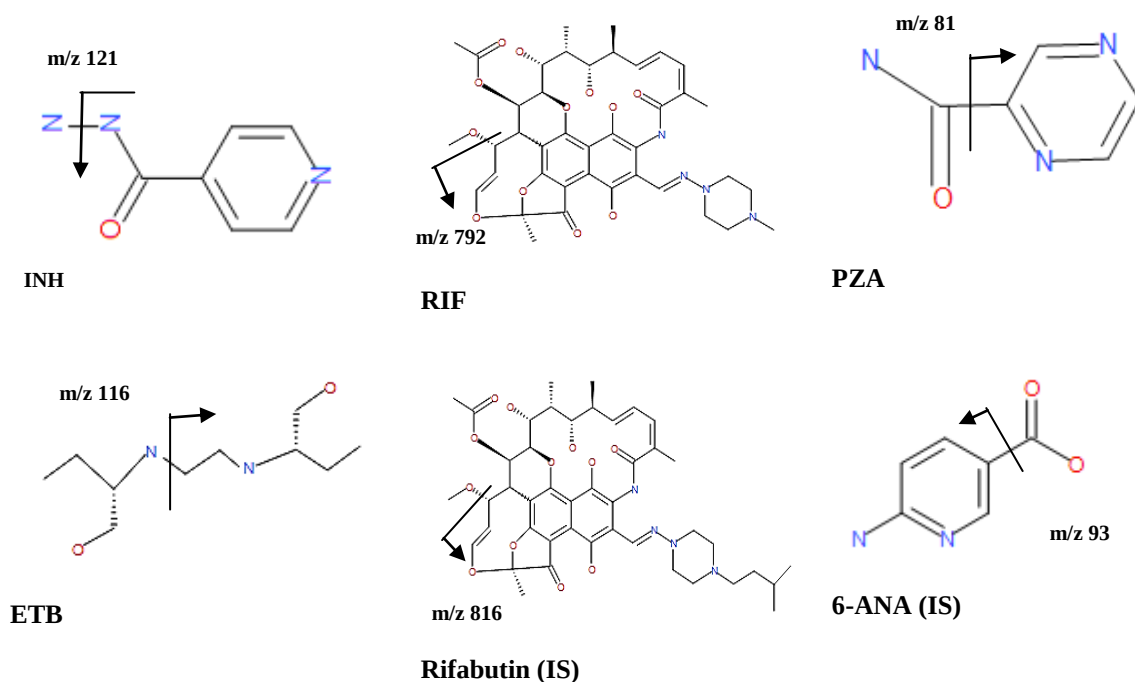


Figure 5.1 Chemical structures of drugs of interest and transition points. IS- internal standard (Song *et al.* 2007:1334).

5.3.8.3 Standard preparations

From stock solutions prepared in 5.3.8.1, standard dilutions were prepared. For analysis of the drugs in plasma, serial dilutions were prepared by spiking blank plasma to reach appropriate concentration (Table 5.3). For tissue analysis, the drugs were spiked into blank (untreated) tissue homogenates and prepared as described in sample preparation. Subsequent to method optimisation in plasma and tissue homogenates, it was observed that a method specifically for ETB was necessary for plasma analysis.

Table 5.3 Standard dilutions for LCMS-MS method development

Concentration of drugs	Volume of each	Final standard concentration	Serum / Tissue Homogenate	Internal std.	1:1	Total vol.	MeOH	Final vol.
ETB/INH/PZA/RIF (ng/ml)	Std. (µl)	ETB/INH/PZA/RIF (µg/ml)	(µl)	72µg/ml Rb; 90µg/ml 6-ANA	MeOH : dH ₂ O	(µl)	0.4% FA	(µl)
*Blank	5	0	100	0	30	130	390	520
*Zero	5	0	100	5 µl of each	20	130	390	520
10/50/25/50	5	0.2/1.0/0.5/1.0	100	5 µl of each	0	130	390	520
50/75/50/100	5	1.0/1.5/1.0/2.0	100	5 µl of each	0	130	390	520
100/100/100/250	5	2.0/2.0/2.0/5.0	100	5 µl of each	0	130	390	520
250/250/500/500	5	5.0/5.0/10.0/10.0	100	5 µl of each	0	130	390	520
500/500/1000/1000	5	10.0/10.0/20.0/20.0	100	5 µl of each	0	130	390	520
1000/1000/2500/2500	5	20/20/50/50	100	5 µl of each	0	130	390	520
2500/2500/5000/5000	5	50/50/100/100	100	5 µl of each	0	130	390	520
5000/5000/7500/7500	5	100/100/150/150	100	5 µl of each	0	130	390	520
7500/7500/10000/10000	5	150/150/200/200	100	5 µl of each	0	130	390	520
10000/10000/12500/12500	5	200/200/250/250	100	5 µl of each	0	130	390	520

5.3.8.4 Data Analysis

Quantitation of the anti-tuberculosis drugs was based on linear standard curves in spiked matrix using the ratio of the drugs' peak area to the internal standard peak area and $1/x^2$ weighting of linear regression with the use of Analyst 1.5 quantitation software. Within-run imprecision, linearity, lower limits of detection (LLODs) and quantification (LLOQs), and matrix effect were evaluated. Within-run accuracy was determined by including low-, medium- and high-concentration quality control samples. Accuracy of the assay method was calculated from the same samples as those used for the within-run imprecision samples. To be acceptable, the values should be within $\pm 15\%$ at all concentrations.

The parameters for the assay performance were calculated with the use of the following equations (Eqn.):

$$\text{Accuracy (\%)} = \left(1 - \left| \frac{\text{Theoretical} - \text{Measured}}{\text{Theoretical}} \right| \right) \times 100 \text{ Eqn 5.1}$$

$$\text{Matrix Factor} = \frac{\text{Drug peak area in post addition spiked sample}}{\text{Drug peak area in solvent sample}} \text{ Eqn 5.2}$$

5.3.8.5 Sample preparation

Plasma (100 µl) and 100 µl of tissue homogenate (200 mg/ml) were used for sample preparation. Tissue homogenate were obtained by weighing the entire organ evaluated and adjusting the volume with deionized water to reach 200 mg/ml. The tissue in water was then placed on ice and homogenized for one minute. Standards were prepared as indicated in Table 5.1 Methanol with 0.4% formic acid was used for protein precipitation. Protein precipitation was facilitated on ice for 20 minutes and vortexed, prior to centrifugation at 13 300rpm for 10 minutes. Unknown plasma and tissue homogenate samples were similarly prepared as for the zero control in Table 5.1. The supernatant (200 µl) was transferred to HPLC vials containing inserts.

5.3.9 *In vivo* pharmacokinetic study of nanoencapsulated RIF, INH, ETB and PZA

The aim of this experiment was to characterize the drug release profile of PLGA nanoencapsulated formulations of the four first-line anti-TB drugs RIF, INH, PZA and ETB. The release kinetics in animals after an oral gavage dose of nanoencapsulated drug was compared to free drugs over time. Plasma drug levels as well as drug distribution to various tissues were evaluated.

A two-part series of experiments were conducted to ensure accurate detection of plasma and tissue concentrations. These will be referred to as PK-Exp. 1 and PK-Exp. 2 in this chapter.

5.3.9.1 PK-Exp. 1

Drug dosing was calculated based on an average mouse weight of 18 g at 10, 25, 100 and 150 mg/kg for RIF, INH, ETB and PZA, respectively. The same doses were used for free-drug. To reach these doses for nanoencapsulated drugs, the percentage drug loading in each formulation was used to determine the amount of nanoparticle required to obtain the correct dose. The drugs were prepared separately at higher concentrations and then combined to result in the accurate doses per 200 µl.

Time course: D=0 the appropriate dose was given by oral gavage. For the nanoparticle combination, plasma samples were taken at 2 hrs., 4 hrs., 8 hrs., 12 hrs. day 1, 3 and 7 following administered dose. Free-drug groups were only collected until day two. At these designated times, three mice from each of these two groups were humanely sacrificed by CO₂ asphyxiation per Colorado State University IACUC guidelines and blood and tissue samples were taken immediately post-mortem via cardiac puncture (terminal bleed) and tissue harvest, respectively. Samples of plasma and tissues of the animals dosed with free-drug combination and nanoparticle combination were collected. Blood samples were collected into plasma separator tubes and centrifuged to collect plasma. Plasma supernatant (200 µl) was immediately frozen at -80 °C. The tissues, i.e. lungs, heart, liver (caudate and left lobe), lymph nodes, spleen, brain, small intestine and kidneys was snap frozen in liquid nitrogen in cryovials and stored at -80 °C. Tissues samples were only collected for day one, three and seven. Plasma and tissue samples were analysed via LCMS-MS analysis described in section 5.3.8.

5.3.9.2 PK-Exp. 2

PK-Exp. 1 was amended to eliminate certain variables. Firstly, dosages for RIF and INH was increased six-fold and PZA and ETB, three-fold. This was to compensate for drug presence in plasma and tissue possibly falling below the LLOQ where it was above the LLOD. There was no concern of overdosing, since previous reports have demonstrated that at even higher doses of these drugs, the mice remained unharmed (Lenaerts *et al.* 2005:153). Secondly, the drugs were administered individually instead of in combination to eliminate possible drug interactions that was not evaluated. The drugs were prepared in 20% Tween 80 and deionized water, since Tween 80 has been reported to act as an absorption enhancer (Chen *et al.* 2006; Junginger, 2008; Yu *et al.* 2004). Another variable that would possibly have affected the PK analysis was using an average mouse weight. Therefore, a dose solution or suspension as in the case of nanoparticles was prepared and each mouse individually weighed prior to dosing and the dosing volume adjusted accordingly. Time points for nanoparticle groups were increased to include two-day and 10-day time points and two-day and seven-day time points for free drugs. The four-hour and 12-hour time points were excluded in this study. Furthermore, sample handling was kept the same as for PK-Exp 1.

5.3.10 Non-compartmental pharmacokinetic analysis

5.3.10.1 Definitions

$AUC_{0 \rightarrow t}$: area under the concentration-time curve for time 0 to time t

$k_{el}(\lambda)$: terminal elimination rate constant

$AUC_{0 \rightarrow \infty}$: area under the concentration-time curve for time 0 to infinity (∞)

$t_{1/2\lambda}$: terminal half life

CL: systemic clearance

C_{max} : maximum concentration

T_{max} : time of maximum concentration (C_{max})

$AUMC_{0-t}$: area under the first moment curve

MRT: mean residence time

V_{ss} : volume of distribution at steady state

5.3.10.2 Equations

The following equations were used for PK analysis using Microsoft Excel 2007:

AUC_{0-t} was geometrically calculated, using the trapezoidal rule illustrated in the following equation:

$$\text{Trapezoid Rule} = \sum_{i=0}^{n-1} [(conc_i + conc_{i+1})/2] \times (time_{i+1} - time_i) = AUC_{0 \rightarrow t}$$

Where n is the number of data points and $conc$ is concentration.

To calculate $k_{el}(\lambda)$, the scatterplot/xy plot of the concentration-time plot (with the y axis on a $\log_{10}$ scale) of each study subject was visually examined. The linear portion of the terminal part of the curve that consists of at least 3 data points* was selected. An exponential regression on these points was performed and the equation of the line was displayed in the following form:

$$C = C_o e^{-k_{el}(\lambda)t}$$

The value of $k_{el}(\lambda)$ is equivalent to the value of k_{el} in this equation.

To calculate $AUC_{0 \rightarrow \infty}$, $AUC_{t \rightarrow \infty}$ was first calculated using the following equation:

$$AUC_{t \rightarrow \infty} = \frac{C_{\lambda}}{k_{el}(\lambda)}$$

Where C_{λ} is the concentration at time t . Next, sum $AUC_{0 \rightarrow t}$ and $AUC_{t \rightarrow \infty}$:

$$AUC_{0 \rightarrow \infty} = AUC_{0 \rightarrow t} + AUC_{t \rightarrow \infty}$$

The following equation was used to calculate $t_{1/2\lambda}$:

$$t_{1/2} \lambda = \frac{0.693}{k_{el}(\lambda)}$$

The following equation was used to calculate CL:

$$CL = \frac{Dose}{AUC_{0 \rightarrow t}}$$

To determine C_{max} , the concentration-time curve was visually inspected and C_{max} as the maximum concentration within this curve was reported. To determine T_{max} , the concentration-time curve was visually inspected and T_{max} as the time at which C_{max} occurs was reported.

The following equation was used to determine MRT:

$$MRT = \frac{AUMC_{0 \rightarrow t}}{AUC_{0 \rightarrow t}}$$

The following equation was used to determine V_{ss} :

$$CL \times MRT$$

5.3.11 Statistical analysis

The statistical analysis was performed using the mean $\pm$ SEM of the values generated for each test group using Microsoft Excel 2010. Non-compartmental analysis was used to calculate the PK parameters for anti-TB drugs in mouse plasma. All values below the LLOQ were not included in the calculations. The equations described in section 5.3.10.2 were programmed in Microsoft Excel 2010 and calculated accordingly.

5.4 Results

5.4.1 Nanoparticle characterization

Nanoparticle characterization was previously described in Chapter 3. Table 5.4 summarizes the results. The PLGA nanoparticles encapsulating anti-tuberculosis drugs yielded an average size of 280 $\pm$ 14 nm and an average PDI of 0.26 $\pm$ 0.02. Since the different formulations were very similar in size and all possessed a positive zeta potential, it would be expected that variations in results for the different formulations would be as a result of the specific drug encapsulated in the formulation. For all the assays conducted in this chapter, the % drug loading was used to determine drug concentrations for dosing.

Table 5.4 Summary of nanoparticle characterization showing the different formulations, size (nm), polydispersity index, encapsulation efficacy (%), drug loading (%) and zeta potential

Sample	size (nm)	PDI	Zeta Potential (mv)	EE (%)	Yield (mg)	Drug loading (%)	Amount of drug in sample (mg)
PLGA-INH (1% PEG)	302 $\pm$ 4.81	0.26 $\pm$ 0.03	+19.45 $\pm$ 4.17	55.2 $\pm$ 2.26	1150	20.68 $\pm$ 1.45	249.9
PLGA-ETB (1% PEG)	260 $\pm$ 8.17	0.27 $\pm$ 0.01	+12.85 $\pm$ 3.46	70.39 $\pm$ 1.68	1005	18.75 $\pm$ 2.61	207.6
PLGA-PZA (1% PEG)	240 $\pm$ 4.57	0.23 $\pm$ 0.02	+17.75 $\pm$ 3.46	73.33 $\pm$ 1.17	1140	22.93 $\pm$ 3.49	290
PLGA-RIF (1% PEG)	253 $\pm$ 3.61	0.27 $\pm$ 0.06	+12.45 $\pm$ 4.53	68.48 $\pm$ 2.09	1470	9.19 $\pm$ 0.12	134
PLGA-DRUG FREE (1% PEG)	238 $\pm$ 3.61	0.23 $\pm$ 0.06	+14.6 $\pm$ 4.53	N/A	1036	N/A	N/A

PDI-polydispersity index; EE- encapsulation efficiency

5.4.2 MIC/MBC assays

Preliminary MIC studies demonstrated similar results when nanoencapsulated and free drug controls were evaluated. This was due to the interference of unencapsulated drug in the

nanoparticle formulation. Therefore, the MIC and MBC assays were conducted using washed nanoparticles where the free (unencapsulated) drug was removed from the formulation. This would ensure that any growth inhibition or bactericidal effects observed would be because of drug released from the nanoparticle.

5.4.2.1 MIC assays

As previously described in Chapter 1, MIC refers to the minimum drug concentration required to exhibit bacterial growth inhibition. For free-drug, MIC should be observed at pre-reported values (chapter 1). Since washed nanoparticles used, MIC would only be observed once the drug was released from the nanoparticle. For reasons discussed in section 5.3.6, PZA was excluded from the study.

For the OD₆₀₀ readings measured on day six of the study, the free drug control wells appeared active with some inhibition observed. No inhibitory activity was observed for any of the nanoparticle formulations. On day eight variations were observed among the six free-INH replicates (average OD₆₀₀ reading 0.0780 ± 0.00026 and 0.1722 ± 0.1595 for 0.12 µg/ml and 0.03 µg/ml control wells, respectively). For INH-NP, three of the 6 replicates demonstrated MIC of 16 µg/ml, but there were large variations in OD₆₀₀ observed for both the control (no drug) wells and the nanoparticles wells. Free ETB MIC control concentrations ranged from 1 to 2 µg/ml. In this group, variation was also observed among the 6 replicates. For the ETB-NP, no activity was observed in any of the wells evaluated. Free RIF MIC concentration was observed at 0.12 µg/ml. No variation was observed among the six replicates. RIF-NP's demonstrated MIC concentrations between 32 and 16 µg/mL, though some variation was observed among replicates.

Figure 5.2 illustrates the MIC profile of INH (free and nanoencapsulated) at observed MIC's. Concentrations selected for graphical representation demonstrated growth inhibition over time based on OD₆₀₀ readings. For free INH, concentration below 0.03µg/ml demonstrated OD₆₀₀ readings comparable to untreated controls. No inhibition for nanoparticles at concentrations below 16 µg/ml was observed. Since the MIC observed for nanoencapsulated drug is dependent on the drug being released from the nanoparticle and the MIC profile observed was comparable to that of the free drug at MIC 0.03 µg/ml, it may suggest that the amount of drug released from the nanoparticles at 16 µg/ml was ~0.03 µg/ml INH. The increase in OD₆₀₀ observed at day 18 was due to decrease in well volume as a result of the

wells drying out. This could suggest that continued MIC could have been observed past the 18-day point because of sustained release of INH from the nanoparticles.

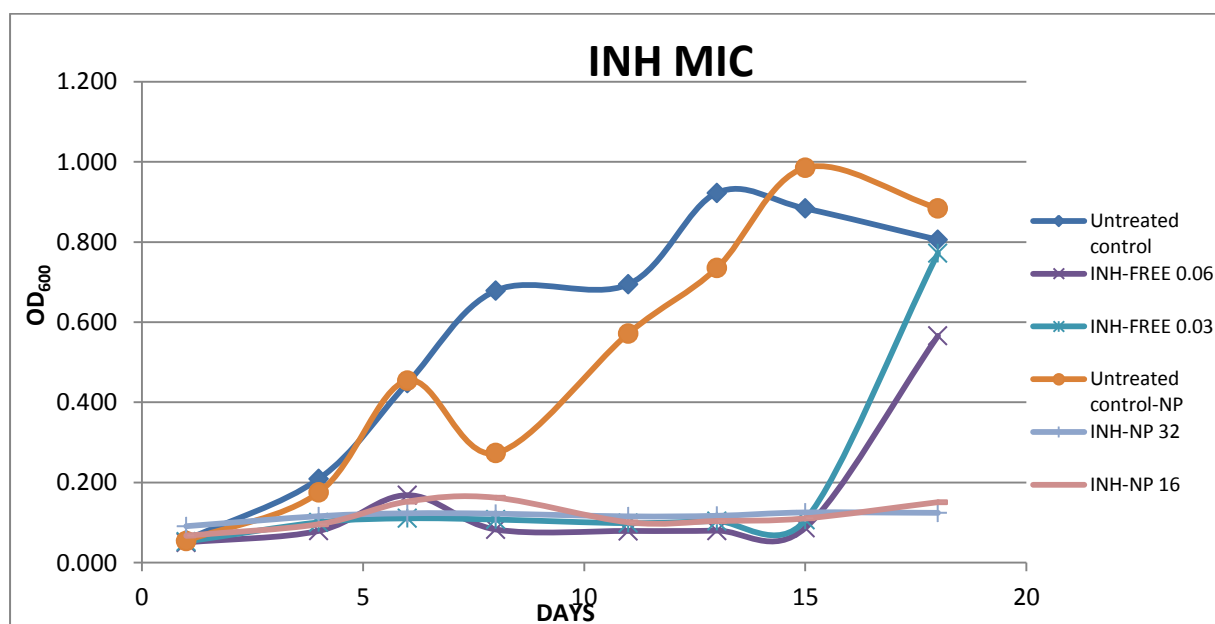


Figure 5.2: MIC profile for INH- free and nanoencapsulated compared against untreated controls. Values calculated as mean± SEM, n=6. SEM values are not displayed in the graph for legibility purposes. Please refer to Appendix C for SEM data values.

RIF demonstrated similar results (Figure 5.3). The MIC for RIF is 0.06 µg/ml and a similar trend was observed for RIF-NP at 8 µg/ml, suggesting that MIC drug concentrations were released from the nanoparticle formulations at this concentration. RIF-NP at 32 µg/ml demonstrated an MIC profile similar to that of RIF-FREE at 0.12, 0.48 and 0.96 µg/ml. Encapsulated ETB displayed no inhibition at the concentrations used (Figure 5.4). However, the MIC for ETB is 1µg/ml, which is significantly higher than for RIF and INH. It is possible that for the media used in this assay, adequate conditions were not created for sufficient drug release to reach MIC.

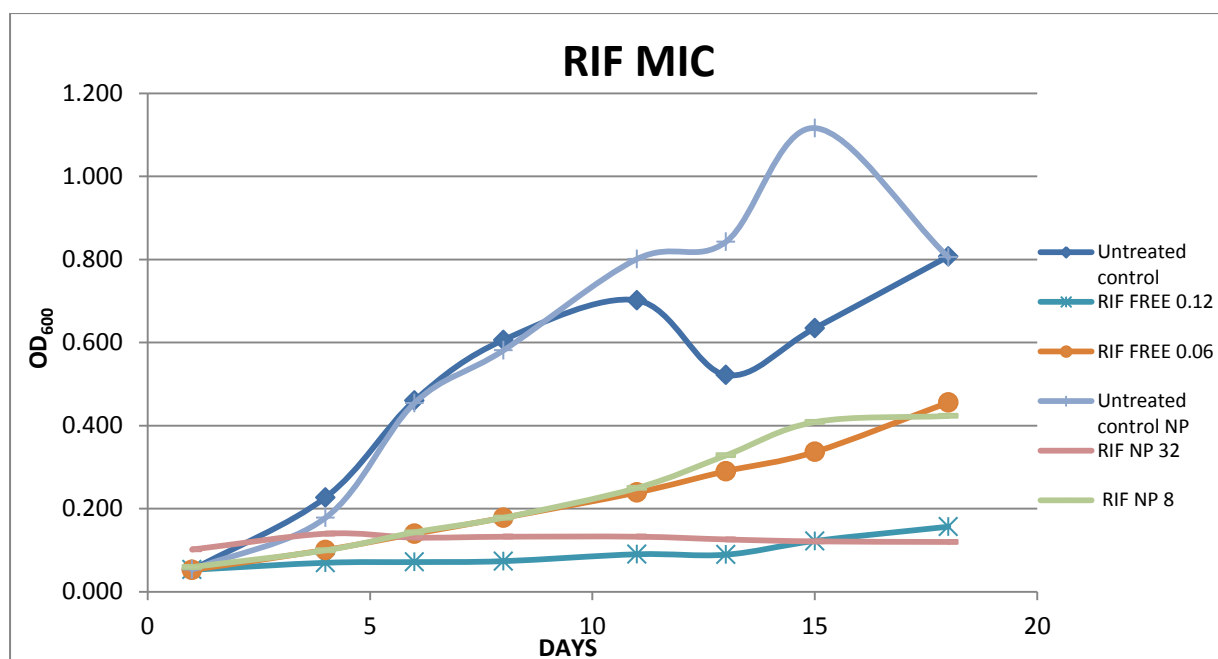


Figure 5.3 MIC profile for RIF- free and nanoencapsulated compared against untreated controls. Values calculated as mean $\pm$ SEM, n=6. SEM values are not displayed in the graph for legibility purposes. Please refer to Appendix C for SEM data values.

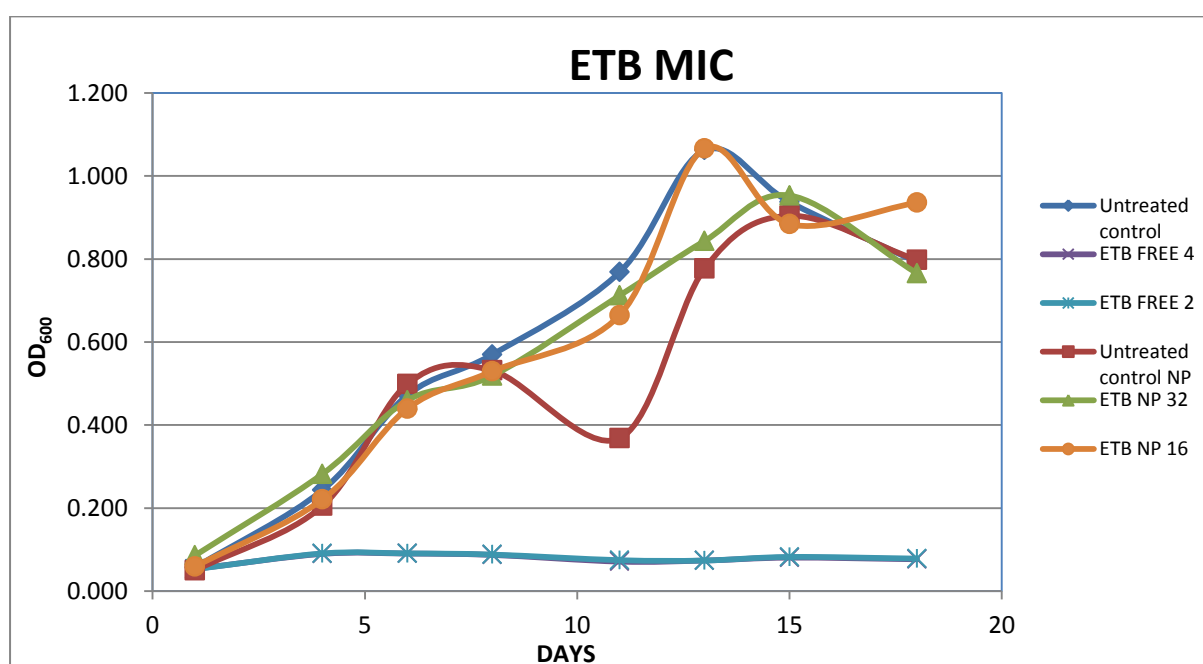


Figure 5.4 MIC profile for ETB- free and nanoencapsulated compared against untreated controls. Values calculated as mean $\pm$ SEM, n=6. SEM values are not displayed in the graph for legibility purposes. Please refer to Appendix C for SEM data values.

Although higher nanoencapsulated concentrations were required to obtain MIC, it remains promising that in the absence of free drug, i.e. washed nanoparticles, MIC could still be

obtained. It is important to note that these results cannot be directly correlated to expected *in vivo* outcomes, since the processes in a biological environment to which nanoparticles are exposed differ from the media in an *in vitro* model.

5.4.2.2 MBC studies

ETB data did not demonstrate inhibitory concentrations for drug released from nanoparticles. Therefore, ETB analysis was not performed for MBC assays. Nanoencapsulated INH and RIF were evaluated to demonstrate whether sufficient drug is released *in vitro* to exhibit bactericidal effects on *M.tb*.

Figure 5.5 illustrates the summarised MIC profile for the MBC study using INH free and INH-NP. Free-INH at 0.03µg/ml (INH MIC) provides a clear distinction between drug concentrations for both free- and nanoencapsulated, demonstrating inhibition and those demonstrating low to no activity compared to untreated controls. Of the INH-NP concentrations evaluated, only 32 µg/ml demonstrated activity over the course of the assay. The growth inhibition was comparable to free drug concentrations of 0.06 µg/ml, suggesting that this was the amount of drug released from the nanoparticles over time. However, this nanoparticle drug concentration was also comparable to 0.48 and 0.12 µg/ml free-drug, suggesting a wider range of drug released from nanoparticles (see Appendix C).

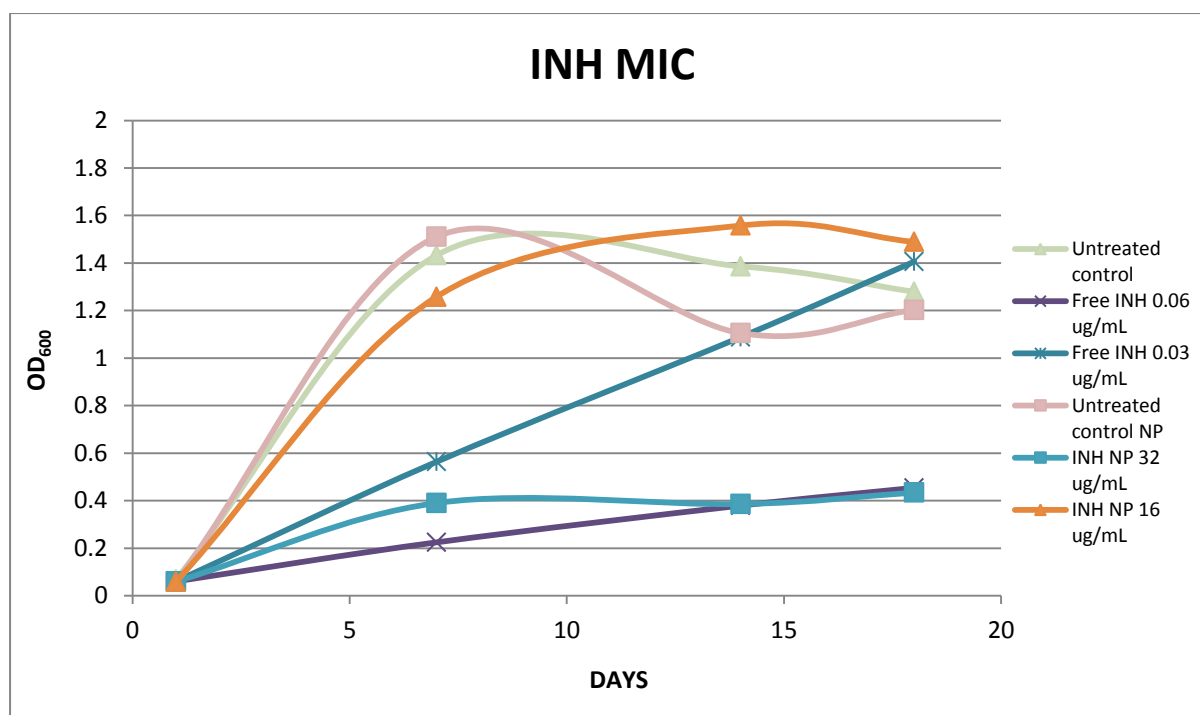
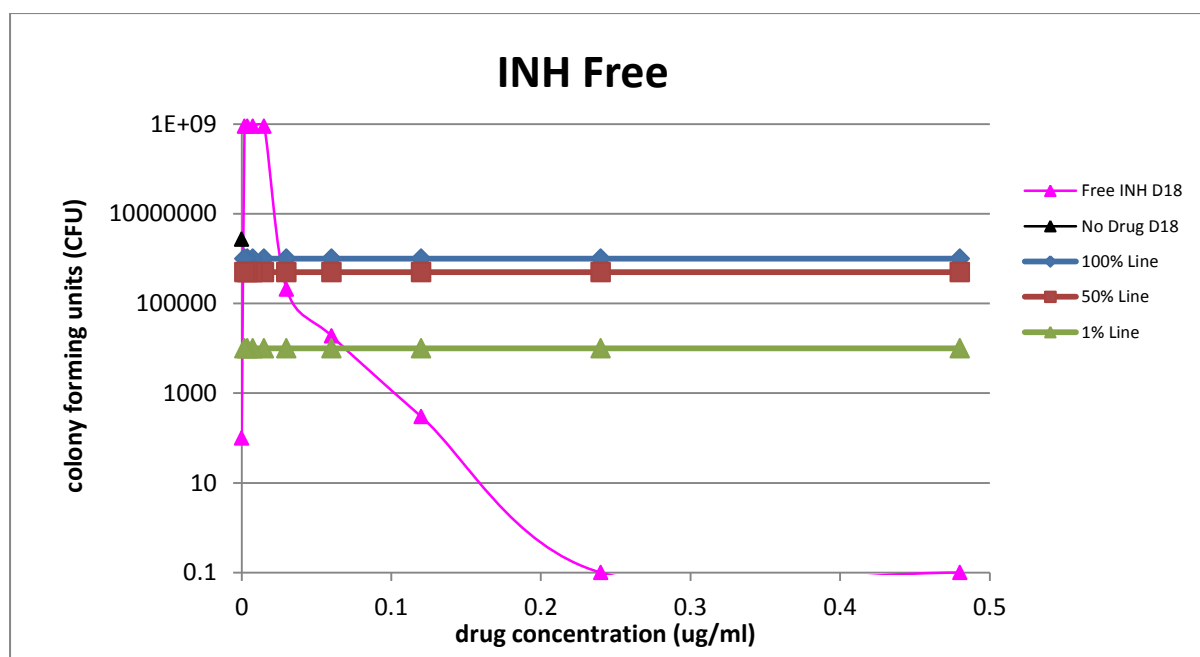
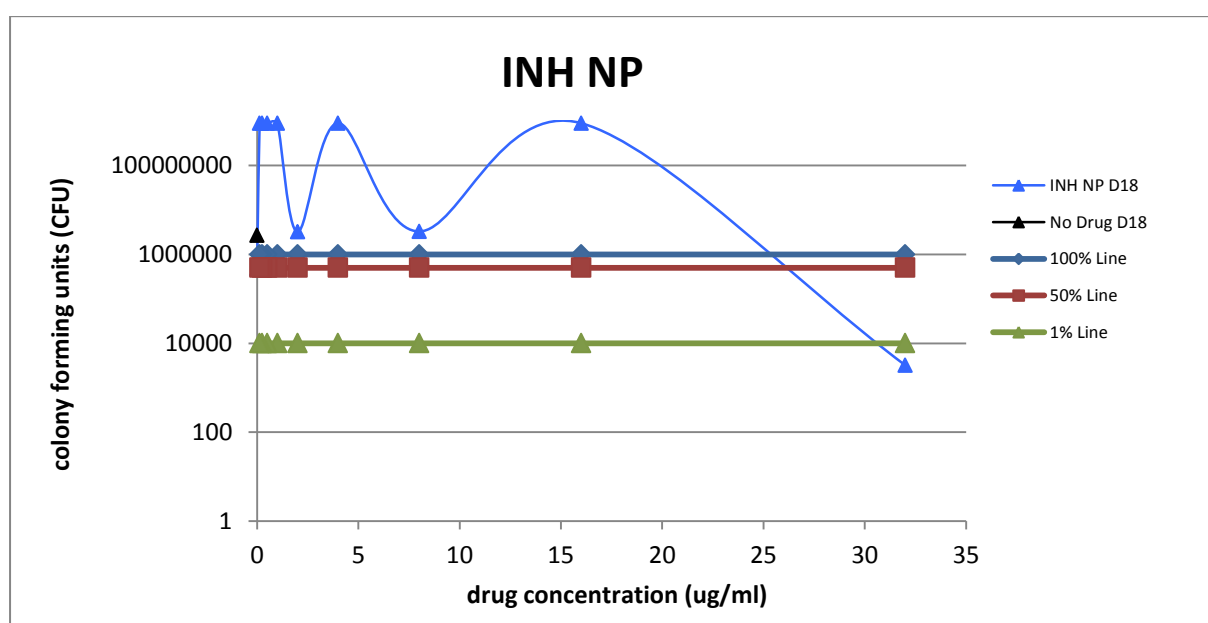


Figure 5.5 MIC profile for INH- free and nanoencapsulated compared against untreated controls in the MBC study. Values calculated as mean± SEM, n=6. SEM values are not displayed in the graph for legibility purposes. Please refer to Appendix C for SEM data values.

Figure 5.6 (a) and (b) summarizes the MBC for INH following plating of MIC samples over 18 days. The untreated controls indicate where 100% bacterial growth is still present (100% line). Below 50% and 1% lines was where 50% and 99% bacterial killing was expected, based on decrease in cfu's. Free-INH exhibited 99% killing at 0.12 µg/ml, which equals four times the MIC of INH with 100% killing at higher concentrations. Figure 5.6 (b) demonstrated that at 30 µg/ml INH-NP, 99% bacterial killing could be achieved.



(a)



(b)

Figure 5.6 (a) MBC profile for free-INH and (b) MBC profile for nanoencapsulated INH at day 18 of MBC analysis. The decrease in colony forming units (cfu) are depicted as the 100% line (no bacterial killing), 50% line (50% killing) and 1% line (99% killing).

MIC evaluated for nanoencapsulated RIF for the MBC assay demonstrated growth inhibition at concentrations of 16 $\mu\text{g/ml}$. This MIC profile was comparable with free drug

concentrations ranging from 0.03 $\mu\text{g/ml}$ (RIF MIC for this assay) to 0.48 $\mu\text{g/ml}$ as shown in Figure 5.7.

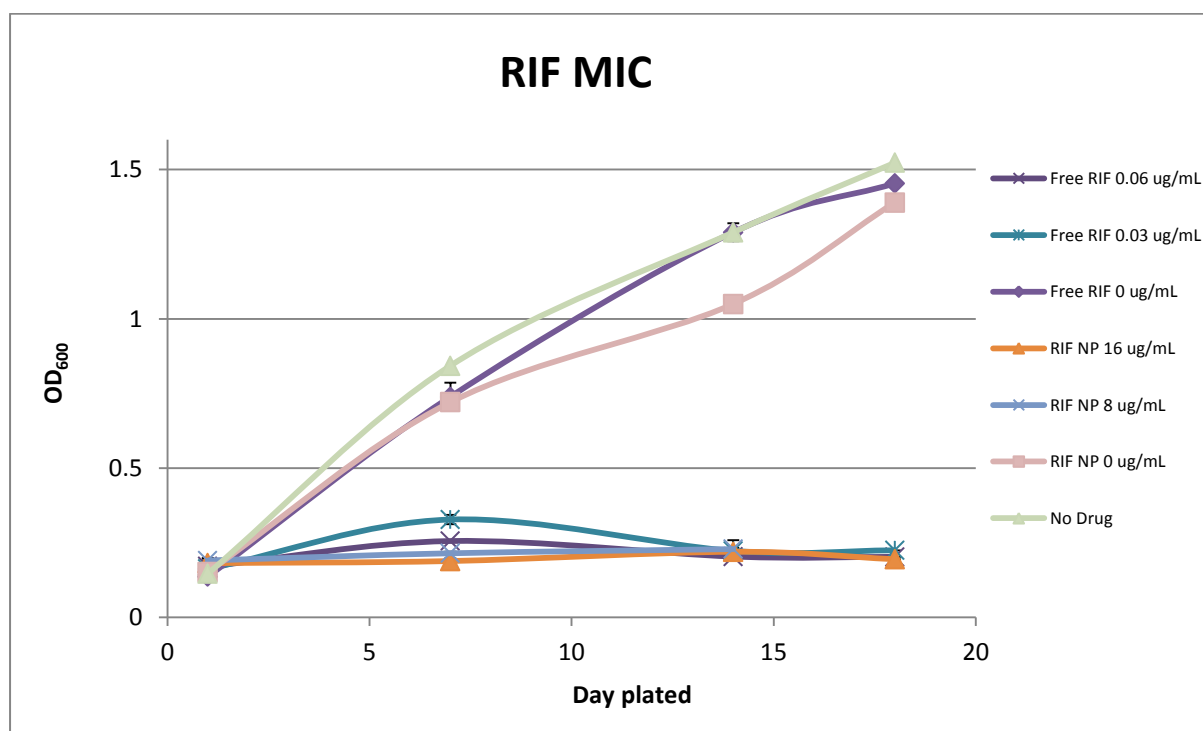
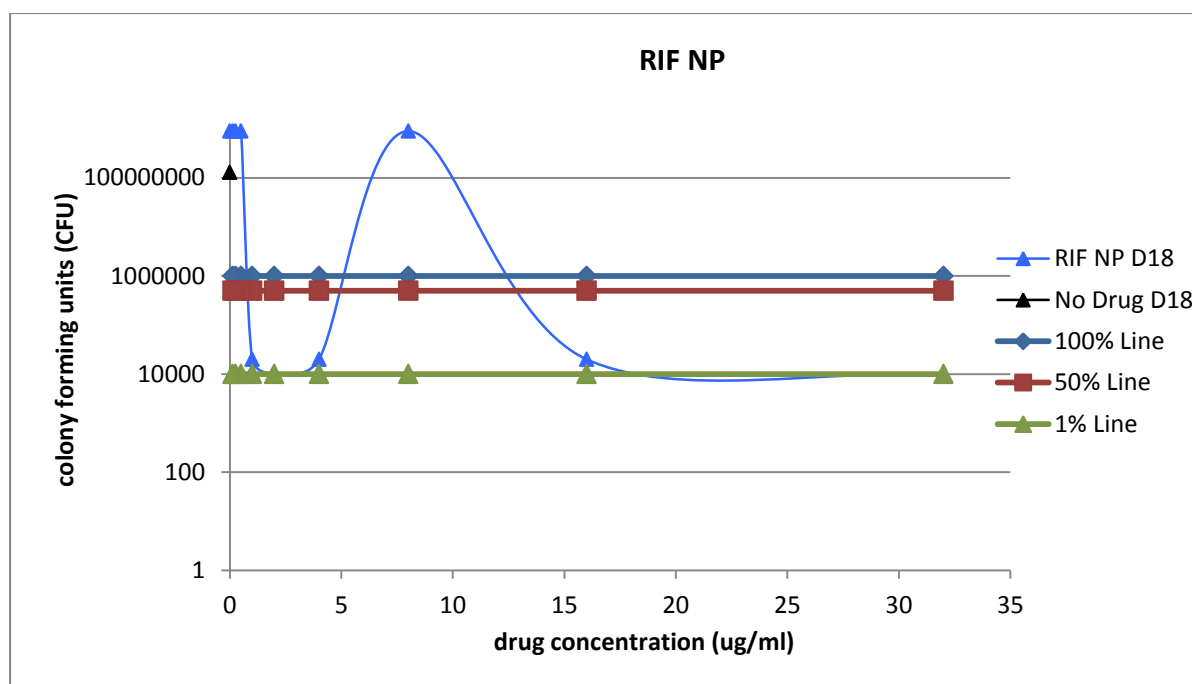
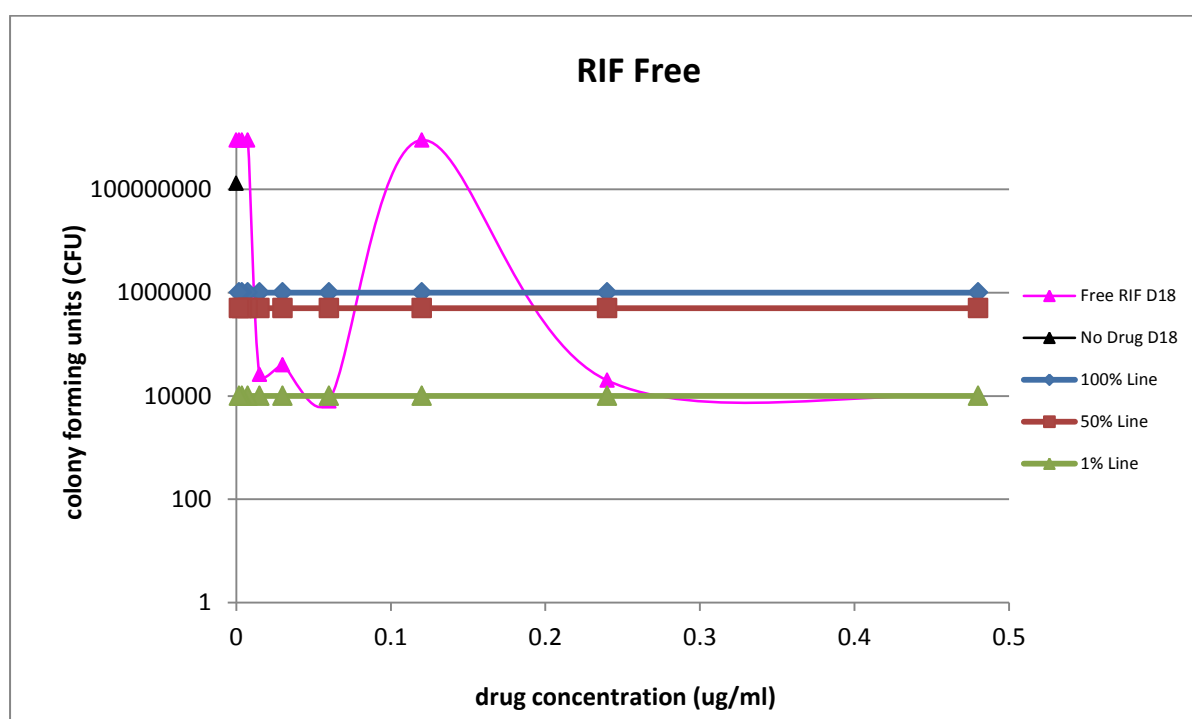


Figure 5.7 MIC profile for RIF- free and nanoencapsulated compared against untreated controls in the MBC study. Values calculated as mean $\pm$ SEM, n=6. SEM values are not displayed in the graph for legibility purposes. Please refer to Appendix C for SEM data values.

Similarly to the INH assay, Figure 5.8 (a) and (b) summarizes the MBC for RIF following plating of MIC samples over 18 days. The untreated controls (no drug) indicate where 100% bacterial growth was still present. The figures are illustrated as cfu versus drug concentrations. Figure 5.8 (a) demonstrates that at 20 $\mu\text{g/ml}$, nanoencapsulated RIF elicited 99% bacterial killing. For free-RIF (figure 5.8 (b)), 99% killing was achieved at 0.05 $\mu\text{g/ml}$ with 100% killing observed at higher concentrations.



(a)



(b)

Figure 5.8 (a) MBC profile for free-RIF and (b) MBC profile for nanoencapsulated RIF at day 18 of MBC analysis. The decrease in colony forming units (cfu) are depicted as the 100% line (no bacterial killing), 50% line (50% killing) and 1% line (99% killing)

5.4.3 *In vivo* bioavailability assays

To determine the bioavailability of INH and RIF in mouse serum samples, an *in vitro* MIC study was performed as described in section 5.3.7. The aim of the study was to demonstrate

that sufficient nanoencapsulated drug was absorbed to reach MIC. Drug levels in the mouse serum were estimated by multiplying the dilution factor by the MIC value of the drug in the absence of serum. The dilution factor (DF) represents the last dilution step of the serum samples in which drug activity was still observed in the bioassay. Bioavailability of the two drugs were rated according to the dilution factor and by correlating the standard drug concentrations used in the assay with the OD₆₀₀ reading obtained for the different wells as shown in Table 5.5. Rating of bioavailability: Low: at a DF of 1:10 and 1:20, Medium: DF is 1:40 or 1:80, High: if DF is 1:160 or 1:320. The MIC is based on the lowest concentration at which growth inhibition was observed.

Table 5.5 Example of RIF OD₆₀₀ readings correlated with drug concentrations used. MIC was expected at 0.041µg/ml, which at 10 times dilution equates to 0.41µg/ml.

Dilution factor (DF)	Drugs Without Serum (OD ₆₀₀)		Drugs With Serum (OD ₆₀₀)		Drugs Without Serum (µg/ml)		Drugs With Serum (µg/ml)	
1/10	0.046	0.09	0.102	0.146	30	0.041	30	0.041
1/20	0.043	0.208	0.077	0.365	10	0.014	10	0.014
1/40	0.042	0.43	0.068	0.317	3.3	0.0046	3.3	0.0046
1/80	0.044	0.386	0.065	0.384	1.1	0.0015	1.1	0.0015
1/160	0.043	0.287	0.071	0.438	0.37	0.0005	0.37	0.0005
1/320	0.053	0.366	0.068	0.559	0.12	0.0	0.12	0.0

Wells were shaded to indicate where there is growth inhibition due to the presence of compound in serum (shading when the OD₆₀₀ of the well is less than 50% of the OD₆₀₀ of the untreated controls). Serum dilutions were made in purchased serum from Balb/C mice (in every well there is a final serum concentration of 10%). The key to interpreting the data displayed below was used to evaluate each plate.

Key to interpreting data:

	Time point 1			Time point 2			Standards (drug dilution µg/ml)			
	m1	m2	m3	m1	m2	m3	without serum		with serum	
10%							30	0.041	30	0.041
5%							10	0.014	10	0.014
2.5%							3.3	0.0046	3.3	0.0046
1.25%							1.1	0.0015	1.1	0.0015
0.625%							0.37	0.0005	0.37	0.0005
0.312%							0.12	0.0	0.12	0.0

m1;m2; m3 indicates mice in triplicate per time point

As expected, both free- nanoencapsulated drugs were bioavailable, based on OD₆₀₀ readings and visual inspection of the microtitre plates. For free-INH, growth inhibition was only observed up to the eight-hour time point indicating the rapid elimination and/or distribution of the free drug. Free-RIF drug levels were observed up to two days in serum and these levels were sufficiently high to exhibit growth inhibition. For both nanoencapsulated formulations of RIF and INH, growth inhibition was observed up to the day 3 time point. This observation suggested at sufficient drug was released into the bloodstream from the nanoparticles to exhibit growth inhibition of *M.tb*. The standard compound lanes summarised in Table 5.5 were used to determine approximate drug levels depicted in Table 5.6. As observed in Table 5.6 drug levels at the two-hour and eight-hour time points for INH and RIF, respectively were high and the sera were not diluted to enough using the described method (dilution was up to 1:320). These drug levels were observed at >13.2 µg/ml. The method described for this study was designed to analyse experimental drugs and since bioavailability for these bioactive drugs are already known, the dilutions used may not have been optimal. However, the aim was to determine the bioavailability of the drugs following release from nanoparticles and this was confirmed (for detailed data see Appendix C). The fact that MIC was the same for all four formulations with and without serum indicates that no significant protein-binding occurred.

Table 5.6 Determination of MIC levels of both free and encapsulated INH and RIF in mouse treated serum over 7 days

Drug	Standard		Mouse data							
	MIC (no serum)	MIC (10% serum)	Drug dose (mg/kg)	Dosing route	Approximate drug level (µg/ml) in serum after:					
					2hr	8hr	1 day	2 days	3 days	7 days
INH-NP	0.041	0.041	150	Oral gavage	>13.12	3.28	BD	0.41	0.41	BD
INH-Free	0.041	0.041	150	Oral gavage	>13.12	0.41	BD	BD	NA	NA
RIF-NP	0.041	0.041	60	Oral gavage	>13.12	>13.12	3.28	0.41	0.41	BD
RIF-Free	0.041	0.041	60	Oral gavage	>13.12	>13.12	>13.12	0.82	NA	NA

BD, below detection limit of the bioassay. The detection limit is 10x the MIC₉₀ of the tested compound in the presence of serum (due to the restriction of using a maximum of 10% serum in the M. tb. NA: not analysed

Based on free drug controls for both RIF and INH, the MIC was calculated at 0.041 µg/ml. Therefore, at 10 times the MIC, 0.41 µg/ml serum concentrations were required to exhibit inhibition. As indicated in Table 5.6, approximate drug levels for encapsulated drug were at 10 times MIC and inhibition was observed.

5.4.4 LCMS-MS method development

Subsequent to compound optimisation, mass spectrometer settings are summarised in Table 5.7. The MRM transitions were selected based on the predominant product ion. The dwell times for each transition were 100 milliseconds (ms). Q₁ and Q₃ were run in unit resolution mode. Linearity was measured with standard curves in spiked mouse plasma over the range 50-1000 ng/ml for RIF and INH, 25-12500 ng/ml for PZA and 10-1000 ng/ml for ETB. The linearity of the standard curves in all matrices evaluated was $\geq r^2=0.99$ using uniform $1/x^2$ weighting. All three forms of weighting resulted in acceptable linear regression, i.e. with r^2 values approaching one, but the $1/x^2$ weighting resulted in a standard curve with higher accuracy, especially for the lower standard concentrations. The LLOQ in mouse plasma was measured as follows; 50 ng/ml for RIF and INH, 25 ng/ml for PZA and 10 ng/ml for ETB. These limits were lower than the limits for the published method used to optimize this method. Matrix effects were quantified by determining the matrix factor, 1 means no MF, > 1 = enhancement and < 1 means suppression. For ETB, the MF appeared to be between 10 and 40. For INH, MF was ~ 0.1, PZA, MF was ~ 0.5 and for RIF, MF was ~2.

Table 5.7 MS/MS conditions and drug MRM transitions

Compound	DP	EP	CEP	CE	CXP	MRM transition (m/z)
Rifampicin	45	4.0	28.87	25	6.1	824→792
Isoniazid	40.08	8.1	101.2	28.5	2.34	138→121
Ethambutol	31	4	11.4	19.1	2.0	205→116
Pyrazinamide	34	4.5	7	20	2.0	124→81
6-ANA	32	5	27	19	2.0	139→93
Rifabutin	38	4.0	27	25	10.0	848→816

DP- declustering potential, EP- entrance potential; CE- collision energy; CXP- collision cell exit potential. For all transitions the following were the same: CUR-curtain gas, 35; IS- ion spray voltage, 4500; and source temperature, 350°C.

The elution time for ETB, INH, 6-ANA, PZA, Rb and RIF were 0.52, 0.74, 0.81, 2.79, 3.37 and 3.60 minutes, respectively. As mentioned previously, measurement of ETB in plasma required a separate method during optimisation. This was, however, not necessary for tissue homogenates. This could be as a result of plasma versus tissue components. Two non-specific peaks were detected in blank plasma and tissue. Since calibration curves were prepared in the same matrix as the unknowns, matrix effects were the same as for standards. Representative chromatographs observed in mouse plasma and mouse kidney are illustrated (Figure 5.9, 5.10 and 5.11).

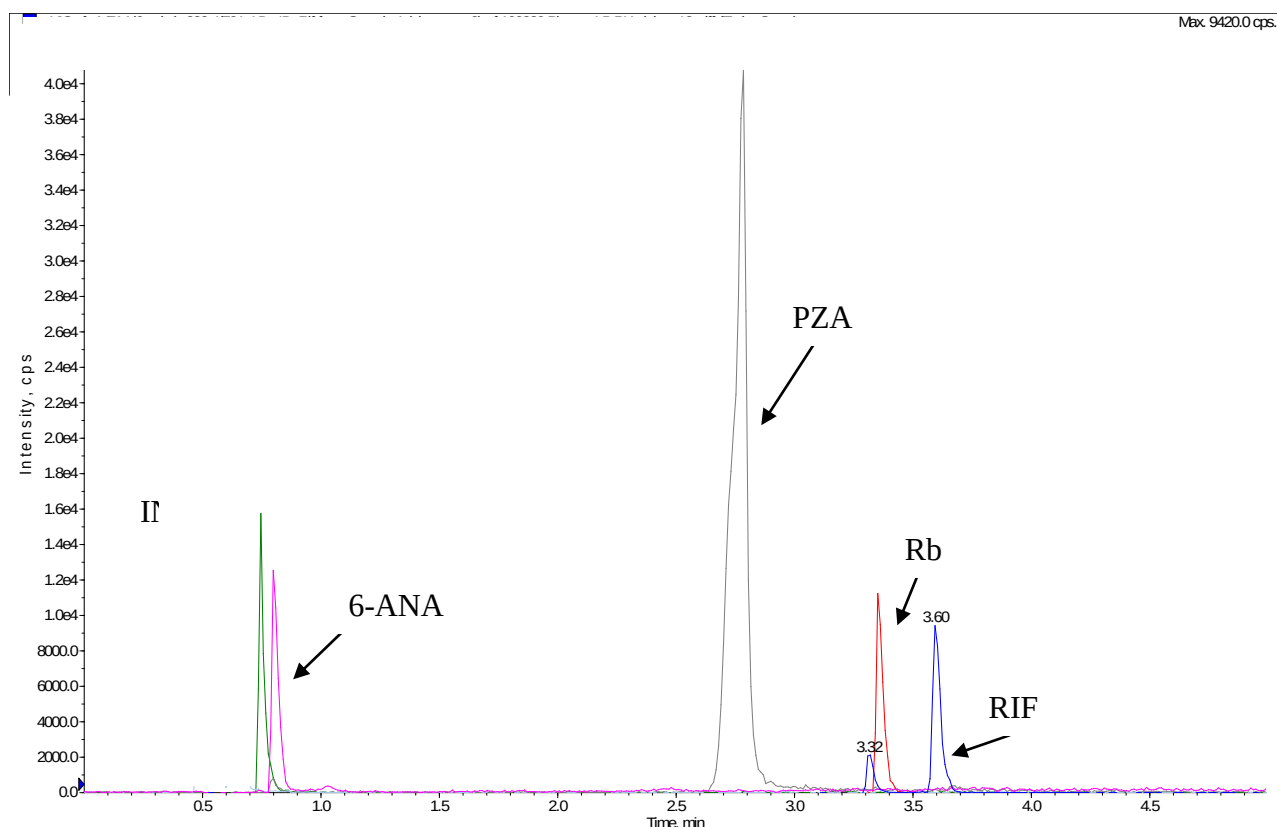


Figure 5.9 Chromatograph of anti-tuberculosis drugs in spiked mouse plasma at 10000/7500/10000ng/ml for RIF, INH and PZA, respectively.

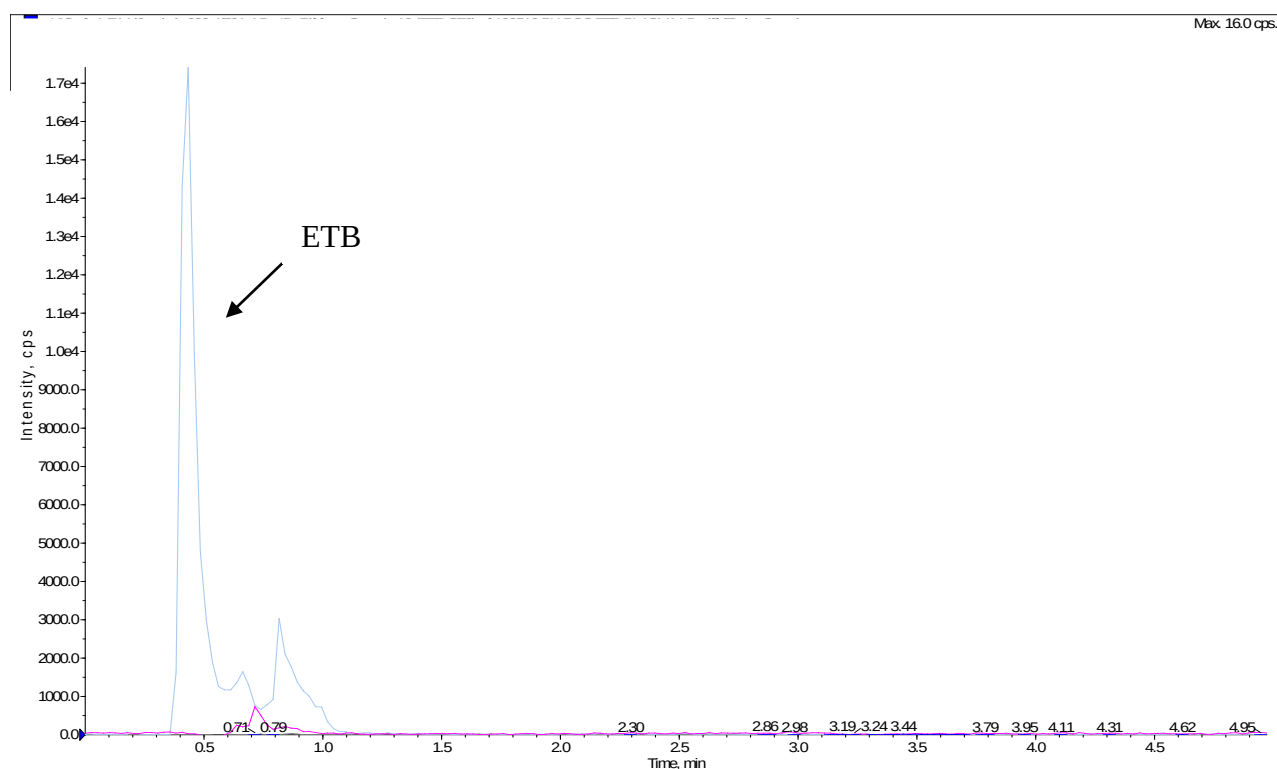


Figure 5.10 Chromatograph of ETB in spiked plasma at 1000ng/ml

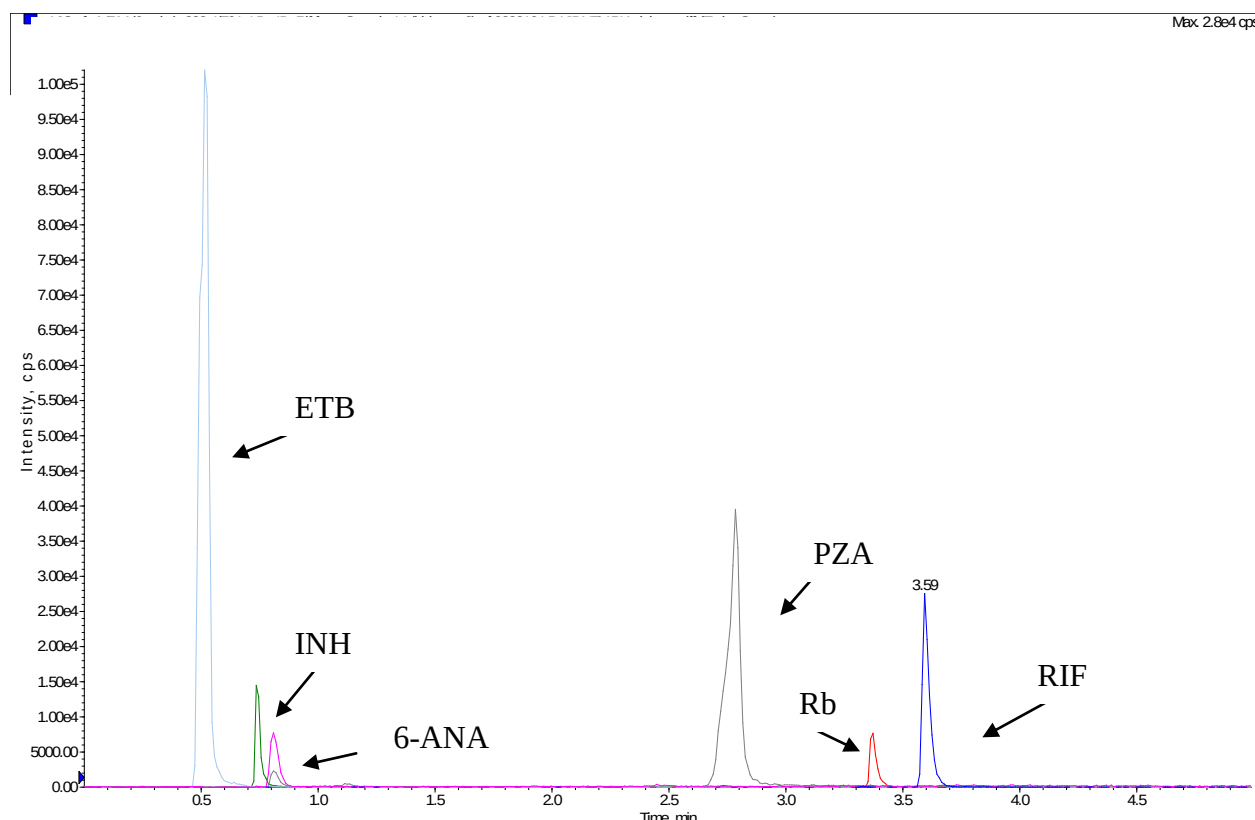


Figure 5.11 Chromatograph of anti-tuberculosis drugs in spiked mouse kidney homogenate at 10000/7500/10000/7500ng/ml for RIF, INH, PZA and ETB, respectively

Accuracy of RIF, INH, PZA and ETB measurement were assessed in spiked mouse plasma (Table 5.8). Within run drug measurements were measured at 50, 200, 500 and 1000ng/ml and accuracy ranged from 84% to 99.5%. Between-run drug measurements were measured at calibration curve concentrations with the LLOQ for each drug as starting concentration up to 12500ng/ml. Accuracy ranged from 85% to 115%. Values outside these ranges were not used in the curve and therefore did not affect linearity. Similar accuracies were observed for spiked tissue homogenates.

Table 5.8 Accuracy assay for RIF, INH, PZA and ETB in mouse plasma

Concentration (ng/ml)	Within representative run		Between run	
	<i>Observed</i>	<i>Accuracy (%)</i>	<i>Observed</i>	<i>Accuracy (%)</i>
Plasma				
RIF				
50	55	98	49	98.0
100			101	99.0
250	281	87.6	330	68.0
500			483	96.6
1000	995	99.5	757	75.7
2500			2000	80.0
5000			4410	88.2
7500			8580.0	85.6
10000			11900	81.0
INH				
50	51	103	52	104.0
75			71	94.7
100			99	99.2
200	184	92.2		
250			236	94.4
500	522.0	104	392	78.5
1000			1150	115.0
2500			2480	99.4
5000			5190	104.0
7500			6720	89.5
10000			9850	98.5
PZA				
25			21	84.6
50	42.0	84	44	87.1
100			98	97.5
200	189.0	94.6		
500	497.0	99.3	518	104.0
1000			1050	105.0
2500			2990	120.0
5000			5670	113.0
7500			7500.0	100.0
10000			9480	94.8
12500			11800	94.4
ETB				

Concentration (ng/ml)	Within representative run		Between run	
	<i>Observed</i>	<i>Accuracy (%)</i>	<i>Observed</i>	<i>Accuracy (%)</i>
10			10	101.0
50	49	98.0	45	89.3
100			122	122.0
200	188	94.0		
250			279	112.0
500	576	87.8	528	106.0
1000			1110	111.0
2500			2650	106.0
5000			4260	85.1
7500			6700	89.3
10000			6970	69.7

The method was observed to be optimized for simultaneous detection in plasma and tissue of all four anti-tuberculosis drugs evaluated along with their respective internal standards. This method was used for all PK analyses.

5.4.5 *In vivo* PK drug release assays

The PK drug release assays were performed in two parts as previously stated. Subsequent to data analysis of PK-Exp. 1, various experimental parameters were adjusted to optimize drug release profile as described in section 5.3.9.2.

5.4.5.1 PK-Exp. 1 and PK-Exp. 2 drug release profiles

The four drugs analysed, i.e. RIF, INH, PZA and ETB were compared for the two experiments. Tables 5.9 and 5.10 summarize the plasma drug levels detected at the different time points analysed. The mean± SEM are shown.

Table 5.9 Summary of plasma drug levels (µg/ml) in PK-Exp. 1. Table depicts the mean± SEM of three mice over 7 day time points for free- and nanoencapsulated RIF, INH, PZA and ETB

		Free RIF	Nano RIF	Free INH	Nano INH	Free PZA	Nano PZA	Free ETB	Nano ETB
2hrs.	mouse 1	19.9	16.7	4.4	5.3	65.9	64.4	6.7	4.9
	mouse 2	21.5	18.1	4.6	2.9	70.1	23.6	7.1	1.3
	mouse 3	27.0	12.5	4.9	2.4	60.9	23.2	5.8	1.6
	MEAN	22.8	15.8	4.6	3.5	65.6	37.1	6.5	2.6
	SEM	3.72	2.91	0.24	1.55	4.61	23.67	0.66	2.01
4hrs.	mouse 1	31.5	11.6	1.01	0.893	24.5	4.56	1.51	0.486
	mouse 2	18.7	18.4	0.615	1.09	21.5	5.48	1.37	0.647
	mouse 3	20.2	17.1	1.03	0.98	24.8	3.71	1.46	0.547
	MEAN	23.5	15.7	0.9	1.0	23.6	4.6	1.4	0.6
	SEM	7.00	3.61	0.23	0.10	1.82	0.89	0.07	0.08
8hrs.	mouse 1	4.71	3.03	<LLOQ	<LLOQ	1.44	0.239	0.526	0.131
	mouse 2	16.8	5.65	<LLOQ	<LLOQ	0.709	0.0747	0.348	0.129
	mouse 3	21.4	2.8	< 0	< 0	1.29	0.134	0.363	0.193
	MEAN	14.3	3.8	0.0	0.0	1.1	0.1	0.4	0.2
	SEM	8.62	1.58	0.00	0.00	0.39	0.08	0.10	0.04
12hrs.	mouse 1	18.6	8.18	<LLOQ	< 0	0.0255	< 0	0.246	0.166
	mouse 2	13.4	0.595	< 0	< 0	0.0478	<LLOQ	0.279	0.051
	mouse 3	17.7	0.815	<LLOQ	< 0	0.0318	< 0	0.141	0.107
	MEAN	16.6	3.20	0.0	0.0	0.0350	0.0	0.222	0.108
	SEM	2.78	4.32	0.00	0.00	0.01	0.00	0.07	0.06
Day 1	mouse 1	2.43	0.729	<LLOQ	< 0	< 0	0.0352	0.0247	0.0729
	mouse 2	1.66	0.142	< 0	< 0	< 0	< 0	<LLOQ	<LLOQ
	mouse 3	0.992	0.529	< 0	< 0	< 0	< 0	0.0451	<LLOQ
	MEAN	1.7	0.47	0.0	0.0	0.0	0.0	0.035	0.073
	SEM	0.72	0.30	0.0	0.0	0.0	0.0	0.01	no SEM
Day 3	mouse 1	< 0	<LLOQ	< 0	< 0	< 0	< 0	0.061	<LLOQ
	mouse 2	< 0	<LLOQ	< 0	< 0	< 0	< 0	< 0	< 0
	mouse 3	< 0	<LLOQ	< 0	< 0	< 0	< 0	< 0	0.031
	MEAN	0.0	0.0	0.0	0.0	0.0	0.0	0.061	0.031
	SEM	0.0	0.0	0.0	0.0	0.0	0.0	no SEM	no SEM
Day 7	mouse 1	< 0	<LLOQ	< 0	< 0	< 0	< 0	< 0	< 0
	mouse 2	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0
	mouse 3	< 0	< 0	< 0	< 0	< 0	<LLOQ	< 0	< 0
	MEAN	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	SEM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

<LLOQ= drug levels detecting below the lower limit of quantitation; <0= no drug detected; no SEM= only 1 out of 3 mice demonstrated quantifiable drug levels.

Table 5.10 Summary of plasma drug levels (µg/ml) in PK-Exp. 2. Table depicts the mean± SEM of three mice over 10 day time points for free- and nanoencapsulated RIF, INH, PZA and ETB

		Free RIF	Nano RIF	Free INH	Nano INH	Free ETB	Nano ETB	Free PZA	Nano PZA
2hrs.	mouse 1	2.4	1.6	40.3	28.4	8.9	5.7	216.0	80.8
	mouse 2	1.2	1.2	43.0	25.6	9.9	6.7	51.1	118.0
	mouse 3	2.4	1.8	32.8	35.2	11.9	7.9	127.0	97.3
	MEAN	2.00	1.50	38.7	29.7	10.2	6.8	131.4	98.7
	SEM	0.68	0.32	5.28	4.94	1.53	1.09	82.54	18.64
8hrs.	mouse 1	1.88	0.86	2.92	4.21	1.41	0.773	1.21	0.677
	mouse 2	5.2	0.74	1.00	2.86	1.26	0.877	0.742	3.51
	mouse 3	4.195	0.575	1.11	5.57	1.45	0.795	0.771	0.416
	MEAN	3.80	0.70	1.70	4.20	1.40	0.80	0.90	1.50
	SEM	1.70	0.14	1.08	1.36	0.10	0.05	0.26	1.72
24hrs.	mouse 1	1.16	8.54	0.346	0.469	0.0394	0.0525	0.0227	0.0488
	mouse 2	0.94	6.51	0.305	0.388	0.0638	0.0567	0.0192	0.0070
	mouse 3	0.94	3.9	0.108	0.466	0.0568	0.0374	0.0114	0.0015
	MEAN	1.00	6.30	0.30	0.40	0.10	0.0489	0.0178	0.0191
	SEM	0.13	2.33	0.13	0.05	0.01	0.01	0.01	0.03
Day2	mouse 1	4.44	0.238	0.206	0.397	0.0236	0.009	0.0029	0.0074
	mouse 2	3.43	< LLOQ	<LLOQ	0.237	0.0124	0.004	0.0051	0.0102
	mouse 3	4.54	0.302	<LLOQ	0.207	0.0045	0.028	< 0	0.002
	MEAN	4.1	0.27	0.21	0.28	0.0135	0.0138	0.004	0.007
	SEM	0.61	0.05	0.00	0.10	0.01	0.01	0.00	0.00
Day3	mouse 1	0	0.0847	< 0	0.301	0.00101	< 0	< 0	0.0124
	mouse 2	0	< LLOQ	< 0	0.231	< 0	0.00363	< 0	< 0
	mouse 3	0	< LLOQ	< 0	0.203	< 0	0.00291	< 0	< 0
	MEAN	0.0	0.08	0.00	0.25	0.001	0.0033	0.00	0.01
	SEM	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00
Day7	mouse 1	< 0	<LLOQ	< 0	0.207	< 0	< 0	0.0009	< 0
	mouse 2	< 0	<LLOQ	< 0	0.162	< 0	< 0	< 0	< 0
	mouse 3	< 0	0.066	< 0	0.241	< 0	< 0	< 0	< 0
	MEAN	0.00	0.066	0.00	0.20	0.00	0.00	0.0009	0.00
	SEM	0.00	no SEM	0.00	0.04	0.00	0.00	no SEM	0.00
Day 10	mouse 1	< 0	<0	< 0	< 0	< 0	< 0	< 0	< 0
	mouse 2	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0
	mouse 3	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0
	MEAN	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	SEM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

<LLOQ= drug levels detecting below the lower limit of quantitation; <0= no drug detected; no SEM= only 1 out of 3 mice demonstrated quantifiable drug levels.

Figure 5.12 (a) and (b) summarizes the plasma drug release profile for RIF in PK-Exp. 1 and PK-Exp. 2, respectively. The release profile in Figure 5.12 (a) observed for the free versus nanoencapsulated RIF were statistically similar up to the eight-hour time point, suggesting that the drug levels detected for the encapsulated drug was the free drug present in the formulation. However, at the 12-hour time point free-RIF was significantly higher than nanoencapsulated RIF ($p \leq 0.01$). Sustained release was only observed up to 24 hours. Since free-drug was also observed up to 24 hours, it cannot be concluded that the sustained release was due to the nanoparticle formulation. Furthermore, some of the LCMS measurements were below the LLOQ, but above the LLOD. The LLOQ for RIF was determined to be 0.05 $\mu\text{g/ml}$ during LCMS method development as previously stated. The MIC for RIF is between 0.03 and 0.06 $\mu\text{g/ml}$. Thus, although the measurements could not be quantified, one cannot conclude that the drug available in plasma would not demonstrate efficacy.

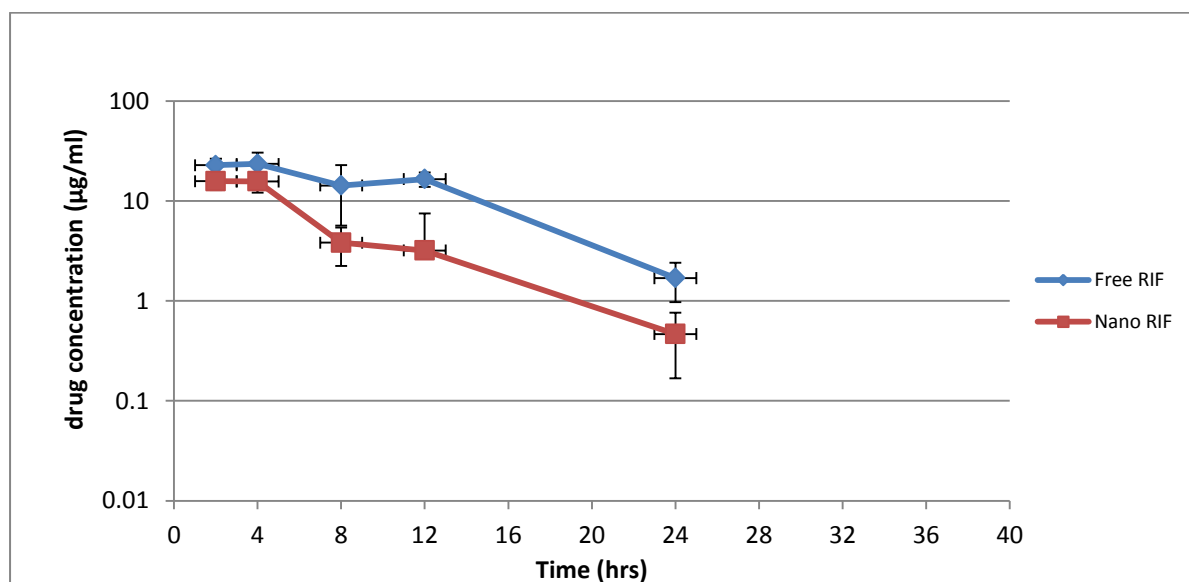


Figure 5.12 (a) Plasma-concentration versus time profile for free and encapsulated RIF in PK-Exp 1 plotted on a logarithmic scale for legibility of the graph. Plasma drug concentrations are depicted as mean $\pm$ SEM.

To address the issue of drug measurements falling below the LLOQ, the dose for RIF was increased six-fold. This adjustment in dose resulted in detection and quantification of nanoencapsulated RIF up to seven days in plasma versus only two days for free RIF. This data provided a clear distinction between nanoencapsulated versus free drug plasma concentrations at the same dose. Furthermore, the observed plasma drug levels up to seven days were above the MIC determined in the *in vitro* studies to be 0.06 $\mu\text{g/ml}$.

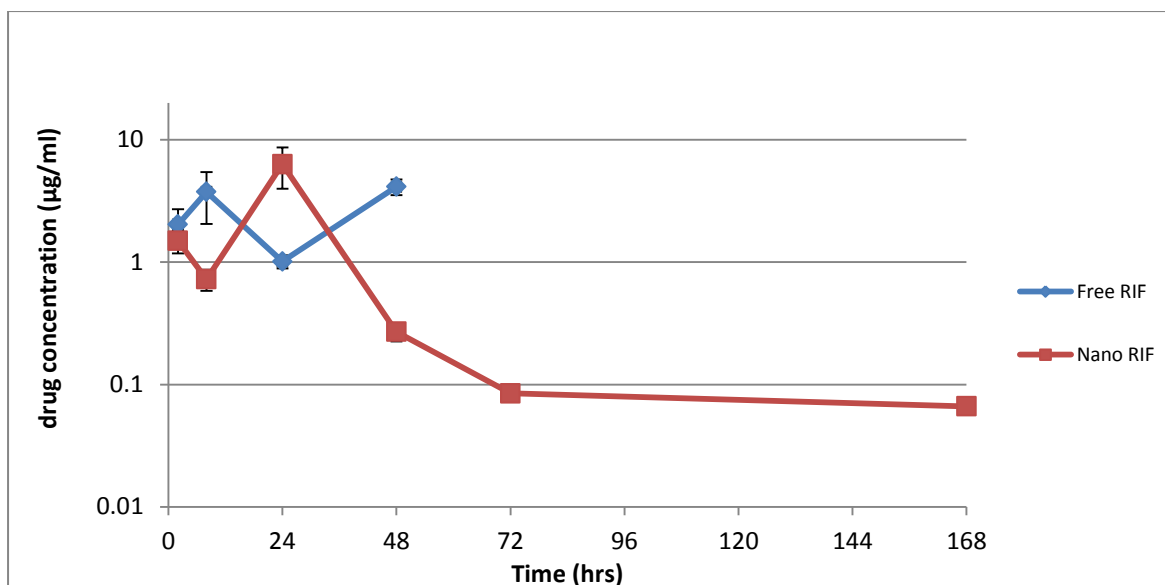


Figure 5.12 (b) Plasma-concentration versus time profiles for free and encapsulated RIF in PK-Exp. 2 plotted on a logarithmic scale for legibility of the graph. Plasma drug concentrations are depicted as mean $\pm$ SEM.

At the standard dose of 10 mg/kg, plasma drug levels of INH were only observed up to 4 hours of the assay (Figure 5.13 (a)). When the dose was increased six times, sustained drug release was maintained for up to seven days at drug levels exceeding 0.2 µg/ml (Figure 5.13 (b)). This may be extrapolated to indicate that at the conventional dose, drug levels can be maintained above the MIC for INH which is 0.03 µg/ml.

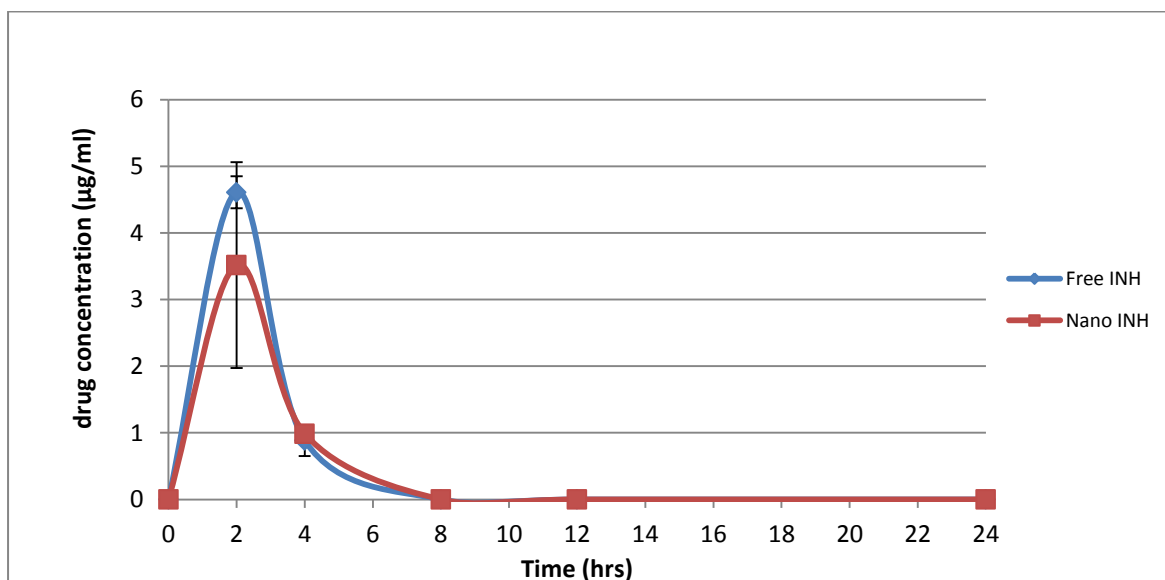


Figure 5.13 (a) Plasma-concentration versus time profile for free and encapsulated INH in PK-Exp. 1. Plasma drug concentrations are depicted as mean $\pm$ SEM.

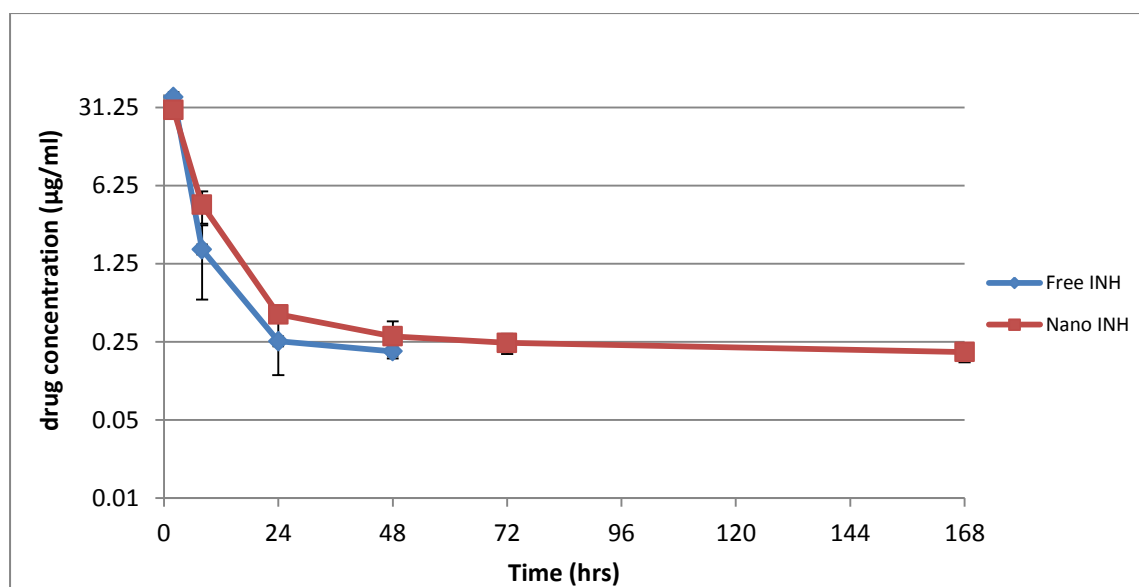


Figure 5.13 (b) Plasma-concentration versus time profile for free and encapsulated INH in PK-Exp. 2 plotted on a logarithmic scale for legibility of the graph. Plasma drug concentrations are depicted as mean $\pm$ SEM.

As stated previously, PZA and ETB were only increased to three times the dose for PK-Exp. 2. These drugs could not be increased six-fold due to high standard doses, i.e. 150 mg/kg and 100 mg/kg, respectively. In the small volume required for dosing, dissolution of free drug and suspension of nanoparticles was not possible. In PK-Exp. 1, plasma drug levels were only observed up to eight hours and 12 hours of analysis for nanoencapsulated and free PZA, respectively (Figure 5.14 (a)). In PK-Exp 2, drug levels were detected beyond eight hours, but these were observed to be below the LLOQ for PZA which was 0.025 µg/ml and could not be used as part of the analysis as observed in Figure 5.14 (b).

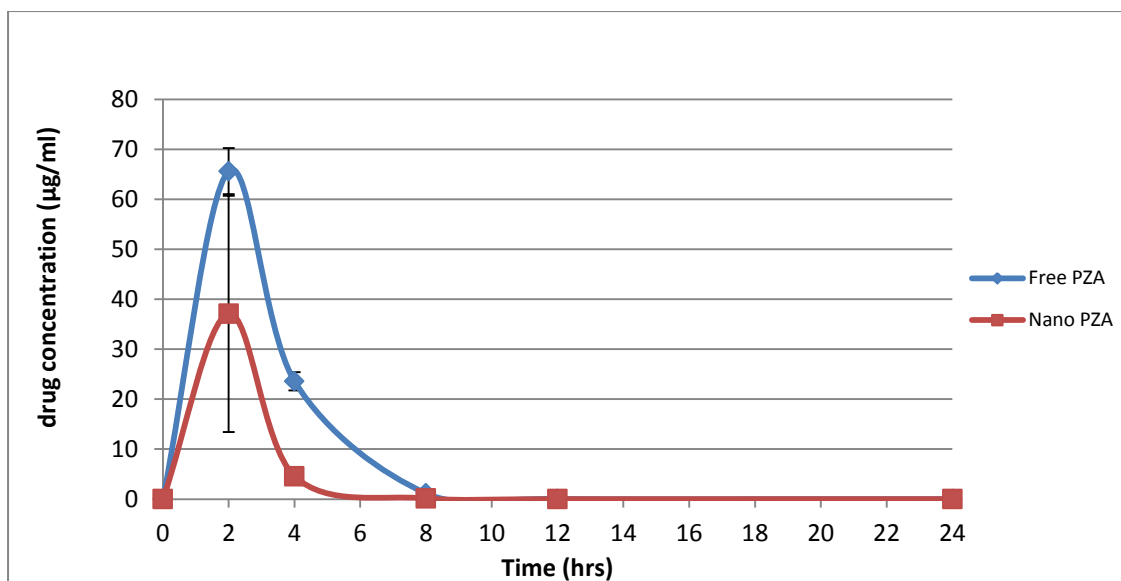


Figure 5.14 (a) Plasma-concentration versus time profile for free and encapsulated PZA in PK-Exp 1. Plasma drug concentrations are depicted as mean $\pm$ SEM.

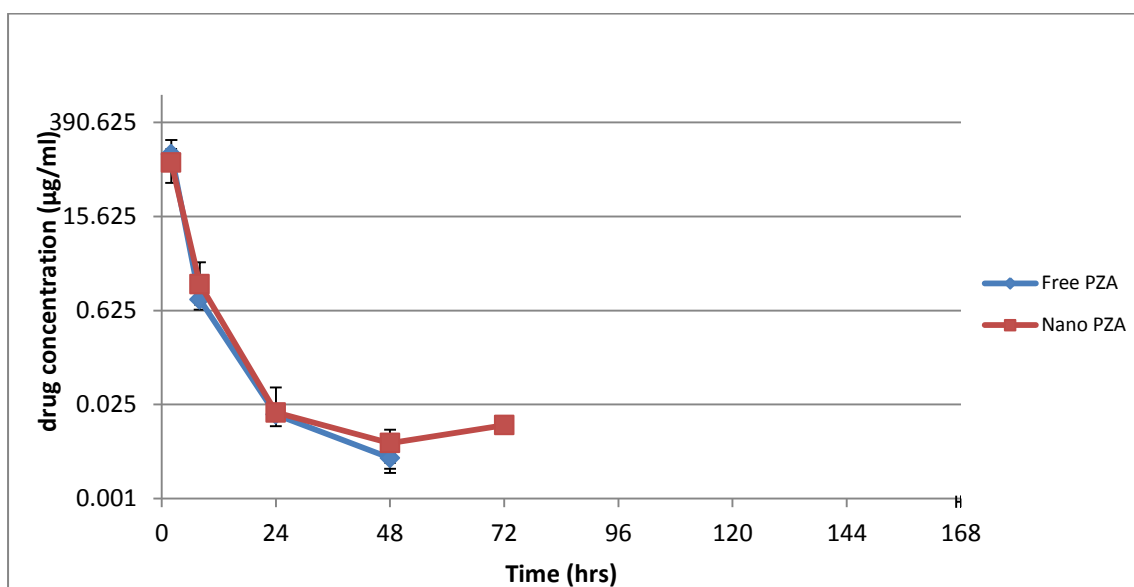


Figure 5.14 (b) Plasma-concentration versus time profile for free and encapsulated PZA in PK-Exp. 2 plotted on a logarithmic scale for legibility of the graph. Plasma drug concentrations are depicted as mean $\pm$ SEM.

The LLOQ for ETB was 0.01 $\mu\text{g/ml}$ and therefore the method of analysis was most sensitive for this drug. In PK-Exp. 1, drug levels were quantified up to three days (Figure 5.15 (a)). However, variation was observed at the three-day time point with only one mouse demonstrating quantifiable drug levels. For PK-Exp. 2 higher and longer plasma drug levels were expected at three times the dose, but drug levels were only observed up to three days of analysis (Figure 5.15 (b)).

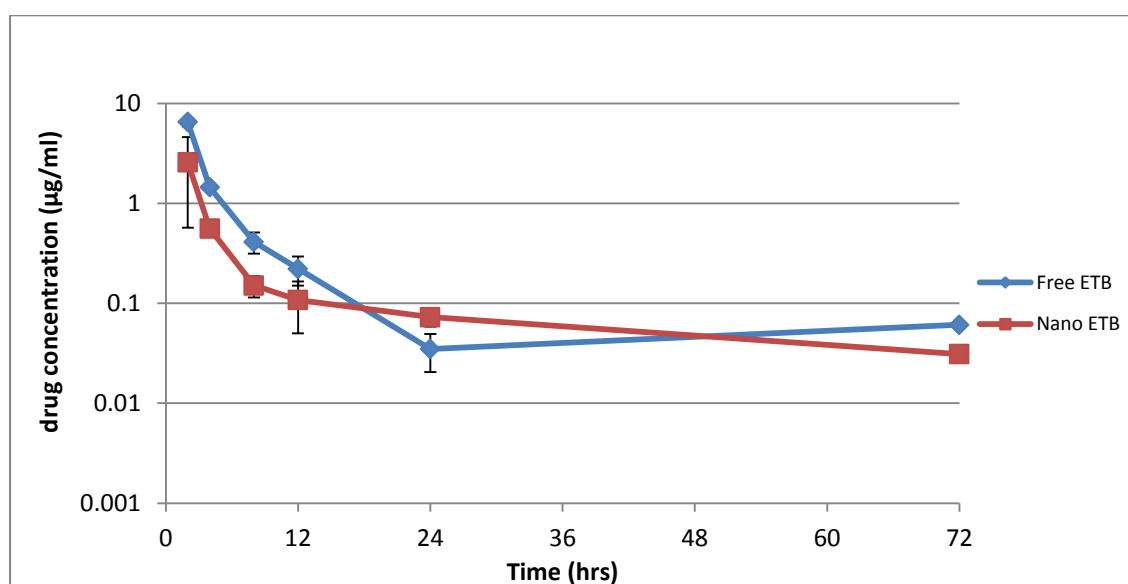


Figure 5.15 (a) Plasma-concentration versus time profile for free and encapsulated ETB in PK-Exp. 1 plotted on a logarithmic scale for legibility of the graph. Plasma drug concentrations are depicted as mean $\pm$ SEM.

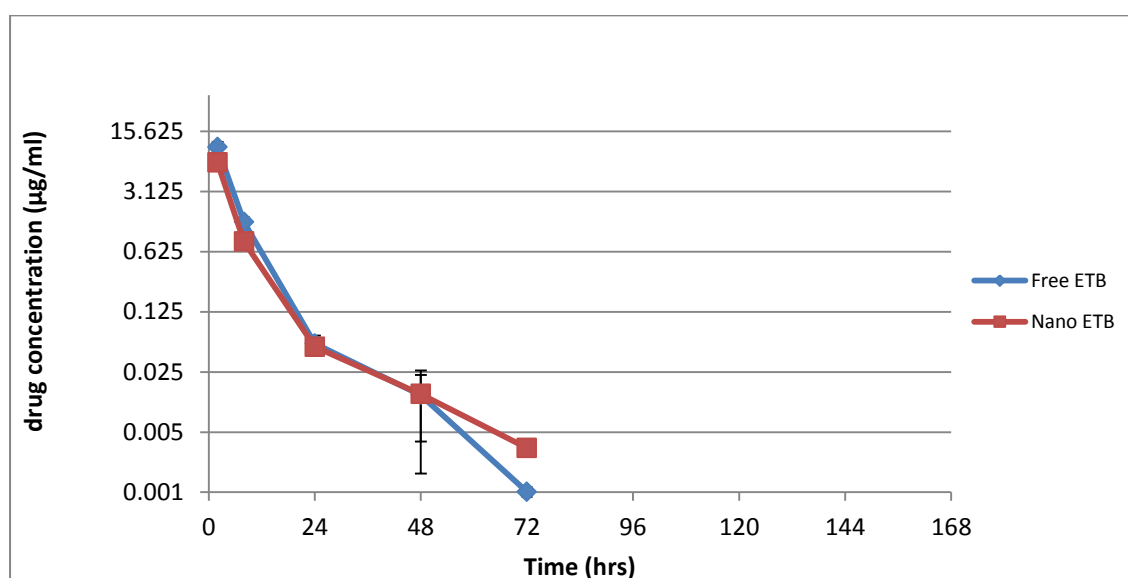


Figure 5.15 (a) Plasma-concentration versus time profile for free and encapsulated ETB in PK-Exp. 2 plotted on a logarithmic scale for legibility of the graph. Plasma drug concentrations are depicted as mean $\pm$ SEM.

5.4.5.2 Pharmacokinetic data analysis

Various pharmacokinetic parameters were evaluated to determine the differences, if any, between free versus nanoencapsulated anti-tuberculosis drugs. PK analysis for PK-Exp. 1 is summarised in Table 5.11. The major difference, besides the lower dose, between this analysis versus that of PK-Exp. 2 (Table 5.12) was that in the former an average mouse weight of 18g was used to calculate the dose. Since this is not very accurate, mice were

weighed individually for PK-Exp. 2 and the dose (mg/kg) adjusted accordingly. The only parameters affected by these differences were AUC, clearance (CL) and volume of distribution at steady state (V_{ss}).

In PK-Exp 1 C_{max} was reached at the 2 hour time point for all four drugs. As observed in the plasma-concentration time curves (Figure 5.12; 5.13; 5.14; and 5.15 (a)) the $AUC_{0 \rightarrow t}$ ($\mu\text{g/ml.min}$) was significantly higher for free RIF, PZA and ETB ($p \leq 0.01$) compared to the nanoencapsulated drugs except in the case of INH, where no significant difference was observed. This indicates a higher extent of absorption for free versus encapsulated drugs, which was expected, since a higher concentration is available faster at the absorption site compared to the controlled release facilitated by the nanoparticles. In all instances, the half-lives were short compared to reported values for these drugs (Budha *et al.* 2008:813). Since the half-life of a drug is directly related to the elimination rate constant, the values observed for these parameters correspond with the release profile. An exception was observed for ETB-NP, with a slightly longer half-life. Of the drugs evaluated, plasma drug levels beyond 24 hours were only observed for ETB in PK-Exp. 1. The V_{ss} was also observed to be slightly higher for ETB-NP (2.1L). ETB-NP also displayed the highest clearance rate (290 ml/min) compared to the other drugs and since ETB is primarily metabolized by the liver and excreted unchanged in the urine (Budha *et al.* 2008:816), it would be expected that ETB drug levels will be observed in the kidneys and liver. Drug distribution assays described in section 5.4.4.2 will provide better insight into the clearance and V_{ss} values observed.

Table 5.12 summarizes the PK analysis for PK-Exp. 2. All drugs reached C_{max} at 2 hours except RIF-free, which only reached C_{max} at the 8-hour time point. RIF-free demonstrated a larger AUC which was significantly higher ($p \leq 0.01$) than what was observed for the other drugs analysed. For RIF-NP and INH-NP, plasma drug levels were detected up to 7 days of analysis. The half-life of these drug formulations were significantly longer ($p \leq 0.01$) compared to free drug at 3.85 and 5.77 hours, respectively. This is based on the determination of the elimination rate constant which is calculated as discussed in section 5.3.10.2. As was observed in PK-Exp. 1, ETB-NP demonstrated faster clearance and large V_{ss} , compared to the ETB-free and the free and nanoencapsulated forms of the other drugs. Although, individual mouse weights were not significantly different, the clearance and V_{ss} values calculated in Table 5.16 are considered to be more accurate since these values were based on the average weight of 3 mice per time point. Higher absorption was observed for PK-Exp. 2

than PK-Exp. 1 based on $AUC_{0 \rightarrow t}$ (ug/ml.min) ($p \leq 0.01$) values. Whether suspension in 20% Tween 80 played a role in the increased absorption could not be concluded since the effect of Tween 80 was not specifically assessed. It could be assumed that since the dose was increased six-fold in INH and RIF and three-fold in PZA and ETB, the rate of absorption was increased as a result of a higher concentration gradient at the absorption site.

Table 5.11 Plasma drug analysis in PK-Exp. 1 for free-and nanoencapsulated RIF, INH, PZA and ETB. PK parameters indicated in the table were calculated as described in section 5.3.10.2.

Drug	Weight (kg)	Dose (mg/kg)	Dose (mg)	$AUC_{0 \rightarrow t}$ (ng/mL * min)	$AUC_{0 \rightarrow t}$ (ug/mL * min)	$k_{el}(\lambda)$ (min ⁻¹)	$AUC_{0 \rightarrow \infty}$ (ng/mL * min)	$t_{1/2\lambda}$ (min)	$t_{1/2\lambda}$ (hr.)	C_{max} (ng/mL)	T_{max} (min)	CL (mL/min)	$AUMC_{0 \rightarrow t}$ (ng/mL * min ²)	MRT (min)	V_{ss} (mL)	V_{ss} (L)
RIF-F	0.018	10.00	0.18	333766.7	334	0.1900	7864069.7	3.6	0.0600	22800.0	120	0.54	3594986.7	10.8	6	0.0
RIF-NP	0.018	10.00	0.18	117746.7	118	0.1380	142336.4	5.0	0.0837	15766.7	120	1.53	985266.7	8.4	13	0.0
INH-F	0.018	25.00	0.45	7265.0	7	0.8250	7265.0	0.8	0.0141	4610.0	120	61.9	19840.0	2.7	169	0.2
INH-NP	0.018	25.00	0.45	6479.7	6	0.6300	6479.7	1.1	0.0183	3516.7	120	69.4	18885.3	2.9	202	0.2
PZA-F	0.018	150.00	2.70	141298.9	141	0.8140	141342.2	0.9	0.0143	65633.3	120	19.1	454512.5	3.2	61	0.1
PZA-NP	0.018	150.00	2.70	65939.3	66	0.0600	65939.3	11.6	0.1925	37066.7	120	40.9	267010.7	4.0	166	0.2
ETB-F	0.018	100.00	1.80	19737.7	20	0.1540	22909.1	4.5	0.0750	6533.3	120	91.2	406285.3	20.6	1877	1.9
ETB-NP	0.018	100.00	1.80	6207.9	6	0.0760	7249.3	9.9	0.1519	2583.3	120	290.0	44655.7	7.2	2086	2.1

Drug-F- unencapsulated (free) drug; Drug-NP- nanoencapsulated drug; $AUC_{0 \rightarrow t}$: area under the concentration-time curve for time 0 to time t; $k_{el}(\lambda)$: terminal elimination rate constant; $AUC_{0 \rightarrow \infty}$: area under the concentration-time curve for time 0 to infinity (∞); $t_{1/2\lambda}$: terminal half life; CL: systemic clearance; C_{max} : maximum concentration; T_{max} : time of maximum concentration (C_{max}); $AUMC_{0 \rightarrow t}$: area under the first moment curve; MRT: mean residence time; V_{ss} : volume of distribution at steady state

Table 5.12 Plasma drug analysis in PK-Exp. 2 for free-and nanoencapsulated RIF, INH, PZA and ETB. PK parameters indicated in the table were calculated as described in section 5.3.10.2.

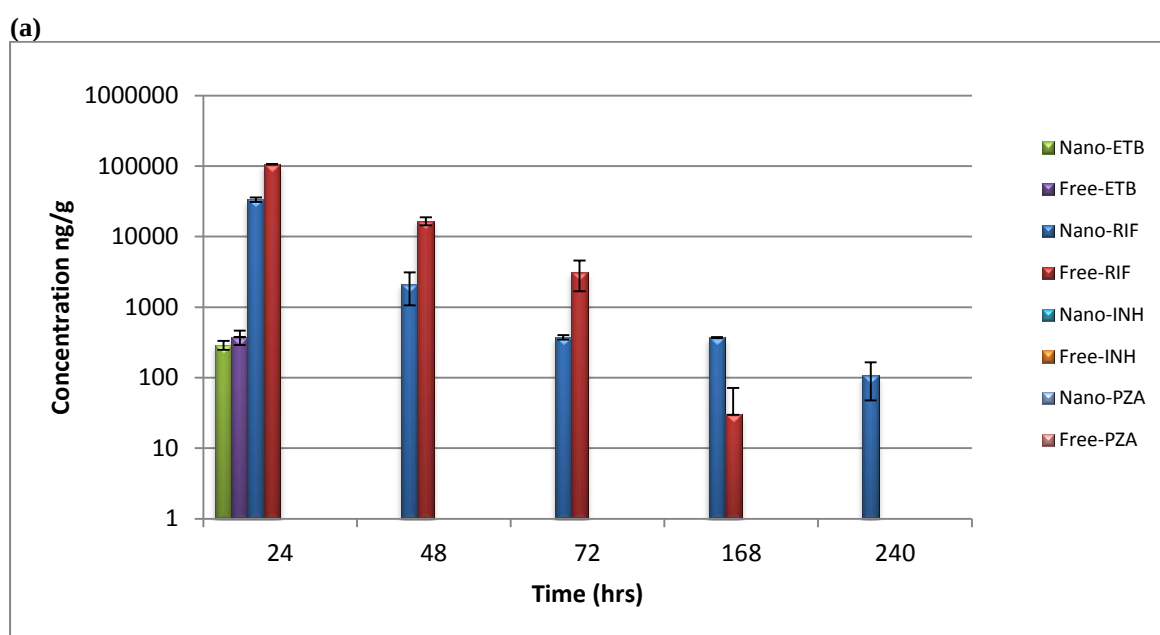
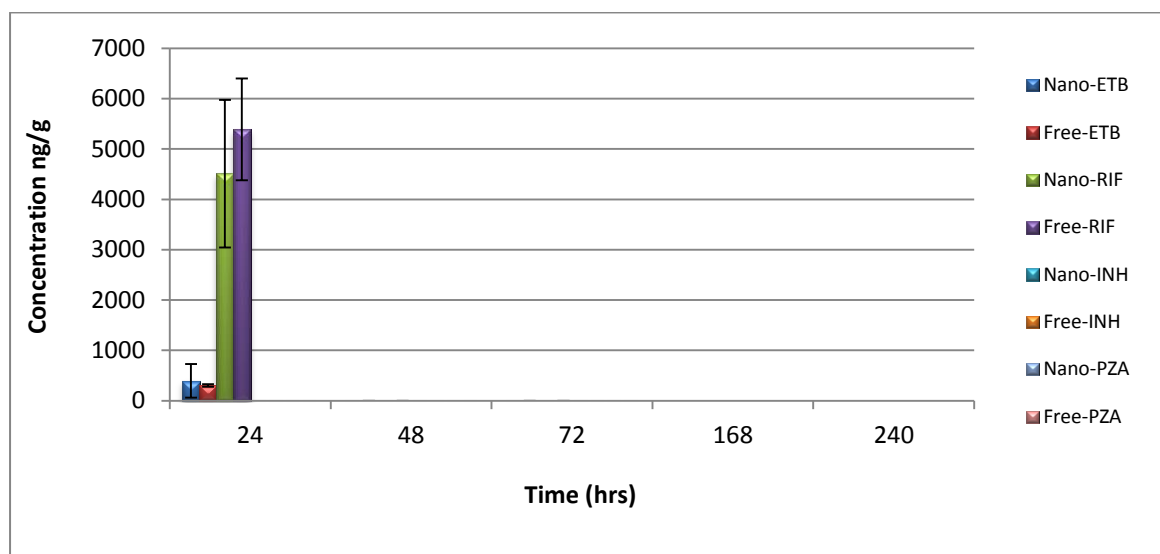
Drug	Weight (kg)	Dose (mg/kg)	Dose (mg)	$AUC_{0 \rightarrow t}$ (ng/mL * min)	$AUC_{0 \rightarrow t}$ (ug/mL * min)	$k_{el}(\lambda)$ (min ⁻¹)	$AUC_{0 \rightarrow \infty}$ (ng/mL * min)	$t_{1/2\lambda}$ (min)	$t_{1/2\lambda}$ (hr.)	C_{max} (ng/mL)	T_{max} (min)	CL (mL/min)	$AUMC_{0 \rightarrow t}$ (ng/mL * min ²)	MRT (min)	V_{ss} (mL)	V_{ss} (L)
RIF-F	0.019	60	1.146	1057231	1057	0.0600	1126175.8	11.0	0.183	55666.67	480	1.08	15565339	14.7	16	0.016
RIF-NP	0.019	60	1.145	408497.7	408	0.0000	498497.7	231	3.85	50200	120	2.80	5479347	13.4	38	0.016
INH-F	0.019	150	2.901	144536.3	145	0.0400	149686.3	77.0	1.283	38700	120	20.1	738410.7	5.1	103	0.1
INH-NP	0.020	150	2.985	362932.6	363	0.0000	503099.3	346.5	5.775	29733	120	8.2	6452101	17.8	145	0.1
PZA-F	0.021	450	9.489	404439.7	404	0.3700	404439.7	2.8	0.047	131367	120	23.5	876602.7	2.2	51	0.1
PZA-NP	0.022	450	9.760	313359.6	313	1.3700	313359.6	0.5	0.008	98700	120	31.3	736387.7	2.3	74	0.1
ETB-F	0.017	300	5.232	47157.17	47	0.5700	47180.8	2.0	0.033	10220	120	110.9	223317.7	4.7	525	0.5
ETB-NP	0.018	300	5.343	30604.41	31	0.5000	30632.1	1.4	0.023	6777	120	174.5	151775.8	5.0	865	0.9

Drug-F- unencapsulated (free) drug; Drug-NP- nanoencapsulated drug; $AUC_{0 \rightarrow t}$: area under the concentration-time curve for time 0 to time t; $k_{el}(\lambda)$: terminal elimination rate constant; $AUC_{0 \rightarrow \infty}$: area under the concentration-time curve for time 0 to infinity (∞); $t_{1/2\lambda}$: terminal half life; CL: systemic clearance; C_{max} : maximum concentration; T_{max} : time of maximum concentration (C_{max}); $AUMC_{0 \rightarrow t}$: area under the first moment curve; MRT: mean residence time; V_{ss} : volume of distribution at steady state

5.4.6 Drug distribution assays

As described in section 5.3.17, mouse organs were harvested in both PK-Exp. 1 and PK-Exp. 2 to evaluate the drug distribution of nanoencapsulated drugs versus free drugs. The major difference between the two studies with regards to drug distribution was sample preparation. In PK-Exp. 1, the organs were weighed to the closest 100mg, filled to volume to obtain 100mg/ml and homogenized. The concern with this practice was that the drug might localise in a section of the organ which was discarded during sample preparation. In a previous study (Chapter 3), nanoparticles were observed to localize in different areas of the organs evaluated and it was expected that the drugs would be released at these sites (Semete *et al.* 2010:669). As a result, sample preparation was amended in PK-Exp. 2 by weighing the entire organ, adjusting the volume to obtain 200 mg/ml, to obtain a more concentrated, representative sample, and then homogenized. Drug distribution for the two studies will now be evaluated. Of the organs harvested, only the organs of interest, namely lungs, liver, kidney and spleen were evaluated.

Figure 5.16 (a) and (b) illustrates liver distribution for the four drugs evaluated, i.e. RIF, INH, PZA and ETB. Similar levels of RIF were observed for encapsulated and free drug in Figure 5.18 (a) at 24 hours. At six times the dose (Figure 5.16 (b)), drug levels were observed up to 10 days for nanoencapsulated and seven days for free drug. RIF is primarily metabolized by the liver and the increased dose may be as a result of drug accumulation in the liver (Budha *et al.* 2008). INH was not quantifiable at the conventional dose in the liver. At seven days, low levels were observed for free INH and only two out of three mice evaluated had detectable nanoencapsulated INH, with one being below LLOQ and one significantly higher ($p \leq 0.01$), which is the reason for the high standard deviation. Free drug was observed between two days and seven days, but the levels at seven days were below the LLOQ. Considering the low residence time of PZA in plasma, higher tissue distribution was expected. However, no tissue drug levels were detected in any of the tissues evaluated for all of the organs evaluated. ETB drug levels were observed at similar concentrations at 24 hours for both experiments.

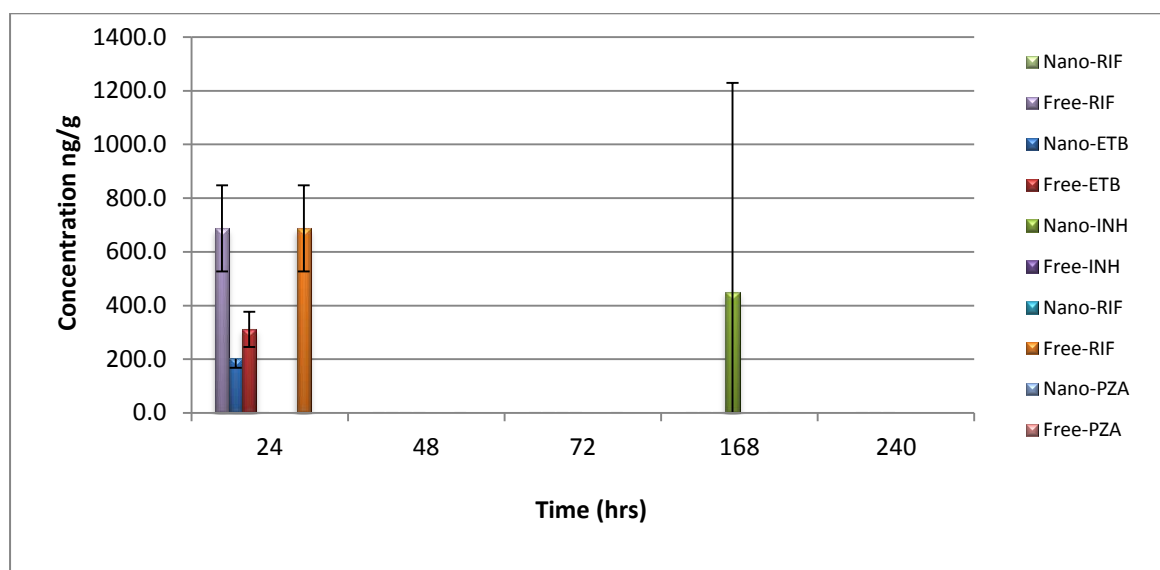


(b)

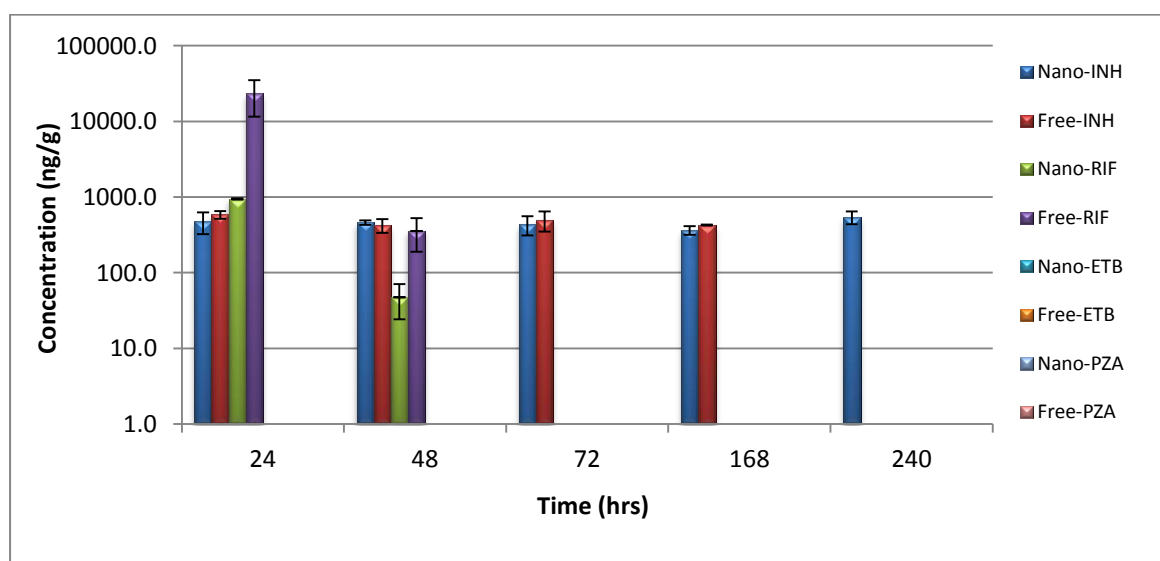
Figure 5.16 Summary of drug distributions of free- and nanoencapsulated RIF, INH, PZA and ETB in mouse liver at (a) conventional dose and (b) logarithmic scale at six times the dose. Mean $\pm$ SEM is shown in the graphs.

Drug levels for RIF were detected in the lungs at 24 hours and up to 48 hours in both PK-Exp. 1 and PK-Exp. 2, respectively. According to literature RIF attains lung concentration equal to or more than serum levels (Budha *et al.* 2008:816). Therefore, for conventional drug administration of RIF (Figure 5.17 (a)) drug accumulation in the lungs was not expected. However, at six times the conventional dose, RIF would be expected to be present for a longer period as was observed in plasma, but this was not the case as demonstrated in Figure 5.17 (b). INH demonstrated similar accumulation in the lung, comparable to liver accumulation of RIF at six times the conventional dose. At the conventional dose, only one

out of the three mice evaluated demonstrated high drug levels in the lungs at seven days, hence the high standard deviation. ETB drug levels in the lungs were only observed until 24 hours at the conventional dose, but no drug was detected in the lungs when the dosage was increased six times.



(a)

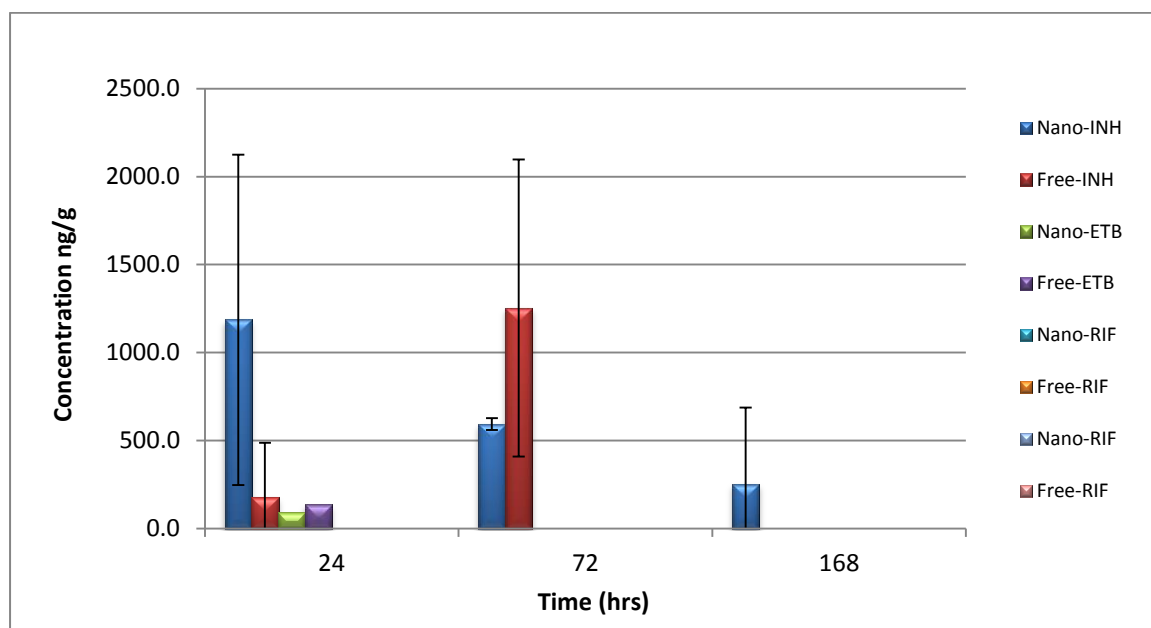


(b)

Figure 5.17 Summary of drug distributions of free- and nanoencapsulated RIF, INH, PZA and ETB in mouse lung at (a) conventional dose and (b) logarithmic scale at six times the dose. Mean $\pm$ SEM is shown in the graphs.

RIF's drug levels were too low to detect in the spleen at the conventional dose (Figure 5.18 (a)). Significantly higher levels were observed for free versus encapsulated RIF at 24 hours ($p \leq 0.01$) (Figure 5.18 (b)). Free and encapsulated INH was observed in the spleen up to three days and seven days, respectively for PK-Exp. 1. At six times the dose, no INH levels were

observed. At time points beyond 24hrs., high standard deviations were observed for ETB in the spleen. For the conventional dose, only free ETB was detected, whereas nanoencapsulated ETB was observed in up to 48 hours at six times the dose.



(a)

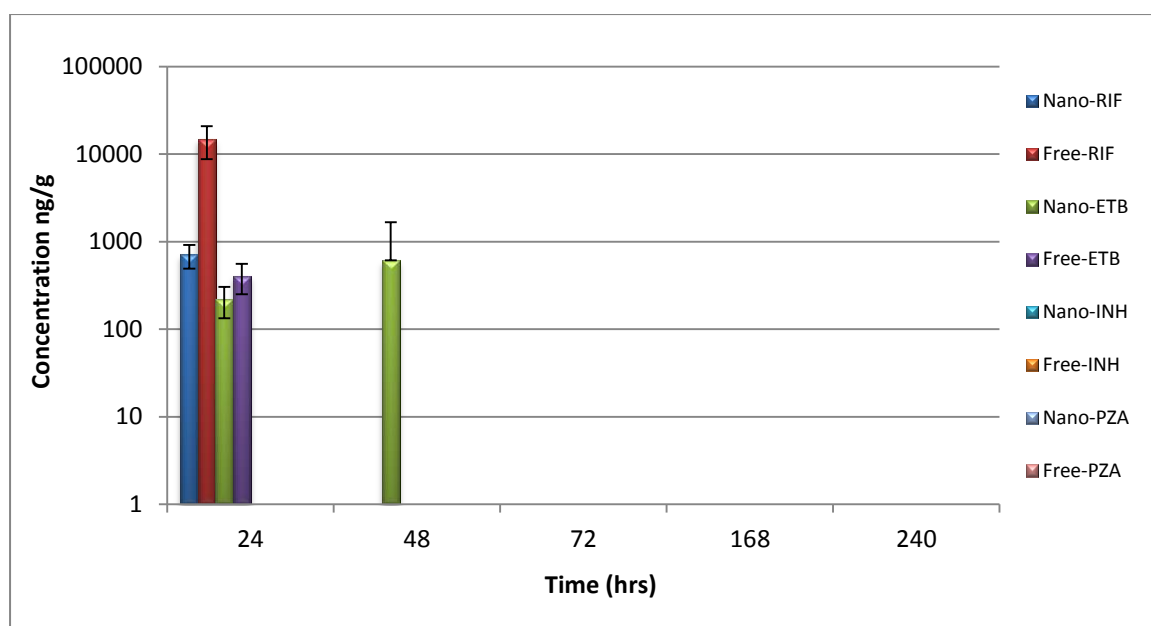
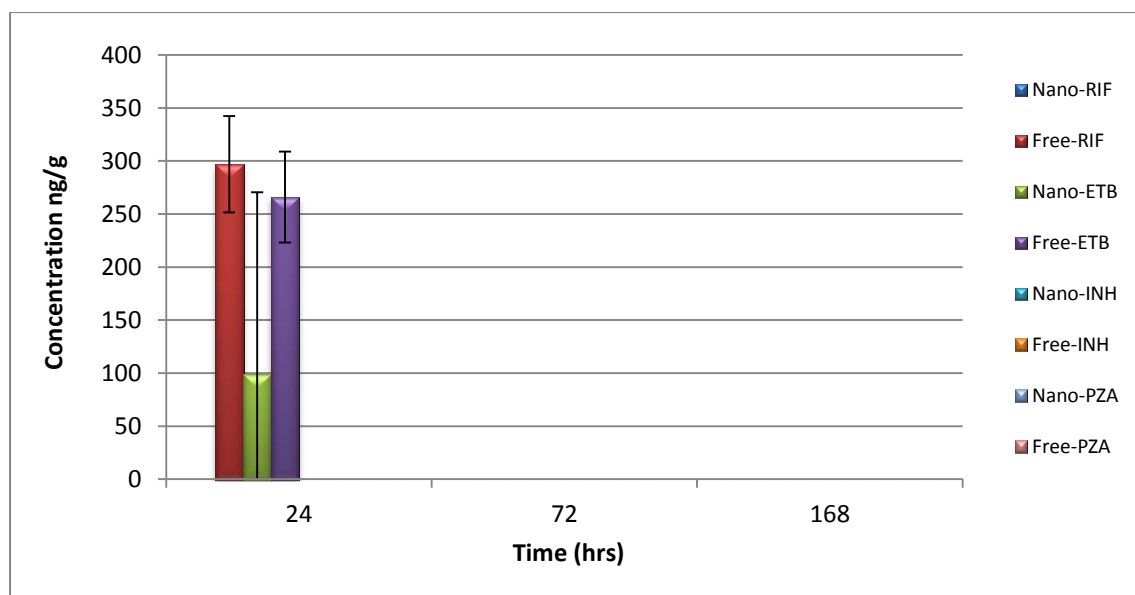


Figure 5.18 Summary of drug distributions of free- and nanoencapsulated RIF, INH, PZA and ETB in mouse spleen at (a) conventional dose and (b) logarithmic scale at six times the dose. Mean $\pm$ SEM is shown in the graphs.

Figure 5.19 (a) and (b) demonstrates RIF levels up to 24 hours with significantly higher concentrations at six times the dose ($p \leq 0.01$). In Figure 5.19 (b) only free RIF was observed at 48 hours and INH at 7 days in the kidney. At higher doses, ETB drug levels were detected

in the kidney up to three days for both free and nanoencapsulated ETB. In PK-Exp. 1, drug levels were only detected up to 24 hours.



(a)

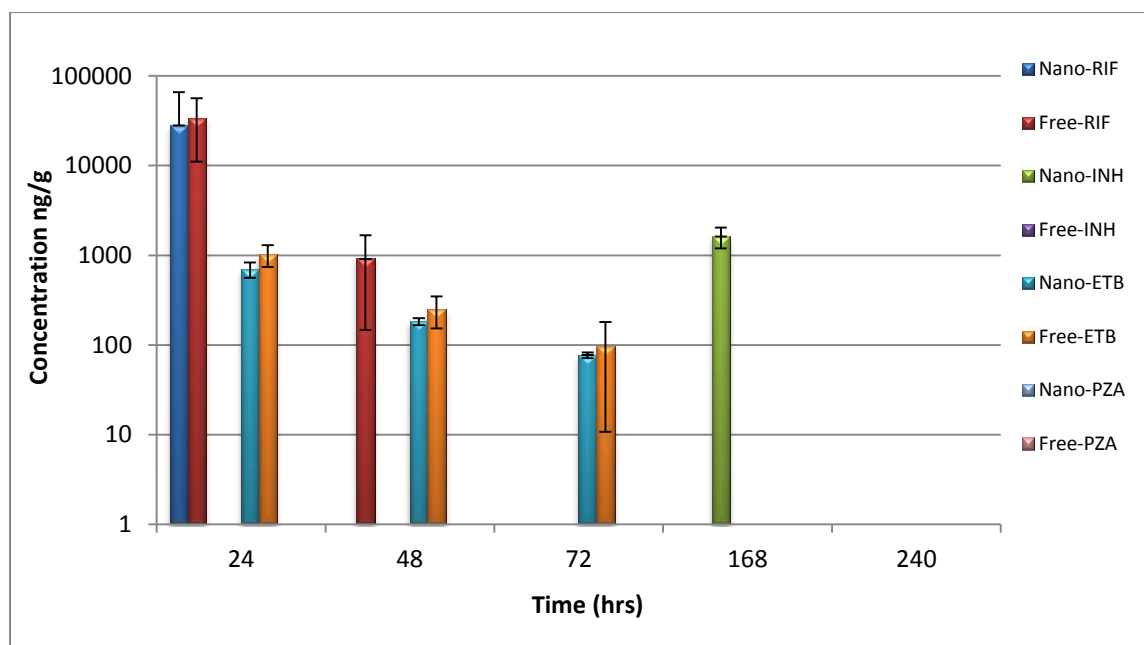


Figure 5.19 Summary of drug distributions of free- and nanoencapsulated RIF, INH, PZA and ETB in mouse kidneys at (a) conventional dose and (b) logarithmic scale at six times the dose. Mean $\pm$ SEM is shown in the graphs.

5.5 Discussion

The primary methods currently used to formulate and manufacture nanoparticles are freeze-drying, emulsion solvent evaporation techniques and/or solvent exchange, salting out (du

Toit *et al.* 2008) and supercritical fluid technology. The challenges in the application of these techniques include aspects such as low drug loading capacity and encapsulation efficiency as well as poor control of the uniformity of particle distribution and the use of solvents (Park 2007), which greatly affect pharmaceutical parameters of the encapsulated drugs. The main objectives of any drug delivery system is to achieve optimum effectiveness, improve bioavailability and ensure maximum drug safety by ensuring cellular uptake of drugs, improve drug solubility and minimizing first pass metabolism (Mahato 2007). Although these are ideal objectives to strive towards, they are not always practically achieved since pharmacokinetic and pharmacodynamic factors and the physicochemical properties of the carrier system greatly influence the success of the drug delivery system (Mahato 2007).

The nanoparticle preparation resulted in small sized (235-298 nm) particles and exhibited satisfactory drug encapsulation efficiency (57-67%). One of the important pharmacodynamic factors to consider is whether the MIC can be maintained over time to ensure maximum growth inhibition in TB chemotherapy. Furthermore, if minimum bactericidal concentration can be reached, it may present the possibility for a faster cure rate. As discussed previously (Chapter 3 and 4), surface-modified nanoparticles are capable of evading recognition by the MPS and thus prolong circulation time. If the nanoparticles remain in circulation for a longer period and is capable of releasing drug levels above MIC and possibly MBC, this supports the potential for nanoparticle formulations to reduce dosing frequency (Ahmad *et al.* 2007). The results in section 5.4.2 demonstrated *in vitro* growth inhibition for nanoparticles encapsulating RIF and INH. ETB nanoparticles demonstrated no growth inhibition in these assays, possibly due to the high ETB MIC (1-2 µg/ml) required to be released from the nanoparticle formulations. Concluding that this cannot be achieved *in vivo* would be inaccurate since the *in vitro* media used (7H9 broth) may not have been ideal for ETB drug release. The starting concentration for the assay was 32 µg/ml since it had to be taken into consideration that sufficient drug had to be released from the nanoparticle during the time course of the study, i.e. 18 days, in the media used. The MIC profiles for RIF-nanoparticle formulations at 8 µg/ml were comparable to the free-drug controls at MIC for RIF as observed in this study (0.06 µg/ml). Similarly, INH nanoparticle formulations (16 µg/ml) demonstrated MIC profiles comparable to free-drug MIC profiles at MIC for INH (0.03 µg/ml). Thus, at the nanoparticle concentrations used sufficient drug was released to facilitate growth inhibition over time *in vitro*. Therefore, based on these *in vitro* assays, nanoparticle-based RIF and INH chemotherapy forms a sound basis for a reduction in dosing

frequency and also offers the possibility of reducing the drug dosage. Confirming these findings with *in vivo* efficacy assays would be the focus for future work. *In vivo* bioassays were conducted to evaluate whether drug release from RIF and INH formulations were sufficiently bioavailable to facilitate MIC. This was a combination *in vitro/in vivo* assay where serum samples from drug treated mice were used to conduct an *in vitro* assay with *M.tb* H37Rv. The results demonstrated that serum drug levels were sufficient up to three days of analysis to facilitate MIC. Although this was considered as a positive result in terms of bioavailability, the data could not be extrapolated to *in vivo* efficacy since the serum drug levels need to be sufficient at the disease sites where *M.tb* resides and this can only be confirmed by *in vivo* efficacy assays.

In a separate assay, MIC profiles were evaluated as part of an MBC study. The difference in the two assays was that the starting concentrations for the *M.tb* inoculant were significantly higher than the MIC-only assay. INH nanoparticles demonstrated similar results to prior MIC assays with the lower concentration inoculant, but for RIF nanoparticles, growth inhibition at 16µg/ml was observed. Thus at 16µg/ml sufficient drug was released from the nanoparticles to facilitate growth inhibition. Another important finding was that for both formulations MBC values were attained. For these MBC studies, 99% killing was achieved at 20µg/ml and 30µg/ml for RIF and INH nanoparticles, respectively. 100% killing was not achieved for either of the formulations, but reaching MBC levels *in vitro* was a very promising result.

Pharmacokinetic parameters such as plasma drug release profile and drug distribution was evaluated. It has been reported that near zero-order release kinetics and modification and improvement of pharmacokinetic parameters such as tissue distribution profile are achieved through nanoparticulate drug delivery (Couvreur & Vauthier 2006:1417). Other advantages include improved bioavailability and decreased toxicity due to high loading efficiency which results in lower doses administered. These factors all culminate in an increase in patient compliance to treatment (Kingsley *et al.* 2006:346). It has also been reported that nanoparticles improve bioavailability by eliminating food effects on absorption (Wu *et al.* 2004:135). Drug release profiles were evaluated in two assays with adjusted variables as discussed in section 5.3.9. In both assays for free and encapsulated drug, a sharp C_{max} was observed with a slow release over 24 hours for the first assay in INH, RIF and PZA and three days for ETB. However, the release profiles at most of the time points were very similar, which suggested that it was possibly a measure of the unencapsulated drug present in the

nanoparticle formulation being detected. In the second assay where the dosage was adjusted, INH and RIF nanoparticles maintained controlled drug release over seven days and free drugs only three days, which provided a clear distinction between nanoparticulate drug release versus free drug. The $AUC_{0 \rightarrow \infty}$ values for these release profiles suggested improved absorption compared to what has been previously reported (Table 2.4. chapter 2) and therefore, improved bioavailability which supports the hypothesis. Furthermore, the mice survived the duration of the assay (seven days) and appeared healthy, i.e. active and feeding well. Thus, it could be concluded that at the higher doses (six times for INH and RIF and three times for PZA and ETB) no toxicity was observed. Besides RIF-NP and INH-NP, the half-lives of the other drugs were significantly shorter than reported values (Budha *et al.* 2008), but these are based on conventional once-daily dosing. Based on these results, INH and RIF nanoparticles demonstrated potential for reduction in dosing frequencies.

Subsequent to entering the vascular system, drugs are distributed to various tissues and body fluids, i.e. extravascular fluid. The differences in tissue distribution exist for different drugs and this is largely attributed to differential tissue affinity, blood flow to tissues, the drugs ability to cross biological membranes and also the drugs' physicochemical properties, i.e. lipophilicity and extent of ionization (Budha *et al.* 2008:812). In PK-Exp. 2, the organs harvested were homogenized as a whole organ and analysed at 200mg/ml of the tissue homogenate. Thus, these data was considered most accurate, since a representative sample of an organ was analysed. ETB drug levels encapsulated in nanoparticles were observed in all organs analysed at up to 24 hours in the liver and lungs, 48 hours in the spleen and up to three days in the kidneys. ETB is metabolized by the liver and excreted unchanged in the urine, thus exhibiting renal clearance (Davies & Nuermberger 2008:S68). With the LCMS method developed ETB could be quantified at 10ng/ml, thus the absence of ETB at other time points were either too low to detect or too low to quantify. RIF drug levels encapsulated in nanoparticles was observed up to 10 days in the liver. Since RIF accumulates and is metabolized by the liver due to first pass metabolism, this was an expected result. The presence of free-RIF was unexpected since clearance within 24 hours was expected. However, the six-fold dose increase may have played a role in this observation. RIF drug levels encapsulated in nanoparticles were observed in the lungs up to two days and in the spleen and kidneys up to 24 hours. RIF is readily distributed into lung parenchyma and kidneys (Budha *et al.* 2008:816), which explains the high drug concentrations in the kidneys at 24 hours, but the low drug concentrations in the lungs cannot be explained. INH

nanoparticles were observed up to 10 days in the lungs which suggests drug accumulation in the lungs. No INH drug levels encapsulated in nanoparticles were observed in the liver and spleen. However, a surprising observation was INH drug concentrations in the spleen up to three days at the conventional dose. At higher doses, no drug was detected. It was not possible to conclude whether this was as a result of variability of *in vivo* assays or as a result of dosage concentration. INH drug levels encapsulated in nanoparticles were observed in the kidneys only at the three days' time point with no drug concentrations observed at earlier time points. Only 5-30% of INH is excreted in urine, this may be why earlier drug concentrations in the kidneys were not detected. PZA was not detected in any of the organs evaluated. The pharmacokinetic parameters calculated did not provide an explanation for the absence of PZA in the organs and the low plasma drug levels. PZA has been documented as being widely distributed throughout the body with excellent tissue penetration in the central nervous system, lungs and liver as well as achieving high drug levels in cerebrospinal fluid (Budha *et al.* 2008:816). Therefore, the absence of PZA in all of the abovementioned tissues could only be explained in that the drug was possibly residing in tissues that were not evaluated and levels in the lungs and liver were too low to detect. Considering the low V_{ss} values calculated for all the drugs in Tables 5.13 and 5.14, it may be assumed that the drugs were residing largely within the vascular space since volume of distribution relates the amount of drug in the body to plasma drug levels. Larger V_{ss} may have resulted in higher drug levels in the tissues.

5.6 Conclusion

Spray-dried PLGA nanoparticle formulations encapsulating ant-tuberculosis drugs demonstrated promising pharmacodynamic properties, *in vitro*. Furthermore, pharmacokinetic data generated resulted in a 7-day sustained release profile for RIF and INH and 3-day profile for PZA and ETB and acceptable bioavailability *in vivo* to facilitate growth inhibition *in vitro*. Although drug-free nanoparticles were detected in organs for up to 7 days in previous studies (Chapter 4, (Semete *et al* 2010) only RIF and INH were detected in the liver and lungs, respectively for up to 7 days. Thus, although nanoparticle tissue distribution has been demonstrated, sufficient drug release of all four drugs was not achieved on a quantifiable scale over 7 days. Overall, this spray-dried formulation demonstrated promising PK/PD data for use in TB chemotherapy, but further studies are warranted to address inexplicable findings.

References

- Ahmad, Z., Pandey, R., Sharma, S., & Khuller, G.K. 2008. Novel chemotherapy for tuberculosis: chemotherapeutic potential of econazole- and moxifloxacin-loaded PLG nanoparticles. *International journal of antimicrobial agents*, 31(2):142-146.
- Ahmad, Z., Pandey, R., Sharma, S., & Khuller, G.K. 2006. Pharmacokinetic and pharmacodynamic behaviour of antitubercular drugs encapsulated in alginate nanoparticles at two doses. *International journal of antimicrobial Agents*, 27(5):409-416.
- Ahmad, Z., Sharma, S., & Khuller, G.K. 2007. Chemotherapeutic evaluation of alginate nanoparticle-encapsulated azole antifungal and antitubercular drugs against murine tuberculosis. *Nanomedicine: nanotechnology, biology and medicine*, 3(3):239-243.
- Budha, N.R., Lee, R.E., & Meibohm, B. 2008. Biopharmaceutics, Pharmacokinetics and Pharmacodynamics of Antituberculosis Drugs. *Current medicinal chemistry*, 15(8):809-825.
- Chen, X.M., Zhu, J.B., Sun, W.D., & Zhang, L.J. 2006. Effect of absorption enhancers on nasal ginsenoside Rg1 delivery and its nasal ciliotoxicity. *Acta pharmaceutica sinica*, 41(2):149-155.
- Couvreux, P. & Vauthier, C. 2006. Nanotechnology: Intelligent design to treat complex disease. *Pharmaceutical research*, 23(7):1417-1450.
- Davies, G.R. & Nuermberger, E.L. 2008. Pharmacokinetics and pharmacodynamics in the development of anti-tuberculosis drugs. *Tuberculosis*, 88(S1):S65-S74.
- Du Toit, L., Pillay, V., Choonara, Y., & Iyuke, S. 2008. Formulation and Evaluation of a Salted-out Isoniazid-loaded Nanosystem. *American association of pharmaceutical scientists (AAPS) Pharmaceutical science & technology*, 9(1):174-181.
- Gruppo, V., Johnson, C.M., Marietta, K.S., Scherman, H., Zink, E.E., Crick, D.C., Adams, L.B., Orme, I.M., & Lenaerts, A.J. 2006. Rapid microbiologic and pharmacologic evaluation of experimental compounds against *Mycobacterium tuberculosis*. *Antimicrobial agents and chemotherapy*, 50(4):1245-1250.
- Jin, Y., Chen, S., Xin, R., & Zhou, Y. 2008. Monolayers of the lipid derivatives of isoniazid at the air/water interface and the formation of self-assembled nanostructures in water. *Colloids and surfaces B: Biointerfaces*, 64(2):229-235.
- Junginger, H. 2008. Excipients as absorption enhancers. (In Krishna, R. & Yu, L., eds., *Biopharmaceutics applications in drug development*. Verlag: Springer US. p. 139-174).
- Kingsley, J., Dou, H., Morehead, J., Rabinow, B., Gendelman, H., & Destache, C. 2006. Nanotechnology: A Focus on nanoparticles as a drug delivery System. *Journal of neuroimmune pharmacology*, 1(3):340-350.
- Kipp, J.E. 2004. The role of solid nanoparticle technology in the parenteral delivery of poorly water-soluble drugs. *International journal of pharmaceuticals*, 284(1-2):109-122.

- Kisich, K.O., Gelperina, S., Higgins, M.P., Wilson, S., Shipulo, E., Oganessian, E., & Heifets, L. 2007. Encapsulation of moxifloxacin within poly(butyl cyanoacrylate) nanoparticles enhances efficacy against intracellular *Mycobacterium tuberculosis*. *International journal of pharmaceutics*, 345(1-2):154-162.
- Lamprecht, A., Ubrich, N., Hombreiro Pérez, M., Lehr, C.-M., Hoffman, M., & Maincent, P. 1999. Biodegradable monodispersed nanoparticles prepared by pressure homogenization-emulsification. *International journal of pharmaceutics*, 184(1):97-105.
- Langer, K., Marburger, C., Berthold, A., Kreuter, J., & Stieneker, F. 1996. Methylmethacrylate sulfopropylmethacrylate copolymer nanoparticles for drug delivery. Part I: Preparation and physicochemical characterization. *International journal of pharmaceutics*, 137(1):67-74.
- Lenaerts, A.J., Johnson, C.M., Marrieta, K.S., Gruppo, V., & Orme, I.M. 2005. Significant increases in the levels of liver enzymes in mice treated with anti-tuberculosis drugs. *International journal of antimicrobial agents*, 26(2):152-158.
- Lockman, P.R., Mumper, R.J., Khan, A., & Allen, D.D. 2002. Nanoparticle technology for drug delivery across the blood-brain barrier. *Drug development and industrial pharmacy*, 28(1):1-13.
- Mahato, R.I. 2007. Pharmaceutical dosage forms and drug delivery. 2nd ed. Boca Raton: CRC Press.
- Pandey, R. & Khuller, G.K. 2005. Solid lipid particle-based inhalable sustained drug delivery system against experimental tuberculosis. *Tuberculosis*, 85:227-234.
- Pandey, R. & Khuller, G.K. 2007. Nanoparticle-based oral drug delivery system for an injectable antibiotic- Streptomycin. *Experimental chemotherapy*, 53:437-441.
- Pandey, R., Ahmad, Z., Sharma, S., & Khuller, G.K. 2005a. Nano-encapsulation of azole antifungals: Potential applications to improve oral drug delivery. *International journal of pharmaceutics*, 301(1-2):268-276.
- Pandey, R. & Khuller, G.K. 2006. Oral nanoparticle-based antituberculosis drug delivery to the brain in an experimental model. *Journal of antimicrobial chemotherapy*, 57(6):1146-1152.
- Pandey, R., Sharma, A., Zahoor, A., Sharma, S., Khuller, G.K., & Prasad, B. 2003a. Poly (DL-lactide-co-glycolide) nanoparticle-based inhalable sustained drug delivery system for experimental tuberculosis. *Journal of antimicrobial chemotherapy*, 52(6):981-986.
- Pandey, R., Sharma, S., & Khuller, G.K. 2005b. Oral solid lipid nanoparticle-based antitubercular chemotherapy. *Tuberculosis*, 85(5-6):415-420.
- Pandey, R., Zahoor, A., Sharma, S., & Khuller, G.K. 2003b. Nanoparticle encapsulated antitubercular drugs as a potential oral drug delivery system against murine tuberculosis. *Tuberculosis*, 83(6):373-378.
- Park, K. 2007. Nanotechnology: What it can do for drug delivery. *Journal of controlled release*, 120(1-2):1-3.

Semete, B., Booysen, L., Lemmer, Y., Kalombo, L., Katata, L., Verschoor, J., & Swai, H.S. 2010. In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. *Nanomedicine: nanotechnology, biology and medicine*, 6(5):662-671.

Sharma, A., Pandey, R., Sharma, S., & Khuller, G.K. 2004a. Chemotherapeutic efficacy of poly (dl-lactide-co-glycolide) nanoparticle encapsulated antitubercular drugs at sub-therapeutic dose against experimental tuberculosis. *International journal of antimicrobial agents*, 24(6):599-604.

Sharma, A., Sharma, S., & Khuller, G.K. 2004b. Lectin-functionalized poly (lactide-co-glycolide) nanoparticles as oral/aerosolized antitubercular drug carriers for treatment of tuberculosis. *Journal of antimicrobial chemotherapy*, 54(4):761-766.

Song, S.H., Jun, S.H., Park, K.U., Yoon, Y., Lee, J.H., Kim, J.Q., & Song, J. 2007. Simultaneous determination of first-line anti-tuberculosis drugs and their major metabolic ratios by liquid chromatography/tandem mass spectrometry. *Rapid communications in mass spectrometry*, 21:1331-1338.

Wu, Y., Loper, A., Landis, E., Hettrick, L., Novak, L., Lynn, K., Chen, C., Thompson, K., Higgins, R., Batra, U., Shelukar, S., Kwei, G., & Storey, D. 2004. The role of biopharmaceutics in the development of a clinical nanoparticle formulation of MK-0869: a Beagle dog model predicts improved bioavailability and diminished food effect on absorption in human. *International journal of pharmaceutics*, 285(1-2):135-146.

Yu, S., Ying, Z., Wu, F., Zhang, X., Lü, W., Zhang, H., & Zhang, Q. 2004. Nasal insulin delivery in the chitosan solution: in vitro and in vivo studies. *International journal of pharmaceutics*, 281:11-23.

Zahoor, A., Sharma, S., & Khuller, G.K. 2005. Inhalable alginate nanoparticles as antitubercular drug carriers against experimental tuberculosis. *International journal of antimicrobial agents*, 26(4):298-303.

Summary and future prospects

Although the absolute number of TB cases has fallen since 2006, the global burden is still considered a crisis since approximately 8.8 million new TB cases were reported in 2010 (WHO, 2011:9). In 2009, approximately 10 million children were orphaned as a result of parent deaths caused by TB. South Africa currently ranks third of the 22 high burden countries worldwide in terms of TB incident cases (WHO, 2001:11). The DOTS program together with the Stop TB Partnership have established systems for addressing the major issue of poor patient compliance with subsequent treatment failure (Jassal & Bishai 2010:S157).

This study aims to contribute to the knowledge base of nanotechnology-based DDS. The research focused on evaluating the efficacy of PLGA nanoparticulate DDS encapsulating the anti-TB drugs RIF, INH, PZA and ETB in reducing the dose frequency and minimising the adverse side effect of the anti-TB drugs. The novelty of this DDS is the patented direct spray-drying technique of a biodegradable polymer formulation to produce smooth, spherical nanoparticles of an acceptable size range for oral delivery (Kalombo, 2008). The *in vitro* analysis of the PLGA nanoparticles encapsulating these drugs, provided an in-depth understanding of the particles uptake by macrophages as well as the protein-binding affinity. On the other hand, *in vivo* analysis of this DDS, led to a better understanding on the immunogenicity, biodistribution, MIC/MBC efficacy, drug release kinetics and drug distribution.

In Chapter 3, the macrophage uptake of PLGA nanoparticles as well as the resulting immune response was evaluated. The study was aimed specifically at analysing the activation of- and uptake of nanoparticles by macrophages since *M.tb* resides and replicates within alveolar macrophages. This would form a platform for predicting efficacy as well as improving the formulation for targeting to macrophages. Flow cytometry analyses demonstrated that PLGA nanoparticles were able to activate and be taken up by macrophages. A similar result was observed in a study by Nicolette *et al* (2001:1559) using PLGA nanoparticles with a size of 389nm. The difference was the method of preparation which was single emulsion evaporation without spray-drying and where a negative zeta potential was observed. Their results demonstrated *in vitro* activation and uptake by J477 murine macrophages of PLGA nanoparticles without any drug. Furthermore, they observed phagocytosis with more avidity than for microparticles (Nicolette *et al.* 2011). It has been reported that size, surface

properties, composition, hydrophobicity and lipophilicity of nanoparticles play an important role in uptake by macrophages (Zhang *et al.* 1998:25). The PLGA nanoparticles' hydrophobic nature were expected to be more susceptible to phagocytosis than their hydrophilic counterparts which is further supported by the positive zeta potential observed for these nanoparticles (Roser *et al.* 1998:255). Maximum phagocytosis has been described for microspheres at 1 to 2 μm (Roser *et al.* 1998:255) therefore these results reflected the extent of uptake by macrophages is unclear since the nanoparticles were below 300nm. It is possible that some of the nanoparticles were pinocytosed instead of phagocytosed because of their small size. In a review by Ahsan *et al.* (2002:35) it was clear that the mechanism of phagocytosis of nanoparticles by macrophages was not clearly understood since different opinions exist (Ahsan *et al.* 2002). Since *M.tb* uses macrophages as a "safe haven" or reservoir (McDonough *et al.* 1993:2765), where they replicate, the uptake of these nanoparticles is a promising result for possible targeting of anti-TB drugs to macrophages.

Since macrophages are the major differentiating cells of the MPS; uptake of these nanoparticles could possibly result in an acute immune response. Thus, further analysis, by means of analysing cytokine expression post exposure to the particles was warranted. The expression of the pro-inflammatory cytokines IL-2, IL-6, IL-12 and TNF- α together with anti-inflammatory cytokines IL-10 and IL-4 and chemokines INF- γ , IL-5 and MCP-1 were evaluated. Plasma and peritoneal lavage samples were collected following oral administration of PLGA nanoparticles and LPS (positive control) against blank (saline treated) samples. The results observed over a 24-hour analysis demonstrated no significant immune response ($p \leq 0.01$) to exposure with PLGA nanoparticles compared to an immune active substance LPS. Instead, results were comparable to observed cytokine concentrations for blank (saline treated) controls. It has been proposed that ambient particulate matter in the nano-size range may be the cause of cardiopulmonary mortality and morbidity (Van Eeden *et al.* 2001:829) and nanotoxicity has been the subject of much debate (Fischer & Chan 2007; Garnett & Kallinteri 2006; Kagan *et al.* 2005; Linkov *et al.* 2008; Oberdörster *et al.* 2005). However, the effects of biodegradable and biocompatible polymeric nanoparticles cannot be compared with these observations since not much information is available in terms of *in vivo* studies. A study reported complete remission of an inflammatory response in inflamed joints of an arthritis mouse model following intravenous administration of PLGA nanoparticles encapsulating glucocorticoids. The study concluded that improved efficacy was as a result of targeted and controlled delivery of the steroids by the nanoparticles (Higaki *et al.*

2005:1135). These particles were formulated with the same PLGA (50:50) composition, but with a different formulation method which produced nanoparticles of a size range between 90– 120 nm. It has been reported that microparticles demonstrate a more pronounced immune response (Nicolette *et al.* 2011:1560), thus it can be postulated that polymer particles in the nano-size range do not elicit an immune response. The results presented in this thesis further supports research that illustrates that due to the biodegradation of PLGA which involves hydrolytic and enzymatic hydrolysis (explained in Chapter 2) resulting in biologically compatible metabolites which are inert. These results were published in Semete *et al.* (2010:665).

Since cellular uptake and absence of an immune response was already confirmed, the *in vivo* biodistribution and *in vitro* protein-binding properties of these nanoparticles were evaluated as discussed in Chapter 4. Protein-binding of unencapsulated (drug-free) PLGA nanoparticles was compared to drug-free nanoparticles coated with two poloxamers, 1% PEG and 1% Pluronic-F127, respectively at different plasma: nanoparticle suspension ratios. At the highest ratio of whole plasma to nanoparticle suspension (40:60% v/v), percentage protein-binding of PLGA-DF ($20.91 \pm 4.44\%$) was comparable to PLGA-DF coated with 1% PEG ($14.32 \pm 7.40\%$). However, a significantly higher protein-binding was observed for PLGA-DF coated with 1% Pluronic ($31.3 \pm 9.76\%$, $p \leq 0.01$). The possibility of passively targeting macrophages by uptake of these PLGA nanoparticles was demonstrated in Chapter 3. The data presented here presents a different perspective to phagocytosis of these particles since the particles in Chapter 3 were not coated. This data presents the possibility that the nanoparticles could circumvent possible opsonisation allowing for longer residence time in plasma with subsequent sustained plasma drug levels over time. The low protein-binding observed for these nanoparticles *in vitro* together with the efficient uptake by macrophage as illustrated in Chapter 3, could suggest that these nanoparticles would provide optimal therapeutic efficacy, possibly by simultaneous site specific targeting as well as increased residence time in the blood. SDS-PAGE analysis, however, revealed that the strongest protein bound was albumin, but since this is the major blood protein, binding of other proteins cannot be excluded. This presents an opportunity for future research into unpacking which specific protein concentrations bind to the particles and to what affinity. PEGylation of these hydrophobic nanoparticles, was expected to demonstrate significantly lower protein-binding than uncoated particles as previously reported (Stolnik *et al.* 2001:268), but the percentage protein-binding was comparable for the un-PEGylated formulations. The nanoparticles evaluated in Stolnik *et*

al (2001:268) were significantly smaller in size (40– 170 nm), suggesting that nanoparticle size played a role in the protein-binding observed. Gref *et al* (2000:306) observed that protein-binding of PEGylated nanoparticles was dependent, not only on the Mw of PEG, but also its concentration in the formulation. PEG (1%, Mw: 10 000Da) was included in the formulation whereas Gref *et al* (2000:307) observed optimal protein resistance by PEGylated formulations between 2 and 5% PEG content with Mw: 5000 Da (Gref *et al.* 2000). In the current formulation used, inclusion of PEG above 1% resulted in a 3-fold increase in nanoparticle size. However, this provides an explanation as to why a significant decrease in protein-binding of PEGylated compared to uncoated PLGA nanoparticles were not observed in this study.

These observations prompted the comparison of these drug-free formulations with formulations encapsulating a drug with high affinity to drugs, RIF and INH, a drug of low protein-binding, respectively. At the same whole plasma to nanoparticle suspension ratio, percentage protein-binding were comparable to PLGA-DF coated with 1% PEG. However, a more important factor to evaluate was the difference in protein-binding of unencapsulated (free) drug versus nanoencapsulated drug. This was to evaluate whether incorporation of a drug in a polymer nanoparticle could possibly improve bioavailability since only unbound drug is able to reach systemic circulation. Equilibrium dialysis of the free drugs resulted in a percentage protein-binding for RIF of $90.0 \pm 1.38\%$ and INH $23.0 \pm 5.2\%$. This fell within the protein-binding range already reported for RIF and INH (Woo *et al.* 1996). For nanoencapsulated INH, a slight decrease in protein-binding was observed ($15.07 \pm 3.4\%$) which was comparable to 1% PEG. A significant decrease in protein-binding was observed for nanoencapsulated RIF ($15.4 \pm 5.5\%$, $p \leq 0.01$) compared to free drug. The protein-binding demonstrated for the encapsulated drugs are based on the assumption that the drugs are protected from plasma proteins by the incorporation within the polymeric shell. The delayed exposure of these drugs to plasma proteins by nanoencapsulation would mean more drug would be available at the target site presenting the possibility for decrease in drug dosage.

Biodistribution of drug-free PLGA nanoparticles labelled with rhodamine 6G were evaluated in the brain, heart, lungs, liver, kidney and spleen using fluorescence detection. These were measured against normalised blank control tissues to eliminate the effect of autofluorescence. At the seven-day point of the analyses, the nanoparticles were detected in the brain ($13.23 \pm 0.11\%$), heart ($16.81 \pm 0.11\%$), kidneys ($54.89 \pm 0.95\%$), lungs ($15.61 \pm 1.15\%$), liver ($48.48 \pm$

2.28%) and spleen ($5.73 \pm 0.21\%$). The highest percentage fluorescence was observed in the kidneys and liver, suggesting that the nanoparticles are able to accumulate in these organs. This observation also suggests uptake by liver macrophages (Kupffer cells) since the slow rate at which PLGA nanoparticles biodegrade within macrophages results in sequestration of these particles in the MPS organs such as the liver and kidneys (Owens & Peppas 2006:94). Confocal imaging clearly depicting the presence of the nanoparticles by fluorescent “hot spots” compared to rhodamine-only images confirmed that the fluorescence detected were not as a result of leaching or degradation of the fluorescent dye. Further analysis with poloxamer coated nanoparticles was performed. Biodistribution for the poloxamer coated nanoparticles were significantly lower ($p \leq 0.01$) compared to the uncoated nanoparticles at day seven of the analyses. For 1% PEG $3.30 \pm 0.89\%$, $6.81 \pm 2.15\%$, $12.57 \pm 3.76\%$, $16.77 \pm 2.82\%$, $4.28 \pm 2.13\%$ and $3.69 \pm 1.85\%$ (percentage fluorescence detected) was observed in the brain, heart, kidneys, liver, lungs and spleen, respectively. These results provided a better understanding of the *in vitro* protein-binding assays performed. The lower percentage biodistribution of coated nanoparticles observed in the organs suggests that the coated nanoparticles administered had a longer residence time than the uncoated nanoparticles. In a study by Panagi *et al* (2001:151) PLGA nanoparticles (154 ± 23.9 nm) were observed to be removed from the systemic circulation rapidly (13-35 seconds) following intravenous administration to mice. This was compared to increased residence time of PEGylated nanoparticles (113.5 ± 14.3 nm) (Panagi *et al.* 2001). This rapid clearance could explain why low plasma detection was observed for the time points in the data presented in this thesis. Their study also demonstrated that the uptake by MPS organs were dose-dependent for nanoparticles and dose-independent for PEGylated nanoparticles. Their analyses were done with ^{125}I -cholesterylamine labelled nanoparticles, making quantification and PK analyses of the nanoparticles-only possible. Stolnik *et al* (2001:276) also demonstrated higher biodistribution of uncoated versus coated nanoparticles. Therefore, despite the bigger size and the percentage PEG content and Mw of the nanoparticles used in this thesis, nanoparticles were detected up to seven days following oral administration whereas the studies cited here were performed over a few hours following intravenous administration. This suggests the potential advantage for oral administration of this formulation. Following the observed data generated thus far, a detailed PK/PD study was warranted as was presented in chapter 5.

It is important to note that all formulations used in the PK/PD study in Chapter 5 were coated with 1% PEG. The *in vitro* MIC/MBC study evaluating nanoencapsulated RIF, INH and ETB and RIF and INH, respectively, was conducted using cultured *M.tb* (strain H₃₇Rv). MIC assays revealed that over a 14-day analysis, RIF and INH encapsulated PLGA nanoparticles were able to release sufficient drug to exhibit growth inhibition. ETB encapsulated nanoparticles exhibited no growth inhibition. However, RIF and INH MICs were determined to be 0.06 and 0.03 µg/ml, respectively compared to 1-5 µg/ml for ETB as literature demonstrates (Chambers, 2001:807). This suggests that for the nanoparticle concentration used, sufficient drug was released from the nanoparticles to reach MIC of INH and RIF but not for ETB at such a high MIC concentration. For this reason ETB was excluded from MBC studies. PZA has an MIC of 8- 20 µg/ml and its evaluation was not compatible with this assay. Therefore, *in vitro* assays demonstrated in this study, shows potential for *in vivo* efficacy. For free-INH in this study, 99% bacterial killing was attained at 0.12 µg/ml compared to 32 µg/ml for nanoencapsulated INH over a 14-day analysis. For free RIF, only 0.01 µg/ml was required to attain 99% bacterial killing compared to 10 µg/ml for nanoencapsulated RIF. Therefore, nanoencapsulation of INH and RIF in the PLGA nanoparticle DDS used in this study shows potential for effective TB chemotherapy. However, *in vivo* studies are needed to conclude this. *In vivo* studies by Pandey *et al* (2003:986) demonstrated no detectable cfu's in lung homogenates after 46 days with 10th day nebulization of RIF and INH encapsulated PLGA nanoparticles. This was compared to once daily dosing of free-drug (Pandey *et al.* 2003). In a similar study Sharma *et al* (2004:601) demonstrated no detectable cfu's after oral dosing every 10 days (five doses) in guinea pigs. The articles cited here, demonstrated no detectable cfu's, which could be interpreted as complete bacterial killing at a similar size range.

Serum recovered from mice treated with RIF and INH encapsulated nanoparticles was used to determine bioavailability using a published rapid assessment *in vitro* MIC method (Gruppo *et al.* 2006). The method is essentially used to determine the bioavailability of experimental drugs. In this instance the bioavailability of RIF and INH are already known. However, whether sufficient drug is bioavailable *in vivo* subsequent to release of nanoparticles to exhibit MIC was still not elucidated. For both nanoencapsulated INH and RIF at 0.41 µg/ml drug in serum exhibited inhibition at the day-seven time point. This observation confirmed the bioavailability of nanoencapsulated drugs *in vivo* with possible subsequent chemotherapeutic efficacy. Furthermore, the inclusion of controls with and without serum to

determine the effect of protein-binding demonstrates no significant protein-binding occurred. This may provide further insight to *in vitro* protein-binding data generated.

Nanoencapsulation of the four first-line anti-TB drugs at standard doses did not exhibit sustained release as was expected from this formulation. In most instances, the drug release profiles were comparable with free-drug, suggesting that the free-drug present in the nanoparticle suspension for administration was detected. Previous reports have demonstrated sustained release of these drugs nanoencapsulated at standard doses and lower (Sharma *et al.* 2004:603) over a number of days (11-14) in guinea pigs. Pandey *et al.* (2003:985) demonstrated sustained release of up to eight days following nebulization of nanoencapsulation RIF, INH and PZA in mice. In this instance, it was assumed that the observation of only 24-hour release following oral administration in mice was possibly due to aerosolized DDS being better absorbed than oral DDS. However, the observation was unexpected since the sustained release of these drugs (nanoencapsulated) following oral administration have been reported (Ahmad *et al.* 2006:414) over 15 days above the MIC compared to only one day for free-drug. It is important to note though that some plasma drug levels at the later time points for this study were above the LLOD but below the LLOQ and could therefore not be confirmed with accuracy. The dose was then increased six-fold for RIF and INH and three-fold for PZA and ETB. There was no concern for drug toxicity at these doses in mice, since even higher doses have been reported with no toxicity (Lenaerts *et al.* 2005). This was confirmed by mice still being alive and in good health 10 days post oral administration. The postulated sustained release was observed up to seven days for RIF and INH at higher doses and three days for ETB and PZA. Based on $AUC_{0 \rightarrow t}$ values, nanoencapsulated INH demonstrated higher absorption compared to free-INH. This was supported with the increased half-life ($t_{1/2}$) observed (3.85 hours). The increased $t_{1/2}$ was also observed for RIF (5.77 hours). The overall PK parameters were improved by the increased dose assay, but although this was a positive finding, it could not be interpolated by assuming that if the LLOQ were lower, sustained drug release would have been observed at standard doses with a high degree of accuracy. These results suggest that the indirect method of determining %EE might not be suitable for PLGA nanoparticles. This indirect method involves “washing” of the nanoparticles, centrifugation and drug analysis of the supernatant to determine the drug concentration in the supernatant and calculate the amount of drug encapsulated in the nanoparticles. As a possible alternative method of determining %EE of nanoparticles was to dissolve or disintegrate the nanoparticles after washing and determining

exact concentrations within the nanoparticles. This was attempted with no success (results not shown) and indicated that this nanoparticle formulation maintains its structure. This may also suggest that biodegradation of this nanoparticle will take very long, but still facilitate sustained release possibly by diffusion through the polymer matrix.

During the sample collection for the drug release assays, organs were harvested from the mice at the 1-day, 2-day, 3-day, 7-day and 10-day time points. The lungs, liver, kidneys and spleen were evaluated for drug distribution of free- and nanoencapsulated drugs. PZA was not detected in any of the organs evaluated for both standard and increased dose studies. The studies cited for the release assays demonstrated tissue levels of nanoencapsulated drug beyond the plasma drug levels for RIF, INH and PZA (Sharma *et al.* 2004:601). For the increased dose assays, nanoencapsulated ETB was detected in the liver, spleen and kidneys at day 1, day 2 and day 3, respectively. RIF was detected up to the 10-day time point in the liver, but free-RIF were also detected up to the 7-day time point, indicating that sustained release was maintained for nanoencapsulated RIF. A similar trend was observed for nanoencapsulated INH in the lungs. In the kidneys, nanoencapsulated INH was only detected at the 7-day time point. This confirms what was observed for the drug-free nanoparticles biodistribution observed in chapter 3 in terms of nanoparticle sequestration in the lungs, kidneys and especially the liver. The observation of INH and RIF in different organs over seven days may be as a result of the hydrophilic and hydrophobic nature of these drugs, respectively in terms of localisation and potentially macrophage uptake. The absence of PZA in all of the organs analysed was an inexplicable result. PZA is generally well absorbed and distributed (Chambers, 2001:808). It has been reported that PZA is well distributed in cerebrospinal fluid (CSF) at concentrations similar to simultaneous serum concentrations (Conte *et al.* 1999). It is therefore possible that the highest concentrations of PZA was localised in the (CSF) with concentration in other organs being below the LLOD.

This study confirmed the uptake of PLGA nanoparticles by macrophages and this could provide a basis for possible drug targeting to treat TB. Furthermore, it was proven that the uptake of these nanoparticles was biocompatible in terms of immunogenicity. The PLGA nanoparticle based drug delivery system formulated did not have the capacity to provide once-a-week dosing of the conventional daily dose through facilitated sustained release of all the anti-TB drugs evaluated in this study. However, once-a-week dosing was possible for RIF and INH at an increased dose. Distribution to various organs whilst maintaining sustained

release as a mechanism for reducing dosing frequency beyond 24 hours could only be confirmed for RIF and INH at an increased dose.

The following recommendations are made for further study to elucidate data generated, especially results that could not be explained:

- Since this work discussed in chapter 3 focused primarily on the acute immune response, i.e. within 24 hours post oral administration, an evaluation of possible long term adverse effects should be evaluated;
- A direct method for determining drug encapsulation, i.e. %EE should be developed to ensure accuracy of drug dosing during *in vivo* studies;
- *In vivo* efficacy studies should be conducted to verify *in vitro* results observed;
- PK analysis should be repeated once direct %EE method has been developed to verify whether results obtained were accurate;
- Possible optimisation of nanoparticle formulation is needed to improve recommendations mentioned.

References

- Ahmad, Z., Pandey, R., Sharma, S., & Khuller, G.K. 2006. Pharmacokinetic and pharmacodynamic behaviour of antitubercular drugs encapsulated in alginate nanoparticles at two doses. *International journal of antimicrobial agents*, 27(5):409-416.
- Ahsan, F., Rivas, I.P., Khan, M.A., & Torres Suárez, A.I. 2002. Targeting to macrophages: role of physicochemical properties of particulate carriers--liposomes and microspheres--on the phagocytosis by macrophages. *Journal controlled release*, 79(1-3):29-40.
- Conte, J., Golden, J, Duncan, S., McKenna, E., & Zurlinden, E. 1999. Intrapulmonary concentrations of pyrazinamide. *Antimicrobial Agents and Chemotherapy*, 43(6):1329-1333.
- Chambers, H. F. 2001. Antimycobacterial drugs. (In Katzung, B. G. ed. Basic & clinical pharmacology, 8 ed. San Francisco: Lange Medical Books/McGraw-Hill. p. 803-814).
- Fischer, H.C. & Chan, W.C. 2007. Nanotoxicity: the growing need for *in vivo* study. *Current opinion in biotechnology*, 18(6):565-571.
- Garnett, M.C. & Kallinteri, P. 2006. Nanomedicines and nanotoxicology: some physiological principles. *Occupational medicine*, 56(5):307-311.
- Gref, R., Lück, M., Quellec, P., Marchand, M., Dellacherie, E., Harnisch, S., Blunk, T., & Müller, R.H. 2000. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): Influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. *Colloids and surfaces B: Biointerfaces*, 18(3-4):301-313.
- Gruppo, V., Johnson, C.M., Marietta, K.S., Scherman, H., Zink, E.E., Crick, D.C., Adams, L.B., Orme, I.M., & Lenaerts, A.J. 2006. Rapid microbiologic and pharmacologic evaluation of experimental compounds against *Mycobacterium tuberculosis*. *Antimicrobial agents and chemotherapy*, 50(4):1245-1250.
- Higaki, M., Ishihara, T., Izumo, N., Takatsu, M., & Mizushima, Y. 2005. Treatment of experimental arthritis with poly(D, L-lactic/glycolic acid) nanoparticles encapsulating betamethasone sodium phosphate. *Annals of the rheumatic diseases*, 64(8):1132-1136.
- Jassal, M.S. & Bishai, W.R. 2010. Epidemiology and challenges to the elimination of global tuberculosis. *Clinical infectious diseases*, 50(S3):S156-S164.
- Kagan, V.E., Bayir, H., & Shvedova, A.A. 2005. Nanomedicine and nanotoxicology: two sides of the same coin. *Nanomedicine: nanotechnology, biology and medicine*, 1(4):313-316.
- Kalombo, L. 2008. Nanoparticle carriers for Drug Administration. (Patent: UK 2469965).
- Lenaerts, A.J., Johnson, C.M., Marrieta, K.S., Gruppo, V., & Orme, I.M. 2005. Significant increases in the levels of liver enzymes in mice treated with anti-tuberculosis drugs. *International journal of antimicrobial agents*, 26(2):152-158.
- Linkov, I., Satterstrom, F.K., & Corey, L.M. 2008. Nanotoxicology and nanomedicine: making hard decisions. *Nanomedicine: nanotechnology, biology and medicine*, 4(2):167-171.

- McDonough, K.A., Kress, Y., & Bloom, B.R. 1993. Pathogenesis of tuberculosis: Interaction of *Mycobacterium tuberculosis* with macrophages. *Infection and immunity*, 61(7):2763-2773.
- Nicolete, R., Santos, D.F., & Faccioli, L.C.H. 2011. The uptake of PLGA micro or nanoparticles by macrophages provokes distinct *in vitro* inflammatory response. *International Immunopharmacology*, 11(10):1557-1563.
- Oberdörster, G., Oberdörster, E., & Oberdörster, J. 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environmental health perspectives*, 113(7):823-839.
- Owens, D.E. & Peppas, N.A. 2006. Opsonisation, biodistribution and pharmacokinetics of polymeric nanoparticles. *International journal of pharmaceutics*, 307:93-102.
- Panagi, Z., Beletsi, A., Evangelatos, G., Livaniou, E., Ithakissios, D.S., & Avgoustakis, K. 2001. Effect of dose on the biodistribution and pharmacokinetics of PLGA and PLGA-mPEG nanoparticles. *International journal of pharmaceutics*, 221, 143-152
- Pandey, R., Sharma, A., Zahoor, A., Sharma, S., Khuller, G.K., & Prasad, B. 2003. Poly (DL-lactide-co-glycolide) nanoparticle-based inhalable sustained drug delivery system for experimental tuberculosis. *Journal of antimicrobial chemotherapy*, 52(6):981-986.
- Roser, M., Fischer, D., & Kissel, T. 1998. Surface-modified biodegradable albumin nano- and microspheres. II: effect of surface charges on *in vitro* phagocytosis and biodistribution in rats. *European journal of pharmaceutics and biopharmaceutics*, 46(3):255-263.
- Semete, B., Booysen, L., Lemmer, Y., Kalombo, L., Katata, L., Verschoor, J., & Swai, H.S. 2010. *In vivo* evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. *Nanomedicine: nanotechnology, biology and medicine*, 6(5):662-671.
- Sharma, A., Pandey, R., Sharma, S., & Khuller, G.K. 2004. Chemotherapeutic efficacy of poly (dl-lactide-co-glycolide) nanoparticle encapsulated antitubercular drugs at sub-therapeutic dose against experimental tuberculosis. *International journal of antimicrobial agents*, 24(6):599-604.
- Stolnik, S., Daudali, B., Arien, A., Whetstone, J., Heald, C.R., Garnett, M.C., Davis, S.S., & Illum, L. 2001. The effect of surface coverage and conformation of poly(ethylene oxide) (PEO) chains of poloxamer 407 on the biological fate of model colloidal drug carriers. *Biochimica et biophysica acta (BBA) - Biomembranes*, 1514(2):261-279.
- Van Eeden, S.T.E.P., Tan, W.A.N., Suwa, T.A.T.S., Mukae, H.I.R.O., Terashima, T.A.K.E., Fujii, T.A.K.E., Qui, D.I.W.E., Vincent, R.E.N.A., & Hogg, J.A.M.E. 2001. Cytokines involved in the systemic inflammatory response induced by exposure to particulate matter air pollutants (PM10). *American journal of respiratory and critical care medicine*, 164(5):826-830.
- Vehring, R. 2008. Pharmaceutical particle engineering via spray-drying. *Pharmaceutical research*, 25(5):999-1022.
- World Health Organization (WHO). 2011. 2011 Global TB Report.

Woo, J., Cheung, W., Chan, R., Chan, S., Cheng, A., & Chan, K. 1996. *In vitro* protein-binding characteristics of isoniazid, rifampicin and pyrazinamide to whole plasma, albumin and [alpha]-1-acid glycoprotein. *Clinical biochemistry*, 29(2):175-177.

Zhang, Q., Liao, G., Wei, D., & Nagai, T. 1998. Increase in gentamicin uptake by cultured mouse peritoneal macrophages and rat hepatocytes by its binding to polybutylcyanoacrylate nanoparticles. *International journal of pharmaceutics*, 164(1):21-27.