

CHITOSAN-NANOPARTICLES AS A DRUG DELIVERY
SYSTEM FOR 5-FLUOROURACIL

by

MUSLIM SUARDI

August 2003

M I L I K	
PERPUSTAKAAN JURUSAN FARMASI FMIPA	
UNIVERSITAS ANDALAS PADANG	
T E N D A F T A R	
TANGGAL :	29-4-2005
NOMOR BI :	20/H-2005
CALL No. :	

Thesis submitted in fulfillment of the requirements
for the degree of Doctor of Philosophy

CONTENTS

	Page
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
CONTENTS	V
LIST OF TABLES	xiv
LIST OF FIGURES	xvi
LIST OF PLATES	xxi
LIST OF SYMBOLS	xxii
LIST OF ABBREVIATIONS	xxv
LIST OF EQUATIONS	xxxv
LIST OF APPENDICES	xxxviii
LIST OF PUBLICATIONS AND SEMINAR	xxxix
ABSTRAK	xl
ABSTRACT	xlii
Chapter 1	
INTRODUCTION	1
1.1 CHITOSAN	1
1.1.1 <i>Properties of Chitosan</i>	1
1.1.2 <i>Biological Activity of Chitosan</i>	4
1.1.3 <i>Utilization of Chitosan</i>	5
1.1.3(a) <i>Pharmaceutical Uses of Chitosan</i>	5

1.1.3(b)	Medical Uses of Chitosan	6
1.1.3(c)	Application to Microparticulate Systems	7
1.1.3(d)	Other Commercial Uses of Chitosan	8
1.2	COLLOIDAL DRUG DELIVERY SYSTEM	9
1.3	MICROSPHERES AND NANOPARTICLES	10
1.3.1	<i>Microspheres</i>	10
1.3.2	<i>Nanoparticles</i>	11
1.3.3	<i>Preparation Methods of Nanoparticles</i>	12
1.3.3(a)	Spontaneous Formation of Nanoparticles from Preformed Polymers	13
1.3.3(b)	Emulsion and Cross-linking Technique	13
1.3.3(c)	Emulsion Polymerization in a Continuous Aqueous Phase	14
1.3.3(d)	Emulsion Polymerization in a Continuous Organic Phase	14
1.3.3(e)	Interfacial Polymerization	15
1.3.3(f)	Solvent Deposition	15
1.3.3(g)	Solvent Evaporation	16
1.3.3(h)	Desolvation from An Organic Polymer Solution	16
1.3.3(i)	Production of Albumin Nanoparticles in An Oil Emulsion	17
1.3.3(j)	Desolvation of Macromolecules	17
1.3.4	<i>Characterization of Nanoparticles</i>	18
1.3.4 (a)	Size of Nanoparticles	18
1.3.4 (b)	Drug Loading Analysis of Nanoparticles	18
1.3.4 (c)	Drug Release from Nanoparticles	19
1.4	5-FLUOROURACIL	20
1.4.1	<i>Physicochemical Characterization of 5-FU</i>	20
1.4.2	<i>Pharmacology of 5-FU</i>	21
1.4.3	<i>Pharmacokinetics of 5-FU</i>	25
1.5	EXPERIMENTAL SCOPE	28

Chapter 2	
CHARACTERIZATION OF CHITOSAN AND FORMULATION OF CHITOSAN NANOPARTICLES	31
2.1 INTRODUCTION	31
2.2 MATERIALS AND METHODS	35
2.2.1 <i>Materials</i>	35
2.2.2 <i>Determination of Degree of Deacetylation of Chitosan</i>	36
2.2.3 <i>Determination of the Viscosity-Average Molecular Mass (Molecular Weight) of Chitosan</i>	37
2.2.3 (a) Determination of Relative Viscosity	37
2.2.3 (b) Estimation of Specific, Reduced and Inherent Viscosity	40
2.2.3 (c) Estimation of Intrinsic Viscosity	40
2.2.3 (d) Estimation of Viscosity-Average Molecular Mass	41
2.2.4 <i>Determination of the Density and pH of Chitosan Solutions</i>	41
2.2.5 <i>Estimation of the Solubility of Chitosan in Various Acidic Solutions</i>	42
2.2.6 <i>Determination of the Viscosity of Chitosan Solutions</i>	42
2.2.7 <i>Influence of Storage Time on the Viscosity and pH of Chitosan in Various Acidic Solutions</i>	42
2.2.8 <i>Formulation of Blank Chitosan Nanoparticles</i>	43
2.2.8 (a) Preparation of Blank Chitosan Nanoparticles	43
2.2.8 (b) Influence of Formulation Variables on Particle Size of Chitosan Nanoparticles	43
2.2.8 (c) Influence of Mixing Method on Particle Size of Chitosan Nanoparticles	44
2.2.9 <i>Particle Size Analysis of Blank Chitosan Nanoparticles</i>	44
2.2.9 (a) Light Microscopy	44
2.2.9 (b) Scanning Electron Microscopy	44
2.2.9 (c) Photon Correlation Spectroscopy	46
2.2.10 <i>Statistical Analysis</i>	46
2.3 RESULTS AND DISCUSSION	46
2.3.1 <i>Degree of Deacetylation of Chitosan</i>	46
2.3.2 <i>Viscosity-Average Molecular Mass of Chitosan</i>	47

2.3.2 (a)	Outflow Time, Density and pH of Chitosan Solutions	47
2.3.2 (b)	Relative, Specific, Reduced, Inherent and Intrinsic Viscosity	53
2.3.2 (c)	Viscosity-Average Molecular Mass	54
2.3.3	<i>Estimated Solubility of Chitosan in Various Acidic Solutions</i>	
2.3.4	<i>Viscosity of Chitosan Solutions</i>	58
2.3.5	<i>Influence of Storage Time on the Viscosity and pH of Chitosan in Various Acidic Solutions</i>	58
2.3.6	<i>Formulation of Blank Chitosan Nanoparticles</i>	61
2.3.6 (a)	Preparation of Blank Chitosan Nanoparticles	61
2.3.6 (b)	Influence of Formulation Variables on Particle Size of Nanoparticles	62
2.3.6 (c)	Influence of Mixing Method on Particle Size of Chitosan Nanoparticles	72
2.3.7	<i>Methods Used for Particle Size Analysis</i>	74
2.3.7 (a)	Light Microscopy	74
2.3.7 (b)	Scanning Electron Microscopy	74
2.3.7 (c)	Photon Correlation Spectroscopy	75
2.4	CONCLUSIONS	75

Chapter 3

FORMULATION AND OPTIMIZATION OF 5-FU LOADED CHITOSAN NANOPARTICLES 77

3.1 INTRODUCTION 77

3.2 MATERIALS AND METHODS 78

3.2.1 Materials 78

3.2.2 Preparation of Drug-Loaded Chitosan Nanoparticles 79

3.2.3 Particles Size Analysis of Drug-Loaded Nanoparticles by Scanning Electron Microscopy 80

3.2.4 Particles Size Analysis of Drug-Loaded Nanoparticles by Photon Correlation Spectroscopy 80

3.2.5	<i>Method of Analysis by High Performance Liquid Chromatography</i>	80
3.2.5 (a)	Instrumentation	80
3.2.5 (b)	Mobile Phase and Solvent	81
3.2.5 (c)	U.V Spectrum of 5-FU in Mobile Phase	81
3.2.5 (d)	Method Validation	81
3.2.6	<i>Determination of Drug-Loaded in Chitosan Nanoparticles</i>	81
3.2.7	<i>Optimization of Drug Loading and Loading Efficiency of Chitosan Nanoparticles Using a 3³ Factorial Design</i>	82
3.2.8	<i>Statistical Analysis</i>	82
3.3	RESULTS AND DISCUSSION	83
3.3.1	<i>Preparation of Drug-Loaded Chitosan Nanoparticles</i>	83
3.3.2	<i>Particles Size Analysis of Drug-Loaded Nanoparticles by Scanning Electron Microscopy</i>	83
3.3.3	<i>Particles Size Analysis of Drug-Loaded Nanoparticles by Photon Correlation Spectroscopy</i>	86
3.3.4	<i>Method of Analysis by High Performance Liquid Chromatography</i>	86
3.3.5	<i>Drug-Loading in Chitosan Nanoparticles</i>	91
3.3.6	Factors Affecting Drug Loading	92
3.3.6 (a)	Washing Process	92
3.3.6 (b)	Particle Size	93
3.3.6 (c)	Residual Organic Solvent	94
3.3.7	<i>Optimization of Drug Loading and Loading Efficiency in Chitosan Nanoparticles</i>	94
3.3.7 (a)	Correlation between Drug Loading, Loading Efficiency and Formulation Variables	96
3.3.7(b)	General Models from Regression Polynomial Equations	96
3.3.7 (c)	Drug Loading and Loading Efficiency at Predetermined Chitosan Concentration	101
3.3.7 (d)	Drug Loading and Loading Efficiency at Predetermined Amount of 5-FU	108
3.3.7 (e)	Drug Loading and Loading Efficiency at Predetermined Glutaraldehyde	115
3.3.7 (f)	Confirmation Study Comparing Predicted and Experimental Values	123

3.4 CONCLUSIONS	123
Chapter 4	
IN-VITRO RELEASE OF 5-FU FROM CHITOSAN NANOPARTICLES	126
4.1 INTRODUCTION	126
4.2 MATERIALS AND METHODS	127
4.2.1 <i>Materials</i>	127
4.2.2 <i>In-Vitro Drug Release Preliminary Studies</i>	127
4.2.3 <i>HPLC Analysis of Released Drug Concentration</i>	129
4.2.4 <i>In-Vitro Drug Release Studies of the 3³ Factorial Design Formulations</i>	130
4.2.5 <i>Statistical Analysis</i>	131
4.3 RESULTS AND DISCUSSION	131
4.3.1 <i>Preliminary Study of In-Vitro Drug Release</i>	131
4.3.2 <i>Kinetic Models</i>	133
4.3.3 <i>In-Vitro Drug Release Studies of the 3³ Factorial Design Formulations</i>	134
4.3.3 (a) Correlation between Drug Release, Drug Loading, Loading Efficiency and Formulation Variables	134
4.3.3 (b) General Models from Regression Polynomial Equations	140
4.3.3 (c) Drug Released at 1 hour at Predetermined Amount of 5-FU or Glutaraldehyde	141
4.3.3 (d) Analysis of Drug Release Kinetics	150
4.4 CONCLUSIONS	158

Chapter 5
HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC
METHOD FOR THE DETERMINATION OF 5-FU IN
RAT PLASMA AND ORGAN TISSUES

5.1	INTRODUCTION	160
5.2	MATERIALS AND METHODS	163
5.2.1	<i>Materials</i>	163
5.2.2	<i>Instrumentation</i>	163
5.2.3	<i>Separation of Uracil, 5-FU, and 5-BU</i>	164
5.2.3(a)	Mobile phase	164
5.2.3(b)	Determination of UV Absorption Spectra of Uracil, 5-FU and 5-BU	164
5.2.3(c)	Choice of Column and Mobile Phase	164
5.2.4	<i>Extraction of 5-FU from Plasma and Organ Tissue</i>	166
5.2.4(a)	Protein Precipitation	166
5.2.4(b)	General Extraction Procedure for Plasma and Tissue Samples	166
5.2.5	<i>Method Validation</i>	167
5.2.5(a)	Preparation of Standard Stock Solutions and Standard Working Solutions	167
5.2.5(b)	Standard Calibration Curves	167
5.2.5(c)	Precision and Accuracy	168
5.2.5(d)	Limit of Detection	169
5.2.5(e)	Recovery	169
5.3	RESULTS AND DISCUSSION	169
5.3.1	<i>Wavelength of Maximum Absorption</i>	169
5.3.2	<i>Influence of Separation Parameters</i>	171
5.3.2(a)	Separation Using PLRP-S Column	171
5.3.2(b)	Separation Using Spherisorb ODS Column	174
5.3.3	<i>Extraction of 5-FU in Plasma and Tissues</i>	178
5.3.3(a)	Protein Precipitation	178
5.3.3(b)	General Extraction Procedure for Plasma and Tissues	178
5.3.4	<i>Chromatographic Analysis</i>	178
5.4	CONCLUSIONS	191

Chapter 6	
IN VIVO DRUG RELEASE OF 5-FU FROM CHITOSAN NANOPARTICLES IN RATS	193
6.1 INTRODUCTION	193
6.2 MATERIALS AND METHODS	194
6.2.1 Materials	194
6.2.2 The Analysis of 5-FU in 5-FU Control Solution	195
6.2.3 In-vivo Study Protocol	195
6.2.3(a) Animals	195
6.2.3(b) Study design	196
6.2.3(c) The Administration of 5-FU Control Solution and Collection of Samples	196
6.2.3(d) The Administration of 5-FU Nanoparticles and Collection of Samples	196
6.2.4 Analysis of 5-FU in Plasma and Vital Organs of Rats	197
6.2.4(a) Preparation of Tissue Samples of Rats	197
6.2.4(b) Extraction Procedure for Plasma Samples	197
6.2.4(c) Extraction Procedures for Tissue/Organ Samples	197
6.2.4(d) Analysis of 5-FU in Plasma and Tissue Organs	198
6.2.5 Data and Statistical Analysis	198
6.3 RESULTS AND DISCUSSION	199
6.3.1 Determination of Drug Content in 5-FU Control Solution	199
6.3.2 Plasma Concentration Profile	199
6.3.3 Heart Tissue Concentration Profile	203
6.3.4 Kidney Tissue Concentration Profile	205
6.3.5 Liver Tissue Concentration Profile	210
6.3.6 Lung Tissue Concentration Profile	213
6.3.7 The Biodistribution of 5-FU	216
6.4 CONCLUSIONS	221

Chapter 7		
SUMMARY AND GENERAL CONCLUSIONS		223
Chapter 8		
SUGGESTIONS FOR FURTHER RESEARCH		224
REFERENCES		230
APPENDICES		265
PUBLICATIONS		279

NANOPARTIKEL KITOSAN SEBAGAI SATU SISTEM PENGHANTARAN DRUG UNTUK 5-FLUOROURASIL

ABSTRAK

Sifat-sifat fizikokimia kitosan dicirikan dengan menentukan darjah deasetilasi (DD), purata kelikatan jisim molekul (Mv) kitosan, kelikatan, kestabilan, kelarutan, dan pH larutan kitosan. DD dan Mv kitosan masing-masing ialah 73 - 76%, dan 0.75 to 0.90 Daltons, manakala kelikatan 1% w/v kitosan di dalam 1 dan 5% v/v asid asetik masing-masing ialah 1.697 ± 0.023 dan 2.23 ± 0.009 Pas. Selepas disimpan selama 10 hari, kelikatan kitosan dalam asid askorbik berkurang 76%, manakala kelikatan di dalam asid asetik, formik, laktik, maleik dan malik menurun dalam julat 21- 37%. Tambahan pula, kitosan lebih larut dalam asid asetik, formik, dan laktik daripada dalam asid maleik, askorbik dan malik. pH larutan 1% w/v kitosan di dalam pelbagai asid ialah dalam julat 1.91-2.62. Untuk tujuan penyediaan nanopartikel kitosan, pengaruh kepekatan kitosan, jenis dan kepekatan pengemulsi, jenis asid, kepekatan asid asetik, dan kaedah pencampuran diselidiki. Keputusan menunjukkan saiz nanopartikel berkadar langsung dengan kepekatan kitosan tetapi berkadar songsang dengan kepekatan pengemulsi. Kaedah pencampuran mengguna sonikator menghasilkan nanopartikel, manakala pencampuran mengguna pengacau magnetik menghasilkan mikrosfera. Pengoptimuman muatan drug dan kecekapan muatan dikaji menggunakan suatu rekabentuk faktorial 3^3 dengan mempelbagaikan amaun kitosan, 5-fluorourasil, dan glutaraldehyd. Muatan drug dan kecekapan muatan masing-masing ialah 1.26 - 5.38% dan 10 - 30%. Muatan drug berkadar secara signifikan dengan amaun permulaan 5-fluorourasil dan kitosan. Kecekapan muatan berkadar dengan kepekatan kitosan dan glutaraldehyd. Kajian kinetik pelepasan drug

dilakukan menggunakan alat Desaga dan dianalisa menggunakan HPLC. Keputusan menunjukkan bahawa, kinetik pelepasan drug mengikuti pola dwifasa iaitu pelepasan drug yang cepat pada permulaan diikuti oleh pelepasan perlahan. Lebih kurang 35 hingga 49% drug dilepaskan dalam jangkamasa 1 jam pertama. Formulasi nanopartikel menggunakan 1.5% w/v kitosan, 35 mg 5-fluorourasil, dan 30 mg glutaraldehid dipilih untuk kajian *in vivo*, kerana muatan drug, kecekapan muatan, dan pelepasan *in vitro* adalah optimum. Kaedah HPLC yang mudah dan spesifik dibangunkan untuk memisahkan urasil, 5-fluorourasil, dan 5-bromourasil dalam plasma, jantung, buah pinggang, hati dan paru-paru. Penemuan semula mutlak 5-fluorourasil dalam plasma dan tisu organ adalah lebih besar daripada 89%. Nilai kejituan dan ketepatan analisa dalam satu hari dan di antara hari ialah kurang daripada 15%. Farmakokinetik dan biodistribusi nanopartikel mengandungi 5-fluorourasil dan injeksi 5-fluorourasil komersil dikaji menggunakan tikus jantan Sprague Dawley. Hasil bahagi luas bawah keluk (AUC), pemalar kadar penyingkiran (k_e), penyingkiran setengah hayat ($t_{1/2}$), masa untuk mencapai kepekatan puncak (T_{max}), dan kepekatan drug puncak (C_{max}) nanopartikel dengan larutan kawalan dalam plasma masing-masing ialah 0.67, 0.058, 17.00, 24, dan 0.16. Dalam organ tisu, AUC nanopartikel lebih tinggi daripada larutan kawalan. Hasil bahagi AUC nanopartikel dengan larutan kawalan ialah 2.04 - 8.78 dan mengikut urutan hati > jantung > buah pinggang > paru-paru.

Abstract

The physicochemical properties of chitosan were characterized by the determination of degree of deacetylation (DD), viscosity-average molecular mass (M_v), viscosity, stability, solubility, and pH of the chitosan solution. DD and M_v of chitosan were about 73 to 76% and 0.75 to 0.90 Daltons, respectively, where as the viscosity of 1% w/v chitosan in 1 and 5% v/v acetic acid were 1.697± 0.023 and 2.23±0.009 Pas, respectively. At the end of 10 storage days, viscosity of chitosan in ascorbic acid decreased by 76%, while in acetic, formic, lactic, maleic, and malic acids, viscosity decreased within a range of 21 – 37%. In addition, chitosan was more soluble in acetic, formic and lactic acids than maleic, ascorbic, and malic acids. The pH of 1% w/v chitosan in the various acids varied from 1.91 to 2.62. In order to formulate chitosan nanoparticles, the influence of chitosan concentrations, type and concentration of emulsifiers, type of acid, concentration of acetic acid, and mixing method were investigated. The results showed that the size of nanoparticles was directly correlated to the chitosan concentration but inversely associated with emulsifier concentrations. Mixing method by sonication produced nanoparticles, while magnetic stirring resulted in microspheres. The optimization of drug loading and loading efficiency was investigated using a 3³ factorial designs by varying the amount of chitosan, 5-FU and glutaraldehyde. Drug loading and loading efficiency were 1.26 to 5.38% and 10 to 30%, respectively. Drug loading was significantly correlated with the initial amount of 5-FU and chitosan. Loading efficiency was correlated with the concentration of chitosan and glutaraldehyde. The drug release kinetics was studied using a Desaga apparatus and analyzed by a HPLC method. The

result showed a biphasic pattern consisting of a fast initial release, followed by a slower release. About 35 to 49% of drug was released within the first one hour. Nanoparticle formulation using 1.50% w/v chitosan, 35 mg of 5-FU, and 30 mg of glutaraldehyde were chosen for the *in vivo* study, since the drug loading, loading efficiency and *in vitro* release were optimum. A simple yet specific HPLC method was developed to separate uracil, 5-FU and 5-BU in plasma, heart, kidney, liver and lung. The absolute recoveries of 5-FU from the plasma and the organ tissues were higher than 89%. All the within-day and between-day precision and accuracy values were less than 15%. The pharmacokinetics and biodistribution of 5-FU loaded nanoparticles and a commercial 5-FU injection were studied using male Sprague Dawley rats. Area under the curve (AUC), elimination rate constant (k_e), elimination half-life (t_e), time to reach the peak concentration (T_{max}), and peak concentration of drug (C_{max}) quotients of nanoparticles to control solution in plasma were 0.67, 0.058, 17.00, 24, and 0.16, respectively. In the organ tissues, AUC of nanoparticles were higher than the control solution. AUC quotients of nanoparticles to control solution varied from 2.04 to 8.78 and followed the order of liver > heart > kidney > lung.