

# CONTENTS

	<b>Page</b>
<b>บทคัดย่อ</b>	(3)
<b>ABSTRACT</b>	(5)
<b>ACKNOWLEDGEMENT</b>	(7)
<b>CONTENTS</b>	(9)
<b>LIST OF TABLES</b>	(16)
<b>LIST OF ILLUSTRATIONS</b>	(19)
<b>ABBREVIATION AND SYMBOLS</b>	(25)
<b>CHAPTER</b>	
<b>1. INTRODUCTION</b>	1
1.1 General Introduction	1
1.2 Objective of the Thesis	3
1.3 Structure of the Thesis	3
<b>2. REVIEW OF LITERATURE</b>	5
2.1 Aetiology of TB	5
2.2 Epidemiology of TB	9
2.3 Pathogenesis	10
2.4 Treatment of TB	16
2.5 Physiology of the Lungs	19
2.5.1 Histological features of the lungs	19
2.5.2 Drug absorption through the lungs	20

## **CONTENTS (Continued)**

	<b>Page</b>
2.5.3 Particle characteristics	21
2.6 New Emerging Alternative Method of TB Therapy	25
2.6.1 Antituberculosis drugs formulated for inhalers	25
2.6.2 Carrier for DPIs	32
<b>3. VALIDATION OF HPLC METHOD FOR DETERMINATION OF RIFAMPICIN AND ISONIAZID</b>	<b>34</b>
3.1 Introduction	34
3.2 Materials	34
3.3 Equipment	34
3.4 Chromatographic Conditions	35
3.5 Methods	35
3.5.1 Stability indicating assay	35
3.5.2 Methods validation of rifampicin and isoniazid	36
3.5.3 Calibration curve for standard rifampicin and isoniazid	37
3.6 Results and Discussion	37
3.6.1 The stability of drugs	37
3.6.2 Method validation of rifampicin and isoniazid	40
3.6.3 Calibration curve for standard rifampicin and isoniazid	43
<b>4. FORMULATION DESIGN OF ANTITUBERCULOSIS DRY POWDER INHALERS BY PHYSICAL MIXING</b>	<b>45</b>
4.1 Introduction	45

## CONTENTS (Continued)

	Page
4.2 Materials	46
4.3 Equipment	47
4.4 Methods	47
4.4.1 Preparation of the micronised and fine particles	47
4.4.2 Particle size distribution measurement	48
4.4.3 Characterization of particle morphology	48
4.4.4 Preparation of formulations	49
4.4.5 Effects of carrier size on deposition <i>in vitro</i>	49
4.4.6 Content uniformity of the powder blends	53
4.4.7 <i>In vitro</i> deposition of drugs in dry powder formulations by ACI	54
4.4.8 Stability of the powder blends after storage	56
4.5 Results and Discussion	56
4.5.1 Particle size distribution measurement	56
4.5.2 Morphology of particles	63
4.5.3 Effects of carrier size deposition <i>in vitro</i>	65
4.5.4 Content uniformity of the powder blends	69
4.5.5 <i>In vitro</i> deposition of drugs by ACI	72
4.5.6 Stability of the powder blends after storage	77
<b>5. FORMULATION DESIGN OF ANTITUBERCULOSIS</b>	
<b>    DRY POWDER INHALERS BY SPRAY DRYING</b>	<b>79</b>
5.1 Introduction	79

## CONTENTS (Continued)

	<b>Page</b>
5.2 Materials	81
5.3 Equipment	81
5.4 Methods	81
5.4.1 Preparation of the formulations	82
5.4.2 Particle size distribution measurement	82
5.4.3 Content uniformity of spray dried formulations	82
5.4.4 Characterization of particle morphology	89
5.4.5 <i>In vitro</i> deposition of drugs in spray dried formulations by TSI	83
5.4.6 <i>In vitro</i> deposition of drugs in spray dried formulations by ACI	83
5.4.7 Stability of the spray dried formulations after storage	84
5.5 Results and Discussion	84
5.5.1 Particle size distribution measurement	84
5.5.2 Content uniformity of spray dried formulations	86
5.5.3 Morphology of dry powder formulations	86
5.5.4 <i>In vitro</i> deposition of isoniazid in spray dried formulations by TSI	88
5.5.5 <i>In vitro</i> deposition of isoniazid in spray dried formulations by ACI	89
5.5.6 Stability of the spray dried formulations after storage	91
<b>6. FORMULATION DESIGN OF ANTITUBERCULOSIS DRY POWDER INHALERS BY SPRAYING INTO ANTISOLVENT</b>	<b>93</b>
6.1 Introduction	93
6.2 Materials	93

## CONTENTS (Continued)

	<b>Page</b>
6.3 Equipment	94
6.4 Methods	95
6.4.1 Production of encapsulated rifampicin	95
6.4.2 Determination of drug encapsulation	97
6.4.3 Effects of cholesterol and lecithin ratios on percent encapsulation	98
6.4.4 The effect of drug loading in formulation sprayed into antisolvent	99
6.4.5 Content uniformity of dry powder formulations	100
6.4.6 Particle size distribution of encapsulated particles	101
6.4.7 Morphology of encapsulated particles	101
6.4.8 Lipid structure of the formulation sprayed into antisolvent by transmission electron microscopy (TEM)	101
6.4.9 Lipid structure of the formulation sprayed into antisolvent using confocal scanning microscopy	102
6.4.10 Determination of chloroform in formulation sprayed into Antisolvent	102
6.4.11 <i>In vitro</i> deposition of rifampicin in dry powder formulations by TSI	104
6.4.12 <i>In vitro</i> deposition of rifampicin in dry powder formulations by ACI	104
6.4.13 Stability of the formulation sprayed into antisolvent after storage	104
6.5 Results and Discussion	105

## CONTENTS (Continued)

	<b>Page</b>
6.5.1 The effects of cholesterol : lecithin ratio on percent encapsulation of rifampicin	105
6.5.2 Study of drug loading	108
6.5.3 Content uniformity of dry powder formulations and determine the chloroform content	109
6.5.4 Particle size distribution measurement	110
6.5.5 Morphology of dry powder formulations	111
6.5.6 Characterization of lipid structure of the formulation sprayed into antisolvent	112
6.5.7 <i>In vitro</i> deposition of rifampicin in dry powder formulation by TSI	114
6.5.8 <i>In vitro</i> deposition of rifampicin in dry powder formulation by ACII	114
6.5.9 Stability of formulation sprayed into antisolvent after storage	115
<b>7. DRUG SUSCEPTIBILITY TESTING OF MYCOBACTERIA</b>	<b>117</b>
7.1 Introduction	117
7.2 Materials	120
7.3 Equipment	120
7.4 Methods	121
7.4.1 Drug susceptibility testing of <i>M. tuberculosis</i> by the broth microdilution method	121
7.4.2 Drug susceptibility testing of <i>M. bovis</i> by using flow cytometry	122
7.5 Results and Discussion	123
	(14)

## CONTENTS (Continued)

	<b>Page</b>
7.5.1 Drug susceptibility testing of <i>M. tuberculosis</i> by broth microdilution method	123
7.5.2 Drug susceptibility testing of <i>M. bovis</i> by flow cytometry	126
<b>8. CONCLUSIONS</b>	<b>131</b>
<b>BIBLIOGRAPHY</b>	<b>132</b>
<b>APPENDIX</b>	<b>146</b>
<b>VITAE</b>	<b>172</b>

## LIST OF TABLES

<b>Table</b>		<b>Page</b>
2.1	The “time table” of primary TB	14
2.2	The WHO-recommended short-course antituberculosis drug regimens	18
2.3	Particle size analysis methods for medical aerosols listed in the current USP and Ph. Eur.	23
2.4	Summary of particle sizing methods used to characterize medical aerosols from inhalers	24
3.1	Linearity of rifampicin standard curve determination by HPLC	42
3.2	Linearity of isoniazid standard curve determination by HPLC	42
3.3	Accuracy of rifampicin standard determination by HPLC	43
3.4	Accuracy of isoniazid standard determination by HPLC	43
4.1	Compositions of the dry powder formulations obtained from physical mixing	50
4.2	The volume median diameter of drugs and carriers (mean $\pm$ SD, n = 3)	58
4.3	Drugs deposition in the TSI after aerosolization of the different blends (mean $\pm$ SD, n = 6)	67
4.4	The summarised of MMAD and GSD as obtained from ACI (mean $\pm$ SD, n = 6)	72



## LIST OF TABLES (Continued)

Table	Page
4.5 Drug contents (mean $\pm$ SD, n = 3) and MMAD (mean $\pm$ SD, n = 6) of dry powder formulation after storage 3 months at room temperature	77
5.1 Compositions of dry powder inhaler formulations obtained from spray drying technique	82
5.2 Drugs deposition in the TSI after aerosolization of spray dried formulations (mean $\pm$ SD, n = 6)	88
5.3 Drug contents (mean $\pm$ SD, n = 3) and MMAD (mean $\pm$ SD, n = 6) of dry powder formulations after storage 3 months at room temperature	91
6.1 Compositions of dry powder formulations obtained from spraying into antisolvent technique	99
6.2 Compositions of dry powder inhaler formulations containing various amounts of rifampicin using cholesterol : lecithin in a ratio of 1 : 3 by weight	100
6.3 Gas chromatographic conditions	103
6.4 %Encapsulation and %yield of dry powder formulations by spraying into antisolvent (mean $\pm$ SD, n = 1-6)	107
6.5 Drug loading of dry powder inhaler formulations (mean $\pm$ SD, n = 6)	109

## LIST OF TABLES (Continued)

<b>Table</b>		<b>Page</b>
6.6	Drug contents (mean $\pm$ SD, n = 3) and MMAD of RIF-1 (50) formulation after storage 3 months (mean $\pm$ SD, n = 6) at room temperature	115
7.1	MICs of standard rifampicin, standard isoniazid and the selected dry powder formulations obtained from physical mixing, spray drying and spraying into antisolvent method	124
A.1	Stability of rifampicin in the solid state at room temperature	150
B.1	Details of TSI	165
B.2	Stage $d_{50}$ values ( $\mu\text{m}$ ) for the various configurations of the Andersen 8-stage cascade impactor at different flow rates	168
B.3	Component units of ACI	170

## LIST OF ILLUSTRATIONS

Figure	Page
2.1 Scanning electron microscope of <i>M. tuberculosis</i>	6
2.2 Mycobacteria cell envelope (does not show LAM)	7
2.3 Estimated TB incidence rate	10
2.4 The example of nebuliser on the market today	27
2.5 The example of MDI on the market today	28
2.6 The example of DPI on the market today	29
3.1 Representative chromatograms obtained from 2 mg/ml of (A) rifampicin and (B) isoniazid	39
3.2 Stability of standard rifampicin (▲) and isoniazid (●) (mean ± SD, n = 3)	40
3.3 Intra-day precision of rifampicin and isoniazid	41
3.4 Inter-day precision of rifampicin and isoniazid	41
3.5 Standard curve of rifampicin	44
3.6 Standard curve of isoniazid	44
4.1 Diagrammatic representation of glass inhaler device showing dimensions which fitted the glass throat of TSI or Andersen cascade impactor (ACI)	51
4.2 Diagrammatic representation of the TSI	52
4.3 Particle size distribution based on volume of micronised rifampicin using Malvern laser light diffraction technique	58

## **LIST OF ILLUSTRATIONS (Continued)**

<b>Figure</b>	<b>Page</b>
4.4 Particle size distribution based on volume of micronised isoniazid using Malvern laser light diffraction technique	59
4.5 Particle size distribution based on volume of fine trehalose using Malvern laser light diffraction technique	59
4.6 Particle size distribution based on volume of micronised trehalose using Malvern laser light diffraction technique	60
4.7 Particle size distribution based on volume of fine mannose using Malvern laser light diffraction technique	60
4.8 Particle size distribution based on volume of micronised mannose using Malvern laser light diffraction technique	61
4.9 Particle size distribution based on volume of fine lactose using Malvern laser light diffraction technique	61
4.10 Particle size distribution based on volume of micronised lactose using Malvern laser light diffraction technique	62
4.11 Electron micrographs of (A) micronised rifampicin $\times$ 1500, (B) micronised rifampicin $\times$ 3000, (C) micronised isoniazid $\times$ 30000 and (D) micronised isoniazid $\times$ 4000	63

## LIST OF ILLUSTRATIONS (Continued)

Figure	Page
4.12 Electron micrographs of (A) fine trehalose $\times$ 4000, (B) micronised trehalose $\times$ 3000, (C) fine mannose $\times$ 500, (D) micronised mannose $\times$ 15000, (E) fine lactose $\times$ 3000 and (F) micronised lactose $\times$ 18000	64
4.13 Content uniformity of formulations RIF-1, RIF-2 and RIF-3 (mean $\pm$ SD, n = 10)	71
4.14 Content uniformity of formulations INH-1, INH-2 and INH-3 (mean $\pm$ SD, n = 10)	71
4.15 Size distribution of rifampicin formulations on each stage of the ACI as aerosolized at a flow rate of 60 l/min (mean $\pm$ SD, n = 6)	74
4.16 Size distribution of isoniazid formulations on each stage of the ACI as aerosolized at a flow rate of 60 l/min (mean $\pm$ SD, n = 6)	74
4.17 Relationship between Z value and cut off aerodynamic diameter (log scale) on each stage of the ACI of formulations ; (A) RIF-1 (A), (B) RIF-2 (C) and (C) RIF-3 (A), as aerosolized at a flow rate 60 l/min	75
4.18 Relationship between Z value and cut off aerodynamic diameter (log scale) on each stage of the ACI of formulations ; (A) INH-1 (A), (B) INH-2 (C) and (C) INH-3 (A), as aerosolized at a flow rate 60 l/min	76

## LIST OF ILLUSTRATIONS (Continued)

Figure	Page
5.1 Particle size distribution based on volume of INH-1 (sd) formulation using Malvern laser light diffraction technique	85
5.2 Particle size distribution based on volume of INH-3 (sd) formulation using Malvern laser light diffraction technique	85
5.3 Content uniformity of INH-1 (sd) and INH-3 (sd) formulations (mean $\pm$ SD, n = 10)	86
5.4 Electron micrographs of (A) INH-1 (sd) $\times$ 300, (B) INH-1 (sd) $\times$ 3000, (C) INH-3 (sd) $\times$ 300 and (D) INH-3 (sd) $\times$ 3000	87
5.5 Size distribution of isoniazid in spray dried formulations on each stage of the ACI as aerosolized at a flow rate 60 l/min (mean $\pm$ SD, n = 6)	90
5.6 Relationship between Z value and cut off aerodynamic diameter (log scale) on each stage of the ACI of formulations (A) INH-1 (sd) and (B) INH-3 (sd)	90
6.1 Schematic diagram of the spraying into antisolvent process	96
6.2 Photographs of dry powder inhaler formulations by spraying into antisolvent (A) using trehalose as carrier and (B) using mannose as carrier	108
6.3 Particle size distribution based on volume of RIF-1 (50) formulation using Malvern laser light diffraction technique	110

## LIST OF ILLUSTRATIONS (Continued)

Figure		Page
6.4	Electron micrographs of (A) RIF-1 (50) × 1500 and (B) RIF-1 (50) × 3500	111
6.5	Photographs of lipid layers of the spraying into antisolvent obtained from confocal laser scanning microscope (A) and (B), obtained from transmission electron microscope (C)	113
6.6	Size distribution of RIF-1 (50) formulation on each stage of ACI as aerosolized at a flow rate of 60 l/min (mean ± SD, n = 6)	114
6.7	Relationship between Z value and cut off aerodynamic diameter (log scale) on each stage of the ACI of formulation RIF-1 (50) as aerosolized at a flow rate 60 l/min	115
7.1	Histogram profiles of the intensity of fluorescence of the number of events (non <i>M. bovis</i> particles or <i>M. bovis</i> cells) in 7H9 medium alone (A), 7H9 medium containing unstained viable <i>M. bovis</i> cells (B), 7H9 medium containing viable <i>M. bovis</i> cells stained with fluorescein diacetate (C), and 7H9 medium containing viable <i>M. bovis</i> cells incubated with 3.0 µg/ml of isoniazid for 24 hours and then stained with fluoresein diacetate (D)	128

## LIST OF ILLUSTRATIONS (Continued)

Figure	Page	
7.2	Viable <i>M. bovis</i> cells after incubated with standard rifampicin and rifampicin dry powder formulations (A), standard isoniazid and dry powder formulations (B),all drugs were incubated for 24 hours and then stained with fluorescein diacetate and detected by flow cytometry	130
A.1	Strucural features of rifampicin and its decomposition producteds in aqueous solution	156
B.1	TSI for the aerodynamic assessment of fine particles (dimension in mm)	164
B.2	Schematic representation of the principle of operation of cascade impactor	167
B.3	Relation ship between Andersen 8-stage cascade impactor cut sizes at 28.3 l/min and likely particle deposition in the respiratory tract	168
B.4	ACI for the aerodynamic assessment of fine particles (Dimentions in mm)	169
B.5	The preseparator of ACI	171



## ABBREVIATIONS AND SYMBOLS

ACI	=	Andersen cascade impactor
API	=	active pharmaceutical ingredient
BCG	=	bacille Calmette-Guérin
°C	=	degree Celcius
CDC	=	the Center of Disease Control
CFC	=	chlorofluorocarbon
CFU	=	colony forming unit
cm	=	centimeter
DNA	=	deoxyribonucleic acid
DOTS	=	Directly Observed Treatment, Short-courses
DPI	=	dry powder inhaler
DPPC	=	dipalmitoylphosphatidylcholine
ED	=	emitted dose
<i>e.g.</i>	=	<i>exempli gratia</i>
FDA	=	fluorescein diacetate
FDA	=	food and drug administration
FPD	=	fine particle dose
FPF	=	fine particle fraction
g	=	gram
GSD	=	geometric standard deviation
HFAs	=	hydrofluoroalkanes

## ABBREVIATIONS AND SYMBOLS (Continued)

HIV	=	human immuno-deficiency virus
HPLC	=	high performance liquid chromatography
i.d.	=	internal diameter
i.e.	=	<i>id est</i>
IPACT	=	International Pharmaceutical Aerosol Consortium of Toxicity Testing
keV	=	kiloelectronvolt
kg	=	kilogram
l	=	liter
LAM	=	lipoarabinomannan
l/mim	=	liter per minute
m	=	meter
M	=	Molar
MDI	=	metered dose inhaler
mA	=	milliAmpere
mg	=	milligram
MIC	=	minimal inhibitory concentration
ml	=	milliliter
ml/min	=	milliliter per minute
mm	=	millimeter
MMAD	=	mass median aerodynamic diameter
µg	=	microgram

## ABBREVIATIONS AND SYMBOLS (Continued)

$\mu\text{l}$	=	microliter
$\mu\text{m}$	=	micrometer
n	=	number of sample
nm	=	nanometer
OADC	=	oleic, albumin, dextrose and catalase
Pa	=	Pascal
Ph. Eur.	=	European Pharmacopoeia
$r^2$	=	correlation coefficient
RNAP	=	RNA polymerase
Rpf	=	resuscitation-promoting cytokine factor
rpm	=	round per minute
RSD	=	relative standard deviation
sd	=	standard deviation
SEM	=	scanning electron microscopy
TB	=	tuberculosis
TOF	=	time-of-flight
TSI	=	twin stage impinger
UDP	=	uracil deoxyphosphate
UK	=	United Kingdom
USA	=	The United States of America
USP	=	The United States Pharmacopoeia
UV	=	ultraviolet

## **ABBREVIATIONS AND SYMBOLS (Continued)**

<b>v/v</b>	<b>=</b>	<b>volume by volume</b>
<b>WHO</b>	<b>=</b>	<b>The World Health Organization</b>
<b>w/v</b>	<b>=</b>	<b>weight by volume</b>