



Analytical Study in Interactions of Certain Antiviral Drugs

**Thesis presented by
Aya Atef Ahmed Youssef**

Demonstrator of Pharmaceutical Analytical Chemistry

Faculty of pharmacy - Ain Shams University

B.Sc. in Pharmaceutical Sciences

Faculty of pharmacy - Ain Shams University

2011

MSc. in Biotechnology

School of Science and Engineering - The American University in Cairo

2016

Submitted for the partial fulfillment of

Master Degree in Pharmaceutical Sciences

(Pharmaceutical Analytical Chemistry)

Under the Supervision of:

Prof. Dr. Amira Mabrouk El-Kosasy

Professor of Pharmaceutical Analytical Chemistry

Faculty of Pharmacy - Ain Shams University

Prof. Dr. Lobna Abd El-Aziz Hussein

Professor and Head of Pharmaceutical Analytical Chemistry Department

Faculty of Pharmacy - Ain Shams University

Associate Prof. Dr. Nancy Magdy Hanna

Associate Professor of Pharmaceutical Analytical Chemistry

Faculty of Pharmacy - Ain Shams University

Pharmaceutical Analytical Chemistry Department

Faculty of Pharmacy - Ain Shams University

2019

Dedication

To my mother; thanks for making me who I am, my father; thanks for supporting and encouraging me all the way, to my brother and sister; thanks for always being there for me, to my beloved husband and my parents in law; this work wouldn't have been accomplished without your support, to my little daughter (Laila); the joy and happiness of my life

Acknowledgment

All Praise is to Allah for Everything

It's my great pleasure to express my sincere thanks and appreciation to **Prof. Dr. Amira Mabrouk El-Kosasy**, Professor of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Ain Shams University. Thanks for being a dedicated professor, for her expert supervision, constructive comments, generous assistance and guidance during the supervision of this work. I will always be indebted to her kindness and continuous support.

I want to express my deep thanks and gratitude to **Prof. Dr. Lobna Abd El-Aziz Hussein**, Professor and Head of Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Ain Shams University, for her kind supervision, continuous encouragement, sincere guidance, endless support throughout my life and her belief in me which always gives me the courage to go forward.

I would like to express my gratefulness and appreciation for **Associate Prof. Dr. Nancy Magdy Hanna**, Associate Professor of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Ain Shams University, for her kind supervision, continuous encouragement, indispensable advice, sincere guidance, constructive criticism, valuable comments and endless support throughout my work in this thesis.

Finally, my sincere appreciation and thanks go to all my colleagues and staff members in Pharmaceutical Analytical Chemistry Department, for their friendly cooperation and constant support.

Aya Atef

List of abbreviations

ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
C _{max}	Peak Plasma Concentration
D ₀	Zero Absorption Spectrum
DAA	Direct Acting Antiviral
DAC	Daclatasvir
DAD	Diode Array Detector
DDI	Drug-Drug Interaction
ENT1	Equilibrative Nucleoside Transporter 1
FDA	US Food and Drug Administration
HCV	Hepatitis C Virus
HETP	Height Equivalent to Theoretical Plates
HPTLC	High Performance Thin Layer Chromatography
HSA	Human Serum Albumin
ICH	Internal Conference on Harmonization
IS	Internal Standard
K _d	Equilibrium Dissociation Constant
K'	Capacity factor
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LLOQ	Lower Limit of Quantitation
LOD	Limit of Detection
LOQ	Limit of Quantitation
MS	Mass Spectrometry
N	Number of theoretical plates
NS5A	Non-structural 5A
NS5B	Non-structural 5B
QCH	Quality Control sample High
QCL	Quality Control sample Low
QCM	Quality Control sample Middle
QCs	Quality Control samples
r	Correlation Coefficient
RBV	Ribavirin
R _s	Resolution
RSD	Relative Standard Deviation
SD	Standard Deviation
SF	Sofosbuvir
T	Tailing Factor
TLC	Thin Layer Chromatography

t_{\max}	Median Time
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
Trizma base	Tris[hydroxymethyl]aminomethane
UPLC	Ultra Performance Liquid Chromatography
UV-Vis absorption	Ultra Violet-Visible Absorption
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength

Table of Contents

List of abbreviations	i
List of Figures	xi
List of Tables	xiv
Summary	xvi

Part I: Introduction and literature review

Section A

General introduction

I.A.1. Antiviral drugs.....	1
I.A.2. Hepatitis C virus (HCV) infection.....	1
I.A.3. Drug interactions	2

Section B

Literature review

I.B.1. Ribavirin (RBV)	6
I.B.2. Sofosbuvir (SF).....	7
I.B.3. Daclatasvir (DAC)	8
I.B.4. Methods of analysis	10
I.B.4.1. Spectroscopic methods	10
I.B.4.2. Separation techniques	13
I.B.4.2. Electrochemical techniques	19

Part II: Investigating the binding interaction of both daclatasvir and ribavirin with serum albumin by spectroscopic methods

Section A

Investigating the binding interaction of both daclatasvir and ribavirin with serum albumin by spectrophotometric method

II.A.1. Introduction.....	22
II.A.2. Experimental	22
II.A.2.1. Instrumentation and software.....	22
II.A.2.2. Materials	23
II.A.2.2.1. Pure standards	23
II.A.2.2.2. Serum albumin	23

II.A.2.2.3. Reagents	23
II.A.2.2.4. Standard solutions	23
II.A.2.3. Procedure	24
II.A.2.3.1. Optimization of measured concentration of serum albumin	24
II.A.2.3.2. Spectral characteristics of serum albumin in presence of daclatasvir and ribavirin	24
II.A.2.3.3. Spectral characteristics and calibration curves of ribavirin and daclatasvir	24
II.A.2.3.4. Effect of BSA and HSA on absorption spectra of ribavirin and daclatasvir	25
II.A.3. Results and discussion	25
II.A.4. Conclusion	29

Section B

Investigating the binding interaction of both daclatasvir and ribavirin with serum albumin by conventional and synchronous spectrofluorimetric methods

II.B.1. Introduction	41
II.B.2. Experimental.....	41
II.B.2.1. Instrumentation and software	41
II.B.2.2. Materials.....	41
II.B.2.2.4. Standard solutions	41
II.B.2.3. Procedure	42
II.B.2.3.1. Fluorescence spectral measurements.....	42
II.B.3. Results and discussion	43
II.B.4. Conclusion.....	48

Part III: Simultaneous determination of studied antiviral drugs by two different techniques (spectrophotometric and HPLC techniques)

Section A

Simultaneous determination of ribavirin and daclatasvir by two spectrophotometric methods

III.A.1. Introduction	63
III.A.2. Experimental.....	63
III.A.2.1. Instrumentation	63
III.A.2.2. Materials	63

III.A.2.2.1. Pure standards.....	63
III.A.2.2.2. Plasma samples.....	64
III.A.2.2.3. Reagents	64
III.A.2.2.4. Standard solutions.....	64
III.A.2.3. Procedure	64
III.A.2.3.1. Spectral characteristics of ribavirin and daclatasvir	64
III.A.2.3.2. Method validation	64
III.A.2.4. Application of the proposed methods for simultaneous determination of RBV and DAC in spiked human plasma samples.....	67
III.A.3. Results and discussion.....	67
III.A.4. Conclusion	71

Section B

Simultaneous determination and bioanalytical validation of ribavirin, sofosbuvir and daclatasvir by HPLC method

III.B.1. Introduction.....	84
III.B.2. Experimental	85
III.B.2.1. Instrumentation	85
III.B.2.2. Materials	85
III.B.2.2.1. Pure standards	85
III.B.2.2.2. Plasma samples	85
III.B.2.2.3. Reagents	85
III.B.2.2.4. Standard solutions	85
III.B.2.3. Procedure	86
III.B.2.3.1. Preparation of standard calibration curve and quality control samples	86
III.B.2.3.2. HPLC conditions.....	87
III.B.2.3.3. Method validation.....	87
III.B.2.3.3.1. Linearity	87
III.B.2.3.3.2. Accuracy and precision	87
III.B.2.3.3.3. Recovery	88
III.B.2.3.3.4. Selectivity	88
III.B.2.3.3.5. Stability.....	88

III.B.2.3.3.6. Robustness	89
III.B.3. Results and discussion.....	89
III.B.4. Conclusion	93

Part IV: Determination of ribavirin by spectrofluorimetric and spectrophotometric methods

Section A

Determination of ribavirin in presence of sofosbuvir by derivative ratio spectra synchronous fluorescence spectroscopy method

IV.A.1. Introduction.....	105
IV.A.2. Experimental	105
IV.A.2.1. Instrumentation	105
IV.A.2.2. Materials	106
IV.A.2.2.1. Pure standards	106
IV.A.2.2.2. Plasma samples.....	106
IV.A.2.2.3. Reagents	106
IV.A.2.2.4. Standard solutions	106
IV.A.2.3. Procedure.....	106
IV.A.2.3.1. Spectral characteristics of ribavirin and sofosbuvir	106
IV.A.2.3.2. Optimization of the assay method.....	107
IV.A.2.3.3. Method validation	108
IV.A.2.3.3.1. Linearity	108
IV.A.2.3.3.2. Accuracy	109
IV.A.2.3.3.3. Precision.....	109
IV.A.2.3.3.4. Specificity	109
IV.A.2.3.3.5. Limit of detection (LOD) and limit of quantitation (LOQ)	109
IV.A.2.3.4. Application of the proposed method for determination of RBV in presence of SF in spiked human plasma samples	109
IV.A.3. Results and discussion.....	110
IV.A.4. Conclusion	114

Section B

Determination and bioanalytical validation of ribavirin in erythrocytes by spectrophotometric method

IV.B.1. Introduction.....	126
IV.B.2. Experimental	126
IV.B.2.1. Instrumentation	126
IV.B.2.2. Materials	126
IV.B.2.2.1. Pure standards.....	126
IV.B.2.2.2. Red blood cells or erythrocytes	127
IV.B.2.2.3. Reagents	127
IV.B.2.2.4. Standard solutions.....	127
IV.B.2.3. Procedure	127
IV.B.2.3.1. Erythrocytes dilution and counting	127
IV.B.2.3.2. Preparation of standard calibration curve and quality control samples	127
IV.B.2.3.3. Spectral characteristics of ribavirin in spiked erythrocytes	128
IV.B.2.3.4. Method validation	128
IV.B.2.3.4.1. Linearity	129
IV.B.2.3.4.2. Accuracy and precision	129
IV.B.2.3.4.3. Recovery.....	129
IV.B.2.3.4.4. Selectivity	129
IV.B.3. Results and discussion	130
III.B.4. Conclusion	132
Part V: General discussion	
General Discussion.....	137
References	142

List of Figures

No.	Title	Page
1	Chemical structure of ribavirin	5
2	Chemical structure of sofosbuvir	6
3	Chemical structure of daclatasvir	7
4	Zero order absorption spectrum of two different concentrations of BSA	27
5	UV absorption spectra of BSA in the presence of DAC	28
6	UV absorption spectra of HSA in the presence of DAC	28
7	UV absorption spectra of BSA in the presence of RBV	29
8	Effect of using water and two types of buffers (at pH 7.4) on the absorbance and λ_{max} of RBV	29
9	Zero-order absorption spectra of different concentrations of RBV in 0.1 M tris-HCl	30
10	Calibration curve correlating absorbance to the corresponding concentrations of RBV at 211.3 nm	30
11	Zero-order absorption spectra of different concentrations of DAC in 0.1 M tris-HCl	31
12	Calibration curve correlating absorbance to the corresponding concentrations of DAC at 315.4 nm	31
13	Zero-order spectrum showing the decrease in absorbance of DAC at 315.4 nm	32
14	Binding isotherms of different concentration of DAC with BSA and HSA	33
15	Scatchard plots of different concentration of BSA and HSA	34
16	Binding isotherm of different concentration of RBV with BSA	35
17	Fluorescence emission spectra of BSA and HSA in the absence or presence of different concentrations of DAC	45
18	Stern–Volmer plot for the quenching of BSA and HSA by DAC at three different temperatures	46
19	Fluorescence emission spectra of BSA in the absence or presence of different concentrations of RBV	47
20	The quenching degree of BSA fluorescence by DAC and RBV	47
21	Stern–Volmer plot for the quenching of BSA by RBV at 300 K	48
22	Plot of $\log[(F_0-F)/F]$ versus $\log[Q]$ of BSA and HSA in presence of DAC at three different temperatures	49
23	Van't Hoff plot for interaction of DAC with BSA and HSA	50
24	Spectral overlap of UV-Vis absorption spectrum of DAC with fluorescence emission spectrum of BSA and HSA	51

25	Synchronous Fluorescence spectra of BSA and HSA in the absence or presence of different concentrations of DAC at $\Delta\lambda = 15$ nm.	52
26	Synchronous Fluorescence spectra of BSA and HSA in the absence or presence of different concentrations of DAC at $\Delta\lambda = 60$ nm	53
27	The quenching degree of BSA and HSA synchronous fluorescence by DAC at $\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm.	54
28	Zero order absorption spectra of DAC and RBV	66
29	Second derivative absorption spectra of DAC showing its amplitude at 310.6 nm and RBV showing its amplitude at 211.6 nm	66
30	First derivative spectrum of ratio spectrum for RBV after division by 10.00 $\mu\text{g/mL}$ DAC showing the measured peak amplitude at 234 nm	67
31	Calibration curve correlating peak amplitude to the corresponding concentration of RBV by second derivative zero-crossing method at 211.6 nm	67
32	Calibration curve correlating peak amplitude to the corresponding concentration of DAC by second derivative zero-crossing method at 310.6 nm	68
33	Calibration curve correlating peak amplitude to the corresponding concentration of RBV by derivative ratio spectra method at 234 nm	68
34	Calibration curve correlating absorbance to the corresponding concentration of DAC from its zero-order spectrum at 312.2 nm	69
35	HPLC chromatogram of blank human plasma sample and HPLC chromatogram of mixture containing RBV, SF, DAC and propyl paraben (IS) at 260 nm in extracted human plasma samples	87
36	Calibration curve representing peak area versus concentration of RBV at 207 nm	88
37	Calibration curve representing peak area versus concentration of SF at 260 nm	88
38	Calibration curve representing peak area versus concentration of DAC at 312 nm	89
39	3D fluorescence scan spectrum of ribavirin showing maximum fluorescence intensity in the red zone of the diagram	106
40	Emission spectrum of ribavirin at 382 nm at λ_{exc} 315 nm in acetonitrile	106
41	Emission spectrum at 382 nm and excitation spectrum at 315 nm of 10 $\mu\text{g/mL}$ ribavirin in acetonitrile	107
42	Synchronous fluorescence spectrum of each of ribavirin and sofosbuvir at $\Delta\lambda = 70$ nm	107
43	Effect of different $\Delta\lambda$ (10-100 nm) of fluorescence of RBV showing maximum intensity at $\Delta\lambda = 70$ nm	108
44	Effect of different solvents on relative fluorescence intensity of RBV at $\Delta\lambda = 70$ nm	109
45	Effect of different pH values of citric-acid phosphate buffer on relative fluorescence intensity of RBV at $\Delta\lambda = 70$ nm	109
46	Effect of different organized media on relative fluorescence intensity of RBV at $\Delta\lambda = 70$ nm	110
47	Derivative ratio synchronous spectrum of RBV after division by 5 $\mu\text{g/mL}$ SF	111

48	Derivative ratio synchronous spectrum of RBV after division by 5 µg/mL SF showing the measured peak amplitude at 435 nm	111
49	Calibration curve correlating peak amplitude to the corresponding concentration of RBV by derivative ratio synchronous spectrum method at 435 nm	112
50	Zero order absorption spectra of RBV in spiked erythrocytes at 232.4 nm	123
51	Calibration curve correlating absorbance to the corresponding concentration of RBV from its zero-order spectrum at 232.4 nm	123

List of Tables

No.	Title	Page
1	Different visible spectrophotometric methods were used for determination of RBV	9
2	TLC methods for the determination of the studied antiviral drugs	12
3	HPTLC methods for the determination of the studied antiviral drugs	13
4	HPLC methods for determination of ribavirin	13
5	HPLC methods for determination of sofosbuvir	15
6	HPLC methods for determination of daclatasvir	15
7	HPLC methods for determination of different mixtures of the studied antiviral drugs	16
8	UPLC methods for determination of the studied antiviral drugs	17
9	Regression parameters of calibration curve of ribavirin	36
10	Regression parameters of calibration curve of daclatasvir	36
11	Binding parameters of DAC with BSA HSA obtained by non-linear regression of Scatchard plot using GraphPad Prism 7 software from UV absorbance data of DAC at 314.8nm	36
12	Stern-Volmer quenching constants for interaction of DAC with BSA and HSA at three different temperatures	55
13	Regression equation, correlation coefficient(r), binding constant (k_b) and the number of binding site (n) between DAC and serum albumins at different temperatures	55
14	Thermodynamic parameters of the interaction between DAC and serum albumins at different temperatures	56
15	Results of assay validation parameters of the proposed second derivative zero-crossing method for simultaneous determination of ribavirin and daclatasvir	70
16	Results of assay validation parameters of the proposed derivative ratio spectra method for determination of ribavirin and zero-order spectrum method for determination of daclatasvir	70
17	Accuracy results for determination of ribavirin and daclatasvir by second derivative zero-crossing method in pure samples	71
18	Accuracy results for determination of ribavirin by derivative ratio spectra method and daclatasvir by zero-order spectrum in pure samples	71
19	Simultaneous determination of ribavirin and daclatasvir in laboratory prepared mixtures using second derivative zero-crossing method	72
20	Simultaneous determination of ribavirin by derivative ratio spectra method and daclatasvir by zero-order spectrum in laboratory prepared mixtures	73
21	Simultaneous determination of ribavirin and daclatasvir in spiked human plasma using second derivative spectra zero-crossing method	74
22	Simultaneous determination of ribavirin by derivative ratio spectra method and daclatasvir by zero-order spectrum in spiked human plasma	74
23	Statistical comparison of the results obtained by applying the proposed second	75

	derivative method and the reported methods ^(41,72) for the determination of RBV and DAC	
24	Statistical comparison of the results obtained by applying the proposed derivative ratio spectra coupled with zero order spectrophotometry method and the reported methodsfor the determination of RBV and DAC	76
25	Preparation of calibrators and quality control samples for ribavirin, sofosbuvir and daclatasvir	79
26	Regression parameters obtained from ribavirin, sofosbuvir and daclatasvir linearity curves	90
27	Accuracy and precision for the HPLC determination of ribavirin, sofosbuvir and daclatasvir in human plasma	91
28	Recovery results for HPLC determination of ribavirin, sofosbuvir and daclatasvir in human plasma	92
29	Stability results of ribavirin ribavirin, sofosbuvir and daclatasvir in human plasma under different stability assessment conditions	93
30	Robustness results of HPLC determination of ribavirin, sofosbuvir and daclatasvir in human plasma	94
31	System suitability parameters of HPLC determination of RBV, SF and DAC	94
32	Statistical comparison of the results obtained by applying the proposed HPLC method and the reported methodsfor the determination of RBV, SF and DAC	95
33	Accuracy results for determination of ribavirin in presence of sofosbuvir by derivative ratio spectra method in pure samples	113
34	Determination of ribavirin in presence of sofosbuvir by derivative ratio spectra method in laboratory prepared mixtures	113
35	Determination of ribavirin in presence of sofosbuvir by derivative ratio spectra method in spiked human plasma	114
36	Results of assay validation parameters of the proposed derivative ratio spectra method for determination of ribavirin in presence of sofosbuvir	114
37	Statistical comparison of the results obtained by applying the proposed derivative ratio spectra method and the reported methodfor the determination of RBV	115
38	Preparation of calibrators and quality control samples for RBV by spiking erythrocytes	118
39	Regression parameters obtained from linearity curve of determination of ribavirin (RBV) in erythrocytes	124
40	Accuracy and precision for the spectrophotometric determination of RBV in erythrocytes	124
41	Recovery results for spectrophotometric determination of RBV in erythrocytes	125
42	Statistical comparison of the results obtained by applying the proposed spectrophotometric method and the reported methodfor the determination of RBV	125
43	Comparison between the proposed methods in terms of linearity and sensitivity	128
44	Results of one way ANOVA for comparison between the proposed methods and the official methodfor determination of ribavirin	129
45	Results of one way ANOVA for comparison between the proposed methods and the reported methodfor determination of daclatasvir	129