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Application of Chemometrics in Determination of Some Drugs via Different Analytical Techniques

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TO

The one who is albeit absent but spiritually present: my beloved father

The light of my sight: my dear mother

My backup and refuge: my brothers Ali and Hashim

My soul mate and the beat of heart: my sister Dhuha

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Summary

The work incorporated in the thesis is an attempt to develop new analytical methods for the determination of four medications belonging to different therapeutic groups namely; furosemide (FURO), carbamazepine (CARB), diazepam (DIAZ) and carvedilol (CARV). The study, which included the estimation of the studied drugs in their pure forms and in pharmaceutical formulations (tablets, ampoule and oral suspension), has been described in four chapters as follows:

Chapter one: Includes an introduction to chromatography as well as basic principles, classification and theory of chromatography. A brief account on basic principles, modes and instrumentation of high performance liquid chromatography have been reported. The basic principles of derivative spectrophotometry and partial least squares method have been described as well as their applications in pharmaceutical analysis. In addition, this chapter gives a brief description of systematic name, structure and literatures on different methods reported for FURO, CARB, DIAZ and CARV.

Chapter two: Covers the experimental part that includes chemicals, solvents and instruments used throughout this work as well as the description of the preparation of standard stock solutions and buffer solutions.

Chapter three: Depicts the development and validation of a simple reverse-phase high performance liquid chromatographic method for the simultaneous analysis (separation and quantification) of furosemide, carbamazepine, diazepam and carvedilol.

The effect of various factors such as mobile phase composition, flow rate, organic modifier and buffer percentage, pH of buffer, temperature and injection volume on the chromatographic performance of the four analytes have been studied. Under optimum conditions, isocratic elution with flow rate of 1.5 $mL.min^{-1}$ was employed on RP-NUCLEODUR® 100-5 C18ec (250 x 4.6 mm, 5µm) column. The

mobile phase composed of 50: 50 acetonitrile: deionized water (pH 3.6, acidified with acetic acid), the column temperature was 40°C and the injection volume 10 µL. The detection was done with UV detector at 226 nm, and the order of elution were FURO, CARB, DIAZ and CARV at 1.90 min, 2.79 min, 5.39 min and 9.56 min respectively. Calibration curves were constructed by plotting the peak area and the peak height against corresponding concentrations in the range of 1.0-100.0 $\mu g.mL^{-1}$ for furosemide, 2.5-100.0 $\mu g.mL^{-1}$ for both carbamazepine and diazepam and 5.0-100.0 $\mu g.mL^{-1}$ for carvedilol. The LOD and LOQ for the cited drugs were between (0.115-0.950 $\mu g.mL^{-1}$) and (0.348-1.440 $\mu g.mL^{-1}$) for peak area and (0.074-2.877 $\mu g.mL^{-1}$) and (0.225-1.599 $\mu g.mL^{-1}$) for peak height respectively. The precision in term of relative standard deviation was calculated and found to be less than 4 %, and accuracy expressed as relative error was less than 5%.

The developed method has been applied successfully for the determination of the cited drugs in their pharmaceutical preparations and the recovery % were found to be (102.3%-106.6%) and (97.5%-103.0%) for furosemide, (94.2%-98.2%) and (88.8%-94.0%) for carbamazepine, (98.4%-102.9%) and (96.9%-107.5%) for diazepam, (92.3%-106.4%) and (93.8%-106.6%) for carvedilol for peak area and peak height respectively.

Chapter four: Entitled “simultaneous determination of furosemide, carbamazepine, diazepam and carvedilol” deals with the simultaneous estimation of the four drugs in their quaternary mixtures via two methods namely: partial least squares regression and derivative spectrophotometry.

Partial least squares (PLS) method was used as chemometric technique for simultaneous spectrophotometric estimation of FURO, CARB, DIAZ and CARV in their quaternary mixtures. Calibration graph for each drug was constructed in the concentration range of (1 – 20 $\mu g.mL^{-1}$) at 233.0 nm, 215.0 nm, 228.5 nm and 242.5 nm for FURO, CARB, DIAZ and CARV respectively and linear relations were obtained in

studied concentration ranges. The first step in PLS methodology involved constructing a calibration (training) set from the spectrophotometric data obtained by careful UV-measurements on a set of known samples, composing of 84 mixtures that selected according to Simplex Lattice Mixture Design.

The absorbance data matrices corresponding to the concentration data matrices for each of the 84 mixtures were obtained by the measurements of absorbance in the range 200-350 nm with a scan speed of 10 $nm.sec^{-1}$, averaging of 1.0 nm, bandwidth of 1.8 nm, and data interval of 0.5 nm against solvent blank. The PLS-1 and PLS-2 regression models were built with the help of OriginPro software version 2015 program and the concentration of the four drugs were then predicted. The result of relative standard deviation percentage (RSD %) and relative error percentage (RE %) were in the ranges (1.2638%-6.0529%) and (-0.0845%-3.8053%) respectively. The proposed method was successfully applied in the simultaneous determination of furosemide, carbamazepine, diazepam and carvedilol in their synthetic sample mixtures.

Derivative spectrophotometric techniques (first, second, third and fourth derivatives) were developed for the simultaneous determination of FURO, CARB, DIAZ and CARV in their quaternary mixtures. It was found that FURO and CARV could be determined by all modes of derivative (i.e. first to fourth order); CARB could be determined by first, second, and third order; while only first and second modes of derivative could be used in the determination of DIAZ in presence of other investigated drugs.

The zero crossing technique was employed in first derivative measurements, using the peak height to baseline at 336.5, 358.5, 306.0 and 331.5 nm for CARV, FURO, CARB and DIAZ respectively, which was in proportion to the drug concentration therefore they are used for the quantitative estimation of the titled drugs. The second derivative spectra of the same mixtures show that peak height at

332.5 nm for CARV, and height to baseline at zero cross (273.5, 226.0 and 218.5 nm) for FURO, CARB and DIAZ respectively, could be used to quantify the corresponding concentration for each drug. While in the third derivative method the peak height at 288.0 nm and height to baseline at zero cross at 237.5 and 224.0 nm were found to be useful for the determination of CARV, FURO and CARB respectively. The careful inspection of the fourth derivative spectra obtained for the mentioned quaternary mixtures reveal that peak height at 288.0 nm and height to baseline at zero cross at 234.0 nm were in proportion to the CARV and FURO concentrations respectively.

The results show no interferences from the excipients on the determination of the four drugs by utilizing these techniques, thus they were suitable to be applied for the estimation of CARV, FURO, CARB and DIAZ in their pharmaceutical preparations.

INDEX

SR. NO.	CONTENT	PAGE NO.
	Summary	I
	Index	V
	List of Tables	IX
	List of Figures	XII
	List of Schemes	XVIII
	List of Abbreviations	XIX
	CHAPTER ONE INTRODUCTION	
1-1	Chromatography	1
1-1-1	Principles of chromatography	1
1-1-2	Classification of chromatography	2
1-1-3	Theory of chromatography	3
1-1-3-1	Plate theory	3
1-1-3-2	Rate theory	4
1-1-4	High Performance Liquid Chromatography	4
1-1-5	Theory of HPLC	5
1-1-6	Modes of HPLC	5
1-1-6-1	Adsorption chromatography	6
1-1-6-2	Ion exchange chromatography	6
1-1-6-3	Size exclusion chromatography	7
1-1-6-4	Affinity chromatography	7
1-1-6-5	Normal phase	8
1-1-6-6	Reversed phase	9
1-1-7	HPLC Instrumentation	10
1-1-7-1	Solvent Delivery System	10
1-1-7-2	Sample Injection System	12
1-1-7-3	Chromatographic Column	12
1-1-7-4	HPLC Detectors	13
1-1-7-5	Data Handling Device	13
1-2	Ultraviolet-Visible absorption spectroscopy	13
1-2-1	Derivative spectrophotometry	15
1-2-1-1	Introduction	15
1-2-1-2	Analytical applications of UV-Vis derivative spectrophotometry	18
1-2-2	Chemometrics	19

INDEX

SR. NO.	CONTENT	PAGE NO.
1-2-2-1	Partial Least Square (PLS) method	19
1-2-2-2	Pharmaceutical Application of PLS regression method	22
1-3	The Drugs	23
1-3-1	Diazepam	23
1-3-2	Carbamazepine	24
1-3-3	Carvedilol	25
1-3-4	Furosemide	26
1-4	Methods for the determination of CARV, FURO, CARB and DIAZ	27
1-4-1	Electroanalytical methods	27
1-4-2	Chromatographic methods	28
1-4-3	Flow injection analysis	30
1-4-4	Spectrophotometric methods	30
1-4-5	High Performance Liquid Chromatography	35
1-5	Objectives	44
	CHAPTER TWO EXPERIMENTAL PART	
2-1	Chemicals and solvents	45
2-2	The Drugs	46
2-3	HPLC system	47
2-3-1	Mobile phase reservoir	47
2-3-2	Connecting tubes and fittings	48
2-3-3	Degassing unit (DGU-20A_5)	48
2-3-4	Solvent delivery unit (LC-20AD)	48
2-3-5	Sample injection unit (SIL-20AC)	49
2-3-6	Column compartment	49
2-3-7	UV-VIS detector (SPD-20A)	49
2-4	Miscellaneous Instruments	50
2-5	Method development	51
2-5-1	Preparation of solutions for pH control	51
2-5-2	Preparation of mobile phase	51
2-5-3	Preparation of standard stock solutions	52
2-5-4	Preparation of samples for linearity	52

INDEX

SR. NO.	CONTENT	PAGE NO.
2-5-5	Preparation of drugs in pharmaceutical formulations	52
2-6	Primary experimental test	53
2-7	Optimization of experimental parameters	56
	CHAPTER THREE RESULTS & DISCUSSION	
3-1	Wavelength selection	58
3-2	Optimization of experimental variables	59
3-2-1	Effect of mobile phase composition	59
3-2-2	Effect of flow rate	60
3-2-3	Effect of organic modifier	64
3-2-4	Effect of buffer percentage	69
3-2-5	pH of buffer	73
3-2-6	Effect of temperature	77
3-2-7	Effect of injection volume	80
3-2-8	Construction of calibration curve	84
3-2-9	Accuracy and precision	92
3-2-10	Application	93
	CHAPTER FOUR SIMULTANEOUS DETERMINATION OF FUROSEMIDE, CARBAMAZEPINE, DIAZEPAM AND CARVEDILOL	
4-1	Simultaneous Spectrophotometric Determination of Quaternary Mixture via Partial Least Squares Method	97
4-1-1	Introduction	97
4-1-2	Spectrophotometric simultaneous determination of Furosemide, Carbamazepine, Diazepam, and Carvedilol by partial least squares in their pure form	98
4-1-3	Experimental	100
4-1-3-1	Instrumentation	100
4-1-3-2	Chemicals	100
4-1-3-3	Pharmaceutical Compounds	100
4-1-3-4	Standard Drugs Solution	100
4-1-3-5	General Recommended Procedures	100

INDEX

SR. NO.	CONTENT	PAGE NO.
4-1-4	Results and Discussion	104
4-1-4-1	Absorption Spectra	104
4-1-4-2	Individual Calibration	105
4-1-4-3	Multivariate -Partial least squares Method	110
4-1-4-4	Application of the method	119
4-1-4-5	Accuracy and Precision	127
4-2	Simultaneous Determination of Furosemide, Carbamazepine, Diazepam and Carvedilol via Derivative Spectrophotometry	130
4-2-1	Introduction	130
4-2-2	Experimental	131
4-2-2-1	Instrumentation	131
4-2-2-2	Chemicals	132
4-2-2-3	Pharmaceutical Compounds	132
4-2-2-4	Standard Drugs Solutions	132
4-2-2-5	Pharmaceutical Product Solution	132
4-2-2-6	General Recommended Procedures	132
4-2-3	Results and discussion	133
4-2-3-1	Absorption Spectra	133
4-2-3-2	First, second, third and fourth derivative modes	134
4-2-3-3	Individual Calibration of Each Drug	136
4-2-3-4	Simultaneous Calibration of Quaternary Mixture via Derivative Mode	137
4-2-3-5	Accuracy and precision	155
4-2-3-6	Application	157
4-3	Statistical evaluation	163
4-4	Conclusion	167
4-5	Suggestions	168
References		169
Appendix		196

LIST OF TABLES

SR. NO.	TITLE	PAGE NO.
1-1	Application of derivative spectrophotometry in pharmaceutical analysis	18
1-2	Different application of PLS technique in pharmaceutical analysis	23
1-3	General properties of Diazepam	24
1-4	General properties of Carbamazepine	25
1-5	General properties of Carvedilol	26
1-6	General properties of Furosemide	27
1-7	Electroanalytical techniques reported for the determination of FURO, CARB, DIAZ and CARV	28
1-8	Chromatographic techniques reported for the determination of FURO, CARB, DIAZ and CARV	29
1-9	Flow injection analysis techniques reported for the determination of FURO, CARB, DIAZ and CARV	30
2-1	Chemical compounds and their provider	45
2-2	The principle pharmaceutical formulations and their manufacturers	46
2-3	The properties of chromatographic peaks	55
3-1	Result of t_R, k', α, N and Rs of drugs mixture at different flow rates	64
3-2	Separation parameters for each drug at different percent of acetonitrile	68
3-3	Separation parameters of each drug at different buffer percentage	72
3-4	Separation parameters of each drug at different pH of the buffer	76
3-5	Separation parameters of each drug at different temperatures	80
3-6	Separation parameters of each drug at different injection volumes	82
3-7	Linearity parameters for FURO, CARB, DIAZ and CARV assay method	92

LIST OF TABLES

SR. NO.	TITLE	PAGE NO.
3-8	Accuracy and precision of the method	93
3-9	Estimation of FURO, CARB, DIAZ and CARV in their pharmaceutical preparations via peak area	95
3-10	Estimation of FURO, CARB, DIAZ and CARV in their pharmaceutical preparations via peak height	96
4-1	Analytical study of PLS regression methods for determination of FURO, CARB, DIAZ and CARV in pharmaceutical products and human fluids	99
4-2	Lists of the coded runs for a Simplex Lattice of four factors for six levels mixture design	102
4-3	Uncoded six level Simplex Lattice mixture design for the composition of the calibration set used to build PLS-2 and PLS-1 models.	103
4-4	Composition of training set, their predictions by PLS-1 model	115
4-5	Composition of training set, their predictions by PLS-2 model	116
4-6	Evaluation of accuracy and precision of the proposed method	117
4-7	F-Test Two-Sample (PLS-1 and PLS-2) for Variances for the predicated results of training sets obtained by PLS-1 and PLS-2 models for the of the analysis of each drug in the presence of the other	118
4-8	Composition of synthetic samples, their predictions by PLS-1 model and statistical parameters for the system	121
4-9	Composition of synthetic samples, their predictions by PLS-2 model and statistical parameters for the system	122
4-10	Goodness of fit statistics for synthetic samples set analyses by PLS-1	123
4-11	Goodness of fit statistics for synthetic samples set analyses by PLS-2	123
4-12	Accuracy and precision of the proposed method	127

LIST OF TABLES

SR. NO.	TITLE	PAGE NO.
4-13	Composition of analyzed pharmaceutical sample solutions with their predictions by PLS-1 model and statistical parameters for the system	128
4-14	Composition of analyzed pharmaceutical samples with their predictions by PLS-2 model and statistical parameters for the system	129
4-15	Application of the PLS methods to the CARV, DIAZ, CARB, and FURO concentration measurements in drugs tablet formulation samples	129
4-16	Analytical study of derivative methods for determination of FURO, CARB, DIAZ and CARV in pharmaceutical formulations and biological fluids	131
4-17	Analytical parameters for the determination of carvedilol using first, second, third and fourth derivative spectrophotometric techniques	145
4-18	Summary of the selected methods for the determination of FURO, CARB and DIAZ and their analytical parameters	146
4-19	Evaluation of accuracy and precision for the determination of CARV, FURO, CARB and DIAZ via derivative spectrophotometry	156
4-20	Application of the derivative spectrophotometry to the CARV, FURO, CARB and DIAZ concentration measurements in their pharmaceutical formulations	158
4-21	Statistical validation data for quantitative assessment of commercial tablet formulation for CARV, DIAZ, CARB, and FURO	159
4-22	Application of standard addition method to the analysis of CARV, FURO, CARB and DIAZ using derivative technique	160
4-23	Statistical comparison of the results obtained by the three proposed methods	165
4-24	One-way ANOVA of the results obtained analyses of drugs mixtures by the proposed methods	166

LIST OF FIGURES

SR. NO.	TITLE	PAGE NO.
1-1	Theoretical plate	3
1-2	Van Deemter plot	4
1-3	Adsorption Chromatography	6
1-4	Ion-exchange Chromatography	7
1-5	Size Exclusion Chromatography	7
1-6	Affinity Chromatography	8
1-7	Bonded silica (C18) illustrating residual silanol groups and Trimethylsilane (TMS) end-capped silanol group	10
1-8	A schematic diagram of typical HPLC system	10
1-11	Differentiation of computed analytical Gaussian curves: (a) the initial curve and its first- to fourth- order derivatives; (b) the initial curve of two superposed Gaussian curves and their first- to fourth- order derivatives	16
1-12	Schematic description of matrix decomposition of PLS data	20
2-1	Shimadzu UFLC system	47
2-2	The reservoir filter	48
2-3	HPLC Chromatograms of individual drugs (20 $\mu g.mL^{-1}$) under preliminary conditions	54
2-4	HPLC Chromatogram of the four drugs mixture (20 $\mu g.mL^{-1}$ of each)	55
3-1	Overlaid UV spectra of the studied drugs (10 $\mu g.mL^{-1}$) in acetonitrile against acetonitrile as a blank	58
3-2	Chromatograms of the drugs mixture (20$\mu g.mL^{-1}$) using different buffers; (The order of elution is: FURO, CARB, CARV, and DIAZ)	60
3-3	Chromatograms of the mixture at different flow rate [a] 0.5 $mL.min^{-1}$, [b] 0.8 $mL.min^{-1}$, [c] 1.0 $mL.min^{-1}$, [d] 1.2 $mL.min^{-1}$, [e] 1.5 $mL.min^{-1}$ and [f] 1.8 $mL.min^{-1}$	62
3-4	The relationship between t_R and Rs as a function of flow rate	63

LIST OF FIGURES

SR. NO.	TITLE	PAGE NO.
3-5	Chromatograms of the mixture at different percentage of acetonitrile (a) 30 : 62.3 : 7.7, (b) 40 : 53.4 : 6.6, (c) 50 : 45 : 5.0, (d) 60 : 35.6 : 4.4 and (e) 70 : 26.7 : 3.3	66
3-6	The relationship between t_R and Rs with the acetonitrile percentage	67
3-7	Chromatograms of the mixture at different percent of buffer (a) 0.5 %, (b) 1.0 %, (c) 1.5 %, (d) 2.0 %, (e) 2.5 %, (f) 3.5 %, (g) 5.0 % and (h) 6.5 %	70
3-8	The relationship between t_R and Rs with the phosphate buffer percentage	71
3-9	Chromatograms of the mixture at different pH [a] 9.48, [b] 10.04, [c] 10.53, [d] 11.0, [e] 11.49 and [f] 11.98	74
3-10	The relationship between t_R and Rs with the pH of buffer	75
3-11	Chromatograms of the mixture at different percent temperature (a) 30 ºC, (b) 35 ºC, (c) 40 ºC, (d) 45 ºC and (e) 50 ºC	78
3-12	The plot of Rs as a function of column temperature for each drug	79
3-13	The van't Hoff's plot for the exothermic drug retention	79
3-14	Overlaid chromatograms of the drugs mixture at different injection volumes (2.5-20 µL)	81
3-15	The relationship between peak area and peak height as a function of injection volume for each drug	83
3-16	Overlaid chromatograms of the four drugs at five concentrations (0.5-5 $\mu g.mL^{-1}$)	84
3-17	Calibration curves, concentration ($\mu g.mL^{-1}$) vs. peak area and peak height for FURO, CARB, CARV and DIAZ	85
3-18	Chromatograms of the drugs mixture at buffer concentration: (a) 0.1M, (b) 0.005 M, and (c) 0.0025 M	86
3-19	The gradient profile used for separation of drugs	86

LIST OF FIGURES

SR. NO.	TITLE	PAGE NO.
3-20	Overlaid isocratic elution of the studied drugs using (50:50) ACN: 0.005M ammonium buffer pH 8.53	87
3-21	Gradient elution of the studied drugs using 0.005M ammonium buffer pH 8.53 (Solvent A) and ACN (Solvent B)	87
3-22	Chromatogram for separation of the four drugs under isocratic mode of elution with (50:50) water acidified with acetic acid; pH= 3.60: acetonitrile	88
3-23	Chromatogram for separation of the four drugs under gradient elution with water acidified with acetic acid; pH= 3.60 (Solvent B), H_2O (Solvent A) and acetonitrile (Solvent C)	89
3-24	The gradient profile used for separation of drugs	89
3-25	Overlaid chromatograms of the four drugs under gradient elution at different concentrations (0.5-100 $\mu g.mL^{-1}$)	90
3-26	Overlaid chromatograms of the four drugs under isocratic elution at different concentrations (0.5-100 $\mu g.mL^{-1}$)	90
3-27	Calibration curves, concentration ($\mu g.mL^{-1}$) vs. peak area and peak height for FURO, CARB, DIAZ and CARV	91
3-28	Chromatograms for separation of the four drugs under isocratic mode of elution with (50:50) water acidified with acetic acid; pH= 3.60: acetonitrile (a) Mix of standard drugs and (b) Mix of pharmaceutical formulations	94
4-1	Overlaid normal mode spectra of 10 $\mu g.mL^{-1}$ of furosemide, carbamazepine, diazepam and carvedilol each against their solvent blanks	104
4-2	Calibration curve of furosemide constructed at 233.0 nm	106
4-3	Calibration curve of carbamazepine constructed at 215.0 nm	107
4-4	Calibration curve of diazepam constructed at 228.5 nm	108
4-5	Calibration curve of carvedilol constructed at 242.5 nm	109

LIST OF FIGURES

SR. NO.	TITLE	PAGE NO.
4-6	Absorbance spectra of eighty-four quaternary mixtures of FURO, DIAZ, CARB, and CARV in acetonitrile measured against solvent blank under experimental condition	112
4-7	Plot of Root Mean PRESS against number of factors for forty CARV, DIAZ, CARB, and FURO mixtures for: (A) PLS-2 model, (B) PLS-1 model for CARV, (C) PLS-1 model for DIAZ, (D) PLS-1 model for CARB, and (E) PLS-1 model for FURO	113
4-8:	Plots of the predicated results of training sets obtained by PLS-1 against those obtained by PLS-2 model for the analyses of: (A) CARV, (B) DIAZ, (C) CARB and (D) FURO in their quaternary mixtures	119
4-9	Plot of Root Mean PRESS against number of factors for eighty-four CARV, DIAZ, CARB, and FURO mixtures: (A) PLS-2 model, (B) PLS-1 model for CARV, (C) PLS-1 model for DIAZ, (D) PLS-1 model for CARB and (E) PLS-1 model for FURO	120
4-10	Plots of PLS-1 predicated vs true drugs concentrations in synthetic quaternary mixtures; (A) CARV, (B) DIAZ, (C) CARB, and (D) FURO	124
4-11	Plots of PLS-2 predicated vs true drugs concentrations in synthetic quaternary mixtures; (A) CARV, (B) DIAZ, (C) CARB, and (D) FURO	125
4-12	plots of standardized concentration residuals vs predicted drugs concentrations for the quaternary synthetic mixture samples, by PLS-1 model	126
4-13	plots of standardized concentration residuals vs predicted drugs concentrations for the quaternary synthetic mixture samples, by PLS-2 model	126
4-14	Zero-order absorption spectra of 3µg.mL^{-1} CARV, 10µg.mL^{-1} DIAZ, 3µg.mL^{-1} CARB, 3 µg.mL^{-1} FURO, and quaternary mixture of 3.3µg.mL^{-1} for CARV, CARB, FURO and 10 µg.mL^{-1} for DIAZ against methanol as a blank	134
4-15	Overlaid first order derivative spectra of 10 µg.mL^{-1} of FURO, CARB, DIAZ, and CARV in methanol	135

LIST OF FIGURES

SR. NO.	TITLE	PAGE NO.
4-16	Overlaid second derivative spectra of 10 μg.mL^{-1} of FURO, CARB, DIAZ, and CARV in methanol	135
4-17	Overlaid third derivative spectra of 10 μg.mL^{-1} of FURO, CARB, DIAZ, and CARV in methanol	136
4-18	Overlaid fourth derivative spectra of 10 μg.mL^{-1} of FURO, CARB, DIAZ, and CARV in methanol	136
4-19	First derivative spectra of (1-20 μg.mL^{-1}) carvedilol and 10 μg.mL^{-1} for FURO, CARB, and DIAZ in methanol for the range (A) 200-380 nm, (B) 323.5- 346.0 nm	139
4-20	Calibration curve obtained via first mode derivative of carvedilol for height measurements at zero cross at 336.5 nm	139
4-21	Second derivative spectra of (1-20 μg.mL^{-1}) carvedilol and 10 μg.mL^{-1} for FURO, CARB, and DIAZ in methanol for the range: (A) 200-380 nm, (B) 322.0- 346.0 nm	140
4-22	Calibration curve obtained via peak height measurements at 332.5 nm in second mode derivative of carvedilol	141
4-23	Third derivative spectra of (1-20 μg.mL^{-1}) carvedilol and 10 μg.mL^{-1} for FURO, CARB, and DIAZ in methanol for the range (A) 200-380 nm, (B) 281.5-297.0 nm	142
4-24	Calibration curve obtained via third mode derivative of carvedilol for peak height at 288.0 nm	143
4-25	Fourth derivative spectra of (1-20 μg.mL^{-1}) carvedilol and 10 μg.mL^{-1} for FURO, CARB, and DIAZ in methanol for the range (A) 200-380 nm, (B) 280.5-292.5 nm	144
4-26	Calibration curve obtained via peak height measurement at 286.0 nm in fourth mode derivative of carvedilol	144
4-27	First derivative spectra of (1-20 μg.mL^{-1}) furosemide and 10 μg.mL^{-1} for CARV, CARB, and DIAZ in methanol for the range (A) 200-380 nm, (B) 325.0-380 nm	147
4-28	Calibration curve obtained via first derivative spectra of furosemide for height at zero cross at 358.5 nm	147

LIST OF FIGURES

SR. NO.	TITLE	PAGE NO.
4-29	Second derivative spectra of (1-20 μg.mL^{-1}) furosemide and 10 μg.mL^{-1} for CARV, CARB, and DIAZ in methanol for the range (A) 200-380 nm, (B) 252.0-295.0 nm	148
4-30	Calibration curve obtained via second derivative spectra of furosemide for height at zero cross at 273.5 nm	148
4-31	Third derivative spectra of (1-20 μg.mL^{-1}) furosemide and 10 μg.mL^{-1} for CARV, CARB, and DIAZ in methanol for the range (A) 200-380 nm, (B) 230.0-246.0 nm	149
4-32	Calibration curve obtained via third derivative spectra of furosemide for height at zero cross at 237.5 nm	149
4-33	Fourth derivative spectra of (1-20 μg.mL^{-1}) furosemide and 10 μg.mL^{-1} for CARV, CARB, and DIAZ in methanol for the range (A) 200-380 nm, (B) 230.0-241.0 nm	150
4-34	Calibration curve obtained via fourth derivative spectra of furosemide for height at zero cross at 234.0 nm	150
4-35	First derivative spectra of (1-20 μg.mL^{-1}) carbamazepine and 10 μg.mL^{-1} for CARV, FURO, and DIAZ in methanol for the range (A) 200-380 nm, (B) 260.0-348.0 nm	151
4-36	Calibration curve obtained via first derivative spectra of carbamazepine for height at zero cross at 306.0 nm	151
4-37	Second derivative spectra of (1-20 μg.mL^{-1}) carbamazepine and 10 μg.mL^{-1} for CARV, FURO, and DIAZ in methanol for the range (A) 200-380 nm, (B) 221.0-240.0 nm	152
4-38	Calibration curve obtained via second derivative spectra of carbamazepine for height at zero cross at 226.0 nm	152
4-39	Third derivative spectra of (1-20 μg.mL^{-1}) carbamazepine and 10 μg.mL^{-1} for CARV, FURO, and DIAZ in methanol for the range (A) 200-380 nm, (B) 216.5-235.0 nm	153
4-40	Calibration curve obtained via third derivative spectra of carbamazepine for height at zero cross at 224.0 nm	153
4-41	First derivative spectra of (1-20 μg.mL^{-1}) diazepam and 10 μg.mL^{-1} for CARV, FURO, and CARB in methanol for the range (A) 200-380 nm, (B) 308.0-352.0 nm	154

LIST OF FIGURES

SR. NO.	TITLE	PAGE NO.
4-42	Calibration curve obtained via first derivative spectra of diazepam for height at zero cross at 331.5 nm	154
4-43	Second derivative spectra of (1-20 μg.mL^{-1}) diazepam and 10 μg.mL^{-1} for CARV, FURO, and CARB in methanol for the range (A) 200-380 nm, (B) 214.0-222.0 nm	155
4-44	Calibration curve obtained via second derivative spectra of diazepam for height at zero cross at 218.5 nm	155
4-45	Derivative spectra of pure CARV (3, 5 and 8 μg.mL^{-1}) and commercial tablet (5 μg.mL^{-1}) (A) 1st derivative, (B) 2nd derivative, (C) 3rd derivative and (D) 4th derivative	161
4-46	Derivative spectra of pure FURO (3, 5 and 8 μg.mL^{-1}) and commercial tablet (5 μg.mL^{-1}) (A) 1st derivative, (B) 2nd derivative, (C) 3rd derivative and (D) 4th derivative	161
4-47	Derivative spectra of pure CARB (3, 5 and 8 μg.mL^{-1}) and commercial tablet (5 μg.mL^{-1}) (A) 1st derivative, (B) 2nd derivative and (C) 3rd derivative	162
4-48	Derivative spectra of pure DIAZ (3, 5 and 8 μg.mL^{-1}) and commercial tablet (5 μg.mL^{-1}) (A) 1st derivative and (B) 2nd derivative	162
4-49	Box plot for the obtained recoveries for (A) FURO, (B) CARB, (C) DIAZ, and (D) CARV via the proposed methods	163

LIST OF SCHEMES

SR. NO.	TITLE	PAGE NO.
1-1	The structural formula of Diazepam	24
1-2	The structural formula of Carbamazepine	25
1-3	The structural formula of Carvedilol	26
1-4	The structural formula of Furosemide	27

LIST OF ABBREVIATIONS

ANN	Artificial Neural Network
API	Active Pharmaceutical Ingredient
B	Longitudinal Diffusion
C	mass transfer
C.V.	Coefficient of variance
C18	Octadecyl groups
C8	Octyl groups
CARB	Carbamazepine
CARV	Carvedilol
CCD	Central composite design
CLS	Classical Least Square
DAD	Diode array detector
DCF	Dichlorofluorescein
DIAZ	Diazepam
DOE	Design of Experiment
EGDMA	Ethylene glycol dimethacrylate
EP	Electrophoresis
ETFE	Ethylene tetrafluoroethylene
FI	Flow injection
FURO	Furosemide
GC	Gas chromatography
GC-MS	Gas chromatograph mass spectrometer system
h	Peak height
HETP	Height equivalent of a theoretical plate
HPAC	High-performance affinity chromatography
HPLC	High pressure liquid chromatography
HPTLC	High pressure thin layer chromatography
i.d.	Internal diameter
IUPAC	International Union of Pure and Applied Chemistry
ḱ	Capacity (retention) factor
LOD	Limit of detection
LOQ	Limit of quantitation
MAA	Methacrylic acid
MIP	Molecularly imprinted polymer
N	Theoretical plate number
NBS	N-bromosuccinimide
^{n}D	Derivative value

NIR	Near-infrared
NPC	Normal phase chromatography
ODS	Octyldecylsilane
P/ACE	Programmable, automated capillary electrophoresis system
PARAFAC	Parallel Factor analysis
PC	Paper chromatography
PCA	Principal Component Analysis
PEEK	Polyether ether ketone
PLS	Partial Least Square regression
Psi	Pounds per square inch
PTFE	Polytetrafluoroethylene
r	Correlation coefficient
R^2	Regression coefficient
RE	Relative error
RP-HILIC	Reversed phase hydrophilic interaction liquid chromatography
RP-HPLC	Reversed phase high pressure liquid chromatography
RP-IEC	Reversed phase ion exchange chromatography
Rs	Resolution
RSD	Relative standard deviation
SPE	Solid phase extraction
t_0	Dead time
$TBAClO_4$	Tetrabutylammonium perchlorate
TLC	Thin layer chromatography
TMS	Trimethylsilane
TPB	Tetraphenylborate
t_R	Retention time
u	linear velocity
UFLC	Ultra-fast liquid chromatography
VS	Versus
W	Peak width
WHO	World Health Organization's List of Essential Medicines
α	Selectivity (separation) factor

CHAPTER ONE

INTRODUCTION

Chapter One

1 Introduction

1-1 Chromatography

In 1903 a Russian botanist, Mikhail Tswett, separate a number of plant pigments through calcium carbonate packed in glass columns. The colored bands he produced on the adsorbent bed evoked the term chromatography to describe this type of separation "means color writing". The term chromatography has persisted and despite its irrelevance, the name is still used for all separation methods that employs the essential requirements for a chromatographic separation, viz. a mobile phase and a stationary phase [(1)].

1-1-1 Principles of chromatography

Chromatography as a term, it is difficult to define strictly, because the name has been applied to numerous systems and techniques. All of the chromatographic methods, however, have in common the use of a stationary phase and a mobile phase. Components of a mixture are transport by the flow of a gaseous or liquid mobile phase through the column (stationary phase), and separations are based upon the differences in migration rates among the mobile phase components. Consequently, the sample components elute from the column with different retention times[(2)].

In all chromatographic systems, the analysis starts when a small volume of sample is introduced at the inlet of a column or another carrier of the stationary phase. The mobile phase bearings the sample components moves in contact with the stationary phase throughout the column. Species in the sample undergo repeated interactions (partitioning) between the mobile phase and stationary phase. Due to this, the sample components migrate through the system at different speeds and elute from the column with different retention times. At the end of the column, a detecting agent provides a signal for all eluting components [(3)].

The unique character of the chromatographic method arises from the dual nature of its function. In a single step process, chromatography can separate a mixture into its individual components and simultaneously provide a quantitative assessment of each component. Samples may be gaseous, liquid or solid in nature and varies in their complexity from a simple mixture of two enantiomers to a multicomponent blend containing widely differing chemical species[4]. In addition, the analysis could be carried out, at one extreme, on a very expensive and complex instrument, and at the other, on a simple, economical thin layer plate. Consequently, chromatography is undoubtedly the most powerful and versatile technique available to the modern analyst [5].

1-1-2 Classification of chromatography [6, 7]

Chromatographic methods can be differentiated into various ways according to:

- ***The scale of operation*:** Chromatography could be classified to analytical and preparative. In the first type, the separated compounds are simply diverted to waste or destroyed by a destructive detection technique. On the other hand, the motivation key in the preparative type is the enrichment or purification and isolation of a target compounds from mixtures for further investigation or for commercial purposes according to the available information on the sample and purpose of analysis.
- ***The geometry of the separation region*:** which based on the physical means of bringing the stationary and mobile phases into contact, i. e. whether the separation process is conducted in a column or in the fibers of a paper or on a flat plate. Examples of column chromatography included simple column chromatography, high-pressure liquid chromatography (HPLC), and gas chromatography (GC). Planar chromatography includes paper chromatography (PC), thin layer chromatography (TLC) and electrophoresis (EP).

- ***The mobile phase*:** In chromatography, the mobile phase could be liquid, gas or a supercritical fluid. Consequently, the method is classified to a liquid, gas, and a supercritical fluid chromatography.
- ***The equilibria involved in solute transfer between the phases*:** According to this type of classification, chromatographic methods could be classified to partition, adsorption, ion exchange, affinity and size exclusion.
- ***The mode of operation*:** Until the late 1980s, chromatography was performed in a packed bed mode, whereas the 1990s saw the development of expanded bed adsorption chromatography wherein bed is allowed to expand to a desired height by pumping the mobile phase upward.

1-1-3 Theory of chromatography

Two theoretical approaches were developed to describe and to explain the process involved in the transport of solutes through the chromatographic system.

1-1-3-1 Plate theory [8]

Based on the work of Martin and Synge, the chromatographic column composed of a number (N) of narrow adjacent contiguous imaginary segments called theoretical plates (Figure 1-1). Between the plates, equilibration of analytes between the stationary and mobile phases occurs. Therefore, the more theoretical plates available within a column, the more equilibrations are possible, and the better quality the separation. According to plate theory, the number of theoretical plates, N, and the height equivalent of a theoretical plate, HETP, are related by the equation (**N = L / HETP)**, where L is the length of the column.

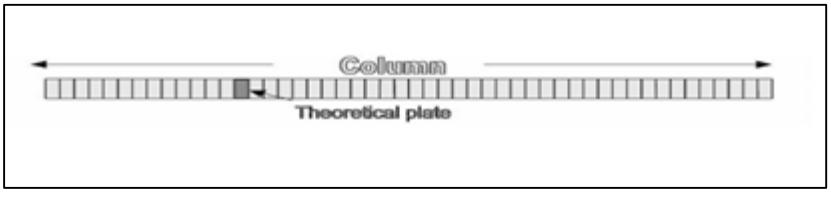


Figure 1-1: Theoretical plate.

1-1-3-2 Rate theory [9]

Rate theory, which was proposed by Van Deemter in 1956, describes the migration of solute particles throughout the column in quantitative terms. This includes band shape, broadening, and the diffusion of a solute. The rate theory is based upon the infinite number of paths that the sample may take in order to elute out of the column. Some solute molecules will transport quickly through the column due to their accidentally inclusion in the mobile phase while other molecules will strictly lag behind because of their accidental inclusion in the stationary phase.

Rate theory follows the Van Deemter equation, which relates the variance per unit length of a chromatographic column to the linear velocity of the mobile phase by considering physical, kinetic, and thermodynamic properties of a separation.

$$H = A + B / u + C u$$

Where: A represent the Eddy-Diffusion, B is the Longitudinal Diffusion, C denote the mass transfer, and u is the linear velocity (Figure 1-2).

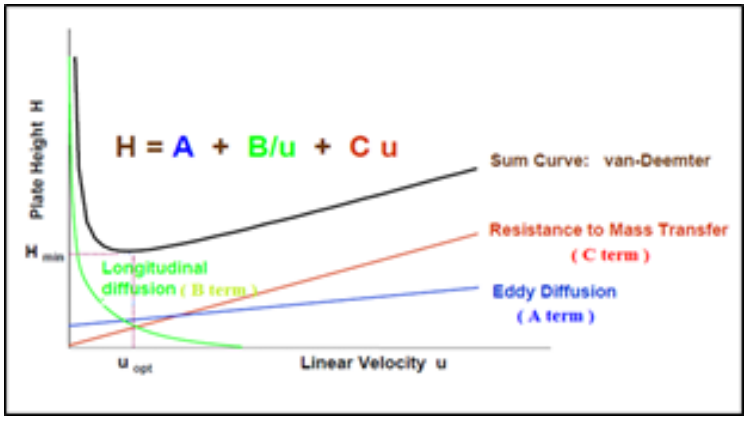


Figure 1-2: Van Deemter plot.

By using Van Deemter equation, it is possible to find the optimum velocity and a minimum plate height and, thence, the maximum efficiency for the column.

1-1-4 High performance liquid chromatography [10]

Until late 1950, liquid chromatography lacked the characteristics of gas chromatography and was being done on columns, papers and thin layer plates.

Modern liquid chromatography got a place in the early 1960's by a combination of the experiences gained with gas chromatography and ordinary column chromatography.

The modern form of liquid chromatography is now referred to as high performance liquid chromatography (HPLC) (formerly referred to as high-pressure liquid chromatography). By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, quantification and purification far above the previous techniques. Computers and automation added to the convenience of HPLC as well as developments in type of columns and thus reproducibility were made as such terms as fast HPLC, micro-column and affinity columns began to emerge.

1-1-5 Theory of HPLC

HPLC is an advanced form of liquid chromatography used to separate, identify, and quantify components that are present in any sample that can be dissolved in a liquid such as pharmaceuticals, forensic samples, food, cosmetics, environmental matrices, nutraceuticals and industrial chemicals[5]. The amount of resolution is dependent upon the interaction between the sample components and the column stationary phase and liquid mobile phase. This interaction can be manipulated through different choices of both mobile (solvent) and stationary phases [11].

1-1-6 Modes of HPLC

A useful classification of the various HPLC techniques is based upon the type of equilibrium that is responsible for the separation processes that occur at the columns. The most known modes of HPLC are adsorption chromatography, ion exchange chromatography, size exclusion chromatography and affinity chromatography[12]. In practice, most HPLC separations are the result of mixed mechanisms therefore, mixed mode phases such as reversed phase ion

exchange chromatography (RP-IEC) and reversed phase hydrophilic interaction liquid chromatography (RP-HILIC) have been developed[13]. A summary of these types is as below:

1-1-6-1 Adsorption chromatography

The principle of adsorption chromatography (Figure 1-3) is known from classical column chromatography and TLC. The main mechanism of adsorption chromatography is based on displacement, i. e. the competition of solute and solvent molecules for adsorbent active sites[14].

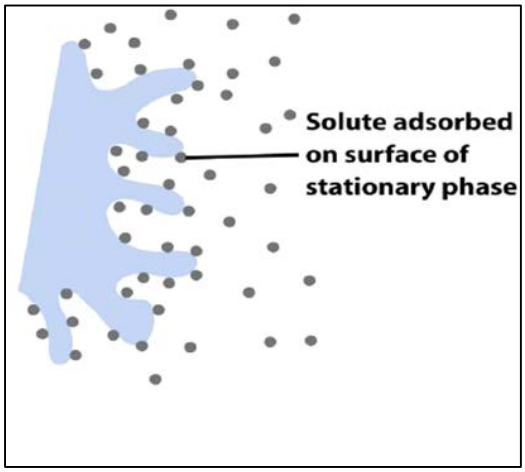


Figure 1-3: Adsorption Chromatography.

1-1-6-2 Ion exchange chromatography

In this type of chromatography, the stationary phase consists of very small polymer resin “beads” which are chemically designed to covalently bind charged ion-exchange groups on the surface of the inert support. The stationary bed has an ionically charged surface of opposite charge to the sample ions. Ion exchange chromatography (Figure 1-4) separates molecules according to the different degrees of sample ions interaction with the exchanger[15].

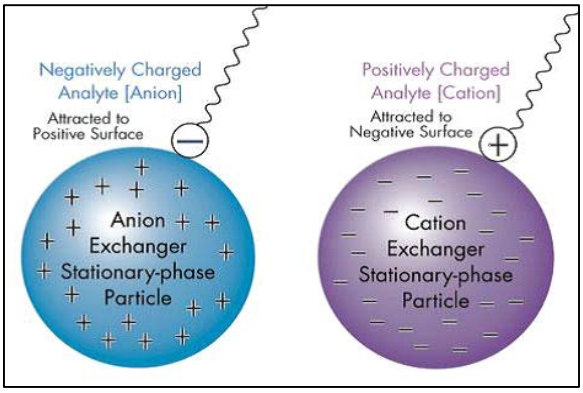


Figure 1-4: Ion-exchange Chromatography.

1-1-6-3 Size Exclusion Chromatography:

In size exclusion chromatography (Figure 1-5), the components of a mixture are separated according to their ability to penetrate into the pores of the stationary phase packing material. The column is packed with material having accurately controlled pore sizes, and the sample is simply filtered or screened according to its solvated molecular size materials[16].

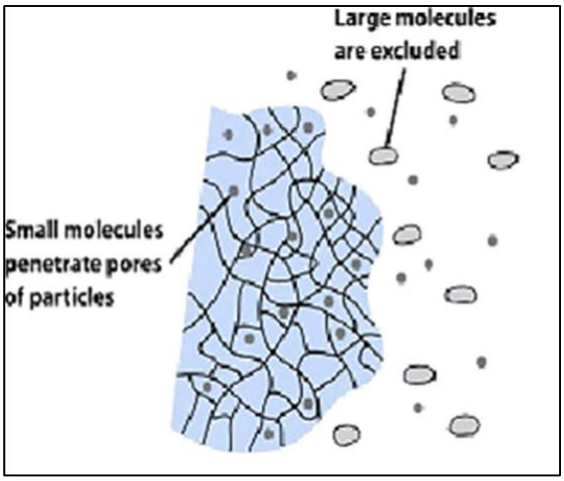


Figure 1-5: Size Exclusion Chromatography.

1-1-6-4 Affinity chromatography

Affinity chromatography (Figure 1-6) is a type of liquid chromatography based on selective non-covalent interaction between an analyte and specific group of molecules immobilized on the stationary phase. This technique is the

most selective type of chromatography employed and is often used in the purification of proteins bound to tags. These fusion proteins are labeled with compounds such as His-tags, biotin or antigens, which bind to the stationary phase specifically[17]. High-performance affinity chromatography (HPAC) is characterized by a support that consists of small, rigid particles that are capable of withstanding high flow rates and/or pressures[18].

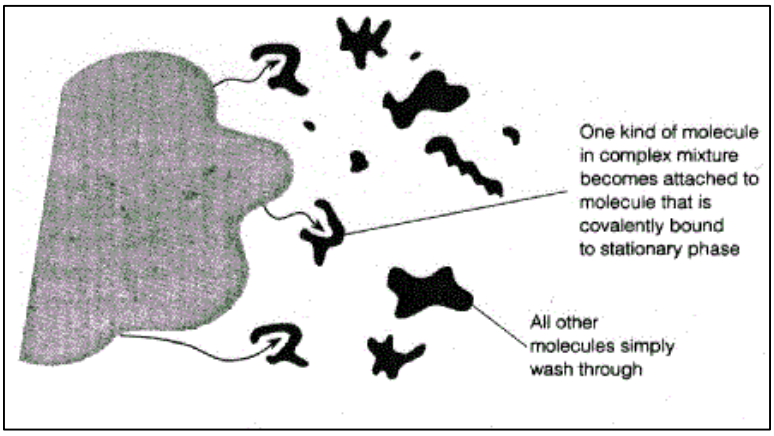


Figure 1-6: Affinity Chromatography

Furthermore, HPLC could be divided into two broad categories depending on the relative polarity of the stationary and mobile phases that are normal phase and reversed phase.

1-1-6-5 Normal phase [11, 19]

The first mode of HPLC to be developed was normal phase chromatography (NPC). Normal-phase HPLC explores the differences in the strength of the polar interactions of the analytes in the mixture with the stationary phase. This mode incorporates a polar stationary phase and non-polar non-aqueous solvents as the mobile phase. A relatively polar material with a high specific surface area is used as the stationary phase, silica being the most popular, but alumina and magnesium oxide are also often used. The retention mechanism is viewed as the competition of analyte molecules with

mobile phase molecules that cover the surface. In NPC, polar analytes are eluted later than non-polar analytes as a result for dipole-dipole interactions. Therefore, normal phase mode of separation is generally not used for pharmaceutical applications due to the polarity of most drug molecules and hence take longer time to elute.

1-1-6-6 Reversed phase

Reversed phase HPLC (RP-HPLC) is now the most widely used in analytical techniques in the European pharmacopoeia (Pharm. Eur.). RP-HPLC is applied for identification; test the purity of bulk drugs and for quantitative analysis of the main compounds in the samples and their related impurities[20]. Reversed-phase chromatography employs mainly dispersive forces (nonspecific hydrophobic or van der Waals interactions). The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP-HPLC is hydrophobic and mobile phase is polar, where mainly water-based solutions are employed. Almost 90% of all analyses of low molecular-weight samples are carried out using RP HPLC. One of the main drivers for its enormous popularity is the ability to discriminate very closely related compounds and the ease of variation of retention and selectivity[21].

The majority of packing materials used in RP-HPLC are chemically modified porous silicas. Silica particles composed of silicon atoms joined by siloxane bonds (≡ Si – O – Si ≡). Bonded silica phases (Figure 1-7) are formed by covalently attaching the desired organic moiety (e.g. C8, C18) with silanol groups on the silica surface [19].

Solute retention in reversed phase liquid chromatography could progress either by partitioning between the hydrocarbonaceous surface layer of the nonpolar stationary phase and the mobile phase or by adsorption of the solute to the nonpolar portion of the stationary phase[22].

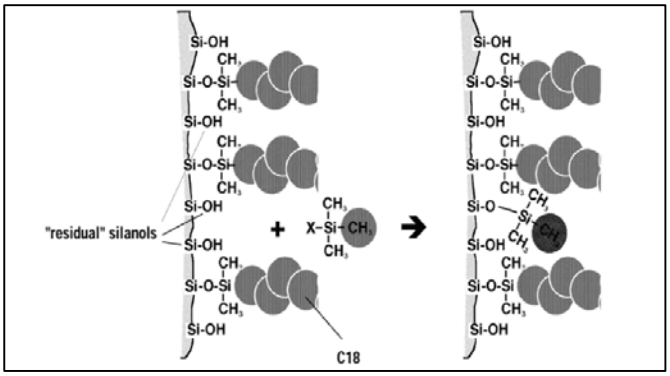


Figure 1-7: Bonded silica (C18) illustrating residual silanol groups and Trimethylsilane (TMS) end-capped silanol group.

1-1-7 HPLC Instrumentation

An illustration of the major components of HPLC system is given in Figure 1-8. The essential features of this assembly are the solvent delivery system (including pump), sample injection system, chromatographic column, detector and data handling system.

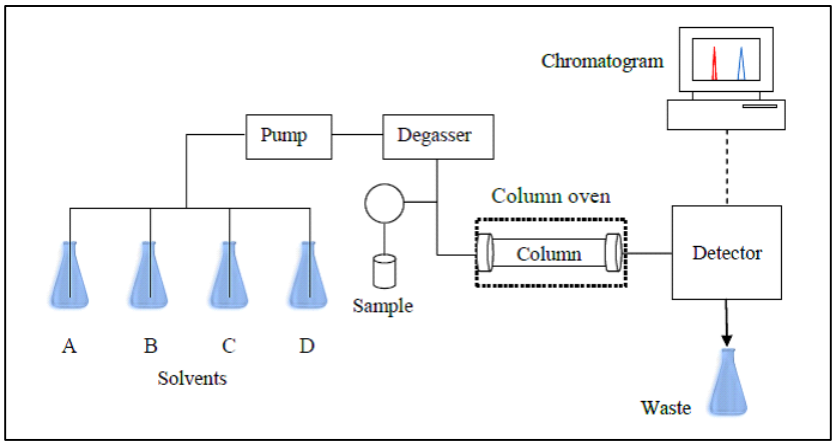


Figure 1-8: A schematic diagram of typical HPLC system

1-1-7-1 Solvent Delivery System

Solvent delivery system consist of:

Mobile phase reservoir:

The mobile phase reservoir could be any clean, inert, glass container with inert lines leading to the pump, typically PTFE tubing. The reservoir also serves to reduce solvent evaporation, allow for pressurization of the bottle, keep out

dust, offer ports for additional inlet lines, and sparging. Each solvent line typically fitted with an inlet filtering device which represents the first line of system defense against particulate contamination from solvents[23].

Offline degassing and online purging:

Offline degassing of the mobile phase could be achieved by agitating the mobile phase in an ultrasonic bath or by bubbling with He or Ar gases for a while of time. The mobile phases are purged before being fed to the pumping system to remove small gas bubbles present in the feeding tubes. Dissolved gases and the presence of small gas bubbles could cause irregular pumping action which fluctuates detector signals [24].

Pump:

Modern liquid chromatography pumps need to operate reliably and precisely at pressures of 10,000 Psi or at least 6,000 Psi. The main purpose of HPLC pump is to pass a constant, pulse-free flow of mobile phase/solvent through the chromatographic column over a wide range of different flows (0.0-10.0 $mL.min^{-1}$ for analytical proposes HPLC and in excess of 100 $mL.min^{-1}$ for preparative HPLC) [25].

Three main types of pumps available for use with HPLC, including reciprocating pumps, displacement type pumps, and constant pressure pumps. Computer or microprocessor-controlled pumping systems are capable of accurately propelling a mobile phase of either constant (isocratic elution) or varying (gradient elution) composition. In the first mode, a mobile phase (eluent) consisting of a single solvent or solvent mixture remains the same throughout the run, While in gradient mode of elution the mobile phase composition (and strength) is changed during the separation in a programmed way and according to a defined program[26].

Online degasser:

Online degassing is the most common technique, since it accomplishes both the filtration and degassing processes at the same time and this process done by passing the mobile phase through a microporous filter under vacuum[24].

1-1-7-2 Sample Injection System [27]

The sample insertion onto the pressurized column must be as a very sharp plug in order to neglect the peak broadening attributable to this step. The role of the injector is to add in the eluent stream a small and precisely measured volume of a solution containing the analyte. Injection reproducibility is of particular prominence, and modern injectors typically show RSD % value less than 0.5 % in the injected volume. Conventional HPLC system have injectors capable to inject sample solution between 1µL to 100 µL and in special system up to 1000 µL.

Introducing the sample into injection port could be done mainly in three ways: injection valve, loop injection, and on column injection. Modern automation injector models, the more common used, can be controlled electronically and have the possibility of injecting any specified sample volume (within a range).

1-1-7-3 Chromatographic Column

The HPLC column is usually made up of stainless steel or plastic (e.g. PEEK) tubing to withstand high pressure. The physical dimensions of common analytical columns vary, values of internal length (L) can be between 30-250 mm, and inside diameter (i.d.) can be between 1-10 mm. It holds the fine, solid particles with special properties to serve as a stationary phase that provides differential retention of sample components. The

packing characteristic such as pore size and bonding chemistry is crucial in determining the performance and resolution of the whole HPLC system [28].

Silica gel with chemically bonded phases is the packing of choice because of rigidity, excellent efficiency, and ability to be functionalized. The most popular bonded phases are alkyl groups. C18 also referred to as octyldecyl silane (ODS) is the most popular. Octyl (C8) groups are the next most popular bonded phase after C18[29].

1-1-7-4 HPLC Detectors

Separations in HPLC occur in a dynamic manner and thus an online detection system is required to produce an instantaneous record of the column events. HPLC detectors are characterized by several specifications: linearity, sensitivity, dynamic range, response, flow sensitivity, noise level, and operating temperature range [1].

There are various types of detectors available; among them are UV-VIS (e.g. fixed wavelength, variable wavelength and diode array), refractive index, fluorescence, electrochemical, evaporative light scattering, conductivity, mass and IR detectors. The UV-VIS spectrophotometric detector is regarded as the most popular one for drug analysis[30].

1-1-7-5 Data Handling Device

Data handling device is a computer-based has a large storage capacity, which helps controls all the modules of the HPLC instrument, takes the signals from the detector and collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data[29].

1-2 Ultraviolet-Visible absorption spectroscopy

Ultraviolet-visible radiation represents only a small portion of the electromagnetic spectrum (between 190 nm to about 800 nm), and have a

sufficient energy to excite valance electrons in atoms, organic molecules and polyatomic ions from their ground state to an excited state. By measuring the variation in light intensity over the entire wavelength range (or frequency), an absorption spectrum is obtained [31]. When a molecule or ion absorbed UV-Vis radiation, three types of electronic transition are possible: Transition involving σ, π and *n* electrons, Transition involving charge transfer electrons, and Transition involving *d* and *f* electrons [32].

UV-VIS spectrophotometry is one of the oldest and the most common simple, accurate, sensitive and rapid spectroscopic techniques in analytical chemistry. In 1852, the definitive formulation of the Bouguer-Lambert-Beer law, created the bases for the quantitative evaluation of absorbance measurement. According to this mathematical law, the absorbance (A) is proportional to the absorption path length and to the amount of the absorbing species in the sample [33]. Therefore, it is one of the most frequently employed technique in routine pharmaceutical analysis [34].

Drug analysis using spectrophotometer may carried out by using of standard absorptivity value, calibration graph and single or double point standardization. For assay of substance/s in multi component mixture by UV-Vis spectrophotometer, the following techniques are being used routinely[35]: dual wavelength method, simultaneous equation (or Vierdott's) method, derivative and ratio derivative methods, absorbance ratio (or q-absorbance) method, h-point standard addition method, and chemometrics methods

Among computer-controlled instrumentation, derivative techniques and multivariate calibration methods are playing a very important role in the multicomponent analysis of mixtures by UV-Visible spectrophotometry [36].

1-2-1 Derivative spectrophotometry

1-2-1-1 Introduction

The basic principles of derivative spectrophotometry were first published in the literature in 1956, when Singleton and Collier published results of taking time derivatives of the output of scanning infrared spectroscopy [37]. Since that time this technique has continued to progress especially with the enormous development that have taken place in spectroscopic instruments, microelectronics, and computers which leads to increasing and revolutionizing the scope for performing derivative spectroscopy [38]. Derivative spectra can be obtained by using electronic method (analog differentiation), optical method (wavelength modulation) and it can be easily obtained by using mathematical techniques. An advantage of the mathematical method is that it can be used to recalculate the derivative spectra with different parameters, and it can be used to smooth the data [39].

Derivative spectroscopy as an analytical technique based on differentiation of the original, zero-order spectrum. The result of derivatization is called the derivative spectrum, which represent the values of absorbance differentials as a function of wavelength (λ), and it can be expressed as:

$$d^nA/d\lambda^n = {}^nD_{x,\lambda} = f(\lambda)$$

Where n denotes the derivative order, ${}^nD_{x,\lambda}$ represents the value of n-order derivative (i.e. the derivative amplitude) of the absorption spectrum of the analyte (x) at the given wavelength (λ), A-absorbance [40].

Derivative Spectra often yield a characteristic profile where refined changes of curvature and gradient in the zero order spectrum are observed as distinctive bipolar functions. The first derivative represent the gradient at all points of the spectrum and can be used to detect hidden peaks, since $dA/d\lambda$ is equal to zero at peak maxima. The distinctive feature of the second derivative

spectrum (as well as all even-order derivatives) is a negative peak with minimum at the λ_{max} of the normal spectrum (Figure 1-9). The derivative spectra are more complicated than the original spectra, and the generation of n^{th} derivative spectrum will produces (n+1) new signals with an intense main signal and weaker peaks, so called satellite signals[37].

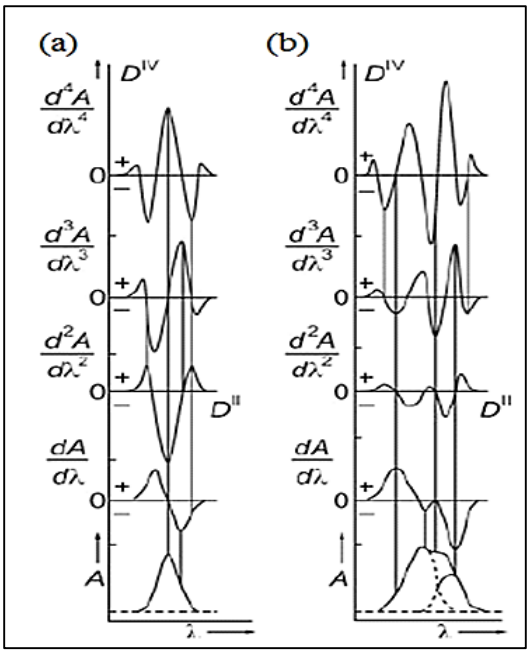


Figure 1-9: Differentiation of computed analytical Gaussian curves: **(a)** the initial curve and its first- to fourth- order derivatives; **(b)** the initial curve of two superposed Gaussian curves and their first- to fourth- order derivatives.

The most important properties of derivative spectrophotometry that, it holds all laws of classical spectrophotometry, e.g. Beer-Lambert law and additivity law. The Beer-Lambert law, in its differential form, expressed as:

$$^{n}D_{x,\lambda} = d^{n}A/d\lambda^{n} = d^{n}\varepsilon/d\lambda^{n}.l.c$$

Where **ε** denotes the molar absorption coefficient (L. mol^{-1}. cm^{-1}), **l** thickness of analyte solution layer (cm), **c** concentration of analyte ($mol.L^{-1}$).

As the additivity law is kept, the derivative spectrum of n-component mixture represents the sum of derivative spectra of individual components:

$$^{n}D_{mix} = {}^{n}D_1 + {}^{n}D_2 + {}^{n}D_3 + \ldots\ldots + {}^{n}D_n$$

An additional property of derivative spectrophotometry is the dependence of derivatization results on the shape (i.e. geometrical characteristic) of zero-order spectra. Due to this property, signals of analyte, which are in zero-order spectra narrow, undergo amplification while the broad signals are quenched with generation of higher orders of derivatives. This property allows reducing or eliminating the effect of background and provide higher sensitivity and/ or selectivity of determination [38].

Derivative spectrophotometry becomes a widely used technique, particularly in UV-Vis absorption [36], infrared spectrophotometry [41] and it was also employed in atomic absorption [42], flame emission spectrophotometry [43] and in capillary electrophoresis [44].

The benefits of this technique include the following:

- Spectral discrimination: this arises from the fact that the derivative value (^{n}D) of a Gaussian band in the n-order derivative is inversely proportional to the n^{th} power of the original peak width (W): $^{n}D = 1/W^{n}$.
- Enhancement of resolution of overlapping spectral peaks.
- Quantification: due to the linear relationship between the amplitude of the derivative (^{n}D) and the concentration levels, the assay of certain analytes from complex mixtures is easily performed via mathematical interpretation of the absorbance signal [38].

The main disadvantage of this technique is the dependence of the recorded signal upon the instrument parameters like scanning rate, distance between measurement points, and degree of amplification and integration time. Reproducible results are obtained on applying identical conditions of spectra recording and using one- and the same- type of spectrophotometer, or by adaptation of a definite method to the available apparatus [45].

1-2-1-2Analytical applications of UV-Vis derivative spectrophotometry

The applications of UV-Vis derivative spectrophotometry have been gathered and reviewed in previously published literatures [46]. Based on these reviews the following areas of analysis can be distinguished: pharmaceutical analysis, inorganic analysis, determination of organic compounds, food, cosmetics colorants and dyes analysis, environmental analysis, and clinical and biological analysis.

Applications in Pharmaceutical Analysis

Pharmaceutical analysis is an important part in drug development by monitoring the assurance of the quality, efficacy and safety of pharmaceutical products. The progress in electronic differentiation techniques have simplified the application of derivative methods to pharmaceutical analysis [47]. Table 1-1 summarized some of the applications of derivative methods in drug analysis.

Table 1-1: Application of derivative spectrophotometry in pharmaceutical analysis.

Pharmaceutical compounds	λ max (nm)	Derivative order and application remark	Ref.
Paracetamol, Chlorpheniramine maleate	257.4,232.0& 215.9,225.3	**1st & 2nd**, Flu-out® tablets.	48
17-Beta-Estradiol, Drospirenone	208.0, 282.0	**1st**, Angeliq tablet	49
Drotaverine hydrochloride, Mefenamic acid	253.8, 304.0	**1st**, combination tablet formulations (Doverin-M and Drofem)	50
Gatifloxacin, Prednisolone Acetate	286.0, 243.0	**2nd**, Gatsun P eye drop	51
Tiemonium methylsulphate	295.6	**2nd**, Viscéralgine tablets	52
Atorvastatin Calcium	236.0	**1st**, bulk drug and tablet dosage form	53
Naphazoline Hydrochloride, Methylene blue	299.0, 337.0	**2nd**, Prisoline Blue® eye drop	54
Acyclovir	295.2	**1st**, in poly (n-butylcyanoacrylate) (PBCA3) nanoparticles	55

1-2-2 Chemometrics

In 1974, Svante Wold, wrote, "Chemometrics is the art of extracting chemically relevant information from data produced in chemical experiments". Chemometrics has given this name in analogy with econometrics, biometrics, and technometrics [56]. Chemometrics is a science of multidisciplinary, which involves information technology, mathematical modeling and multivariate statistics, specifically applied to chemical data [57].

Rapid technological developments, especially in the area of computerized instruments have enabled and necessitated phenomenal growth in the field of chemometrics in several areas such as process monitoring, biology, food analysis, environmental monitoring and analytical chemistry [58].

The most common chemometrics methods implement on: Classical Least Square (CLS), Principal Component Analysis (PCA), Design of Experiments (DOE), Parallel Factor analysis (PARAFAC), Partial Least Square regression (PLS), and Artificial Neural Network (ANN).[59]

1-2-2-1 Partial Least Square (PLS) method [60-62]

PLS regression is a powerful multivariate statistical tool that refers to a set of repetitious algorithms based on least squares that apply a broad spectrum of both explanatory and exploratory multivariate techniques. The objective of PLS is that it modifies relations between sets of the observed variables (i. e. independent variable (X matrix) and dependent variable (Y matrix)) through the extraction of a small number of latent variables. In PLS the variance in a data matrix X (its dimension n × m) and a dependent matrix Y (its dimension n × p) is decomposed into a new latent variables by successively estimating PLS components that capture the variance and correlation between X and Y matrices. The matrices in PLS model are shown schematically in Figure (1-10).

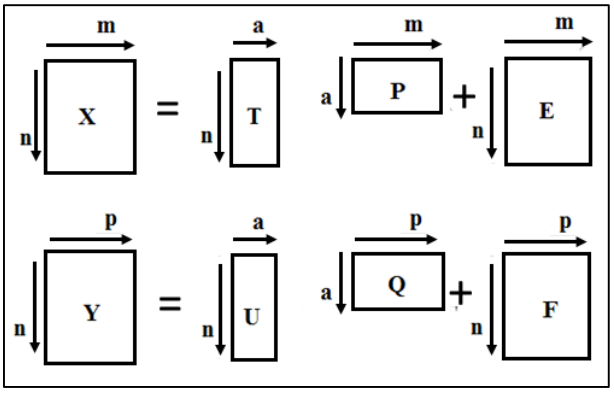


Figure 1-10: Schematic description of matrix decomposition of PLS data.

The PLS regression can be considered as involving of outer relations, for X and Y matrices individually, (Equations (**1**) and (**2**) respectively) and an inner relation links both matrices together (Equation (**3**)).

$$\mathbf{X = TP' + E} \quad \ldots\mathbf{1}$$

$$\mathbf{Y = UQ' + F} \quad \ldots\mathbf{2}$$

$$\mathbf{U = BT + H} \quad \ldots\mathbf{3}$$

Where **T** and **U** are score matrices, **P′** and **Q′** are loading matrices, **E** and **F** are matrices residual of **X** and **Y** block respectively, **B** is an identity matrix and **H** is a residual matrix.

In analytical chemistry, where PLS is mainly used in multivariate calibration, **X** matrix contains the digitized spectral data (i.e. absorbance matrix **A**) while **Y** matrix contains the analyte concentration (i.e. concentration matrix **C**). Calibration with using PLS model is done by decomposition of both the concentration matrix **C** and absorbance matrix **A** of the mixture, standard solutions are estimated simultaneously in calibration set into latent variables, as follows:

$$\mathbf{C = F_c L_c + E_c} \quad \ldots\mathbf{4}$$

$$\mathbf{A = F_A L_A + E_A} \quad \ldots\mathbf{5}$$

where $\mathbf{F_c}$ represent the latent concentration matrix, $\mathbf{L_c}$ is the concentration-loading matrix, $\mathbf{F_A}$ denote the latent absorbance matrix, $\mathbf{L_A}$ is the absorbance

loading matrix, and $\mathbf{E_c}$ and $\mathbf{E_A}$ are error matrices that have the same dimensions as the original concentration and absorbance matrices, respectively. Relating the latent variable matrix from Equation (**4**) to that in Equation **(5),** one obtains a diagonal regression matrix **V**:

$$\mathbf{F_c = F_A\, V + E_d} \quad \ldots\mathbf{6}$$

where $\mathbf{E_d}$ is an error matrix.

The unknown concentration of the sample could be estimated from the absorbance spectrum $\mathbf{a_0}$ by using the matrix **V**, as follows:

$$\mathbf{c_0 = a_0\, (F^t c\, A)^t\, V\, L_c} \quad \ldots\mathbf{7}$$

Here the matrices **Fc** and **Lc** and **A** are known from calibration, **t** represent the transpose of the matrix.

PLS as a full- spectrum multivariate calibration method, its only need to know the concentration of the interest analyte in the calibration samples and it is unnecessary to know the concentration of other analytes. An important feature of PLS is that it uses both spectral and concentration data, that means it takes into account errors in both the concentration estimates and the spectra. Therefore, the directly related decompositions of the two matrices (concentration and absorbance) create an algorithm that is robust to calibration changes [(63)].

Chemometrics models, particularly PLS, have been rapidly progressed with the evolution of computerized digital instruments that enable obtaining large amounts of data. Consequently, PLS in combination with various techniques, has found wide scope application in various fields such as medicine [(64)], geology [(65)], water pollution [(66)], agricultural [(67)] and food science [(57)].

PLS is a useful tool in spectral analysis of multicomponent mixtures because of the simultaneous inclusion of multiple spectral intensities instead of single intensity, which can greatly improve the precision, and applicability of quantitative analysis. Therefore, PLS has been widely used in the quantitative application of multivariate chemical data, owing to the availability of the digitized spectroscopic data and commercial software for laboratory computers [(68)].

The principles of chemometrics methods however extend beyond their application to spectroscopy; virtually every branch of analytical chemistry has been affected significantly by chemometrics. Computerization of analytical instrumentation provides an opportunity to acquire enormous amounts of data on chemical systems due to the commercial software that implements chemometrics methods has become commonplace in analytical instruments. So in the field of analytical chemistry, various chemometrics techniques, particularly PLS, could be used to extract valuable quantitative information by circumventing the difficult mathematics, by focusing on data and by arguing practicalities rather than dwelling on theory [65, 69].

1-2-2-2 Pharmaceutical application of PLS regression method

Recently, an increasing attention has been paid to several multivariate calibration methods, especially to those using the PLS method in the biomedical and pharmaceutical analysis [70]. Table (1-2) illustrate some of the different application of PLS technique in pharmaceutical analysis by using various analytical instruments.

Table 1-2: Different applications of PLS technique in pharmaceutical analysis.

Pharmaceutical compound/s	Analytical technique	Application remark	Ref.
Paracetamol and Ibuprofen	Spectrofluorometry	Marketed tablets and urine samples	60
Esomeprazole magnesium trihydrate and Naproxen	HPLC-DAD	Vimovo® tablet and synthetic mixtures	71
Isoniazid and Rifampicin	Differential Pulse Polarography	Synthetic mixtures	72
Ciprofloxacin, Diclofenac and Tetracycline	UV-Vis spectrophotometer	Synthetic mixture and chitosan dental films	73
Amino acids	P/ACE system/ DAD	Synthetic mixtures	74
Metoprolol tartrate	NIR spectroscopy	API samples	75
Levodopa and Carbidopa	Potentiometer	Pharmaceutical formulations	76

1-3 The Drugs

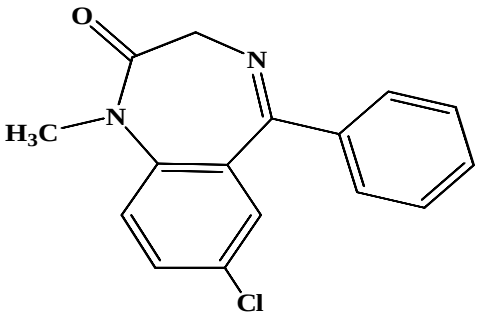
The objects of analysis were Diazepam (DIAZ), Carbamazepine (CARB**)**, Carvedilol (CARV), and Furosemide (FURO). These medications are belonging to four different therapeutic groups with some specific similarities in their medical uses. DIAZ, which is a type of Benzodiazepine, and CARB both are considered as anticonvulsant drugs, while CARV and FURO both act as antihypertensive agents, although DIAZ may be used in certain circumstances for same reason.

1-3-1 Diazepam

DIAZ is a medication of benzodiazepine derivatives, belongs to a group of psychoactive drugs used to treat a range of medical conditions like anxiety, which is the main indication for its use, also for acute alcohol withdrawal and status epilepticus and other convulsive states [(77)].

Since its synthesis by Leo Sternbach, being the second benzodiazepine following chlordiazepoxide (Librium), and manufactured by Hoffmann-La Rache, it has been and still the most frequently prescribed medication in the world. DIAZ is on the World Health Organization's List of Essential Medicines (WHO), the most important medications needed in a basic health system [(78)].

The structural formula of DIAZ is shown in Scheme (1-1) and the general properties are listed below in Table (1-3) [(79)].



Scheme (1-1): The structural formula of Diazepam.

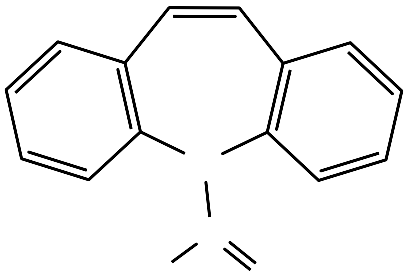
Table 1-3: General properties of Diazepam

Systematic IUPAC name	7-chloro-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one
Trade name	Valium
Formula	$C_{16}H_{13}ClN_2O$
Molecular mass	284.7 $g.mol^{-1}$
Properties	A white or almost white, crystalline powder, very slightly soluble in water, soluble in ethanol.
Preparations	Tablets, injection, oral solution, rectal solution
Storage	It should be stored in well-closed airtight containers and protected from light.
Metabolism	Hepatic
Therapeutic	Sedative, anticonvulsant

1-3-2 Carbamazepine

CARB is a medication primarily used in treatment of epilepsy and neuropathic pain. It is a lipophilic tricyclic compound used as a first choice antiepileptic drug, and in the management of simple and complex seizures. CARB was discovered in 1953 by a Swiss Chemist Walter Schindler and marketed as a drug to treat epilepsy under its brand name Tegretol [(80)]. According to WHO Model List of Essential Medicines, CARB considered one of the most important medication needed in a basic health system [(78)].

Scheme (1-2) illustrate the structural formula of Carbamazepine and the general properties are listed below in Table (1-14) [(79)].



Scheme (1-2): The structural formula of Carbamazepine.

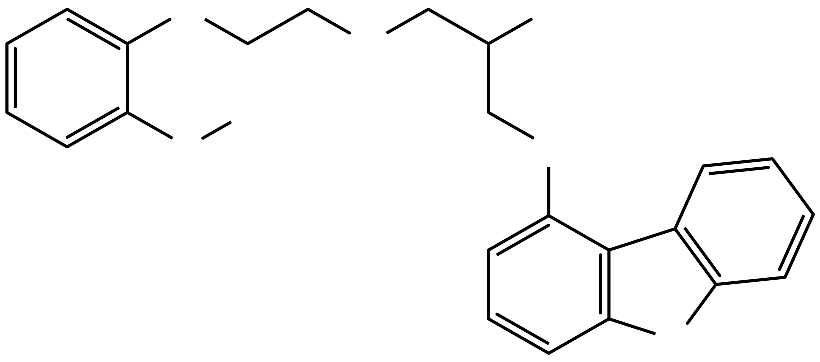
Table 1-4: General properties of Carbamazepine

Systematic IUPAC name	5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide
Trade name	Tegretol, Epitol, Carbatrol and Equetro
Formula	$C_{15}H_{12}N_2O$
Molecular mass	236.3 $g.mol^{-1}$
Properties	A white or yellowish – white crustal, very slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in acetone and in ethanol.
Preparations	Tablets, oral suspension
Storage	It should be stored in well-closed airtight containers and protected from light.
Metabolism	Hepatic
Therapeutic	Anticonvulsant, antiepileptic, antimanic

1-3-3 Carvedilol

It is a nonselective beta-blocker/ alpha-blocker antihypertensive agent, widely used in the treatment of hypertension, congestive heart failure, cardiac arrhythmia, and angina pectoris. It can be prescribed alone or together with other antihypertensive or with diuretic [(81)].

The structural formula of CARV is revealed in Scheme (1-3) and the general properties are reported below in Table (1-5) [(79)].



Scheme (1-3): The structural formula of Carvedilol.

Table 1-5: General properties of Carvedilol

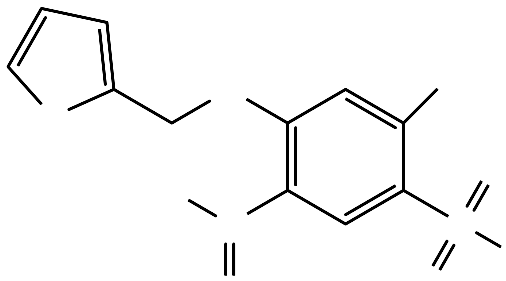
Systematic IUPAC name	(2RS)-1-(9*H*-Carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy) ethyl] amino] propan-2-ol.
Trade name	Coreg, Carvil, Dilatrend, Carloc
Formula	$C_{24}H_{26}N_2O_4$
Molecular mass	406.5 $g.mol^{-1}$
Properties	A white or yellowish – white crustal, very slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in acetone and in ethanol.
Preparations	Tablet
Storage	It should be stored in well-closed airtight containers and protected from light.
Metabolism	Hepatic
Therapeutic	Antihypertensive

1-3-4 Furosemide

FURO is a type of loop diuretics that get their name from loop shaped part of kidney where they have their effect. It is considered as a powerful diuretic and mainly used for the treatment of hypertension and edema. FURO is also used to treat fluid buildup caused by heart failure, liver cirrhosis, and chronic kidney failure [82].

According to the World Health Organization's List of Essential Medicines, it is one of the most important medications needed in the basic health system [78]. Furosemide was discovered in 1962, and it is on the World Anti-Doping Agency's banned drug list due to concerns that it may mask other drugs [83].

Scheme (1-4) depicted the structural formula of Furosemide and the general properties are listed below in Table (1-6) [79].



Scheme (1-4): The structural formula of Furosemide.

Table 1-6: General properties of Furosemide

Systematic IUPAC name	4-Chloro-2-[(furan-2-ylmethyl) amino]- 5-sulfamoylbenzoic acid
Trade name	Lasix
Formula	$C_{12}H_{11}ClN_2O_5S$
Molecular mass	330.7 $g.mol^{-1}$
Properties	A white or almost white crustal, practically insoluble in water, soluble in acetone, practically insoluble in methylene chloride, sparingly soluble in ethanol. It dissolves in dilute solution of alkali hydroxide.
Preparations	Tablets, injection
Storage	It should be stored in well-closed airtight containers and protected from light.
Metabolism	Hepatic and renal
Therapeutic	Diuretic

1-4 Methods for the determination of CARV, FURO, CARB and DIAZ

Review of literature revealed that a large number of analytical methods have been used to quantify CARV, FURO, CARB and DIAZ in their bulk and pharmaceutical formulations as well as in biological fluids.

1-4-1 Electroanalytical methods

The determination of the studied drugs by applying modern electroanalytical methods, namely: potentiometry, cyclic, linear sweep, differential pulse and adsorptive stripping voltammetric techniques, are presented in Table 1-7.

Table 1-7: Electroanalytical techniques reported for the determination of FURO, CARB, DIAZ and CARV.

Technique	Drug	Method	Conc. range	LOD	Application	Ref.
Potentiometry	DIAZ	The electrode active material: DIAZ-TPB ion pairs	$5x10^{-5}$–$1x10^{-2}$ $mol.L^{-1}$	$8x10^{-7}$ $mol.L^{-1}$	Pharmaceutical formulations	84
	FURO	Ion sensor immobilized in a graphite matrix Pt|Hg|$Hg_2(Fur)_2$ |Graphite	$5x10^{-7}$–$1x10^{-2}$ $mol.L^{-1}$	$8x10^{-7}$ $mol.L^{-1}$	Tablets, blood serum, urine and bovine milk	85
	CARB	Molecularly imprinted polymer (MIP) with methacrylic acid (MAA) as the functional monomer and ethylene glycol dimethacrylate (EGDMA) as the crosslinker	-----	-----	Aqueous solutions	86
	CARV	The electrode active material: CARV-phosphotungstate ion association complex	$3x10^{-7}$–$1x10^{-3}$ $mol.L^{-1}$	$1.5x10^{-7}$ $mol.L^{-1}$	Commercial tablets, urine and blood serum samples.	87
Cyclic and linear sweep voltammetry	CARV	At platinum electrode in acetonitrile solution containing 0.1 $mol.L^{-1}$ $TBAClO_4$	5–40 $\mu g.mL^{-1}$	1.32 $\mu g.mL^{-1}$	Pharmaceutical preparations	88
Differential pulse voltammetry (DPV)	CARB	At glassy carbon electrode	2–12 $\mu g.mL^{-1}$	-----	Human serum	89
Cyclic voltammetry	FURO	At modified glassy carbon electrode in phosphate buffer solution, pH=6.8	Peak I: 15–340 µM Peak II: 17–100µM	Peak I: 0.1 µM Peak II: 0.11 µM	Spiked human serum and urine fluids	90
Adsorptive stripping voltammetric	DIAZ	Using a disposable Screen-Printed Sensor	7.1–285 $mg.mL^{-1}$	1.8 $mg.mL^{-1}$	Beverages samples	91

1-4-2 Chromatographic methods

In the context of this review, elaborate discussion on HPLC method has been attempted rather than GC-MS and HPTLC (Table 1-8) thus HPLC method is individually reviewed.

Table 1-8: Chromatographic techniques reported for the determination of FURO, CARB, DIAZ and CARV.

Technique	Drug	Method	Conc. range	LOD	Application	Ref.
GC-MS	CARV	Extraction process have been done by using liquid–liquid extraction technique in presence of atenolol as internal standard	15–500 $ng.mL^{-1}$	5 $ng.mL^{-1}$	Human plasma	92
	CARB	Extraction process have been done by using solid phase extraction (SPE) technique	25–4000 $ng.g^{-1}$	12.27 $ng.g^{-1}$	Plant tissue (leafy lettuce)	93
	FURO	Using probenecid as a masking agent	-----	0.050 $\mu g \cdot mL^{-1}$	Human urine	94
	DIAZ	Solid-phase extraction (SPE) have been used for the preparation and purification of the samples	10–500 $ng.mL^{-1}$	2.0 $ng.mL^{-1}$	Pork samples	95
HPTLC	FURO	Stationary phase: silica gel GF254 precoated on aluminum sheets, mobile phase: ethyl acetate- hexane (80: 20, v/v), UV detection at 254nm	0.016-0.064 mg ml^{-1}	-----	Tablet formulation	96
	CARB	Stationary phase: silica gel 60 F254 TLC plate, mobile phase: ethyl acetate-toluene-methanol (5.0: 4.0: 1.0 v/v), the spots were analyzed at a wavelength of 285 nm	100–600 ng/spot	16.7 ng/spot	Bulk and commercial tablet (Mazetol 100, India)	97
	CARV	Stationary phase: aluminum cards (20x20 cm), 0.2 mm thickness, mobile phase: chloroform–methanol (8:2 v/v) with fluorescent indicator at 254 nm	0.05–1 µg/spot	0.01 µg/spot	Pharmaceutical tablets	98
	DIAZ	Stationary phase: silica gel 60 F254 precoated on aluminum plate, mobile phase: ethylacetate-methanol-toluene-triethylamine (1.0: 3.0: 6.0: 0.1, v/v), UV detection at 235 nm	25 – 250 ng/spot	0.23 ng/µL	Tablet formulation	99

1-4-3 Flow injection analysis

Applications of flow injection analysis for the determination of CARB, FURO, CARV and DIAZ in bulk and pharmaceutical formulations are reported in Table 1-9.

Table 1-9: Flow injection analysis techniques reported for the determination of FURO, CARB, DIAZ and CARV.

Drug	Method	Conc. Range	LOD	Application	Ref.
CARB	Based on the CARB enhancement for the weak chemiluminescence reaction of Ce (IV) and Na_2SO_3 in an acidic medium	$4.2x10^{-11}$ – 4.2×10^{-9} $mol.L^{-1}$	2.5×10^{-11} $mol.L^{-1}$	Pharmaceutical preparations	100
CARV	based on CARV inhibition for the chemiluminescence of luminol H_2O_2 system catalyzed by ZnO nanoparticles (ZnO-NPs)	$5.0x10^{-8}$ – $1.0x10^{-6}$ $mol.L^{-1}$	$3.25x10^{-9}$ $mol.L^{-1}$	Bulk and pharmaceutical tablets	101
DIAZ	Based on the reaction of diazepam with N-bromosuccinimide (NBS) in alkaline medium in the presence of dichlorofluorescein (DCF) as an effective energy-transfer agent	2.0×10^{-6} – 2.0×10^{-4} $mol.L^{-1}$	5.0×10^{-7} $mol.L^{-1}$	Pharmaceutical formulations, plasma and urine samples	102
FURO	The carrier solution was 10^{-2} $mol.L^{-1}$ ethanolic Fe (III). The best parameters for FI analysis; flow-rate (1.0 $mL.min^{-1}$), injection volume (250 µL) and detection wavelength of 513 nm	1.0×10^{-4} – 1.0×10^{-2} $mol.L^{-1}$	3.0×10^{-5} $mol.L^{-1}$	Four brands of FUR tablets and ampules, synthetic urine sample spiked with FUR	103

1-4-4 Spectrophotometric methods

Harshad and Sugandha [104] developed an accurate, reproducible, precise and economical UV spectrophotometric determination of carbamazepine in methanol as a solvent. The proposed method based on calculating the area under curve of UV spectrum between 275 to 295 nm. The calibration curve was found to be linear in the range of 2-12 $\mu g.ml^{-1}$ (r=0.999) with detection and quantification limits of 0.2984 $\mu g.ml^{-1}$ and 0.9042 $\mu g.ml^{-1}$, respectively. Results of the recovery studies (99.67% to 101.18%) showed accuracy of the

method. The proposed method is suitable in practical routine estimation of carbamazepine in bulk and tablet dosage forms.

Jayanna et. al. [105] described a simple spectrophotometric method for the determination of carbamazepine in pharmaceutical formulation. Carbamazepine, in the proposed method, is react with nitrite under acidic condition to form a greenish yellow nitrosamine product. Under the optimum conditions, the absorbance was measured at 417 nm and obeys Beer's law over concentration ranging between 0.2 and 10 $\mu g.mL^{-1}$. The proposed method was successfully applied to assay the drug in tablet formulation and the results was compared statistically with those obtained with reference methods.

Borse and Mulgund[106] proposed a simple, fast, precise, and economical method spectrophotometric estimation of carbamazepine (CBZ) in 60 % v/v aqueous methanol as a solvent. The method employs quantitative determination of CBZ at 284 nm by regression equation obtained from calibration curve (Abs = 0.049 [CBZ] + 0.0098) with correlation coefficient value of 0.999. The method obeys Beer's law over the concentration range of 8-18 $\mu g.ml^{-1}$. The proposed method was applied to the determination of CBZ in tablet formulation (TEGRITAL®) and the results obtained show good repeatability and accuracy.

Shivashankar et. al.[107] developed a simple method for the spectrophotometric estimation of carvedilol phosphate in bulk and pharmaceutical preparations. The method is based on the estimation of carvedilol in 70 % v/v aqueous methanol as a solvent at 241 nm. Beer's law was obeyed over concentration range of 5-30 $\mu g.mL^{-1}$ with correlation coefficient of 0.993. The obtained results were processed statistically in terms of RSD % (< 2.0) and recovery percentage (range between 97.0 % to107 %). The proposed method show no interference from any common pharmaceutical excipient and additive.

Rad et. al.[108] developed a simple, accurate, highly sensitive, and cost-effective method for the spectrophotometric determination of carvedilol (CAR) in its pharmaceutical formulation and in urine samples. The proposed method is based on the reaction of CAR with sodium nitrite in acidic medium followed by the addition of sodium hydroxide to form a yellow colored product has a maximum absorption located at 250-280 nm. The study involves the optimization of factors affecting the reaction (i. e. concentrations of hydrochloric acid, sodium nitrite and sodium hydroxide) by using a central composite design (CCD). Under the optimum conditions, 0.05–0.20 and 1.5–3.5 $mg.L^{-1}$ are the two linear ranges of calibration curves at 250 and 278 nm respectively. At the mentioned wavelengths, the values of ε_{max} for the product are $3.41x10^6$ and $3.19x10^5$ $L.mol^{-1}.cm^{-1}$, and the detection limits are $1.76x10^{-4}$ and $8.12x10^{-4}$ $mg.L^{-1}$. The method was applied successfully for the estimation of CAR in two brands of CAR tablets and human urine samples. A comparison was made between the developed method with the reported methods which revealed that there were no significant differences between either methods.

Two simple, fast and accurate spectrophotometric methods for the quantifying of carvedilol (CAR) have been reported. In method A, the presence of secondary amino group in carvedilol enables the use of condensation reaction with *p*-dimethylaminobenzaldehyde (PDAB) to form a blue colored product exhibits maximum absorbance at 610 nm. In method B, the forming of charge transfer complex is based on that *p*-chloranil as π-acceptor react with CAR as n-donor to form a bluish green colored product in acetone and the color developed is measured at 662 nm. The calibration plots were found to be linear in the ranges of 50.00–250.00 and 20.00–100.0 $mg.L^{-1}$ with ε_{max} values $0.920\ x10^3$ $L.mol^{-1}.cm^{-1}$ and $0.257\ x10^4$ $L.mol^{-1}.cm^{-1}$ for methods A and B respectively. The proposed methods were applied for the analysis of

CAR in three brands of carvedilol tablets and good values of recovery were obtained. Moreover, statistical comparisons with other standard method are performed by using Student's *t*-test and *F*-test and the calculated values agree favorably with those obtained by the reference method [109].

Mohamed et. al.[110] investigated the molecular interaction between diazepam (DZP) as electron donor with each of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone (*p*-CLA) as π -acceptors spectrophotometrically. The formed CT complexes were measured at 550 and 480 nm. All the optimum conditions were identified. Job's and molar ratio methods were applied to study the stoichiometric ratio of the formed complexes. The obtained results indicated the formation of 1:1 adducts of DZP with both of DDQ and *p*-CLA. Calibration curves obeyed Beer's law at 10-150 and 10-250 $\mu g.mL^{-1}$ with molar absorptivities of $5.20x10^2$ $L.mol^{-1}.cm^{-1}$ and $5.41\ x10^2$ $L.mol^{-1}.cm^{-1}$ and detection limits of 6.83 and 9.60 $\mu g.mL^{-1}$ for DDQ and *p*-CLA complexes respectively. The proposed methods were applied to the assay of DZP in pharmaceutical tablet preparation with good accuracy and precision.

Khan et. al.[111] reported new spectrophotometric method for the determination of diazepam. The method is based on specific reaction for diazepam with ninhydrin in an alkaline medium. Under optimum condition, diazepam reacts with ninhydrin when the system is heated for 60 seconds at 60 ^oC at pH range 12-12.5 to give violet colored product exhibits a maximum absorbance at 530 nm. Beer's law limit is ranging from 10-200 $\mu g.mL^{-1}$ and the values of coefficient of variation and relative standard deviation are 0.06 and 1.5 % respectively. The method was found to be simple, accurate, not requiring many chemicals and the quantitative estimations of tolerable amount of other drugs like chloromazine HCl, buscopan, phenobarbiturates, flauxitine

HCl, prioxicam have been studied and no interference was found even if these drugs are present as 21, 19.3, 39.6, 38.7 and 18.2 folds respectively.

Daharwal[112] introduced a simple, precise and efficient Vierdott's method for the simultaneous determination of diazepam (DZ) and propranolol (PL) in bulk drug and in fixed dose combination. The method is based on the formation of simultaneous equation at the absorbance maxima of DZ and PL at 241 and 289 nm respectively. Beer's law was obeyed over the concentration ranged of 2-25 μg.mL^{-1} and 1-45 μg.mL^{-1} and the accuracy in term of recovery was found to be 100.14 ± 0.219 % and 100.17 ± 0.134 % for DZ and PL respectively.

Hassouna et. al.[113] developed a simple, precise and reproducible spectrophotometric method for the assay of furosemide. The proposed method based on the formation of Schiff's bases coupling with aromatic aldehydes such as salicylaldehyde, benzaldehyde, 2,3,4- chlorobenzaldehyde, vanilline, anisaldehyde, dimethylaminobenzaldehyde, 2-methoxy benzaldehyde and 4-nitro benzaldehyde in presence of sulphuric acid. The optical characteristics such as Beer's law limits, molar absorptivity and Sandell's sensitivity for each aldehyde are given. The recommended method has been applied for the determination of furosemide in bulk form and in pharmaceutical formulations.

Tharpa et. al.[114] analyzed furosemide (FUR) spectrophotometrically by using diazocoupling reaction. The two methods are based on the hydrolysis of furosemide in acidic media and diazotization followed by coupling with either N-1-napthylethylene diamine (NEDA) (method I) or 4,5-dihydroxynaphthalene-2,7-disulfonic acid (chromotropic acid, CTA) (method II) to yield colored products have absorption maxima at 520 and 500 nm with molar absorptivities of 1.34 x10^4 and 8.50 x10^3 L.mol^{-1}.cm^{-1} respectively. Linear relationships were found in the ranges of 1.75- 21.0 μg.mL^{-1} and 2.5–30.0 μg.mL^{-1} with good correlation coefficients of 0.9979 and 0.9984 for

methods I and II respectively. Limiting logarithmic method was used to evaluate the reaction stoichiometry in both methods and the study shows that the two complexes were formed in a ratio 1:1 for both reagents. The proposed methods have been successfully applied to the estimation of furosemide in three brands of FUR tablets (Frunex, Lasix and Amifru) and in spiked human urine and the obtained results show good reproducibility and accuracy.

Narayana and Ashwini[115] have proposed the determination of furosemide using a known excess of ceric ammonium sulphate (CAS) followed by the determination of residual oxidant using two reagents: xylene cyanol FF (XC) in method A and safranin O (SAF) in method B. The obtained products exhibit maximum absorbance at 612 and 526 nm with ε_{max} values 1.160 $x10^4$ and 2.025 $x10^4$ $L.mol^{-1}.cm^{-1}$ and the linearity of the calibration were in concentration ranges of 2-30 $\mu g.mL^{-1}$ and 6–16 $\mu g.mL^{-1}$ for both methods respectively.

1-4-5 High Performance Liquid Chromatography

Thejaswini and Gurupadayya[116] described a simple, rapid and accurate RP-HPLC method for simultaneous estimation of amitriptyline, carbamazepine and flunarizine in human plasma. The separation was achieved by using C8 (250 × 4.6 mm, 5µ) column with a mobile phase composing of acetonitrile, methanol and phosphate buffer (pH=3) in proportion of 40:50:10 (v/v) and mitrazapin as an internal standard at flow rate of 0.8 $mL.min^{-1}$. The detection were carried out at 210 nm; the retention time of amitriptyline, carbamazepine and flunarizine were 3.6 ± 0.1, 4.1 ± 0.2 and 4.6 ± 0.2 min respectively. The RSD % for precision and accuracy of the method was found to be < 1. The proposed method is suitable for routine quality control analysis.

Aydomuş et. al.[117] developed and validated a simple, fast and accurate HPLC method for the simultaneous determination of carbamazepine (CBZ) and nilotinib (NIL) in their tablets. In vitro drug interaction studies was

applied in different pH environments, simulating empty and full stomach juice (pH 1 and 4), blood pH (7.4) at 37°C and intestinal juice (pH 9). Isocratic elution at flow rate of 1.0 $mL.min^{-1}$ was employed on RP-C18 HPLC column with mobile phase composed of water and acetonitrile (30:70 v/v). The detection was done by using a UV photo diode array detector at 281 nm and linear calibration curves were obtained in concentration ranges of 0.0125-5 $\mu g.mL^{-1}$ for both drugs. The availability of CBZ in all interaction medium was increased in the presence of NIL in different pH medium from most to least were pH 7.4 > pH 4 > pH 9 > pH 1.2 > pH 6.8. While the availability of NIL in the presence of CBZ was mostly increased in pH 4 and moderate in pH 1.2 and pH 6.8 and showed decrease in pH 7.4 and 9.

An ultra-sensitive reverse phase liquid chromatographic method was described by *Sultana et. al.*[118] for the simultaneous determination of carbamazepine and NSAIDs in pure, pharmaceutical preparation and human serum. A Bondapak C18 (250 × 4.6 mm, 10 μm) column was used with mobile phase comprised of methanol: water pH 3.0 (80:20 v/v). The quantitative determination was achieved at isobestic point of 220 nm with a flow rate of 1.0 $mL.min^{-1}$ at ambient temperature. The calibration curves were linear over the concentration range of 0.4-12 $\mu g.mL^{-1}$ for carbamazepine, 0.5-16 $\mu g.mL^{-1}$ for meloxicam and 0.25-8.0 $\mu g.mL^{-1}$ for ibuprofen and mefenamic acid with detection limits of 4.0, 3.0, 1.0, and 13.0 $ng.mL^{-1}$ respectively. A comparison have been done with another approach by programming the detector at the λmax of each components and the obtained results were more sensitivity, with linear range of 0.10-3.0, 0.15-5.0, 0.10-3.0 and 0.125-4.0 $\mu g.mL^{-1}$ and limits of detection 2.0, 2.0, 1.0, and 3.0 $ng.mL^{-1}$ respectively. The method is accurate, precise and can be used

for analysis of pharmaceutical formulations and human serum without interference of endogenous components of serum or excipients.

Ibrahim[119] has reported a simple and precise RP-HPLC chromatographic method for the simultaneous analysis of carvedilol (CRV) and glimepiride (GMP) or glibenclamide (GBD). This method was validated on a Zorbax Eclipse XDB-C18 column (250 × 4.6 mm, 5 μm) using UV detection with mobile phase composed of 0.2 $mol.L^{-1}$ phosphate buffer (pH 3.5): methanol in a ratio of 30:70 (v/v) at flow rate of 1.0 $mL.min^{-1}$ and column temperature adjusted to 30 ^oC. The detection was done at 220 nm and linear calibration curve was obtained in concentration ranges 2-75 $\mu g.mL^{-1}$ for CRV and 5-300 $\mu g.mL^{-1}$ for each GMP and GBD. The developed procedure has been applied successfully for the estimation of CRV and GMP or GBD in laboratory prepared binary combination tablets with RSD % value <1% and assay percent range 99.49- 99.95 %. In vitro interaction studies have also been carried out at 37 ^oC in simulated human blood pH (pH 7.4) or simulated stomach environments pH 1 and % availability has been calculated. This method was found to be rapid, accurate and sensitive as well as it is suitable for routine quantitative analysis of CRV and GMP or GBD in their binary mixtures.

Naidu et. al.[120] developed a precise, simple, selective and accurate RP-HPLC method for estimation of carvedilol in API and pharmaceutical formulations. Hypersil ODS C18 (150 ×4.6 mm, 5μ) column was used to achieved the effective separation with a mobile phase consist of acetonitrile: phosphate buffer pH 3.0 (50: 50 v/v) with flow rate of 1.0 $mL.min^{-1}$ using UV detection at 240 nm. The method was linear over the range 25-150 $\mu g.mL^{-1}$ ($r^2 = 0.9999$). The limits of detection and quantification were found to be 0.8346 $\mu g.mL^{-1}$ and 2.5292 $\mu g.mL^{-1}$.

Elezovic et. al.[121] developed a new direct chiral HPLC method for the separation and determination of carvedilol enantiomers. Achiral method was validated on a Hypesil Gold-C8 column (150 ×4.6 mm, 5μm) with chromatographic conditions as in Ph. Eur. The chiral method was achieved on Chirobiotic V column (250 ×4.6 mm, 5μm) at flow rate of 0.8 $mL.min^{-1}$ and 20°C with a mobile phase consisted of methanol with added 160 μL diethyl amine and 160 μL acetic acid/ 1000 mL of methanol. UV detection was performed at 242 nm and fluorescence detection at ex.280/ em.340 nm. The linearity of the achiral method was in the concentration range of 4.0-50 $\mu g.mL^{-1}$, while chiral method was linear in the concertation range of 18.0-100 $\mu g.mL^{-1}$ (UV-Vis detector) and 12.0-60 $\mu g.mL^{-1}$ (fluorescence detector). Achiral method has a chromatographic run time 7-8 times shorter than in chiral method but it is demands more manual labor. Both methods were found to be adequate for the determination of carvedilol in pharmaceutical formulations in presence of commonly used tablet excipients.

Srikantha and Raju[122] described a simple and fast RP-HPLC method for simultaneous determination of imipramine hydrochloride and diazepam in pharmaceutical preparations. Chromatographic separation was carried out isocratically on Chromosil C18 column with a mobile phase comprising of methanol: water: 0.1M sodium acetate (30:50:20 v/v/v) at a flow rate of 1.0 $mL.min^{-1}$ using UV detection at 243 nm. The retention times for imipramine hydrochloride and diazepam were 3.33±0.02 min and 4.64±0.02 min respectively. Linearity range was 25-150 $\mu g.mL^{-1}$ for imipramine hydrochloride (r^2 =0.999) and 5-30 $\mu g.mL^{-1}$ for diazepam (r^2 =0.9994) with limits of detection and quantitation of 0.03 and 0.10 $\mu g.mL^{-1}$ for imipramine hydrochloride and 0.02 and 0.07 $\mu g.mL^{-1}$ for diazepam. The recovery were found to be 100.95-101.52% for imipramine hydrochloride and 99.47-

100.33% for diazepam. The reported method is appropriate for routine quantitative determination of imipramine hydrochloride and diazepam in tablets.

Shar et. al.[123] developed and validated a specific and fast HPLC method for determination of diazepam in bulk and pharmaceutical formulations. The assay was employed on two columns; Zorbax Eclipse XDB – C8 column (150 x 4.6 mm, 5 μm) with mobile phase comprising acetonitrile - methanol - potassium dihydrogen phosphate (22: 34: 44 v/v) and ACE – 5 – C8 column (150 x 4.6 mm, 5 μm) with mobile phase containing acetonitrile - methanol - potassium dihydrogen phosphate (20: 32: 48 v/v). With utilizing the second column a better results can be achieved. The proposed method have been successfully applied to the determination of diazepam in its related substances (Generics® tablets and injection) and the obtained results revealed good reproducibility and accuracy.

Sruthi et. al.[124] have performed quantitative analysis of diazepam using RP-HPLC in pharmaceutical dosage form. The assay was achieved using a Hypersil ODS C-18 column (250 x 4.6 mm, 5 μm) with a mobile phase comprising of 1% phosphate buffer (pH 3.0): acetonitrile: methanol (24: 18:58 v/v) at a flow rate of 1 $mL.min^{-1}$. The detection was done at 232 nm; the retention time was 6.23±0.002 min. The calibration curve was linear in the concentration range 2-20 $mg.mL^{-1}$, with limits of detection and quantitation of 0.898 $mg.mL^{-1}$ and 2.72 $mg.mL^{-1}$, respectively. The percent recovery for diazepam ranged between 99.4-100.3 %.

A simple, fast, reliable and economical method for the simultaneous analysis (separation and quantification) of amlodipine and furosemide in pharmaceutical formulation using RP-HPLC has been reported. The separation was made by a Fortis™ C18 column (250 x 4.6 mm, 5 μm) using

a mobile phase consisted of acetonitrile and water (pH 5.0) in a ratio 50:50 v/v, at 45 ºC. The flow rate was affixed at 1.0 $mL.min^{-1}$ and the detector response was monitored at 238 nm. Under the conditions used, amlodipine and furosemide were eluted at 4.28 and 3.68 min, respectively. Calibration curves were obtained in the range of 1.0 -16.0 $\mu g.mL^{-1}$ and 0.1-12.0 $\mu g.mL^{-1}$ for amlodipine and furosemide, respectively (r^2 >0.999). Limits of detection and quantitation were 0.642 $\mu g.mL^{-1}$ and 2.139 $\mu g.mL^{-1}$ for amlodipine 0.010 $\mu g.mL^{-1}$ and 0.031 $\mu g.mL^{-1}$ for furosemide, respectively. The developed method was successfully applied for the analysis of amlodipine and furosemide in commercially available tablets[125].

Baranowska et. al.[126] proposed an ultra-high performance liquid chromatography (UHPLC) method for the simultaneous estimation of furosemide, spironolactone, terbinafine, vancomycin and their metabolites in human plasma and urine. The chromatographic separation was achieved on a Hypersil GOLD C18e column (50 x 2.1 mm, 1.7 μm) with the mobile phase composed as 0.1% formic acid and acetonitrile, by gradient elution in 3.3 min. All of the calibration curves were linear over a wide range of drugs concentrations as follow: spironolactone (SPR) and its active metabolite carnenone (CAR) were 0.23-20 and 0.17-20 $\mu g.mL^{-1}$ respectively, vancomycin was 0.36-20 $\mu g.mL^{-1}$, terbinafine (TER) and its metabolite N-desmethylcarboxy terbinafine (DMT) were 0.03-20 and 0.10-20 $\mu g.mL^{-1}$ respectively, saluamine the furosemide metabolite was 0.16-20 $\mu g.mL^{-1}$, finally furosemide have two linear ranges: 0.04-5 and 5-20 $\mu g.mL^{-1}$. The LOD values varied from 0.01 to 0.07 $\mu g.mL^{-1}$, with vancomycin as an exception (0.11 $\mu g.mL^{-1}$). This method can be applied to routine clinical

analysis due to the short analysis time and small quantities of plasma or urine needed.

Ram et. al.[127] have developed a specific method for the simultaneous quantification of spironolactone and furosemide in tablet formulation using RP-HPLC. An isocratic mode was achieved on a Wakosil II 5 C8 RS column (150 x 4.6 mm, 5 μm) using a mobile phase of acetonitrile–ammonium acetate buffer in ratio of 50:50 (v/v) at a flow rate of 1.0 $mL.min^{-1}$. The quantification was done with PDA detector at 254 nm. The linearity was obtained in the concentration range of 40–160 $mg.mL^{-1}$ with $R^2 = 0.9977$ and 0.9953 for spironolactone and furosemide, respectively. The precision in term of RSD (n = 6) was 0.87 % and 1.1 % for spironolactone and furosemide, respectively. Intermediate precision and repeatability expressed as RSD (n = 6) were 0.46 % and 0.20 % for spironolactone and furosemide, respectively. The recovery was ranged between 98.05-100.17 % and 99.07-100.58 % for spironolactone and furosemide, respectively. The drug was subject to stress conditions such as oxidation, hydrolysis, photolysis and heat. The degradation products that produced as a result of stress studies did not interfere with the detection of furosemide and spironolactone; therefore, the evaluate can be considered to be stability-indicating.

Simultaneous analysis (HPLC):

Patil et. al.[128] described a simple, sensitive and fast RP-HPLC method for the simultaneous estimation of carbamazepine (CBZ), furosemide (FSD), antipyrine (ANT) and phenytoin (PHTN). Chromatographic analysis was achieved on a C18 column (250 x 4.6 mm, 5 μm), using mobile phase composed of 50:50 mixtures of methanol and acetonitrile and water pH 3.0 in a ratio (42: 58 v/v), at a flow-rate of 1.0 $mL.min^{-1}$ and column temperature adjusted to 40 °C. Detection was effected by ultra-violet absorption at 230

nm for ANT and FSD and at 205 nm for CBZ and PHTN. Elution of ANT, FSD, PHTN, and CBZ was detected at 4.1, 5.1, 12.3 and 13.5 min, respectively. The method was found to be linear in the concentration range of 5–100 μmol.L^{-1} for all analytes with $R^2 \geq 0.999$. The values of LOD were 3.28, 0.67, 0.13 and 0.73 μmol.L^{-1} while for LOQ were 5.28, 2.24, 0.58 and 2.04 μmol.L^{-1} for ANT, CBZ, FSD and PHTN, respectively. Intra- and inter-day results (n=3) showed that the method is efficient for routine determination of these penetrability markers in Caco-2 cell monolayer permeability studies.

Soltani and Jouyban[129] have reported a simple isocratic HPLC-UV method for simultaneous determination of carvedilol, furosemide, losartan, diltiazem and propranolol in spiked human plasma. The separation was carried out using a MZ-analytical column (15 x 4.6 mm, 5 μm) under an isocratic condition of 15 mmol.L^{-1} phosphate buffer (pH 2.0): 2-propanol: acetonitrile (65: 2.5: 32.5 v/v) mobile phase with UV detection at 225 nm. The method was validated according to the FDA guidance for bioanalytical method validation and showed acceptable accuracy, precision, and the linearity was as follow: carvedilol (0.025-0.800 μg.mL^{-1}), furosemide (0.025-0.800 μg.mL^{-1}), diltiazem (0.050-0.800 μg.mL^{-1}), losartan (0.050-0.800 μg.mL^{-1}), and propranolol (0.025-0.800 μg.mL^{-1}). The mean recoveries were in the range 99.0-104.4 % that indicate the method is robust and reproducible.

Baranowska and Kowalski[130] have developed an HPLC method for simultaneous determination of carvedilol (CAR) and carbamazepine (CBM). For the best enrichment of all drugs from waters samples, three different SPE columns achieved extraction and pre-concentration of the drugs. Optimum separations (as well as great sensitivity) of the analytes was attained with the

gradient RP-HPLC-DAD with the mobile phase composed as acetonitrile, methanol and 0.1% formic acid at ambient temperature. The determined substances were eluted from a Develosil C30 (250 x 4.6 mm, 5.8 μm) column in 25 min. Retention times were 20.564 min for CBM and 18.524 min for CAR. The linearity ranges were found to be 0.09-10 $\mu g.mL^{-1}$ and 0.32-10 $\mu g.mL^{-1}$ for CBM and CAR respectively. The limits of detection were 0.029 and 0.105 $\mu g.mL^{-1}$ and the limits of quantification were 0.086 and 0.315 $\mu g.mL^{-1}$ for CBM and CAR respectively. The proposed method has been applied successfully for the estimation of the cited drugs in tab water, surface water and wastewater.

Zhang et. al.[131] have reported a novel method for the simultaneous determination of carbamazepine and diazepam as a part of 61 central nervous system (CNS) drugs in plasma. The method utilized weak cation exchange (WCX) column for solid phase extraction (SPE), HPLC system (Beckman Coulter with DAD-detector) and a column (Agilent TC-C18, 250 x 4.6 mm, 5 μm). The acetonitrile-phosphate buffer elution gradient has been applied for the separation at flow rate of 1.5 $mL.min^{-1}$ and the injection volume was 50 μL. The detection wavelength was 210 nm, and the full spectra were recorded from 200 to 364 nm. The absolute recoveries of carbamazepine and diazepam were 89.82 % and 63.51 % (for 3 replicate of 10 $\mu g.ml^{-1}$). In conclusion, the WCX SPE preparation combined with HPLC-DAD, is suitable for a broad drug screening for CNS drugs.

1-5 Objectives

The objective of the work is to develop new methods for the simultaneous determination of FURO, CARB, DIAZ and CARV.

1-Reverse phase-high performance liquid chromatographic method:

The aim of the suggested work is to develop and validate a simple and suitable HPLC method for simultaneous determination of the cited drugs and to evaluate the performance features of the proposed method comprising linearity range, detection and quantification limits, accuracy, precision and recovery. Moreover, the study is extended to check the applicability of the proposed method for the determination of pure drugs and to assay the contained of the four stated drugs in their pharmaceutical preparations and for routine quality control analysis. The estimation include tablets, ampoule and oral suspension dosage forms (either individually or in mixture).

2- Simultaneous determination of the studied drugs

- Derivative spectrophotometry: Establish a new method relying on different modes of derivative spectrophotometry for simultaneous determination of the examined drugs in their quaternary mixture. The study includes the evaluation of the analytical results and examining the possibility of the implementation of the developed method for the analysis of the drugs in their pure form and pharmaceutical preparations.
- Partial least squares methods (PLS-1 and PLS-2): To find PLS-1 and PLS-2 models for simultaneous estimation of drugs in their quaternary mixture without prior separation and the utilization of the models to estimate the analytes in their pure form and pharmaceutical formulations.

CHAPTER TWO

EXPERIMENTAL PART

Chapter 2

2-Experimental part

2-1 Chemicals and solvents

The solvents and chemicals, which were used throughout the work and their providers, are listed in Table 2-1.

Table 2-1: Chemical compounds, purity and their providers.

Materials	Assay (Purity %)	Provider
Acetonitrile	99.9 % for HPLC	SIGMA-ALDRICH (Germany)
Methanol	99.9 % for HPLC	SIGMA-ALDRICH (Germany)
Acetic acid	99-100 %	SIGMA-ALDRICH (Germany)
Deionized (DI) water	Residue < 0.0001% For HPLC (Ω >18 megaohm)	ROMIL (United Kingdom)
Disodium hydrogen phosphate $2H_2O$	99.0 %	Hopkin &Williams (England)
Potassium dihydrogen phosphate	98 %	Hopkin &Williams (England)
Formic acid	90% General purpose reagent	Hopkin &Williams (England)
Sodium hydroxide	99.9 % w/w	Panreac
Hydrochloric acid	37 %	SIGMA-ALDRICH (Germany)
Ortho phosphoric acid	85 % Analar	CHEM-SUPPLY (Australia)
Potassium hydroxide	Analytical grade AG	Fluka
Ammonium hydroxide	32.0 % w/w	Scharlau (Spain)

2-2 The Drugs

Pharmaceutical grade Furosemide (FURO), Carbamazepine (CARB), Diazepam (DIAZ) and Carvedilol (CARV) powders received in pure form (99.99 %) were provided as a gift from State Company for Drug Industries and Medical Appliances Samara-Iraq (SDI). The principle pharmaceuticals formulations that were used during this work with their corresponding manufacturers are summarized in Table 2-2.

Table 2-2: The principle pharmaceutical formulations and their manufacturers.

pharmaceutical formulation	Manufacturer
Lasix 40 mg furosemide / tablet	SDI, Iraq
Lasix 40 mg furosemide / tablet	SWI, France
Valiapam 2 mg diazepam / tablet	SDI, Iraq
Valiapam 10 mg /2mL diazepam / injection ampoule	ALSAVAL, Syria
Carbamazepine 200 mg // tablet	TAVER, Cyprus
Tegretol 2 % (100 mg /5 mL) carbamazepine /oral suspension	Novartis, Switzerland
Carvedilol 25 mg // tablet	Pharma International, India
Carvedilol 6.25 mg // tablet	EMESSA, Syria

2-3 HPLC system

The chromatographic study of the selected drugs were carried out on a Shimadzu UFLC system (Kyoto, Japan) which is shown in Figure 2-1.

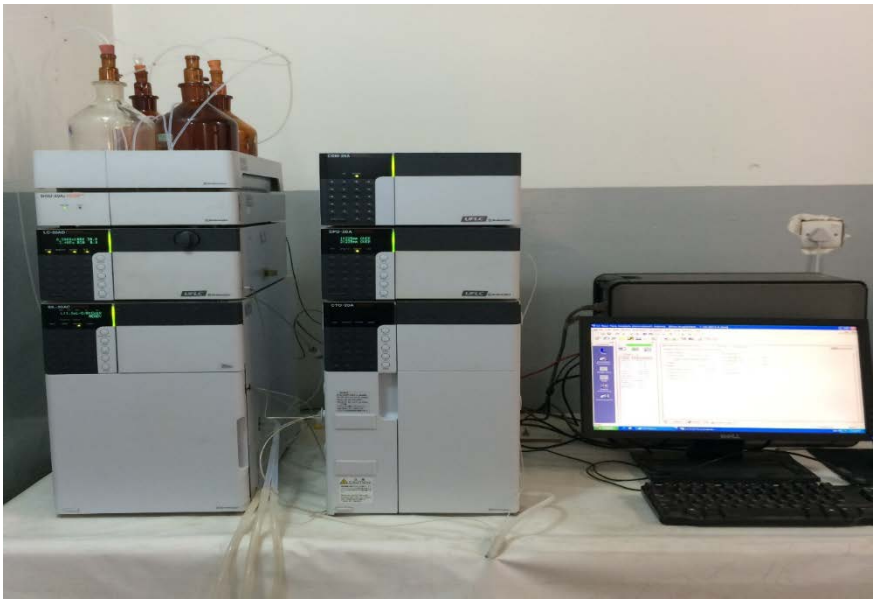


Figure 2-1: Shimadzu UFLC system

The modules of HPLC system are:

2-3-1 Mobile phase reservoir

The reservoir holds the solvent, which is referred to as the mobile phase because it moves through the pump by gravity and it will be pumped into the column. There are usually a minimum of two reservoirs in a HPLC system, with each holding up to 1000 mL of solvent, constructed from inert borosilicate glass container, and holds in a special compartment called reservoir tray elevate above pumps. The solvents were passed through inert PTFE tubes supplies at their ends with a stainless steel filter of 0.45µm porosity (Figure 2-2) dips into the reservoir to facilitated a hygienic condition by removing particulates from the solvents, preventing

them to pass through the pump toward the column and causes clogging the tubes, destroy the pump, and decrease the column lifetime.

Figure 2-2: The reservoir filter

2-3-2 Connecting tubes and fittings

The connections between mobile phase containers, pump, injector, column, and detector may compromise the overall efficiency of the system and assures the circulation of the solvents between the HPLC modules. There are several types of connectors and plugs like stainless steel, titanium, and flexible polymers (PTFE, ETFE, and PEEK) which recommended to have the minimum lengths with a narrower internal diameter (≤ 0.5 mm) as possible to minimize band broadening and to provide very low, or zero dead volume. The transfer tubes used in this system were made from stainless steel and PEEK of 0.5 mm i.d., which capable of withstanding high-pressure up to 350 bars (35 MPa).

2-3-3 Degassing unit (DGU-20A_5)

The DGU-20A_5 is an on-line vacuum degasser unit in UFLC system used to remove dissolved gasses by passing the solvents through a porous fluoroethylene membrane (with small internal capacity about 0.4 mL) and this accomplishes both the degassing and filtration processes at the same time.

2-3-4 Solvent delivery unit (LC-20AD)

The main function of this unit is to convey metered amount of solvents at a specific, constant flow rate from one reservoir or more via a liquid chromatograph. UFLC system consist of two LC-20AD with a flow rate range

from 0.0001 to 10.0 mL.min^{-1} and a maximum discharge pressure 40 MPa which achieves the highly stable solvent delivery performance demanded by this system.

2-3-5 Sample injection unit (SIL-20AC)

This unit is an auto sampler model with applicable pressure ≈ 35MPa Max, injection volume range 0.1-100 µL (accuracy 1% max, and precision RSD: 0.3% under specified condition, for 10 µL injection).The other advantage of this unit is the sampler cooler with block cooling/heating system , that features a dehumidifier function for storing samples between 4 ºC to 40 ºC.

2-3-6 Column compartment

i. Column:

A NUCLEODUR® 100-5 C_{18} ec (250 × 4.6 mm i.d. 5µm) (MACHEREY-NAGEL Germany) stainless steel, reversed phase column, was used in this study to achieve separation of selected drugs.

ii. Column oven (CTO-20A)

To perform reproducible results, a constant temperature for solvent and column is required. This achieved by using CTO-20A a forced-air circulation, type column oven. It can regulate the temperature with a high-performance thermistor from 10 ºC below room temperature to 85 ºC. Rapid temperature equilibration allows complex temperature programs such as linear and/or step-wise heating and cooling (from −10°C below room temperature to 85°C).

2-3-7 UV-Vis detector (SPD-20A)

This type of detectors can measure two wavelengths simultaneously, with a high level of sensitivity, stability, and superior signal processing technology upholds the linearity to the ASTM standard: 2.5AU. Wavelength range 190 to

700 nm, bandwidth 8 nm, wavelength precision 0.1 nm max, and the flow cell: optical path length 10mm, capacity 12 µL.

2-4 Miscellaneous Instruments

UV-VIS spectrophotometer

The spectrophotometric measurements were done with Cecil CE 7200 UV-Visible double beam spectrophotometer with 10 mm quartz cell, (Cambridge-England).

Ultrasonic water bath

A Power Sonic model (LUC-405) (40 KHz frequency) was used for dissolution of the powdered sample and degassing of the mobile phase to remove the dissolved gases that affect the column performance and disrupted the detector.

pH meter

The pH meter used was iTRANS BP3001 professional benchtop pH meter, equipped with iTRANS PP 4010-glass pH electrode.

Sensitive Digital balance

All weighing was carried out using a sensitive balance ± 0.0001g Sartorius BL 210 S scientific balance, Gottingen – Germany.

Adjustable micropipette

For transferring micro volumes of standard and working solutions, an adjustable micropipette was used, with a volume range 100-1000 µL from ISOLAB / Germany.

Syringe filter

Minisart ® CE Nylon syringe filters with mesh size of 0.20 µm were used for particulate filtration of samples before injection.

2-5 Method development

2-5-1 Preparation of solutions for pH control

1. Disodium hydrogen phosphate buffer (0.1M) was prepared by dissolving 17.80 g of $Na_2HPO_4.2H_2O$ in 1000 mL HPLC grade water. The pH was adjusted to 11.0 by addition of drops of NaOH solution ≈ (5M) [132].
2. Potassium dihydrogen phosphate buffer (0.1M) was prepared by dissolving 13.60 g of KH_2PO_4 in 1000 mL HPLC grade water and adjusting to pH 7.0 by addition of drops of NaOH solution ≈ (5M) [132].
3. Formic acid buffer (0.1M) was prepared by dissolving 4.2 mL of formic acid in 1000 mL HPLC grade water to get solution have pH 2.2
4. Sodium hydroxide ≈ (5M): 20 g of NaOH was dissolved in 100 mL volumetric flask with HPLC grade water and the solution was completed to the mark.
5. Hydrochloric acid ≈ (5M): 41.64 mL of hydrochloric acid (37%) was slowly added to approximately 40 mL of HPLC grade water in 100 mL volumetric flask and the solution was completed to the mark.
6. Ammonium buffer (0.005M) was prepared by dissolving 0.3 mL of ammonium hydroxide (32%) in 1000 mL HPLC grade water and adjusting to pH 8.50 by addition of drops of HCl solution ≈ (5M).

2-5-2 Preparation of mobile phase

Throughout this study, two kinds of mobile phase were prepared:

1. A mobile phase consisted of acetonitrile, deionized water, and 0.1M phosphate buffer, which have been filtered and then degassed in an ultrasonic bath for at least 10 min, prior to use.

2. A mobile phase consisted of a mixture of acetonitrile and deionized water acidified with acetic acid (pH 3.60) in the ratio of (50:50 v/v). The mobile phase was filtered and degassed as mentioned before.

2-5-3 Preparation of standard stock solutions

Standard stock solutions containing 1000 $\mu g.mL^{-1}$ of furosemide, carbamazepine, diazepam and carvedilol were prepared separately in different 50 mL volumetric flasks. These solutions were prepared by dissolving exactly 50 mg of each drug in acetonitrile, and diluted to the mark with acetonitrile. The stock solutions were protected from light and were stored at 4 ºC.

For the preparation of working solutions, further dilution was done to get (100 $\mu g.mL^{-1}$) of each drug. Standard mixture (contain 20 $\mu g.mL^{-1}$, of each drug) was prepared by mixing 2 mL from 100 $\mu g.mL^{-1}$ solution of each drug in 10 mL volumetric flask then made up to the volume with acetonitrile.

2-5-4 Preparation of samples for linearity

In order to ascertain there is a direct proportional relationship between the analyte response and its concentration, mixtures, containing different concentrations of the cited drugs, were prepared by serial dilutions at the concentration levels of (1-100) $\mu g.mL^{-1}$ for the studied drugs.

2-5-5 Preparation of drugs in pharmaceutical formulations

I. Tablets

Ten tablets from each drug were separately weighed, finely powdered in a mortar and the average weight was determined. From these, a portion of the powder equivalent to 0.0380 g and 0.0400 g for Lasix (FURO) (Iraq, France) respectively, 0.3310 g and 0.1520 g for Carvedilol (CARV) (Syria, India) respectively, 0.0130 g for Tegretol (CARB) (Cyprus) and 0.6010 g for Valiapam (DIAZ) (Iraq) were weighed, dissolved in about 8 mL of acetonitrile and sonicated for a minimum 10 min, with intermittent shaking. The content of each was transferred quantitatively into a separate 10 mL volumetric flask,

shaken well and diluted to mark with acetonitrile to get 1000 µg.mL^{-1}. The solutions were filtered through Whatman filter paper No. 41 and stored as the standard stock solutions for further dilution in subsequent uses.

II. Ampoule

The 1000 µg.mL^{-1} Valiapam (DIAZ) solution was prepared by quantitatively transferring the content of 1 ampoule (10 mg /2 mL) to 10 mL volumetric flask, diluted to the mark with acetonitrile and stirred for 5 min, for complete dissolution (mixing, homogenous) the drug. The resulted solution was filtered and further diluted with acetonitrile before its application to HPLC system for analysis.

III. Oral suspension

The content of one container of Tegretol (CARB) oral suspension (100 mg /5 mL) (2 %) was mixed well and 0.5 mL of the suspension was quantitatively transferred into 10 mL volumetric flask and dissolved in 10 mL of acetonitrile, shaken well and sonicated for 5 min, then made up to the mark with acetonitrile to get 1000 µg.mL^{-1}. An aliquot of the resulted filtered solution was diluted for subsequent uses.

2-6 Primary experimental test

Preliminary tests for the determination of the studied drugs (FURO, CARB, CARV and DIAZ) were performed and carried out under a simple isocratic elution using a NUCLEODUR® C18 ec column. The mobile phase utilized was 50:45:5 (acetonitrile-water-0.1M phosphate buffer) at flow rate 1.0 mL.min^{-1}. Ten microliters of each reference drug solution (20 µg.mL^{-1}) was injected onto the column. Figure 2-3 illustrates the HPLC chromatogram for each drug.

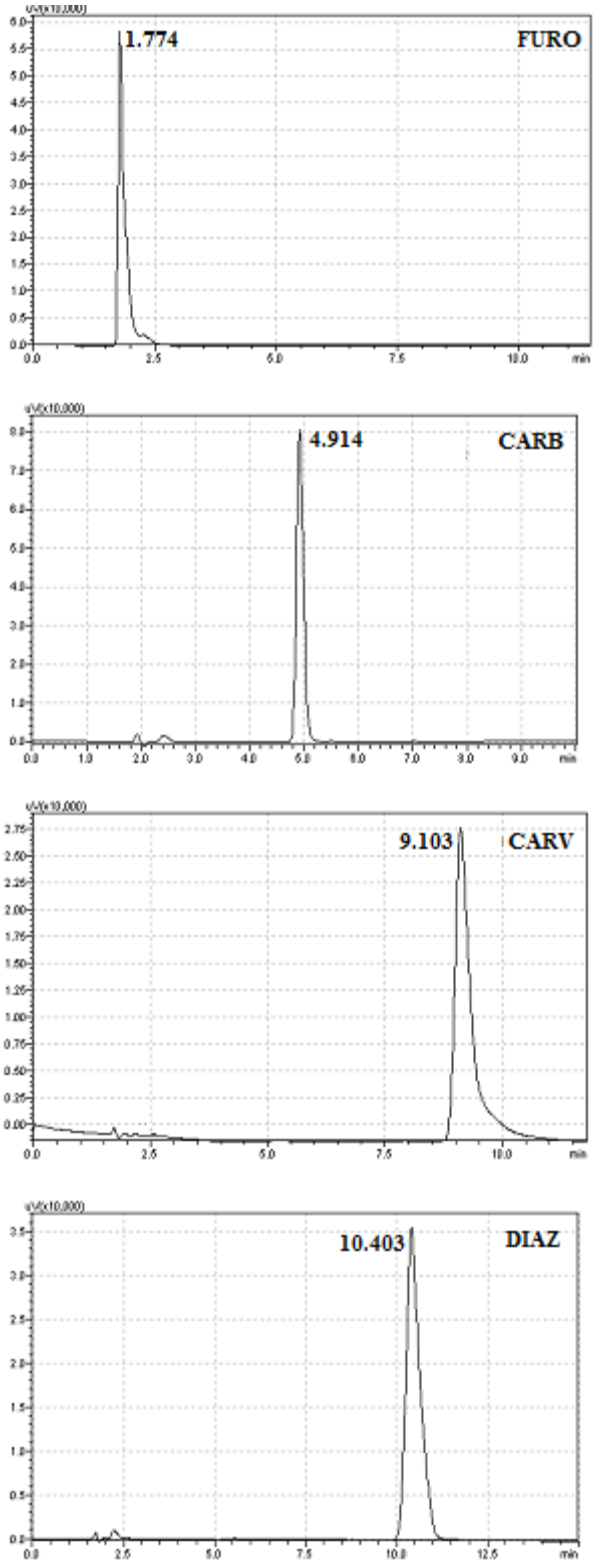


Figure 2-3: HPLC Chromatograms of individual drugs (20 μg.mL^{-1}) under preliminary conditions.

In order to investigate the possibility of simultaneous separation of the titled drugs in their mixtures, an aliquot (10 µL) of the standard mixture containing 20 µg.mL^{-1} of each, was injected under the same outlined chromatographic conditions. The elution sequence of the drugs was in the following order: FURO, CARB, CARV and DIAZ as shown in Figure 2-4, and the chromatographic peak properties (retention time, peak area, and peak height) illustrated in Table 2-1.

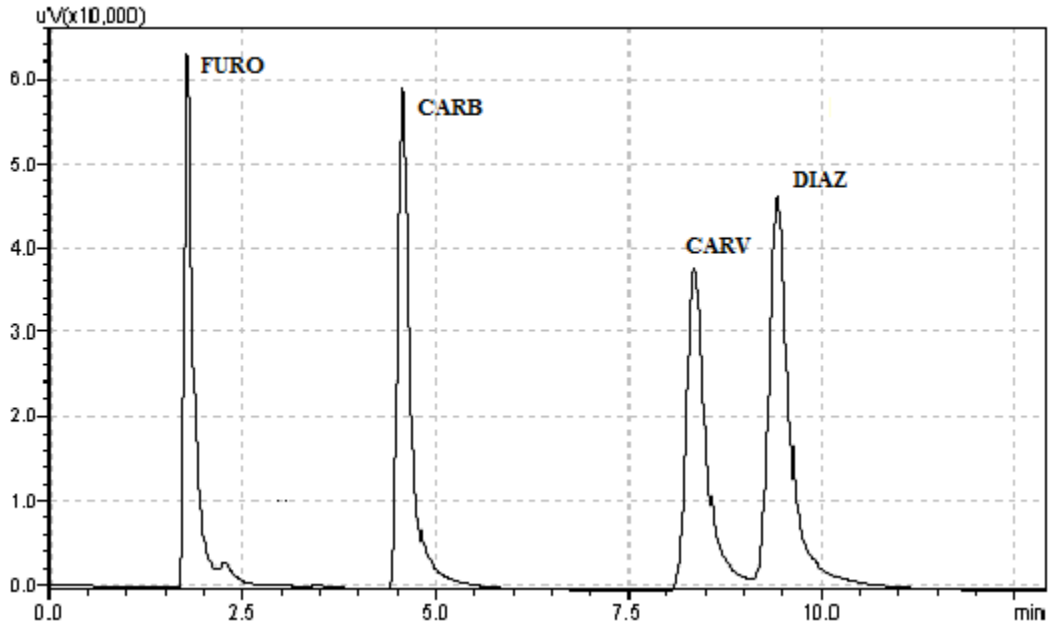


Figure 2-4: HPLC Chromatogram of the four drugs mixture (20 µg.mL^{-1} of each).

Table 2-3: The properties of chromatographic peaks.

Peak	Drug	Retention time (t_R) min	Area (µv.s)	Height (µv)
1	FURO	1.766	587505	63290
2	CARB	4.563	740452	59554
3	CARV	8.345	714137	38245
4	DIAZ	9.423	976434	46764

2-7 Optimization of experimental parameters

Influencing and optimization of various experimental operating factors were studied to obtain better separation (i.e. higher resolution), to separate faster (i.e. shorter retention time) and lower detection limit. This work were done by using a mixture of the four drugs (contain 20 $\mu g.mL^{-1}$) under isocratic elution at 226 nm.

1. Effect of mobile phase composition

Several experiments were performed using acetonitrile as the organic modifier with aqueous buffer at a ratio 50:45:5 (ACN: H_2O: buffer), using either disodium hydrogen phosphate buffer (0.1M, pH 11), potassium dihydrogen phosphate buffer (0.1M, pH 7), or formic acid buffer (0.1M, pH 2.2). Figure 3-2 illustrates the effect of buffer type on the separation of (20 $\mu g.mL^{-1}$) mixture of FURO, CARB, CARV and DIAZ at a flow rate of 1.0 $mL.min^{-1}$, injection volume 10 µL and 226 nm for detection of chromatographic peaks.

2. Effect of flow rate of mobile phase

To observe the effect of flow rate on the separation of the titled drugs, changing the rate from 0.5 to 1.8 $mL.min^{-1}$ was made, while the other chromatographic conditions were held constant. The obtained results are given in Figures 3-3, 3-4 and in Table 3-1.

3. Effect of organic modifier percentage

Different compositions of mobile phase (ACN : H_2O : Na_2HPO_4 buffer 0.1M, pH11.0) namely 30:62.3:7.7 , 40:53.4:6.6 , 50:45:5 , 60:35.6:4.4 , 70:26.7:3.3 were investigated under isocratic elution at flow rate 1.5 $mL.min^{-1}$. Results are illustrated in Figures 3-5, 3-6 and in Table 3-2.

4. Effect of buffer percentage

To verify the effect of buffer percentage on elution of the cited drugs, a sequence of experiments were done with varying the buffer ratio in a range (0.5% - 6.5%) at a flow rate 1.5 $mL.min^{-1}$, injection volume 10 µL, and 226 nm as the choosing wave length for detection. The chromatograms in Figure 3-7 as well as Figure 3-8 and Table 3-3, demonstrate the impact of this factor on chromatographic separation.

5. Effect of pH of the buffer

The influence of pH on the chromatographic separation of drugs mixture was studied by recording the chromatograms of drugs mixture concentration (20 $\mu g.mL^{-1}$) over pH range (9.5-12.0) .The pH was adjusted by addition of few drops of 0.1M NaOH or 0.1M HCl solution as appropriate. Results are portrayed in Figures 3-9, 3-10 and Table 3-4.

6. Effect of temperature

The temperature used for the separation of (20 $\mu g.mL^{-1}$) mixture of FURO, CARB, CARV and DIAZ ranged from 30°C to 50°C. 50:47.5:2.5(ACN: H_2O: 0.1M Na_2HPO_4) was used as mobile phase and the other conditions were held constant. Results are depicted in Figures 3-11, 3-12, 3-13 and in Table 3-5.

7. Effect of injection volume

The relationship between injection volume and the elution of the cited drugs from RP-HPLC NUCLEODUR column was investigated using volumes of 2.5, 5, 7.5, 10, 15, 20 µL respectively. Figures 3-14, 3-15 and Table 3-6 represented the obtained results.

CHAPTER THREE

RESULTS

&

DISCUSSION

Chapter 3

3 Results and discussion

3-1 Wavelength selection

To find the proper wavelength that used to recognize the chromatographic bands of **FURO**, **CARB**, **CARV**, and **DIAZ** which gave approximately equal reasonable values of absorbance for the four analytes, the spectrum of (10 μg.mL^{-1}) solution of each drug in acetonitrile was recorded against solvent blank solution in the wavelength range of 200 - 400 nm. Figure 3-1 shows the overlaid UV spectra of the four medications from which the wavelength 226 nm was chosen for qualitative evaluation of HPLC chromatogram and quantification of the cited drugs.

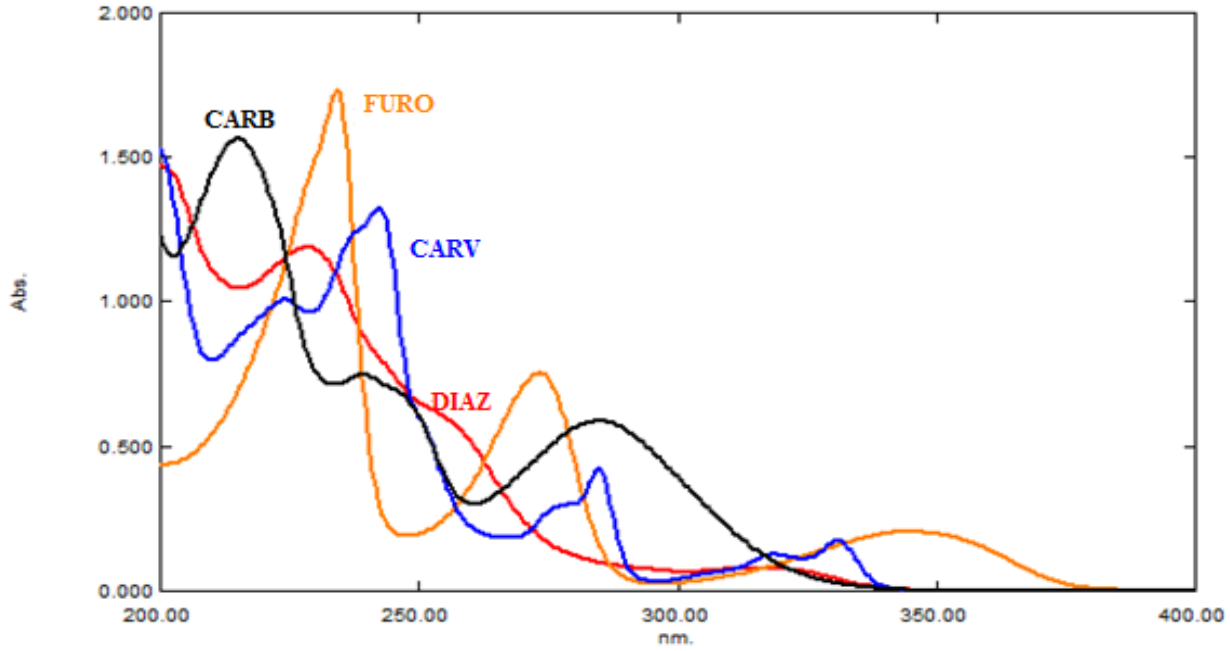


Figure 3-1: Overlaid UV spectra of the studied drugs (10 μg.mL^{-1}) in acetonitrile against acetonitrile as a blank.

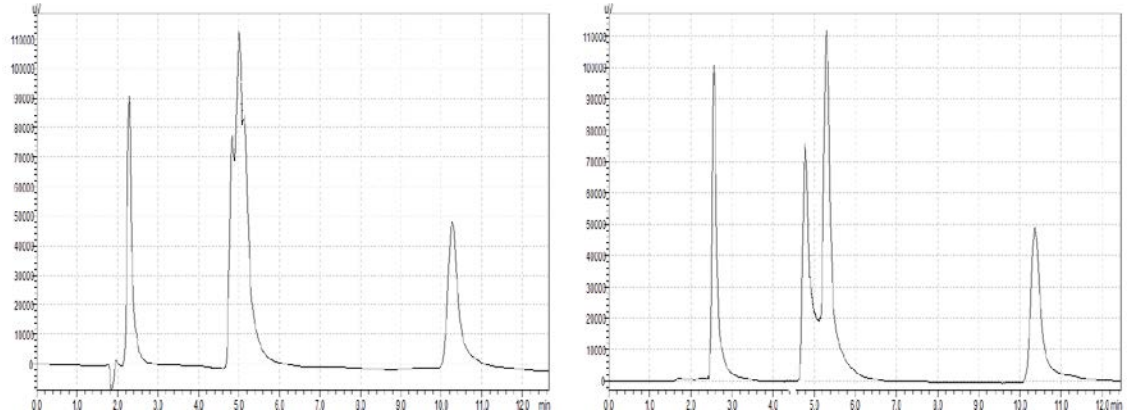
3-2Optimization of experimental variables

The system parameters namely mobile phase composition, flow rates, gradient, run time, and sample volume to be injected, were carefully studied to choose the most suitable conditions for subsequent drug assay execution.

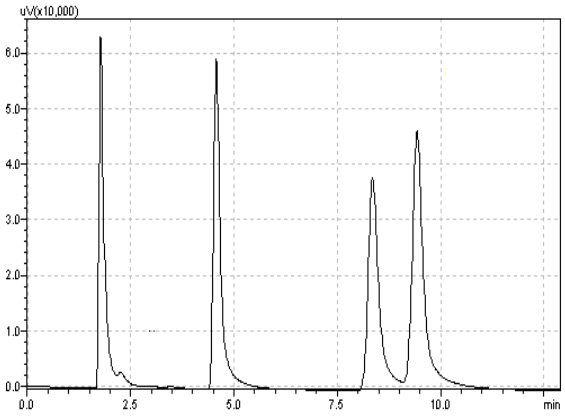
3-2-1 Effect of mobile phase composition

Generally, drugs bearing acidic or basic functional groups are present in their solutions in completely, partially, or non-ionized form. Their state and degree of ionization critically affect their retention in RP-HPLC. The retention of ionizable analytes, basic and acidic compounds, show significant changes with aqueous/organic mobile phases and is dependent upon the type and amount of organic eluent. Therefore, developing rugged procedure not sensitive to small variations in conditions requires controlling the pH and ionic strength of the mobile phase [133].

In this preliminary investigation the mobile phase composition for isocratic reversed-phase separation was acetonitrile : H_2O : buffer (50: 45: 5). Three types of buffers at different pH values namely; (0.1M solution of KH_2PO_4 (pH=7.0), 0.1M solution of HCOOH (pH=2.2) and 0.1M solution of Na_2HPO_4 (pH=11.0) were used with mobile phase flow rate of 1.0 $mL.min^{-1}$, sample injection volume of 10 µL, column temperature of 40°C and UV detection at 226nm, to evaluated the system suitability parameters and overall chromatographic performance. Figure 3-2 shows the chromatograms of the eluted drugs from their mixture (20 $\mu g.mL^{-1}$).



0.1M KH_2PO_4 (pH=7.0) 0.1M HCOOH (pH=2.2)



0.1M Na_2HPO_4 (pH=11.0)

Figure 3-2: Chromatograms of the drugs mixture (20µg.mL^{-1}) using different buffers; (The order of elution is **FURO**, **CARB**, **CARV** and **DIAZ**).

It is obvious that using 0.1M solution of Na_2HPO_4 as pH buffer at pH=11.0 gave the well defined separated chromatographic bands for the four drugs, therefore; this buffer was selected for the subsequent studies.

3-2-2 Effect of flow rate

In HPLC, one of the important factors that can affect the chromatographic behavior and alters the efficiency is mobile phase flow rate [134]. Moreover, the

retention time of an analyte is inversely proportional to the mobile phase flow rate, hence decrease the analysis time i.e. using high flow rates decrease the retention time because the eluent carries the drugs through the column faster after desorption and vice versa[135].

According to Van Deemter equation, the height equivalent to a theoretical plate "H" is related to the linear velocity (flow rate) of the mobile phase.

$$\mathbf{H = A + B/u + Cu}$$

where **u** represents the linear velocity of the mobile phase, **A**, **B** and **C** are constants representing eddy diffusion, longitudinal diffusion, and mass transfer in mobile and stationary phases respectively. As it is obvious from the equation, eddy diffusion is independent of **v**, whereas the other two terms behaves differently with mobile phase linear velocity. Accordingly, optimizing of the flow rate possibly leads to increase throughput, and thus the speed of analysis without affecting the chromatographic performance [136].

A study was performed to evaluate the influence of the mobile phase flow rate on the separation process over the range of 0.5–1.8 $mL.min^{-1}$. Chromatograms depicted in Figure 3-3 shows that upon increasing the flow rate within the studied range, the time analysis starts to decrease from 18.054 to 5.363 min.

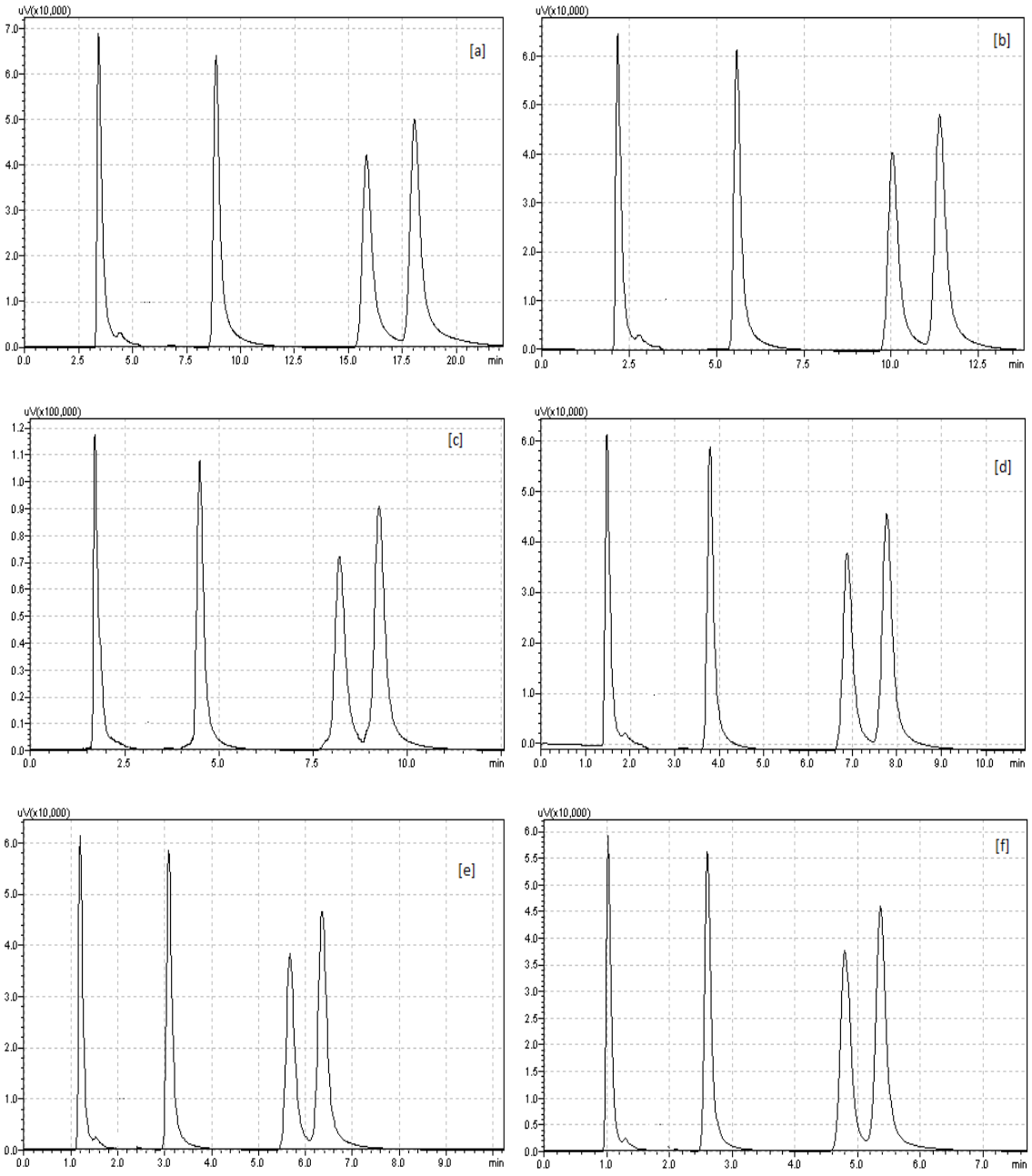


Figure 3-3: Chromatograms of the mixture at different flow rate [a] 0.5 mL.min^{-1}, [b] 0.8 mL.min^{-1}, [c] 1.0 mL.min^{-1}, [d] 1.2 mL.min^{-1}, [e] 1.5 mL.min^{-1} and [f] 1.8 mL.min^{-1}.

The flow rate 1.5 mL.min^{-1} was chosen to be the optimum since reasonable resolution (Rs) values for the separated drugs with a short analysis time were obtained. Figure 3-4 and Table 3-1; illustrate the results for the HPLC separation

with different values of mobile phase flow rates while all other operating parameters were held constant.

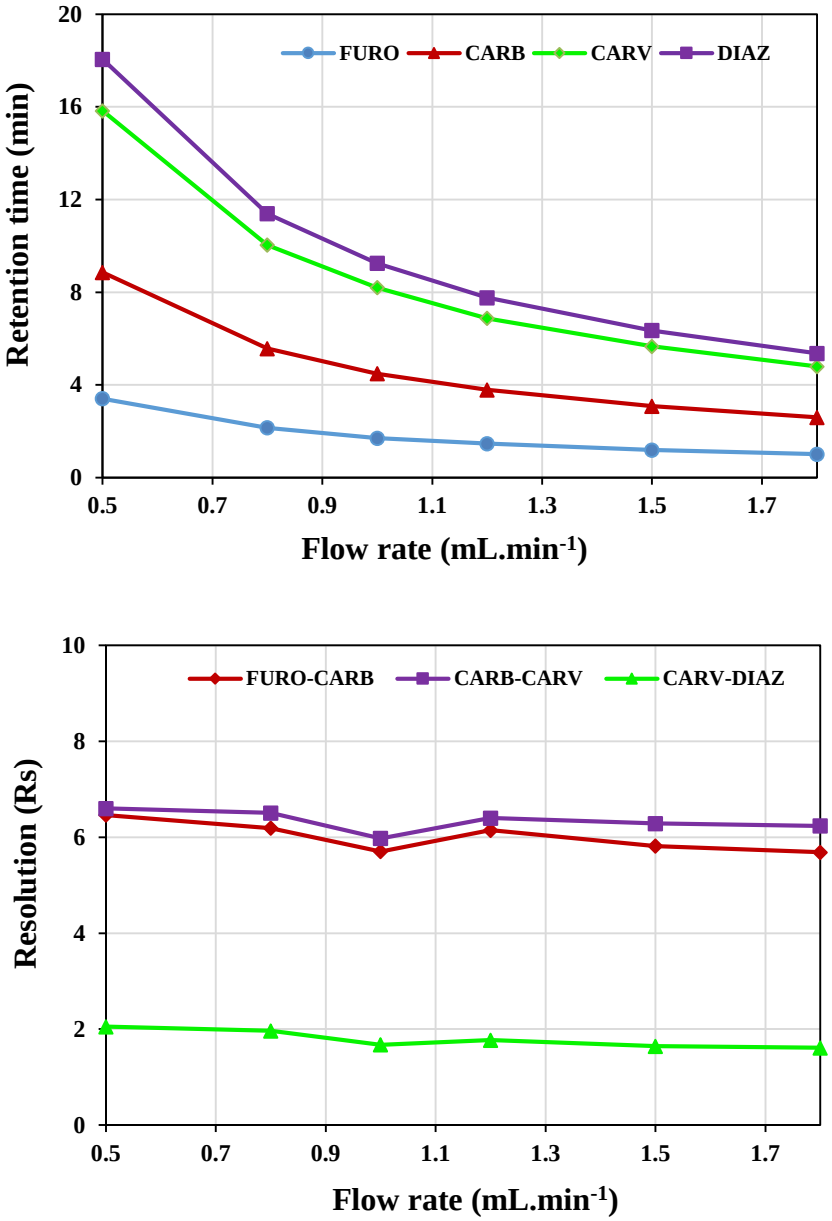


Figure 3-4: The relationship between t_R and R_s as a function of flow rate.

Table 3-1: Results of t_R, k', α, N and Rs of drugs mixture at different flow rates.

Flow rate ($mL.min^{-1}$)	Drug	t_R	k'	α	N	R_s
0.5	FURO	3.409	1.166	1.798	782.37	4.257
	CARB	8.854	4.625	1.958	2752.55	6.600
	CARV	15.826	9.055	1.156	4430.35	2.051
	DIAZ	18.054	10.470		4410.54	
0.8	FURO	2.153	1.188	1.755	696.00	3.863
	CARB	5.569	4.660	1.974	2563.34	6.507
	CARV	10.035	9.198	1.149	4277.44	3.863
	DIAZ	11.387	10.572		4482.33	
1.0	FURO	1.702	1.163	1.674	575.50	3.506
	CARB	4.486	4.700	2.004	2126.91	5.977
	CARV	8.199	9.418	1.142	3448.01	1.675
	DIAZ	9.250	10.753		3505.04	
1.2	FURO	1.468	1.238	1.714	641.48	3.857
	CARB	3.786	4.771	1.987	2584.74	6.402
	CARV	6.876	9.482	1.143	3910.50	1.772
	DIAZ	7.764	10.835		3771.56	
1.5	FURO	1.189	1.265	1.644	622.06	3.355
	CARB	3.080	4.867	2.011	2250.60	6.288
	CARV	5.664	9.789	1.133	3829.31	1.646
	DIAZ	6.349	11.093		3614.20	
1.8	FURO	1.009	1.009	1.638	597.30	3.268
	CARB	2.599	2.599	2.016	2168.42	6.238
	CARV	4.796	9.198	1.130	3765.67	1.611
	DIAZ	5.363	10.572		3662.45	

3-2-3 Effect of organic modifier

Generally, organic solvent is added to pure water as organic modifier when it is used as mobile phase on RP- chromatographic columns. The most common organic modifiers used to this purpose are methanol, acetonitrile, and to less extent,

tetrahydrofuran[137]. The choice of the mobile phase modifier is generally simple in RPHPLC. Usually acetonitrile or methanol is the preferred choice [138].

Methanol and acetonitrile were tried as organic modifiers to affect the retention mechanism of the studied drugs. The preliminary results show a significant variation in the obtained chromatograms when the mobile phase composition was changed from methanol : H_2O : Na_2HPO_4 buffer 0.1M pH=11 (50: 45: 5) to acetonitrile : H_2O : Na_2HPO_4 buffer 0.1M pH=11 (50: 45: 5). Using acetonitrile as a modifier gave much more reasonably acceptable separation. This could be due to the fact that when acetonitrile is used as organic modifier, faster separation method could develops because the viscosity of acetonitrile-aqueous mixtures is approximately 2.5 times lower than the corresponding methanol-water mixtures [139]. Furthermore, acetonitrile has a little ability to form weak hydrogen bond but has usually a larger influence on the solute solvation, in comparison to methanol. The high dipole-moment of acetonitrile enables this solvent to participate in selective dipole-dipole interaction with certain solutes [140].

The relative proportion of acetonitrile as organic modifier in the mobile phase was changed to decide the optimum value. For this purpose, different proportions of acetonitrile : H_2O: (0.1M) Na_2HPO_4 buffer (viz; **30** : **62.3** : **7.7**, **40** : **53.4** : **6.6**, **50** : **45** : **5**, **60** : **35.6** : **4.4**, **70** : **26.7** : **3.3**) were examined by inspection the values of chromatographic parameters and the bands shape of the obtained chromatograms, Table 3-2 and Figure 3-5.

Usually in RP-HPLC, when the percentage of the more polar component of the mobile phase is increased the retention time tends to increase[133]. It was found that the best separation was obtained when the modifier percent in the mobile phase ranged between 50 – 60 %. On the other hand, although increasing the percent of acetonitrile above these values tend to decrease the analysis time and increase the

peaks height but the resolution and the baseline start to degrade, Figure 3-6. Therefore, a mobile phase with 50% organic modifier was chosen for subsequent work since it gave acceptable resolution values and reasonable analysis time.

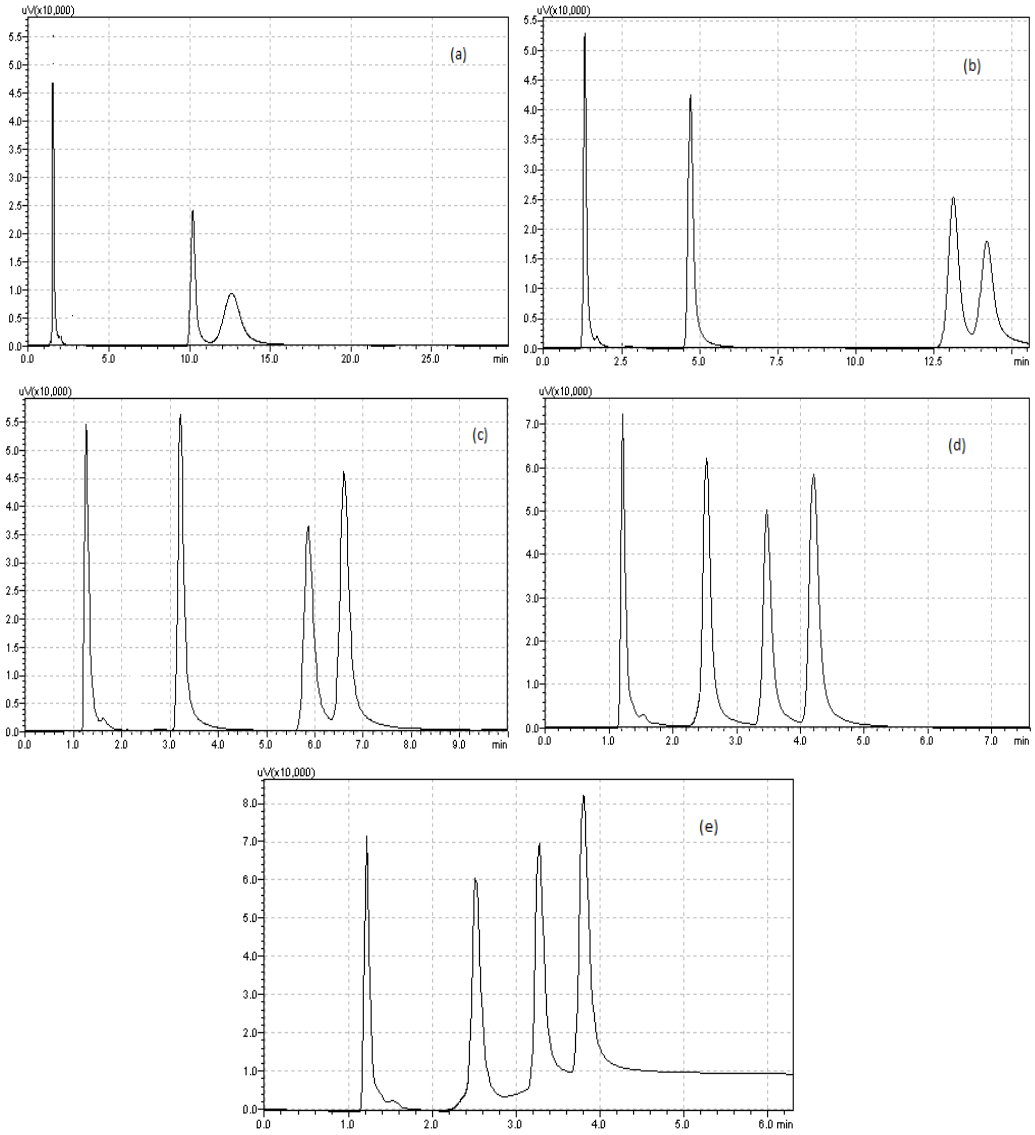


Figure 3-5: Chromatograms of the mixture at different percentage of acetonitrile (a) 30 : 62.3 : 7.7, (b) 40 : 53.4 : 6.6, (c) 50 : 45 : 5, (d) 60 : 35.6 : 4.4 and (e) 70 : 26.7 : 3.3

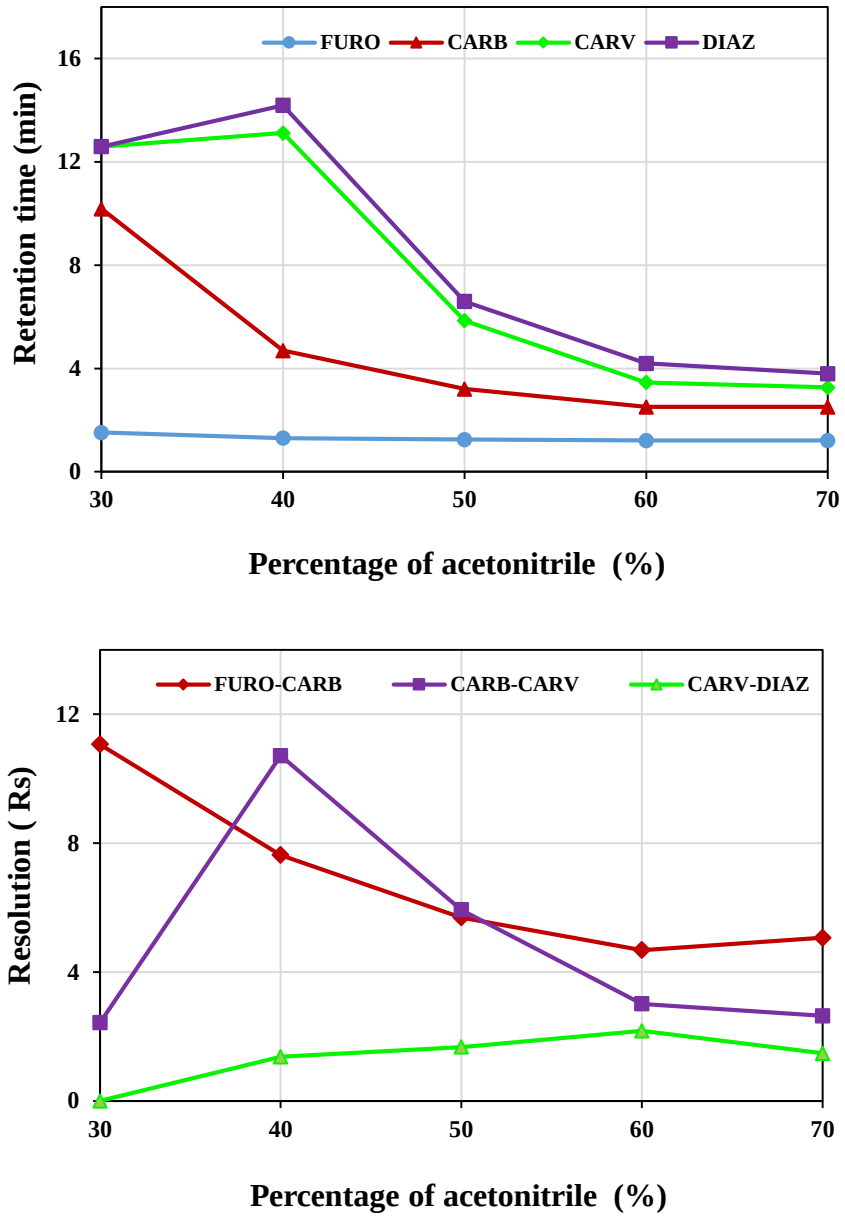


Figure 3-6: The relationship between t_R and R_s with the acetonitrile percentage.

Table 3-2: Separation parameters for each drug at different percent of acetonitrile.

% ACN	Drug	t_R	k'	α	N	R_s
30:62.3:7.7	FURO	1.523	1.901	9.676	842.00	11.069
	CARB	10.182	18.394	1.250	4579.42	2.429
	CARV	12.592	22.985	1.000	573.25	0.000
	DIAZ	12.592	22.985		573.25	
40:53.4:6.6	FURO	1.303	1.482	5.356	635.28	7.632
	CARB	4.692	7.937	3.022	2937.82	10.715
	CARV	13.117	23.985	1.085	5968.12	1.367
	DIAZ	14.188	26.025		4518.45	
50:45:05	FURO	1.248	1.377	3.707	624.63	5.693
	CARB	3.205	5.105	1.987	2156.88	5.932
	CARV	5.851	10.145	1.140	3348.49	1.671
	DIAZ	6.595	11.562		3668.92	
60:35.6:4.4	FURO	1.209	1.303	2.908	992.20	4.680
	CARB	2.514	3.789	1.475	1608.53	3.015
	CARV	3.459	5.589	1.251	2288.79	2.176
	DIAZ	4.196	6.992		2620.63	
70:26.7:3.3	FURO	1.211	1.307	2.899	1061.55	5.065
	CARB	2.514	3.789	1.380	1993.90	2.640
	CARV	3.269	5.227	1.193	2187.12	1.476
	DIAZ	3.799	6.236		1397.12	

3-2-4 Effect of buffer percentage

The pH, of the mobile phase, is usually controlled by using a suitable buffer to affect the separation of such analytes [21, 141]. The amount of buffer used in the preparation of mobile phase and its pH are very important factors that controls the chromatographic results [142, 143]. Reasonable separation of drugs mixture usually achieved by making a proper mixture containing component of different polarities to modify the distribution of the drugs in the mixture between the none-polar stationary phase and the mobile phase (namely acetonitrile - phosphate buffer). On the other hand, the buffering capacity may alter with changing the percentage of organic modifier. Therefore, using mobile phase containing high percent of buffer may overcome this phenomenon and better resolution with more symmetrical peaks could be obtained [144].

Different percent (0.5 – 6.5 %) of phosphate buffer were used in preparation of the mobile phase. Figures (3-7 and 3-8) and Table 3-3 illustrates the effect of buffer percentage on the bands shape and the chromatographic parameters. The best separation with acceptable analysis time was attained when the phosphate buffer percent in the mobile phase was 2.5%; therefore, this composition of mobile phase was used for following work.

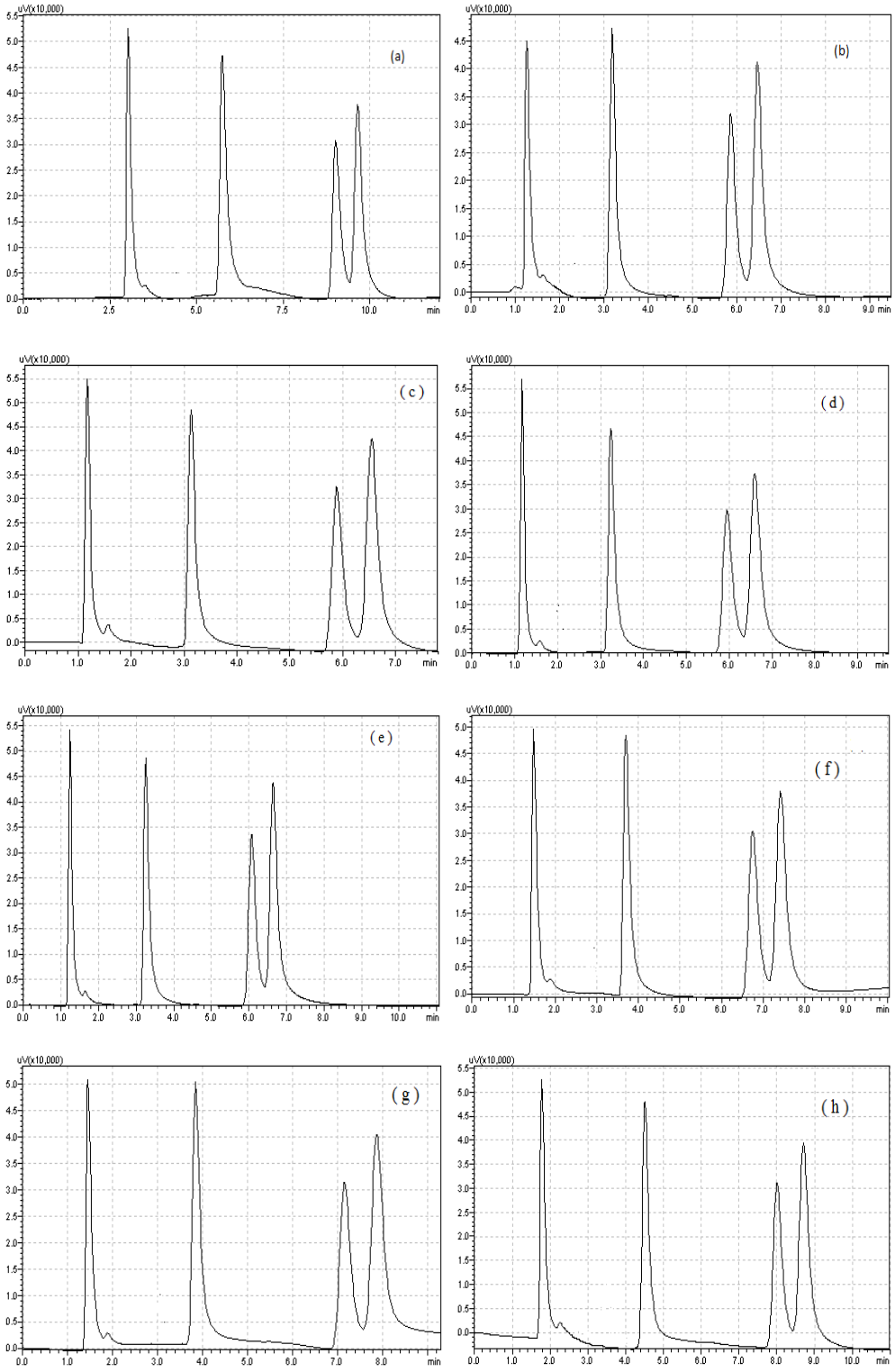


Figure 3-7: Chromatograms of the mixture at different percent of buffer (a) 0.5 %, (b) 1.0 %, (c) 1.5 %, (d) 2.0 %, (e) 2.5 %, (f) 3.5 %, (g) 5.0 % and (h) 6.5 %.

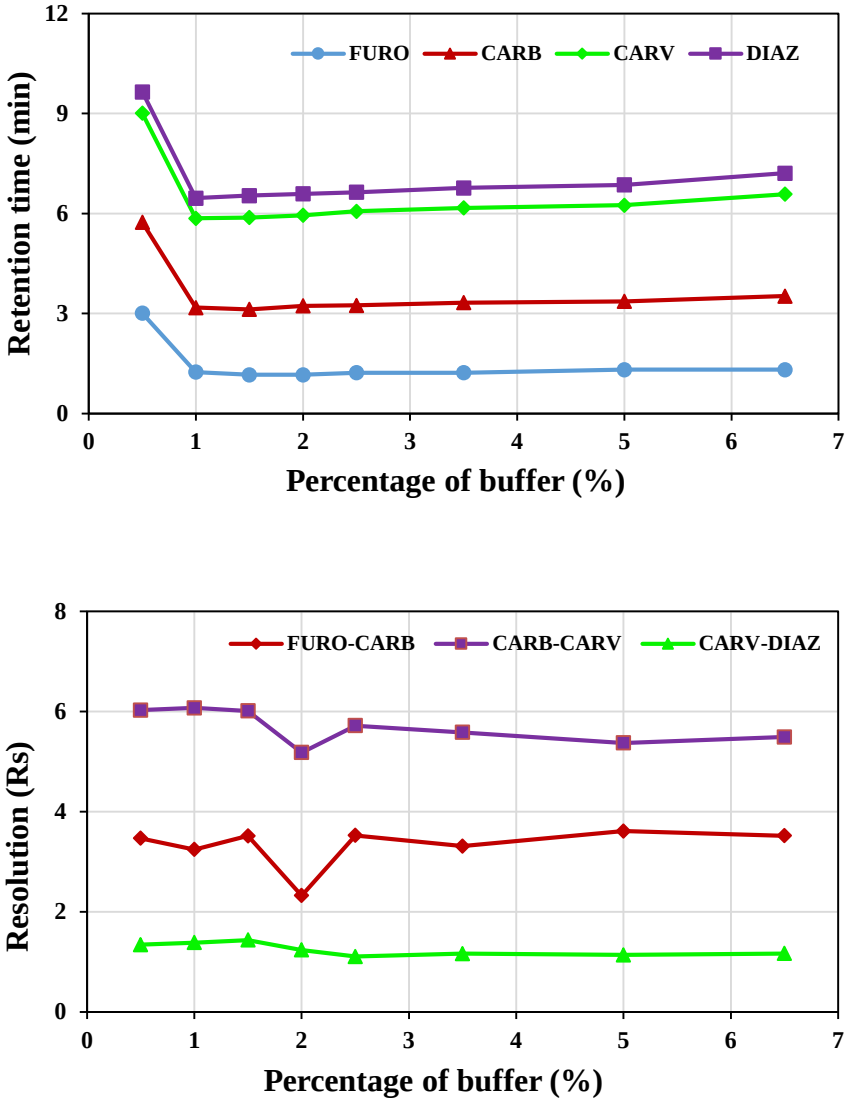


Figure 3-8: The relationship between t_R and R_s with the phosphate buffer percentage.

Table 3-3: Separation parameters of each drug at different buffer percentage**.**

% Buffer	Drug	t_R	k'	α	N	R_s
0.5	FURO	3.007	4.728	2.099	1625.71	5.002
	CARB	5.734	9.922	1.628	1914.27	6.027
	CARV	9.007	16.156	1.075	6887.84	1.342
	DIAZ	9.644	17.370		6323.28	
1.0	FURO	1.244	1.370	3.687	352.39	5.132
	CARB	3.176	5.050	2.011	1925.08	6.072
	CARV	5.856	10.154	1.113	3708.54	1.381
	DIAZ	6.460	11.305		3275.97	
1.5	FURO	1.162	1.213	4.082	540.46	5.501
	CARB	3.125	4.952	2.059	1914.07	6.009
	CARV	5.878	10.196	1.123	3353.87	1.434
	DIAZ	6.538	11.453		3101.89	
2.0	FURO	1.159	1.208	4.268	535.87	4.873
	CARB	3.231	5.154	2.003	1311.92	5.179
	CARV	5.944	10.322	1.119	2808.08	1.235
	DIAZ	6.590	11.552		2272.56	
2.5	FURO	1.225	1.333	3.884	514.30	5.199
	CARB	3.244	5.179	2.038	1718.82	5.717
	CARV	6.067	10.556	1.103	3111.62	1.105
	DIAZ	6.637	11.642		2187.61	
3.5	FURO	1.222	1.328	4.016	471.37	4.949
	CARB	3.324	5.331	2.016	1488.87	5.582
	CARV	6.168	10.749	1.106	3201.62	1.161
	DIAZ	6.765	11.886		2339.55	
5.0	FURO	1.313	1.501	3.604	454.06	3.993
	CARB	3.365	5.410	2.015	1370.59	5.371
	CARV	6.247	10.899	1.107	2966.85	1.135
	DIAZ	6.860	12.067		2200.08	
6.5	FURO	1.313	1.501	3.806	429.90	3.928
	CARB	3.524	5.712	2.019	1252.99	5.489
	CARV	6.579	11.531	1.104	3218.87	1.164
	DIAZ	7.210	12.733		2439.86	

3-2-5 pH of buffer:

The mobile phase pH plays an important role in determining the retention of pharmaceutical compounds that contain ionizable functional groups. Normally, the ionized form of an analyte does not retained very much by hydrophobic column packing and therefore has significantly lower k' than the non-ionized neutral form.

The investigated compounds contain ionizable functionalities (viz: amine, amide, carboxyl, sulfonamide, hydroxyl) groups. Therefore, the pH of mobile phase is one among those factors that mostly used to control HPLC retention of such compounds and must be optimized[145].

The mobile phase pH within the recommended values of the column manufacturer generally provides stable conditions for retention versus small changes in pH, and this pH range is recommended for starting method development with most samples, including basic compounds and typical weak acids[146]. The packing of the used NUCLEODUR® 100-5 C18 ec column is stable over a wider pH range up to about pH 10, which allows a wide scope of mobile-phase pH as a retention and selectivity adjustment parameter.

To recognize the influence of mobile phase pH on retention of the studied drugs, the pH of phosphate buffer was varied from 9.5 – 12, and keeping other separation conditions constant. Figures 3-9, 3-10 and Table 3-4 show that there was no significant influence on the chromatographic behaviors of the compounds under investigation in the studied range of pH. This could be attributed to the fact that within this interval of mobile phase pH values no change in the ionization behavior of the mentioned drugs was happened (i.e. the ionized and non-ionized forms of the drugs remain unchanged with changing the buffer pH). Phosphate buffer with pH = 11 was chosen because

since it gave well defined chromatographic bands with reasonable analysis time and used for subsequent work.

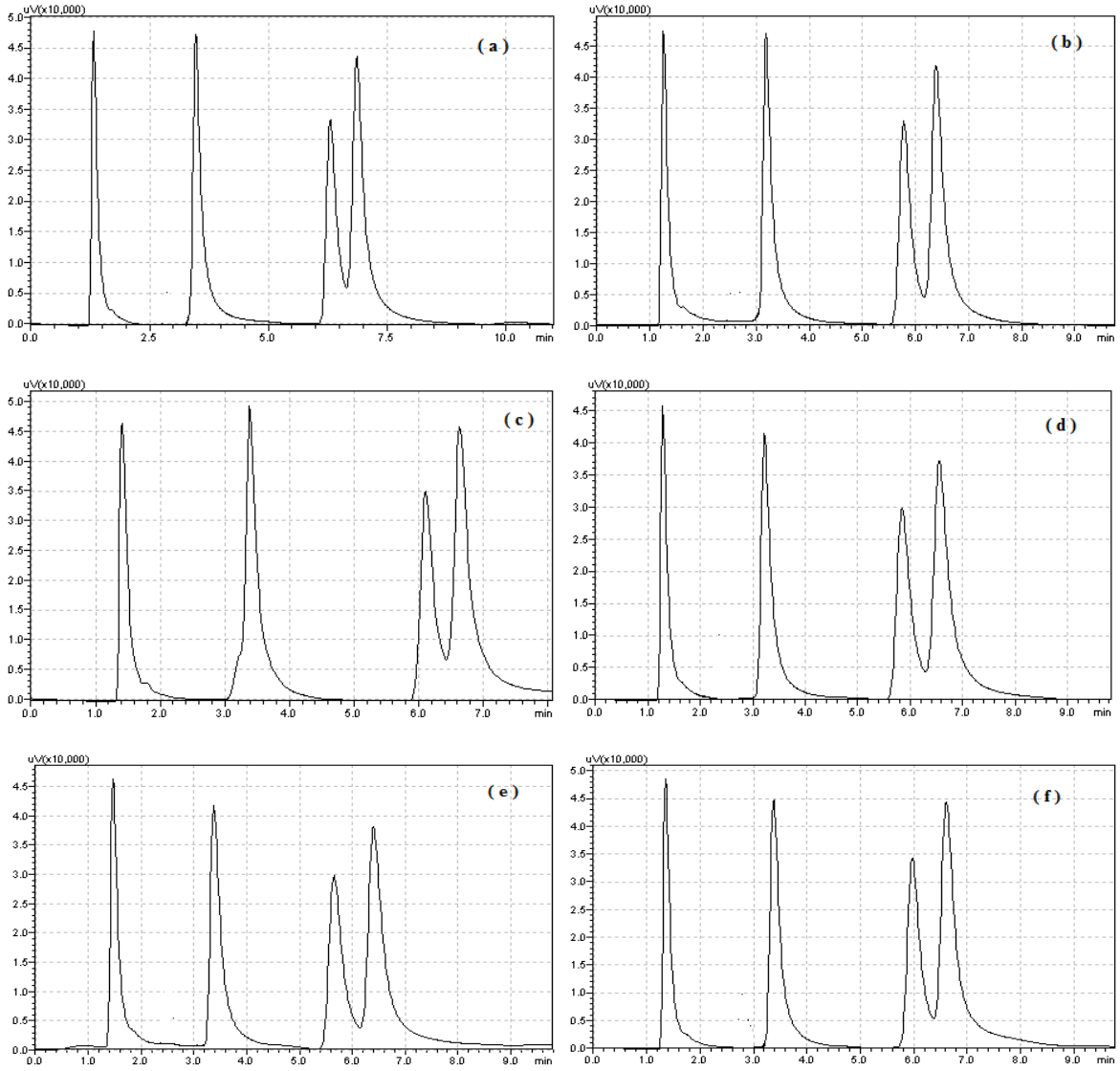


Figure 3-9: Chromatograms of the mixture at different pH [a] 9.48, [b] 10.04, [c] 10.53, [d] 11.0, [e] 11.49 and [f] 11.98.

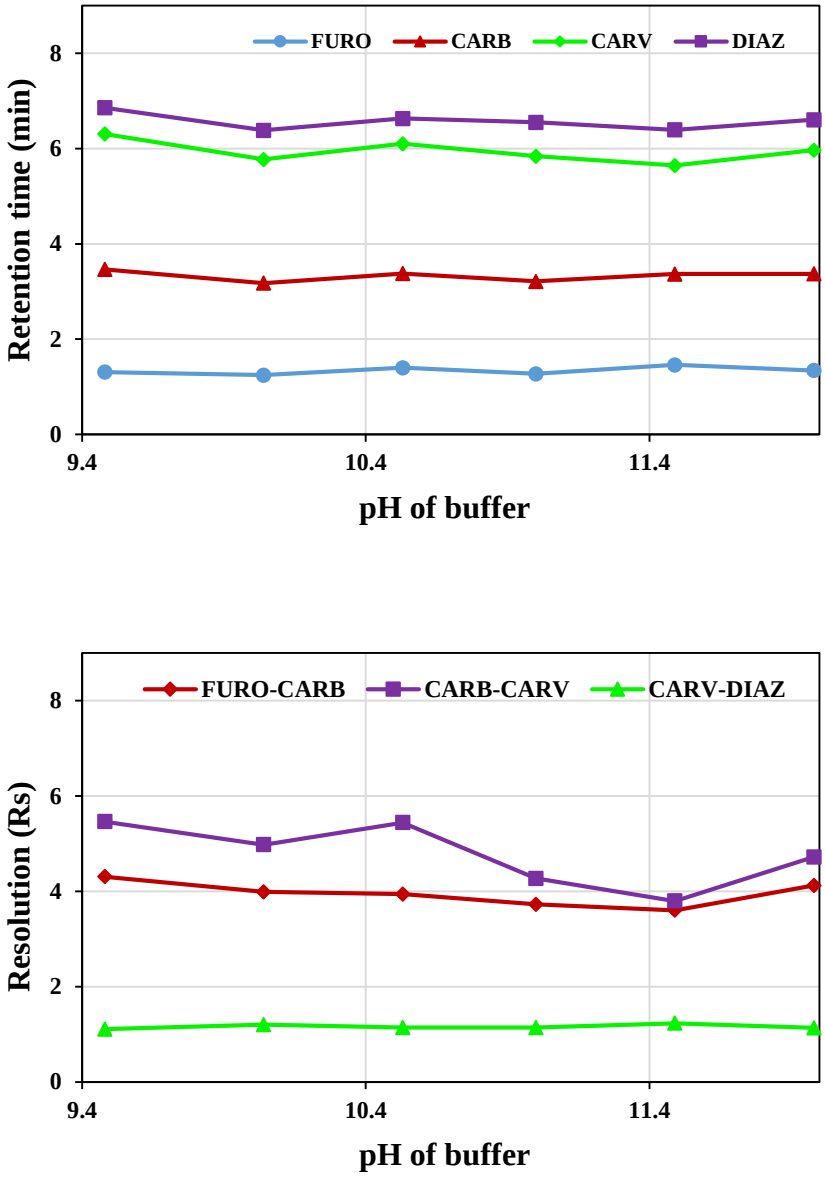


Figure 3-10: The relationship between t_R and R_s with the pH of buffer.

Table 3-4: Separation parameters of each drug at different pH of the buffer.

pH	Drug	t_R	k'	α	N	R_s
9.48	FURO	1.310	1.495	3.744	377.24	4.307
	CARB	3.464	5.598	1.967	1157.75	5.461
	CARV	6.305	11.010	1.096	3543.23	1.107
	DIAZ	6.857	12.061		2504.41	
10.04	FURO	1.247	1.375	3.672	323.08	3.988
	CARB	3.176	5.050	1.979	1056.61	4.978
	CARV	5.771	9.992	1.116	2864.61	1.201
	DIAZ	6.382	11.156		2171.72	
10.53	FURO	1.402	1.670	3.254	390.92	3.943
	CARB	3.379	5.436	1.954	1062.25	5.443
	CARV	6.102	10.623	1.095	3698.43	1.141
	DIAZ	6.631	11.630		2843.46	
11.00	FURO	1.274	1.427	3.589	335.97	3.728
	CARB	3.213	5.120	1.977	885.04	4.270
	CARV	5.838	10.120	1.134	2000.90	1.142
	DIAZ	6.550	11.476		1528.41	
11.49	FURO	1.462	1.785	3.035	366.41	3.601
	CARB	3.369	5.417	1.800	928.72	3.796
	CARV	5.643	9.749	1.146	1911.24	1.231
	DIAZ	6.392	11.175		1617.63	
11.98	FURO	1.344	1.560	3.474	365.43	4.122
	CARB	3.370	5.419	1.913	1139.25	4.719
	CARV	5.967	10.366	1.117	2623.28	1.135
	DIAZ	6.603	11.577		1823.42	

3-2-6 Effect of temperature:

Separation temperature is one of the factors that affect chromatographic behavior of the analyte and the chromatographic separation[134]. The performance of a RPHPLC-column generally increases with increasing separation temperature while the retention time of the separated bands decrease. This may be attributed to the decrease in mobile phase viscosity which improves mass transfer and lowering the column head pressure. It is also possible that performance decreases, therefore, the separation factor can increase or decrease [147].

The effect of column temperature on the separation was checked by changing temperature to ± 5 ºC for the range (30-50 ºC). Figure 3-11 depicts the obtained chromatograms, while Figure 3-12 shows that increasing columns temperature leads to an increase in the resolution of the method, but the column efficiency for most analytes reduced up to 40 ºC. This may be due to the fact that the change in temperature differently affect the analyte mass transfer and its longitudinal diffusion through the column i.e. lowering the first factor and increasing the second one and the resultant is poorer column efficiency. Consequently, for columns operating at elevated temperatures, the longitudinal diffusion may dominate, thus increasing the B term on the expenses the already decrease C term in van Deemeter equation [148].

The van't Hoff's plot of natural logarithm of the distribution coefficient (K_D) for the obtained results versus the inverse of absolute temperature yields a straight line (Figure 3-13). As it is obvious the values of capacity factor (k`) for the separated drugs are inversely proportional to the decreased column temperature, Table 3-5. This may be attributed to the depression of mobile phase viscosity, and to the fact that usually the exothermic behavior of these analytes partition to the stationary phase i.e. negative retention enthalpy [149, 150]. Forty centigrade was chosen to accomplish the separation process in the forthcoming works.

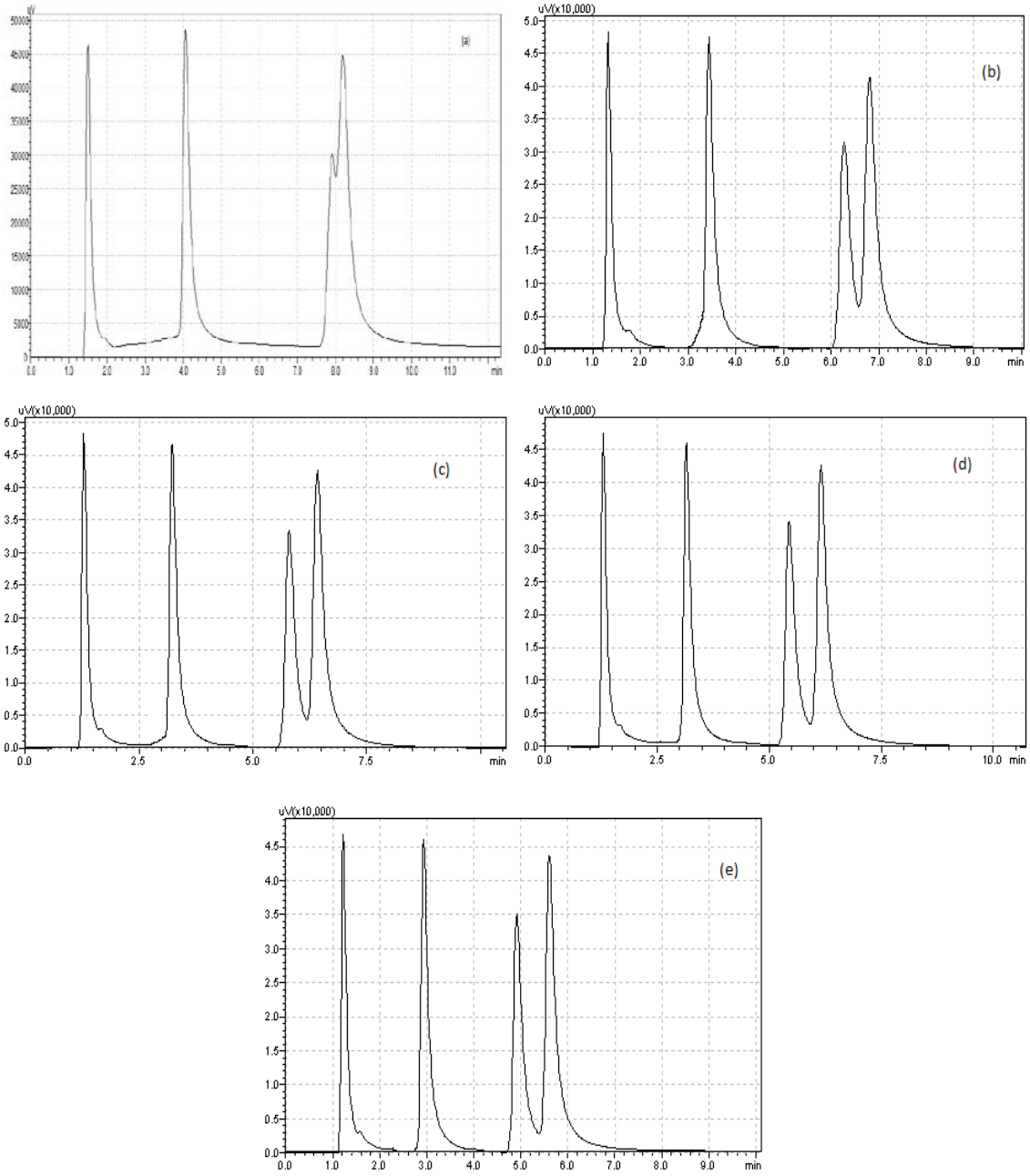


Figure 3-11: Chromatograms of the mixture at different temperatures (a) 30 ºC, (b) 35 ºC, (c) 40 ºC, (d) 45 ºC and (e) 50 ºC.

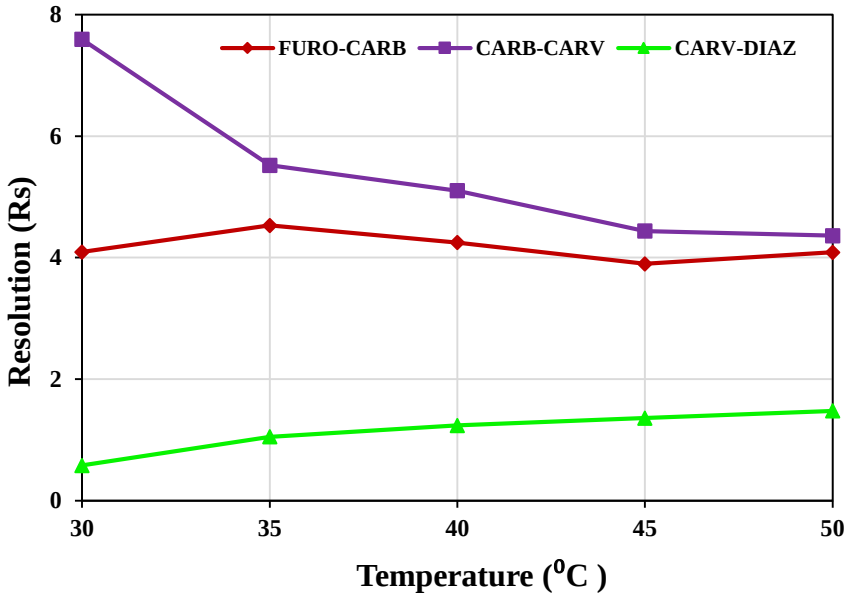


Figure 3-12: The plot of Rs as a function of column temperature for each drug.

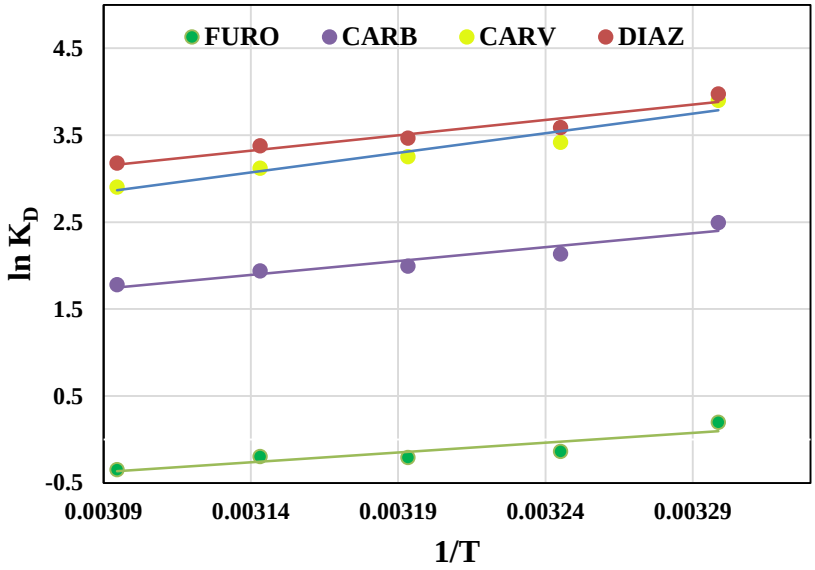


Figure 3-13: The van't Hoff's plot for the exothermic drug retention.

Table 3-5: Separation parameters of each drug at different temperatures**.**

Temp °C	Drug	t_R	k'	α	N	R_s
30	FURO	1.489	1.836	3.658	406.98	4.094
	CARB	4.051	6.716	2.093	933.81	7.592
	CARV	7.906	14.059	1.038	6824.41	0.580
	DIAZ	8.188	14.596		2265.77	
35	FURO	1.310	1.495	3.699	372.28	4.531
	CARB	3.429	5.531	1.977	1348.14	5.522
	CARV	6.267	10.937	1.093	3409.16	1.051
	DIAZ	6.802	11.956		2299.77	
40	FURO	1.276	1.430	3.585	367.16	4.247
	CARB	3.217	5.128	1.956	1218.42	5.101
	CARV	5.791	10.030	1.118	2995.98	1.238
	DIAZ	6.410	11.210		2264.15	
45	FURO	1.281	1.440	3.455	326.60	3.897
	CARB	3.137	4.975	1.881	1061.90	4.438
	CARV	5.438	9.358	1.145	2458.56	1.361
	DIAZ	6.148	10.710		1985.32	
50	FURO	1.212	1.309	3.491	355.05	4.088
	CARB	2.923	4.568	1.828	1206.07	4.362
	CARV	4.908	8.349	1.157	2516.11	1.051
	DIAZ	5.598	9.663		2080.56	

3-2-7 Effect of injection volume:

In HPLC, many experimental factors (such as instrument type, detector's sensitivity, column-loading capacity, and the effect of sample solvent on peak shape) affect the volume of injected sample [(151)]. Moreover, the chromatographic detection limits of the separated analytes are functions of injection volume. Generally, using small volumes of samples leads to prolonging the lifetime of column and resulting in well-defined narrower peaks [(152)]. On the other hand, when very small volumes of samples are utilized; problems concerning reproducibility, sensitivity of the detector

or even sample loss in the chromatographic column may arise, while larger volumes of samples than that required can degrade the chromatographic peak shape [28].

Injections of sample with different volumes were made at 40 °C via an automatic sample injection valve in the range 2.5 µL to 20 µL to find the optimum value. Figure 3-14 shows clearly that this factor significantly affects the peaks criteria i.e. retentions, areas, heights, and shapes. Therefore, adequate volume of sample should be used so the peak area of the smallest concentration is easily measured taking in the account the other criteria [150]. Consequently; 10 µL was chosen to be the best results, Table 3-6.

Figure 3-15 shows plots of the measured peak area and peak height for the chromatographic band of each drug against the injected sample volume. All plots represent strong linear relationships with correlation coefficient values ranging between 0.9928-0.9990 and 0.9961-0.9990 for peak area and peak height respectively.

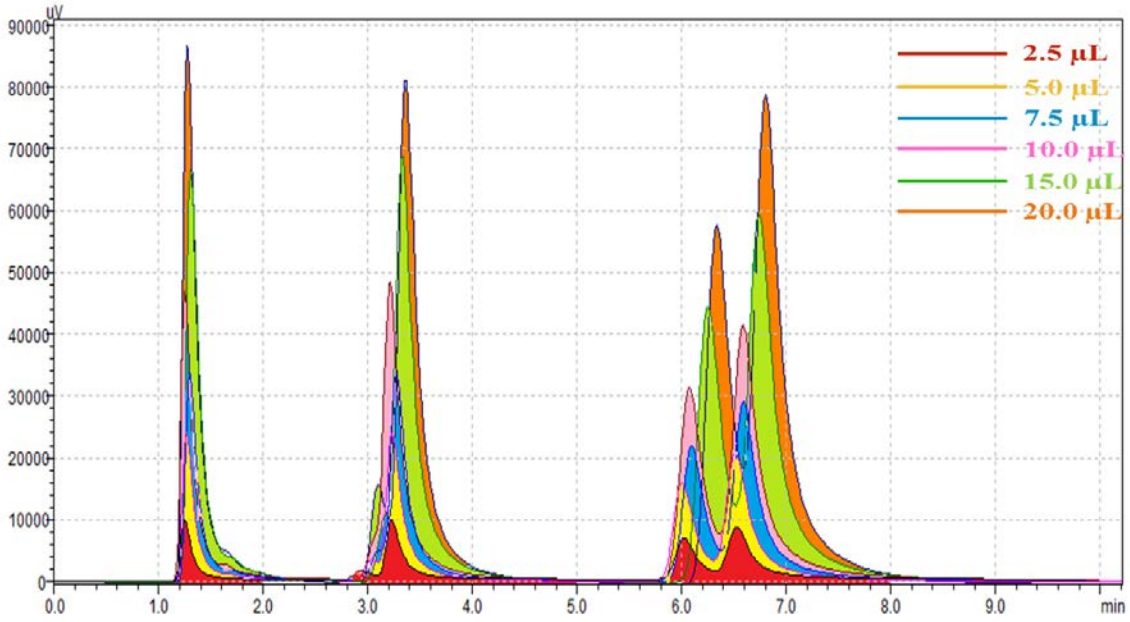


Figure 3-14: Overlaid chromatograms of the drugs mixture at different injection volumes (2.5-20 µL).

Table 3-6: Separation parameters of each drug at different injection volumes

Injection volume (µL)	Drug	t_R	Peak area (µv.s)	Peak height (µv)
2.5	FURO	1.242	92544	9944
	CARB	3.219	131468	9814
	CARV	6.016	96346	6490
	DIAZ	6.519	151544	8351
5.0	FURO	1.261	237669	24140
	CARB	3.224	354454	24194
	CARV	5.996	243629	15899
	DIAZ	6.507	429405	20862
7.5	FURO	1.289	339456	33525
	CARB	3.264	496671	33742
	CARV	6.087	341405	22038
	DIAZ	6.582	603319	29135
10.0	FURO	1.243	471876	47049
	CARB	3.203	735861	48568
	CARV	6.064	509318	31258
	DIAZ	6.578	901665	41429
15.0	FURO	1.297	727605	66743
	CARB	3.319	1101459	69067
	CARV	6.242	721574	44652
	DIAZ	6.731	1260575	59561
20.0	FURO	1.265	935875	87053
	CARB	3.349	1304216	81737
	CARV	6.326	946102	58310
	DIAZ	6.794	1737263	79453

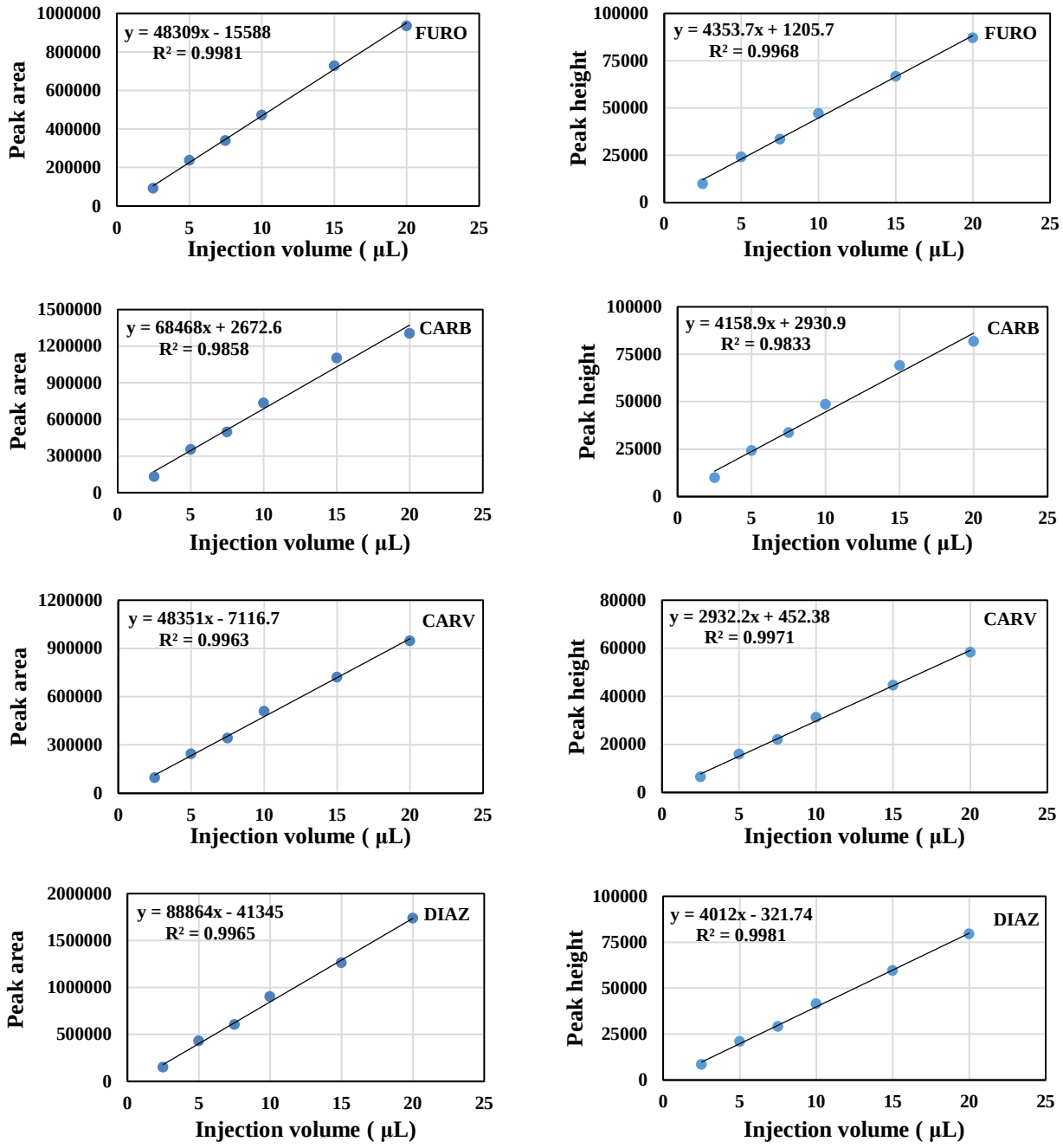


Figure 3-15: The relationship between peak area and peak height as a function of injection volume for each drug.

3-2-8 Construction of calibration curve

Under the studied experimental conditions, linearity for each drug was tested by injecting FURO, CARB, CARV, and DIAZ mixtures containing drugs in the concentration range 0.5-20 µg.mL^{-1}. Figure 3-16 shows the overlaid chromatograms of the four drugs. Separated calibration plots were constructed for all four drugs by plotting the peak area and peak height vs. concentrations as shown in Figure 3-17.

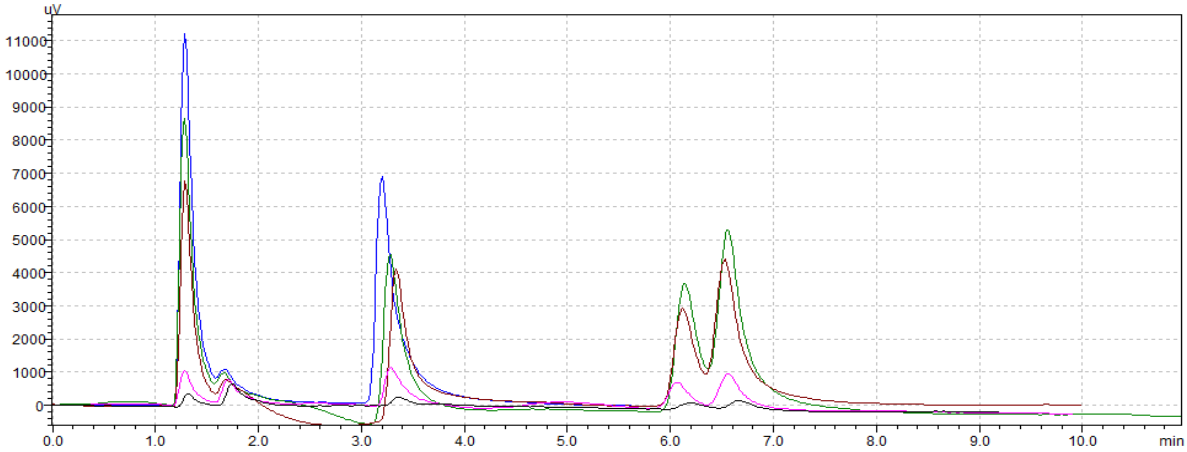
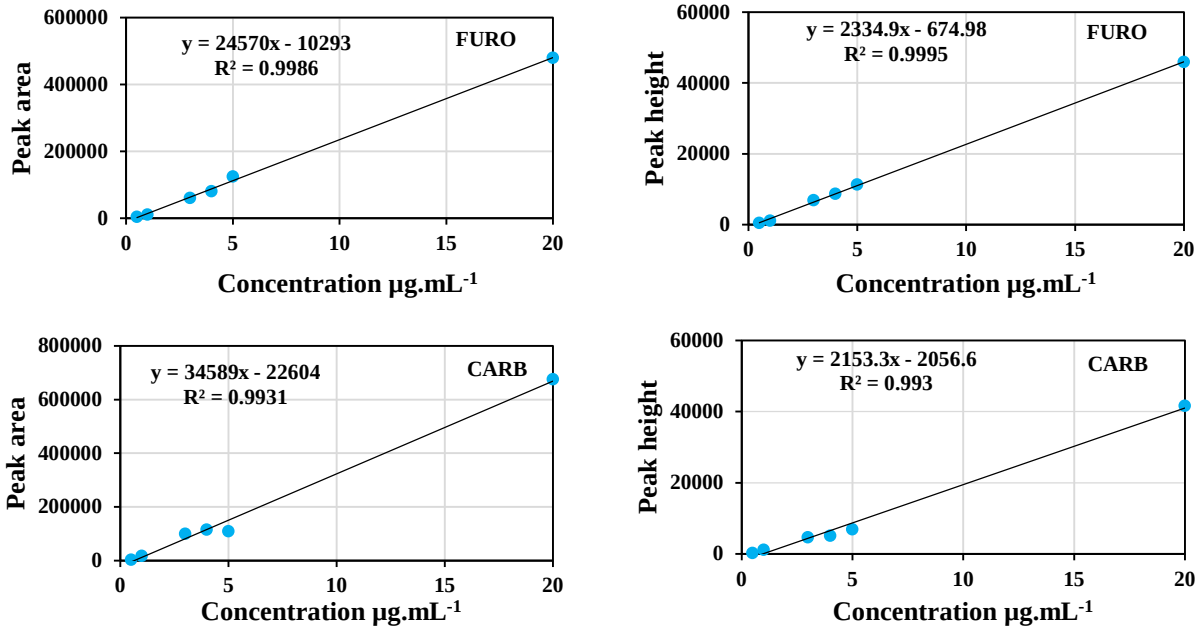


Figure 3-16: Overlaid chromatograms of the four drugs at five concentrations (0.5-5 µg.mL^{-1}).



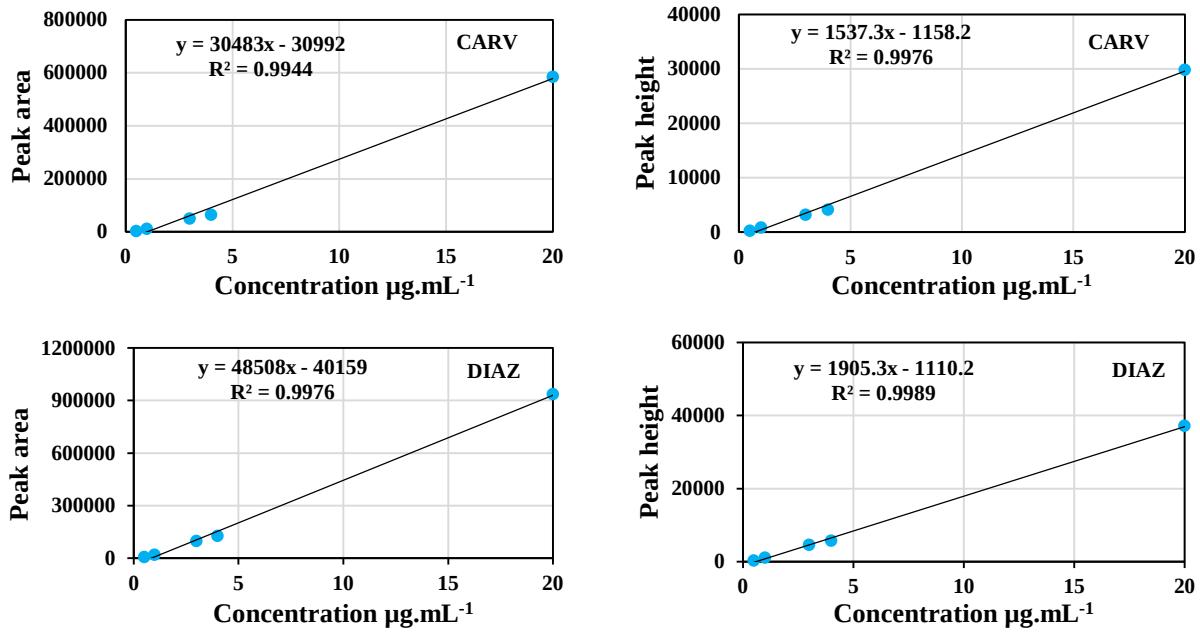


Figure 3-17: Calibration curves, concentration (µg.mL^{-1}) vs. peak area and peak height for FURO, CARB, CARV and DIAZ.

Although, the obtained results were resonably accepted but after this period of work a problem arise where the back pressure of the column starts to increase so much that it was not possible to conduct analyses and the column starts to clog. Therefore, different concentrations of buffer solution (viz: 0.1M, 0.005M and 0.0025M) and even different buffer percenct were tried, after rinsing the column with water to reduce the pressure to its normal value, Figure 3-18, but the problem presist i.e. the column pressure in each case start to increase and remain above the higher pressure limit 25 MPa. This may be due to the fact that the solubility of inorganic buffer may limited in mobile phases which are predominantly organic, so there is a danger of buffer precipitation, which could lead to blockage of the HPLC column. Moreover, above pH 8, the silica backbone becomes increasingly soluble, therfore, phosphate buffer should be avoided, because it enhances silica dissolution at high pH [28, 153].

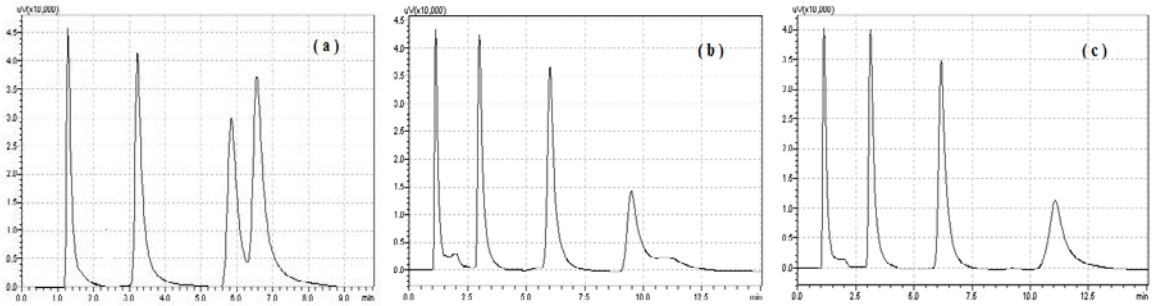


Figure 3-18: Chromatograms of the drugs mixture at buffer concentration: (a) 0.1M, (b) 0.005 M, and (c) 0.0025 M.

To overcome this problem, the pH of mobile phase was adjusted with ammonium buffer to pH 8.50 and the drug mixture was eluted using both isocratic and gradient mode of elution. Gradient elution program was carried out with aqueous 0.005 M ammonium buffer pH 8.50 (Solvent A) and pure acetonitrile (Solvent B), according to the following program. Figure 3-19.

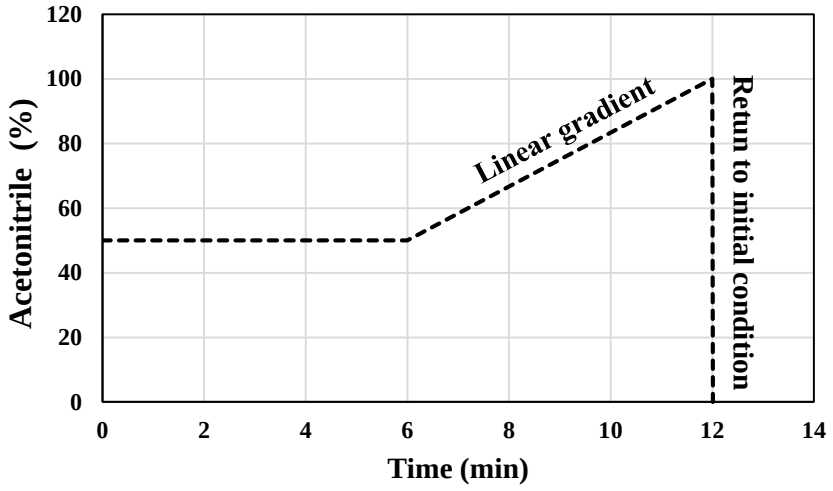


Figure 3-19: The gradient profile used for separation of drugs.

Figures 3-20 and 3-21 show the overlaid chromatograms for three replicates obtained under both modes of elution. The order of eluted drugs was FURO, CARB, DIAZ and CARV respectively.

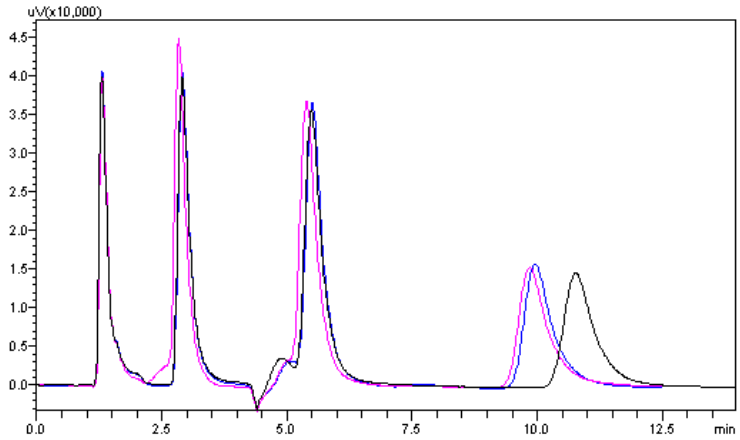


Figure 3-20: Overlaid Isocratic elution of the studied drugs using (50:50) ACN: 0.005M ammonium buffer pH 8.53.

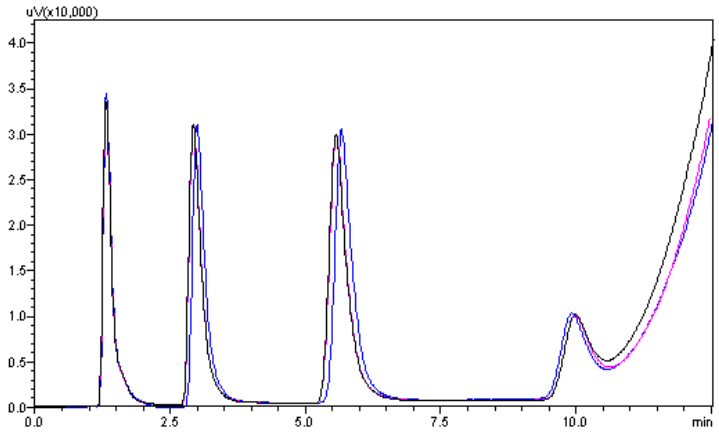


Figure 3-21: Gradient elution of the studied drugs using 0.005M ammonium buffer pH 8.53 (Solvent A) and ACN (Solvent B).

Even though, using ammonium buffer improve, to some extent the performace of the column i.e. minify the problem of column pressure elevation, the obtained chromatograms loss their reproducibility especially for the CARV band in the case of isocratic elution while with gradiant elution the base-line starts to degrade near the

end of elution. Therefore, to increase the column performace and to maintain the pressure unchanged during the separation, the pH of mobile phase was change to 3.60 by using acetic acid and trying both modes of elutions i.e isocratic and gradient.

When acetic acid was used to adjust the pH of mobile phase, the obtained chromatograms were improved in terms of separation performance and column back-pressure which remain unchanged and at its normal value during the period of the analysis.

Figures 3-22 and 3-23 show the chromatograms obtained via isocratic elution with mobile phase composition of 50:50 (water acidified with acetic acid; pH= 3.60: acetonitrle) and by gradient elution using mobile phases: (Solvent A) water, (Solvent B) water acidified with acetic acid; pH= 3.60, and (Solvent C) acetonitrile according to the proposed gradient profile (Figure 3-24).

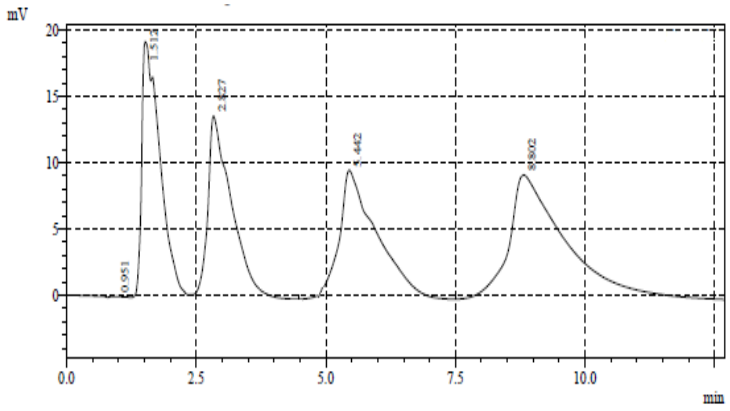


Figure 3-22: Chromatogram for separation of the four drugs under isocratic mode of elution with (50:50) water acidified with acetic acid; pH= 3.60: acetonitrile.

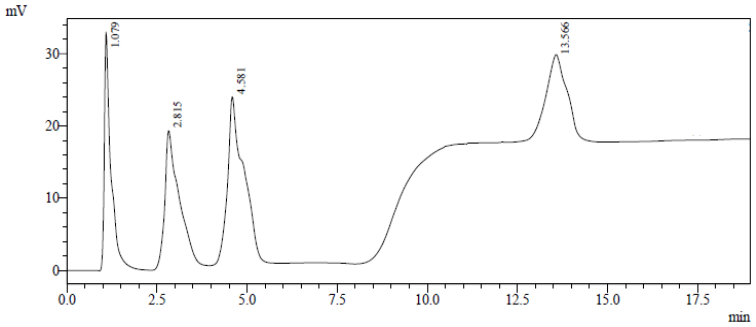


Figure 3-23: Chromatogram for separation of the four drugs under gradient elution with water acidified with acetic acid; pH= 3.60 (Solvent B), H_2O (Solvent A) and acetonitrile (Solvent C).

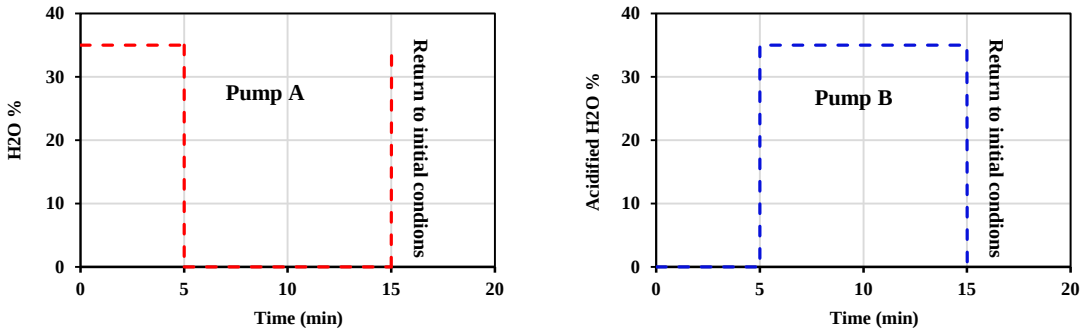


Figure 3-24: The gradient profile used for separation of drugs.

The results attaineded by following the two modes of elution were compared to each other by checking the linearities for determination of the studied drgus in their mixture in the concentration range (0.5-100) µg.mL^{-1}. Figures 3-25 and 3-26 show the overlaid chromatograms for the analysis of drgus mixture by applying gradient and isocratic modes respectively. Although the results dipicated in table 3-7 shows that both elution modes gave resonable results in terms of linearity range and correlation coefficient, the gradient mode was ruled out due to the time needed for column re-equilibrium and baseline drift on varying the mobile phase. The isocratic mode was selected for further study of the accuracy and precision of the proposed method as well as for application study. Figure 3-27 demonstrates the calibration

graphs for individual all four drugs. The regression equation, correlation coefficient, limits of detection and limits of quantification are given below in Table 3-7.

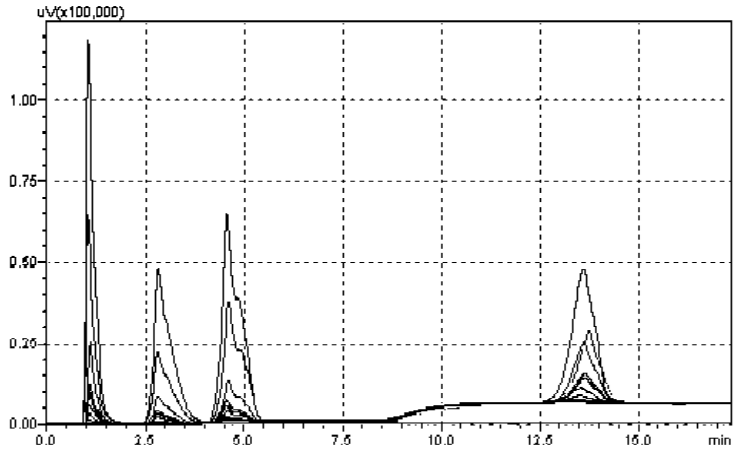


Figure 3-25: Overlaid chromatograms of the four drugs under gradient elution at different concentrations (0.5-100 µg.mL^{-1}).

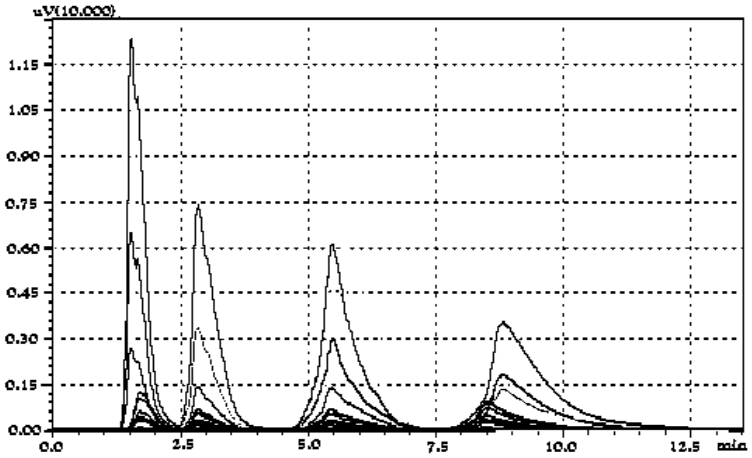


Figure 3-26: Overlaid chromatograms of the four drugs under isocratic elution at different concentrations (0.5-100 µg.mL^{-1}).

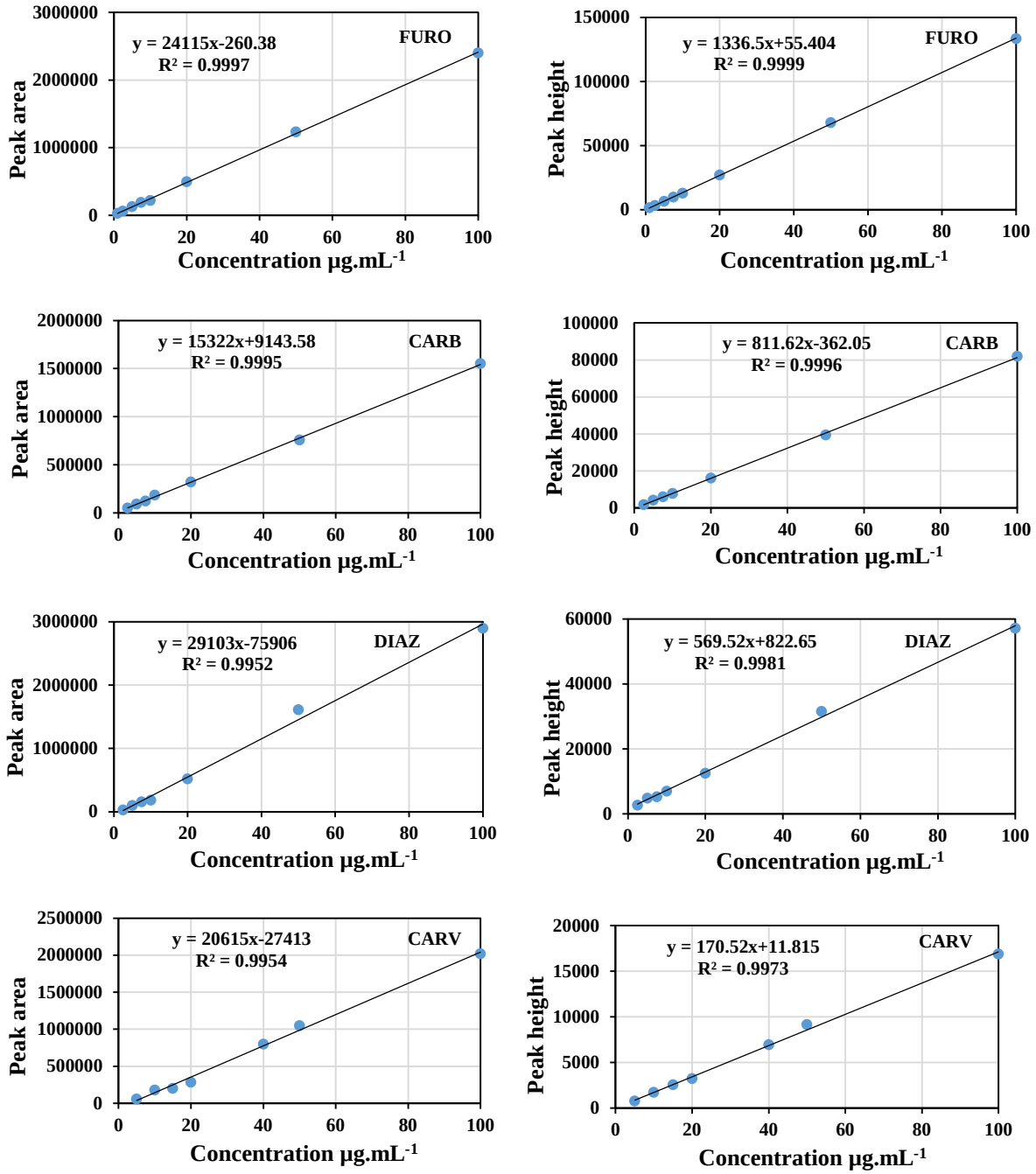


Figure 3-27: Calibration curves, concentration (µg.mL^{-1}) vs. peak area and peak height for FURO, CARB, DIAZ and CARV.

Table 3-7: Linearity parameters for FURO, CARB, DIAZ and CARV assay method.

Drug	Linearity range ($\mu g.mL^{-1}$)	Calibration curve	r	Slope	Intercept	LOD* ($\mu g.mL^{-1}$)	LOQ+ ($\mu g.mL^{-1}$)
Gradient elution							
FURO	1.5-100	Peak area	0.99935	20489	9080	0.396	1.200
		Peak height	0.99940	1493	433	0.378	1.145
CARB	2.5-100	Peak area	0.99855	23202	5978	0.634	1.921
		Peak height	0.99925	711	425	0.403	1.221
DIAZ	5-100	Peak area	0.99524	22391	77880	1.056	3.200
		Peak height	0.99685	682	1091	0.536	1.624
CARV	10-100	Peak area	0.99161	19703	708758	1.331	4.033
		Peak height	0.99393	373	4869	0.664	2.012
Isocratic elution							
FURO	1-100	Peak area	0.99985	24115	260	0.115	0.348
		Peak height	0.99995	1337	55	0.074	0.225
CARB	2.5-100	Peak area	0.99975	15322	9144	0.210	0.636
		Peak height	0.99980	812	362	0.171	0.519
DIAZ	2.5-100	Peak area	0.99760	29103	75906	0.475	1.440
		Peak height	0.99905	570	823	0.462	1.399
CARV	5-100	Peak area	0.99770	20615	27413	0.950	2.877
		Peak height	0.99880	171	12	0.528	1.599

***Detection limit = 3.3 (SD / slope)**

+Quantification limit = 10 (SD / slope)

3-2-9Accuracy and precision:

Accuracy of the proposed method was done by determining the values of relative error percentage (RE %) and precision was evaluated by calculating the values of relative standard deviation percentage (RSD %) of the obtained results for the

studied drugs. Three replicate analyses were carried out for each drug at two different levels of concentrations within the linearity range for each drug. The obtained results indicate good accuracy and precision of the recommended procedure at the studied concentration levels (Table 3-8).

Table 3-8: Accuracy and precision of the method.

Drug	Injected ($\mu g.mL^{-1}$)	Calculated Conc. from peak area			Calculated Conc. from peak height		
		Mean*	RSD%	RE%	Mean*	RSD%	RE%
FURO	20	19.9346	1.167	-0.327	20.221	0.7229	1.107
	80	81.056	0.9628	1.32	80.96	0.9732	1.2
CARB	20	19.8258	1.5263	-0.871	19.054	1.5356	-4.729
	80	78.4613	1.5018	-1.923	82.664	1.1275	3.33
DIAZ	20	19.0398	2.0011	-4.801	20.339	2.7821	1.6951
	80	79.2053	2.6548	-0.993	80.754	1.3357	0.9429
CARV	20	19.3214	3.9056	-3.393	20.981	2.2751	4.9066
	80	81.3142	3.0715	1.6428	81.93	1.9848	2.4127

*Average of three measurements.

3-2-10 Application

The quantitative determination of FURO, CARB, DIAZ and CARV in commercially available pharmaceutical preparations (tablets, syrup and ampoule) was carried out following the proposed HPLC procedure. Figure 3-28 shows the chromatograms obtained via isocratic elution with mobile phase composition of 50:50 (water acidified with acetic acid; pH= 3.60: acetonitrle) for the drugs in their pure and pharmaceutical forms. Two concentration levels for each drug were separately injected in triplicate into the HPLC system and analyzed under the optimum conditions. The obtained values of recovery percentage (Tables 3-9 and 3-10)

revealed the reliability of the recommended method for the determination of the cited drugs in their pharmaceutical formulations.

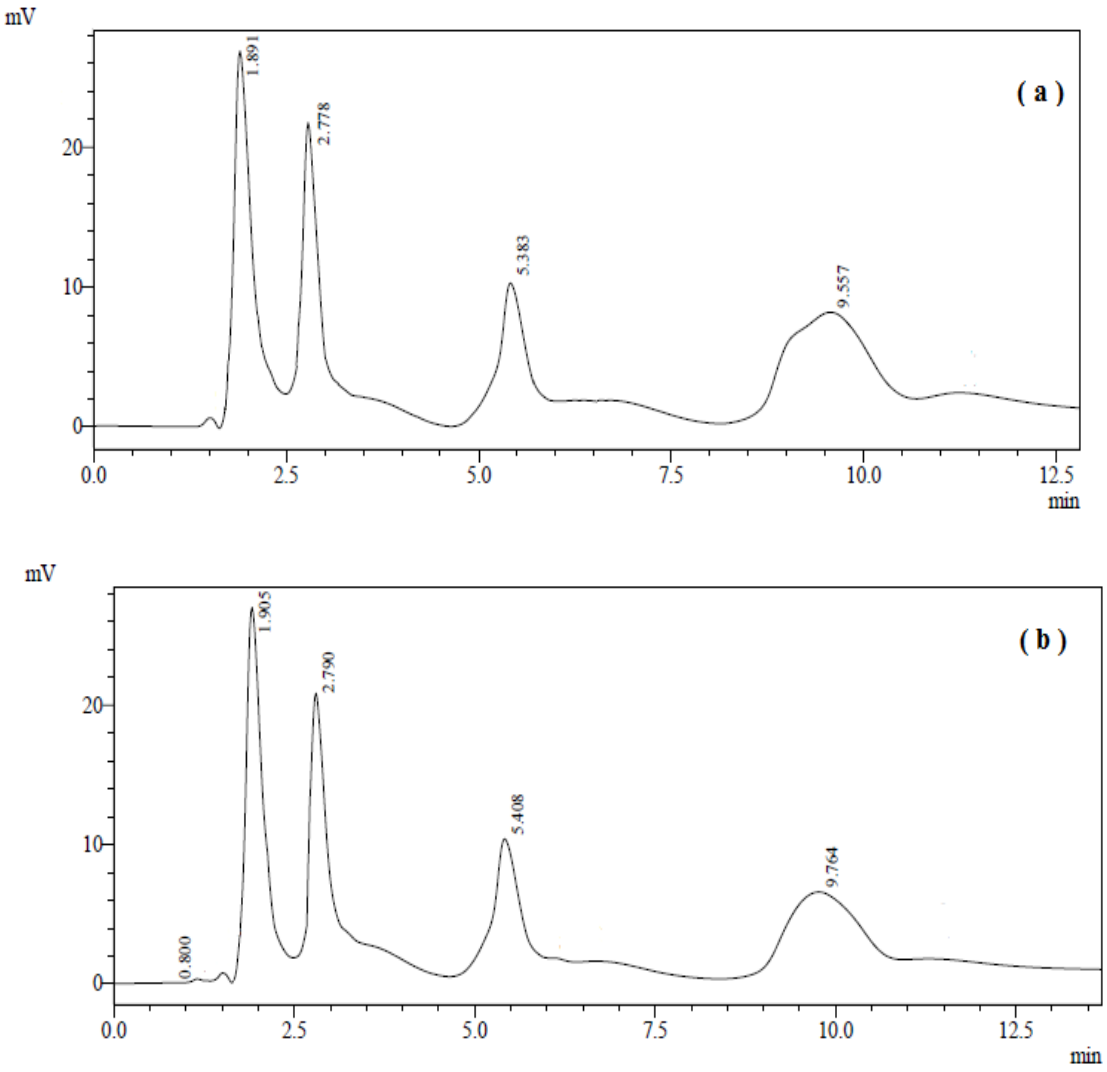


Figure 3-28: Chromatograms for separation of the four drugs under isocratic mode of elution with (50:50) water acidified with acetic acid; pH= 3.60: acetonitrile (a) Mix of standard drugs and (b) Mix of pharmaceutical formulations.

Table 3-9: Estimation of FURO, CARB, DIAZ and CARV in their pharmaceutical preparations via peak area.

Sample	Conc. ($\mu g.mL^{-1}$)		Calculated Conc. from peak area		
	Taken	Found	Weight* found (mg/dosage)	Recovery %	RSD %
FURO (Iraq) tablet 40 mg	20	21.313	42.622	106.6	1.628
	80	83.845	41.923	104.8	1.247
FURO (France) tablet 40 mg	20	20.453	40.906	102.3	1.201
	80	82.699	41.349	103.4	1.080
CARB (Cyprus) tablet 200 mg	20	19.493	194.930	97.5	1.131
	80	78.570	196.425	98.2	1.294
CARB (Switzerland) syrup 100 mg	20	19.355	96.775	96.8	1.075
	80	75.325	94.156	94.2	0.987
DIAZ (Iraq) tablet 2 mg	20	20.339	2.034	101.7	1.548
	80	78.693	1.967	98.4	1.066
DIAZ (Syria) ampoule10mg	20	20.304	10.152	101.5	1.267
	80	82.323	10.290	102.9	0.942
CARV (Syria) tablet 6.25 mg	20	21.277	6.649	106.4	2.348
	80	83.279	6.506	104.1	2.075
CARV (India) tablet 25 mg	20	18.467	23.084	92.3	2.540
	80	74.885	23.402	93.6	1.774

*Average of three measurements.

Table 3-10: Estimation of FURO, CARB, DIAZ and CARV in their pharmaceutical preparations via peak height.

Sample	Conc. (µg.mL^{-1})		Calculated Conc. from peak height		
	Taken	Found	Weight* found (mg/dosage)	Recovery %	RSD %
FURO (Iraq) tablet 40 mg	20	19.850	39.700	99.3	1.447
	80	82.408	41.204	103.0	1.070
FURO (France) tablet 40 mg	20	20.289	40.578	101.4	0.698
	80	78.004	39.002	97.5	0.681
CARB (Cyprus) tablet 200 mg	20	18.807	188.070	94.0	1.043
	80	74.803	187.008	93.5	1.237
CARB (Switzerland) syrup 100 mg	20	18.240	91.201	91.2	1.008
	80	71.024	88.780	88.8	0.946
DIAZ (Iraq) tablet 2 mg	20	21.503	2.150	107.5	1.282
	80	77.565	1.940	96.9	1.040
DIAZ (Syria) ampoule10mg	20	20.995	10.498	105.0	1.147
	80	77.480	9.685	96.9	0.931
CARV (Syria) tablet 6.25 mg	20	21.315	6.661	106.6	2.696
	80	85.010	6.641	106.3	1.680
CARV (India) tablet 25 mg	20	20.449	25.561	102.2	2.304
	80	75.070	23.460	93.8	1.307

*Average of three measurements.

CHAPTER FOUR
SIMULTANEOUS DETERMINATION OF FUROSEMIDE, CARBAMAZEPINE, DIAZEPAM & CARVEDILOL

Chapter Four

4 Simultaneous Determination of Furosemide, Carbamazepine, Diazepam and Carvedilol

4-1 Simultaneous Spectrophotometric Determination of Quaternary Mixture via Partial Least Squares Method

4-1-1 Introduction

Chemometrics multivariate procedures have an expanding significance in the analysis of multi-components systems; particularly those use the Partial Least Square (PLS) technique [154]. Partial Least Squares is a linear regression method that forms components (factors, or latent variables) as new independent variables (explanatory variables, or predictors) in a regression model. The components in this technique are determined by both the response variable(s) (q) and the predictor variables (p) by iterative calculations in view of minimum squares that execute a wide range of both informative and exploratory multivariate systems. A regression model from partial least squares can be expected to have a smaller number of components without an appreciably smaller R-square value. Herman Wold [155], who invented partial least squares (PLS) approach, conceived the nonlinear iterative least squares (NILES) calculation [156]. PLS is an algorithm for estimation, regression, and classification, whose goal is to foresee a set of dependent variables (responses) from a set of independent variables (predictors). Two PLS models of calculations are available, in the PLS1 regression (PLS univariate regression), there is only one variable to explain (q=1) and there are explanatory variables (p>1), whilst in the PLS2 regression (PLS multivariate regression) there are q variables to explain (q>1) and explanatory variables (p>1) [157]. Both chemometric techniques are useful for investigations of complex mixtures. In

light of the fact that they enables quick and simultaneous analysis of each species in the mixture, with slightest sample pre-treatments or need for pre-separation [158, 159].

4-1-2 Spectrophotometric simultaneous determination of Furosemide, Carbamazepine, Diazepam, and Carvedilol by partial least squares in their pure form

The spectrophotometric study of several species in their mixture to discriminate between them depending on differences in their spectral properties is often suffer from having overlapping spectral characteristics. Such studies in which heavily overlapping responses of the studied components are present were possible to be carry out relying on chemometrics procedures [160].

Chemometrics as a term was first used in 1971 to describe the developing utilization of mathematical and statistical models, principles, and other rationale strategies in the field of chemistry especially those used in analytical chemistry. These models were used to design experiments to optimize analytical procedures and to provide maximum chemical information by analyzing chemical data [161].

Chemometrics have got quick development and varieties of application in the field of analytical chemistry, principally due to the computerization of analytical instrumentation. Computerization gives a chance to obtain colossal amount of information on studied system. For all intents and purposes, all branches of analytical chemistry significantly influenced by chemometrics; especially after availability of commercial software that implements chemometrics methods in the field of analytical instruments[160].

- ***Literature review for the determination of the titled drugs via PLS regression method***

The pronounced advent and wide spread of the laboratory computers, has been allowed analytical researchers to use multivariate calibration methods for accurate and facile analysis of drug mixtures. PLS regression is one of the most commonly used methods, which can be performed with simply accessible statistical software, and they are currently widely used in pharmaceutical analysis [(162)]. Although there are several developed methods for the determination of FURO, CARB, DIAZ and CARV, simultaneous determination of these drugs via PLS method is not presented. However, review of literature revealed that some PLS methods have been reported for estimation of the cited drugs in pure form, pharmaceutical preparations and human biological fluids (Table 1).

Table 4-1: Analytical study of PLS regression methods for determination of FURO, CARB, DIAZ and CARV in pharmaceutical products and human fluids.

Pharmaceutical compound	Mode	Analytical technique	Application remark	Ref.
Carbamazepine	PLS-1	UV-Vis spectrophotometer	*Human plasma*	163
	PLS	NIR spectroscopy	*Synthetic mixtures*	164
	PLS	Raman spectroscopy	*Synthetic mixtures and laboratory prepared tablets*	165
Furosemide	PLS	Fluorimeter	*Synthetic mixtures*	166
	PLS-1 PLS-2	UV-Vis spectrophotometer	*Synthetic mixtures and combined tablets*	82
Carvedilol	PLS	HPLC, CE system /UV detector	*Human serum samples*	167
	PLS	UV-Vis spectrophotometer	*Pharmaceutical products*	168
Diazepam	PLS	NIR spectroscopy	*Marketed tablet*	169
	PLS	UV-Vis spectrophotometer	*Synthetic mixtures and pharmaceutical formulation*	170

4-1-3 Experimental

4-1-3-1 Instrumentation

Cecil CE7200 UV-Visible double beam spectrophotometer with 10 mm quartz cell equipped with Cecil Instrument-DataStream Software Version: 5.1, Windows 8.1 Laptop (DELL).

4-1-3-2 Chemicals

All chemicals used in this investigation are described in section 2-1.

4-1-3-3 Pharmaceutical Compounds

Table 2-2, section 2-2, illustrates the pharmaceutical compounds used and their manufactures.

4-1-3-4 Standard Drugs Solutions

The procedures used for the preparation of the standard pharmaceutical solutions which were used throughout the investigation are given in section 2-5-3.

4-1-3-5 General Recommended Procedures

Assay procedure for individual determination furosemide, carbamazepine, diazepam, and carvedilol

1.0 mL aliquots, of each drug standard solution containing 5-100 μg were transferred into a series of 5 mL volumetric flasks and diluted with methanol. The spectrum for each solution was recorded against solvent blank. The amounts of drug were computed from the standard calibration graphs.

Assay procedure for determination each drug in the presence of the others

A Simplex Lattice Mixture Design was used to prepare a set of calibration mixtures for the simultaneous determination of furosemide, carbamazepine, diazepam, and carvedilol. “The simplex lattice design is a space filling design

that creates a triangular grid of runs. The design is the set of all combinations where the factors values are (***i /m***); where ***i*** is an integer from ***zero*** to ***m*** such that the sum of the factors is ***one"*** [171].

Eighty-four mixtures combination were prepared according to optimal mixture design type (Simplex Lattice) which lets to specify the degree to which the factor combinations are made (i.e. to specify number of levels per factor) create by JMP® 11.0.0 SAS Institute Inc. software program. Table (4-2) shows the mixture design in coded values.

Aliquots, of each drug standard solution containing (0, 16.5, 33.5, 50.0, 66.5, and 83.5 μg), were transferred into a series of 5 mL volumetric flasks containing (0, 16.5, 33.5, 50.0, 66.5, and 83.5 μg of the other drugs). Each mixture was then diluted to the mark with methanol (Table 4-3). The spectrum for each solution was recorded for wavelength range of 200-350 nm against methanol as a blank.

Table 4-2: Lists of the coded runs for a Simplex Lattice of four factors for six levels mixture design.

No.	FURO	DIAZ	CARB	CARV	No.	FURO	DIAZ	CARB	CARV
1	0.1667	0.5000	0.1667	0.1667	**43**	0.0000	0.0000	0.1667	0.8333
2	0.0000	0.5000	0.1667	0.3333	**44**	0.3333	0.5000	0.1667	0.0000
3	0.1667	0.8333	0.0000	0.0000	**45**	0.3333	0.6667	0.0000	0.0000
4	0.1667	0.1667	0.0000	0.6667	**46**	0.3333	0.0000	0.0000	0.6667
5	0.5000	0.3333	0.0000	0.1667	**47**	1.0000	0.0000	0.0000	0.0000
6	0.0000	0.3333	0.1667	0.5000	**48**	0.0000	0.3333	0.0000	0.6667
7	0.0000	0.3333	0.5000	0.1667	**49**	0.1667	0.0000	0.3333	0.5000
8	0.0000	0.5000	0.3333	0.1667	**50**	0.0000	0.1667	0.6667	0.1667
9	0.6667	0.0000	0.1667	0.1667	**51**	0.0000	0.5000	0.0000	0.5000
10	0.5000	0.1667	0.0000	0.3333	**52**	0.3333	0.3333	0.1667	0.1667
11	0.8333	0.1667	0.0000	0.0000	**53**	0.3333	0.1667	0.5000	0.0000
12	0.1667	0.0000	0.8333	0.0000	**54**	0.0000	0.1667	0.0000	0.8333
13	0.3333	0.0000	0.5000	0.1667	**55**	0.0000	0.3333	0.3333	0.3333
14	0.0000	0.0000	0.0000	1.0000	**56**	0.5000	0.0000	0.5000	0.0000
15	0.6667	0.1667	0.1667	0.0000	**57**	0.5000	0.5000	0.0000	0.0000
16	0.1667	0.3333	0.5000	0.0000	**58**	0.1667	0.0000	0.0000	0.8333
17	0.1667	0.0000	0.1667	0.6667	**59**	0.0000	0.8333	0.0000	0.1667
18	0.5000	0.0000	0.3333	0.1667	**60**	0.3333	0.1667	0.0000	0.5000
19	0.8333	0.0000	0.0000	0.1667	**61**	0.8333	0.0000	0.1667	0.0000
20	0.3333	0.0000	0.3333	0.3333	**62**	0.0000	1.0000	0.0000	0.0000
21	0.0000	0.6667	0.3333	0.0000	**63**	0.6667	0.1667	0.0000	0.1667
22	0.1667	0.5000	0.0000	0.3333	**64**	0.0000	0.6667	0.1667	0.1667
23	0.3333	0.1667	0.3333	0.1667	**65**	0.5000	0.0000	0.0000	0.5000
24	0.0000	0.0000	1.0000	0.0000	**66**	0.1667	0.3333	0.3333	0.1667
25	0.0000	0.1667	0.3333	0.5000	**67**	0.1667	0.6667	0.0000	0.1667
26	0.3333	0.5000	0.0000	0.1667	**68**	0.3333	0.1667	0.1667	0.3333
27	0.6667	0.0000	0.0000	0.3333	**69**	0.1667	0.1667	0.1667	0.5000
28	0.1667	0.1667	0.5000	0.1667	**70**	0.0000	0.0000	0.3333	0.6667
29	0.1667	0.3333	0.1667	0.3333	**71**	0.1667	0.1667	0.3333	0.3333
30	0.3333	0.0000	0.1667	0.5000	**72**	0.1667	0.3333	0.0000	0.5000
31	0.5000	0.1667	0.1667	0.1667	**73**	0.3333	0.3333	0.3333	0.0000
32	0.3333	0.0000	0.6667	0.0000	**74**	0.0000	0.0000	0.5000	0.5000
33	0.1667	0.1667	0.6667	0.0000	**75**	0.0000	0.8333	0.1667	0.0000
34	0.0000	0.0000	0.8333	0.1667	**76**	0.0000	0.0000	0.6667	0.3333
35	0.6667	0.0000	0.3333	0.0000	**77**	0.1667	0.5000	0.3333	0.0000
36	0.5000	0.3333	0.1667	0.0000	**78**	0.1667	0.6667	0.1667	0.0000
37	0.0000	0.1667	0.1667	0.6667	**79**	0.0000	0.3333	0.6667	0.0000
38	0.1667	0.0000	0.5000	0.3333	**80**	0.6667	0.3333	0.0000	0.0000
39	0.0000	0.1667	0.8333	0.0000	**81**	0.5000	0.1667	0.3333	0.0000
40	0.3333	0.3333	0.0000	0.3333	**82**	0.1667	0.0000	0.6667	0.1667
41	0.0000	0.6667	0.0000	0.3333	**83**	0.0000	0.1667	0.5000	0.3333
42	0.5000	0.0000	0.1667	0.3333	**84**	0.0000	0.5000	0.5000	0.0000

Table 4-3: Uncoded six level Simplex Lattice mixture design for the composition of the calibration set used to build PLS-2 and PLS-1 models.

No.	FURO ($\mu g.mL^{-1}$)	DIAZ ($\mu g.mL^{-1}$)	CARB ($\mu g.mL^{-1}$)	CARV ($\mu g.mL^{-1}$)	No.	FURO ($\mu g.mL^{-1}$)	DIAZ ($\mu g.mL^{-1}$)	CARB ($\mu g.mL^{-1}$)	CARV ($\mu g.mL^{-1}$)
1	3.3	10.0	3.3	3.3	**43**	0.0	0.0	3.3	16.7
2	0.0	10.0	3.3	6.7	**44**	6.7	10.0	3.3	0.0
3	3.3	16.7	0.0	0.0	**45**	6.7	13.3	0.0	0.0
4	3.3	3.3	0.0	13.3	**46**	6.7	0.0	0.0	13.3
5	10.0	6.7	0.0	3.3	**47**	20.0	0.0	0.0	0.0
6	0.0	6.7	3.3	10.0	**48**	0.0	6.7	0.0	13.3
7	0.0	6.7	10.0	3.3	**49**	3.3	0.0	6.7	10.0
8	0.0	10.0	6.7	3.3	**50**	0.0	3.3	13.3	3.3
9	13.3	0.0	3.3	3.3	**51**	0.0	10.0	0.0	10.0
10	10.0	3.3	0.0	6.7	**52**	6.7	6.7	3.3	3.3
11	16.7	3.3	0.0	0.0	**53**	6.7	3.3	10.0	0.0
12	3.3	0.0	16.7	0.0	**54**	0.0	3.3	0.0	16.7
13	6.7	0.0	10.0	3.3	**55**	0.0	6.7	6.7	6.7
14	0.0	0.0	0.0	20.0	**56**	10.0	0.0	10.0	0.0
15	13.3	3.3	3.3	0.0	**57**	10.0	10.0	0.0	0.0
16	3.3	6.7	10.0	0.0	**58**	3.3	0.0	0.0	16.7
17	3.3	0.0	3.3	13.3	**59**	0.0	16.7	0.0	3.3
18	10.0	0.0	6.7	3.3	**60**	6.7	3.3	0.0	10.0
19	16.7	0.0	0.0	3.3	**61**	16.7	0.0	3.3	0.0
20	6.7	0.0	6.7	6.7	**62**	0.0	20.0	0.0	0.0
21	0.0	13.3	6.7	0.0	**63**	13.3	3.3	0.0	3.3
22	3.3	10.0	0.0	6.7	**64**	0.0	13.3	3.3	3.3
23	6.7	3.3	6.7	3.3	**65**	10.0	0.0	0.0	10.0
24	0.0	0.0	20.0	0.0	**66**	3.3	6.7	6.7	3.3
25	0.0	3.3	6.7	10.0	**67**	3.3	13.3	0.0	3.3
26	6.7	10.0	0.0	3.3	**68**	6.7	3.3	3.3	6.7
27	13.3	0.0	0.0	6.7	**69**	3.3	3.3	3.3	10.0
28	3.3	3.3	10.0	3.3	**70**	0.0	0.0	6.7	13.3
29	3.3	6.7	3.3	6.7	**71**	3.3	3.3	6.7	6.7
30	6.7	0.0	3.3	10.0	**72**	3.3	6.7	0.0	10.0
31	10.0	3.3	3.3	3.3	**73**	6.7	6.7	6.7	0.0
32	6.7	0.0	13.3	0.0	**74**	0.0	0.0	10.0	10.0
33	3.3	3.3	13.3	0.0	**75**	0.0	16.7	3.3	0.0
34	0.0	0.0	16.7	3.3	**76**	0.0	0.0	13.3	6.7
35	13.3	0.0	6.7	0.0	**77**	3.3	10.0	6.7	0.0
36	10.0	6.7	3.3	0.0	**78**	3.3	13.3	3.3	0.0
37	0.0	3.3	3.3	13.3	**79**	0.0	6.7	13.3	0.0
38	3.3	0.0	10.0	6.7	**80**	13.3	6.7	0.0	0.0
39	0.0	3.3	16.7	0.0	**81**	10.0	3.3	6.7	0.0
40	6.7	6.7	0.0	6.7	**82**	3.3	0.0	13.3	3.3
41	0.0	13.3	0.0	6.7	**83**	0.0	3.3	10.0	6.7
42	10.0	0.0	3.3	6.7	**84**	0.0	10.0	10.0	0.0

4-1-4 Results and Discussion

4-1-4-1 Absorption Spectra

Figure 4-1 shows the spectra of FURO, CARB, DIAZ, and CARV in the 200-350 nm wavelength range recorded with scan speed of 10 nm.sec^{-1}, averaging of 1.0 nm, bandwidth of 1.8 nm, and data interval of 0.5 nm. It is obvious that the absorption spectra of the studied drugs are strongly overlaps with each other. Therefore, the direct UV-spectrophotometric determination of FURO, CARB, DIAZ, and CARV simultaneously in their quaternary mixture is not possible.

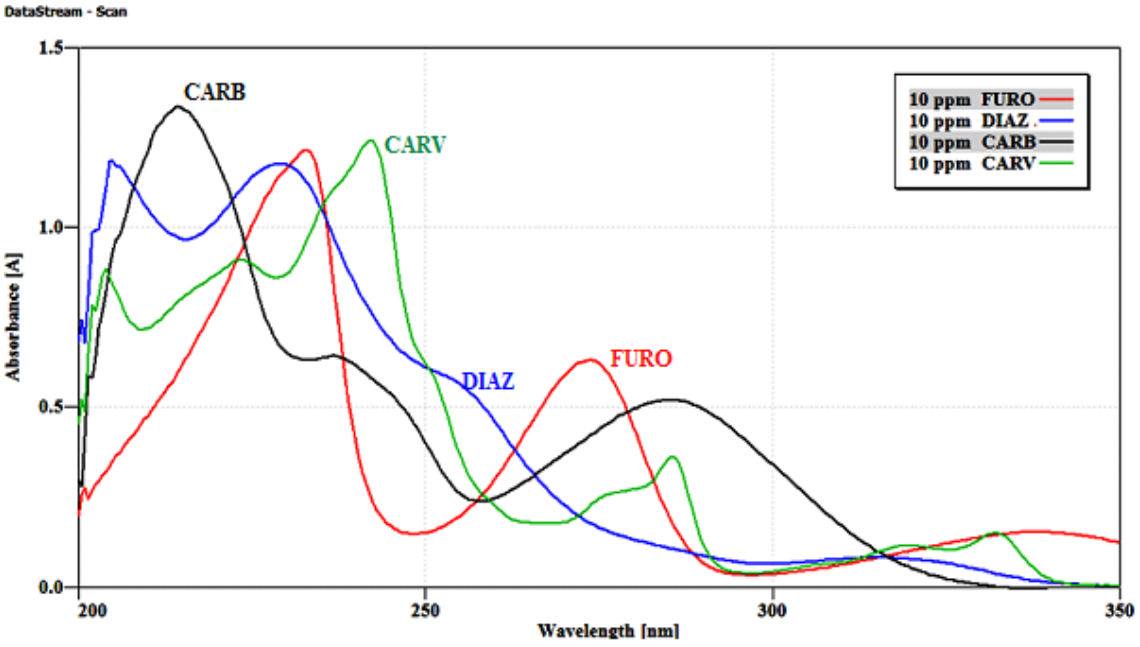


Figure 4-1: Overlaid normal mode spectra of 10 µg.mL^{-1} of furosemide, carbamazepine, diazepam and carvedilol each against their solvent blanks.

Both predictive and descriptive issues of life sciences could be solved by chemometrics. Chemometrics is utilized for multivariate information gathering especially in the field of simultaneous determination of the

organic components. Partial least squares methods are proficient of being full-spectrum techniques, and can be utilized to decipher qualitative spectral analyses [172] and quantitative analysis of chemical samples when using near-infrared [173] and UV spectra data [174].

PLS is more powerful tool to extract quantitative spectral information than the ones based on measurement at only one wavelength, such as the direct spectrophotometric method, because the consideration of full spectral intensities enhance the precision and applicability of the quantitative spectral analysis of mixtures [175].

To carry out simultaneous and multicomponent analysis utilizing UV-VIS measurements and PLS it is necessary first to prepare calibration matrix that provide the best predictions.

4-1-4-2 Individual Calibrations

Calibration curves for the cited drugs in their concentration range 1.0-20.0 $\mu g.mL^{-1}$, as absorbance versus drugs concentrations, at 233 nm, 228.5 nm, 215.0 nm, and 242.5 nm for FURO, DIAZ, CARB and CARV respectively and statistically evaluated by linear regression (Figures 4-2 – 4-5).

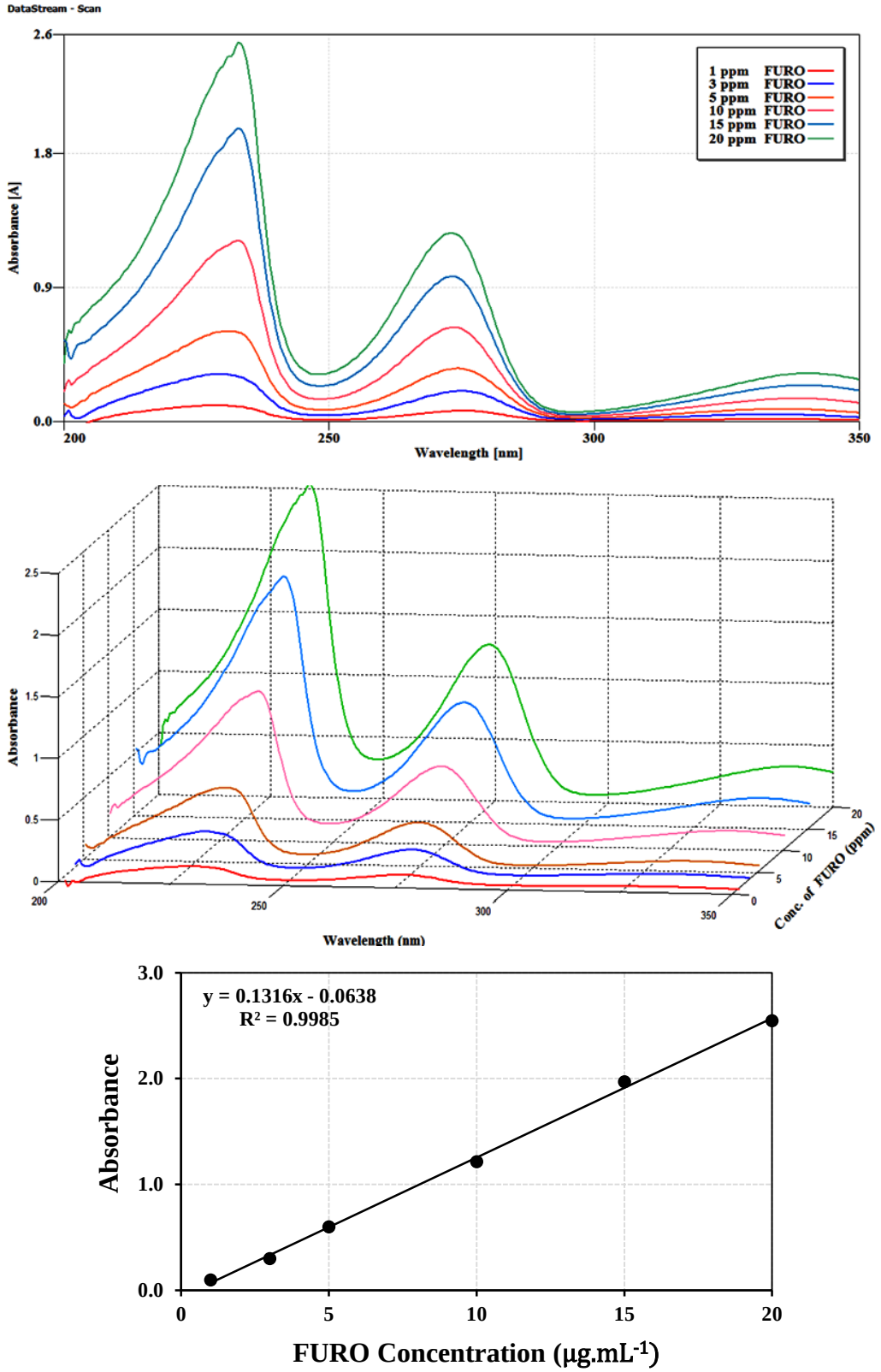


Figure 4-2: Calibration curve of furosemide constructed at 233.0 nm.

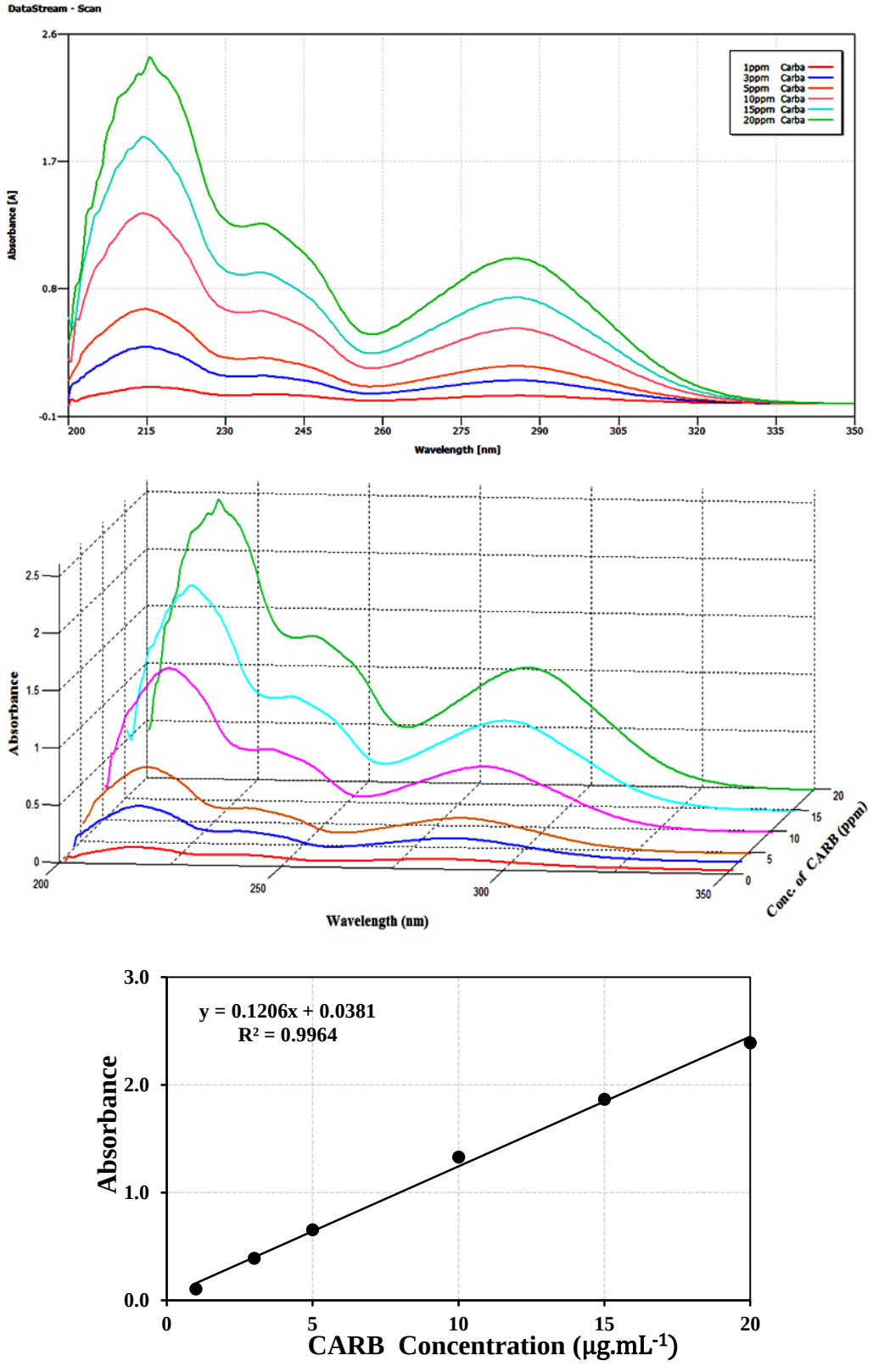


Figure 4-3: Calibration curve of carbamazepine constructed at 215.0 nm.

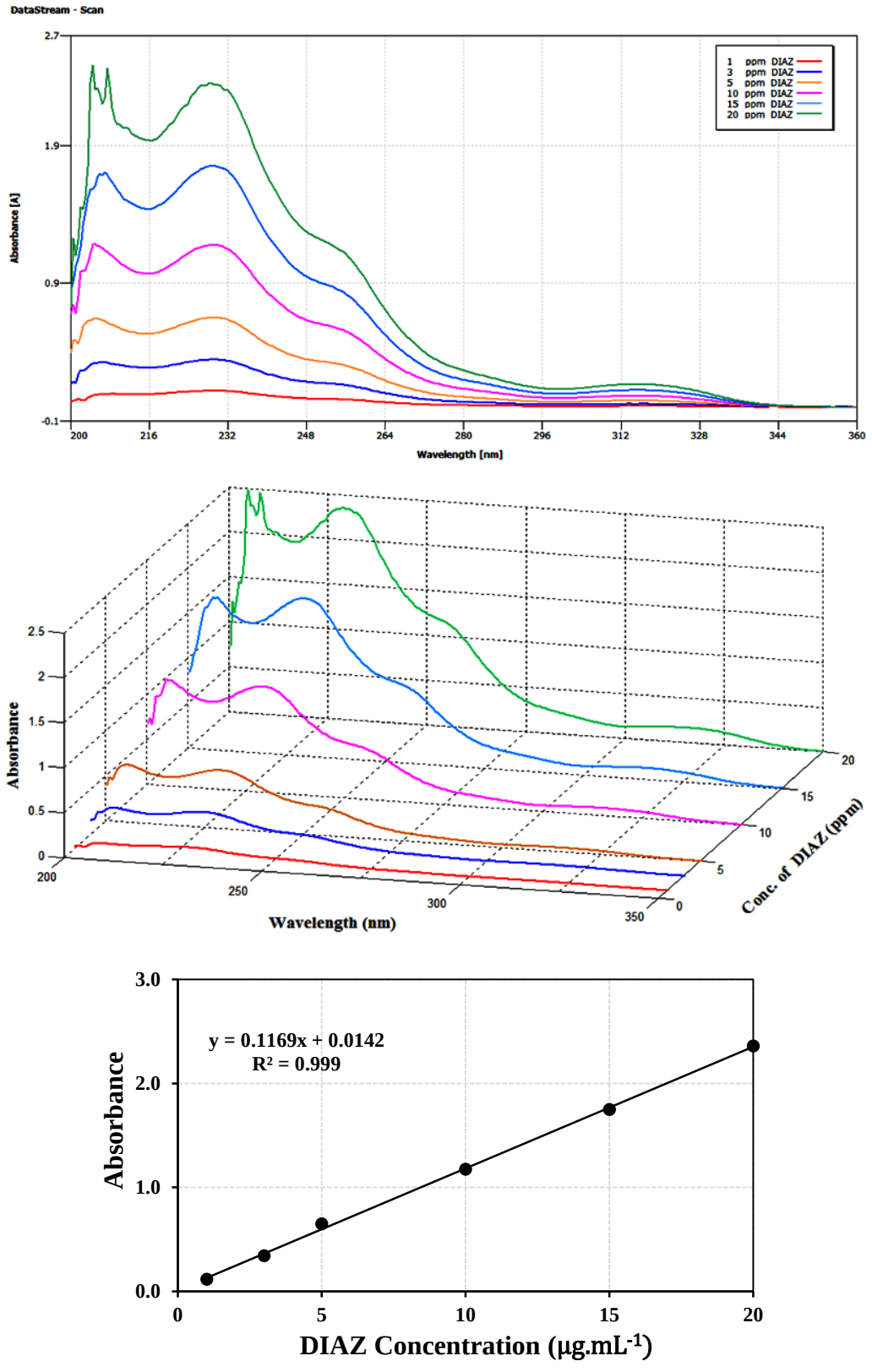


Figure 4-4: Calibration curve of diazepam constructed at 228.5 nm.

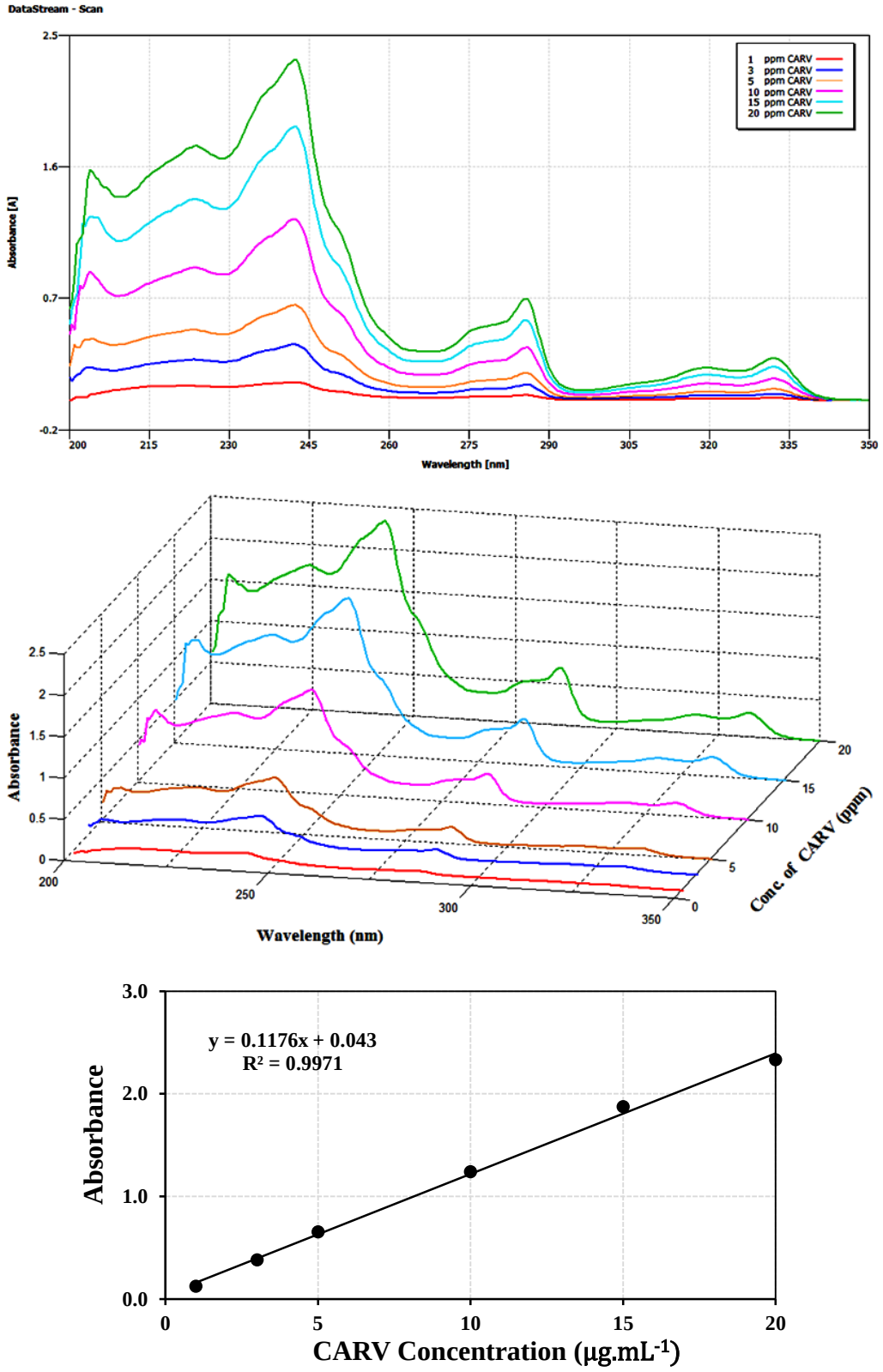


Figure 4-5: Calibration curve of carvedilol constructed at 242.5 nm.

4-1-4-3 Multivariate -Partial least squares Method

Partial least squares is a statistical method make use of a mathematical algorithm, which regress the relation between predictive variables and multiple response variables, i.e. input–output samples (xi, yi) by a linear multivariate model. This method is widely used in the field of chemometrics in general, and especially in spectrophotometry [176].

The way to deal with factor development gives the portrayal of data utilizing least number of adjustable parameters and, therefore, greatest precision and stability of regression model. On the other hand, incorporation of much more factors in the build model rise the accuracy of the model however may diminish its productivity as model begins used to characterize the pattern of relation between descriptors and activity but also random noise and individual features of the training set [177].

Training set was obtained from the spectrophotometric data obtained by careful UV-measurements on a set of known samples. These data were arranged as matrix, which organized into pairs i.e. each absorbance matrix is paired with its corresponding concentration matrix. The formed matrix was used in calibration, i.e. using known samples spectra, to predict the concentrations of unknown samples. This could be carried out on one condition that these known samples must be representative, in all cases, of the unknown samples on which the analysis will be used. In practical terms, this means that training sets should contain all expected components, span the concentration ranges of interest, span the conditions of interest, and contain mutually independent samples.

On the other hand, there are three rules to guide the selection of the number of calibration samples, which included in a training set.

Rule of three, which is the minimum number of samples required for calibration, it simply 3 times the number of samples as there are components.

The Rule of five; i.e. using 5 times the number of samples, it allows to use enough samples to reasonably represent all possible combinations of concentrations values for not more than a 3-components system.

The Rule of ten; When 10 times the number of samples as there are components is used, a solid calibration for typical applications is usually could be obtained [178].

According to rule of ten, forty quaternary mixtures were selected, from the eighty-four mixtures prepared according to Simplex Lattice mixture (Table 4-3) and depicted in Table (4-4) randomly, (mixture No. 1 to mixture No. 40 in Table 4-4) as calibration or training set, so to fulfill the non-correlated concentration matrix. These mixtures were used to build up PLS models for determination of drugs in their pure forms. On the other hand, training set for PLS models used for the determination of the cited drugs in their pharmaceutical preparation was required the all the eighty-four mixture.

The calibration matrix was obtained by recording the absorption spectrum for each of the eighty-four quaternary drugs mixtures, in the range of 200-350 nm with scan speed of 10 $nm.sec^{-1}$, averaging of 1.0 nm, bandwidth of 1.8 nm, and data interval of 0.5 nm Figure (4-6). The resulted spectra were stored as digital data in a Microsoft Excel file.

In the selected region, the spectral information in the range of 200-350 nm (301 experimental points per spectrum) was used for the calibration. These data were then arranged as row absorbance matrix paired with their corresponding concentration matrix and used to build up PLS-1 and PLS-2 regression modes using OriginPro 2015- OriginLab Corporation.

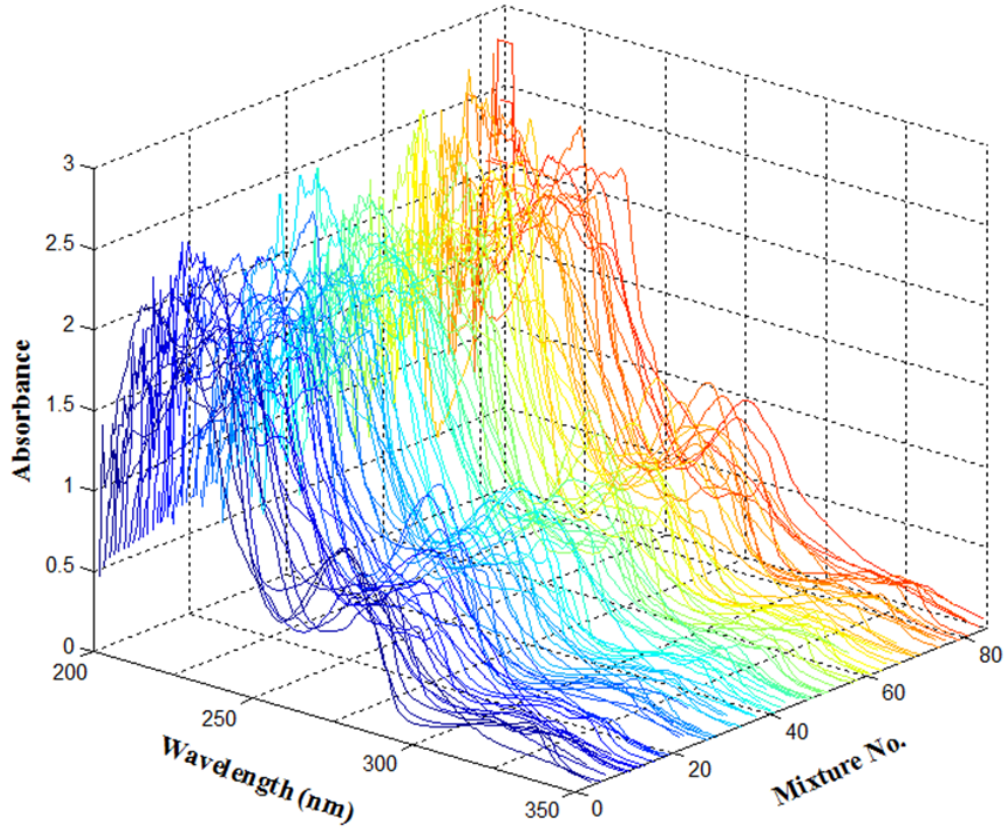


Figure 4-6: Absorbance spectra of eighty-four quaternary mixtures of FURO, DIAZ, CARB and CARV in methanol measured against solvent blank under experimental condition.

The OriginPro software was programmed to use a leave-one-out cross-validation technique for evaluating estimator performance of the constructed PLS models. This technique is carried out by letting the software to predict the value of residual error sum of squares (**PRESS** $=\sum(\boldsymbol{y_i} - \boldsymbol{\hat{y}_i})^{\mathbf{2}}$) (where $\boldsymbol{y_i}$ and $\boldsymbol{\hat{y}_i}$ are the known and predicted analyte concentrations, respectively) and use it as an indicator for appropriateness of models (the smallest model with fewest numbers of factors). The spectral data of one sample at a time is randomly eliminated and the other remaining samples spectral data is then used by PLS model as standards. By using this calibration, the concentration of the sample left out was predicted. This process was repeated as the number of samples [154].

Figure (4-7) shows the plot of PRESS against the number of factors for PLS-2 and PLS-1 model for determination of drugs in their pure forms.

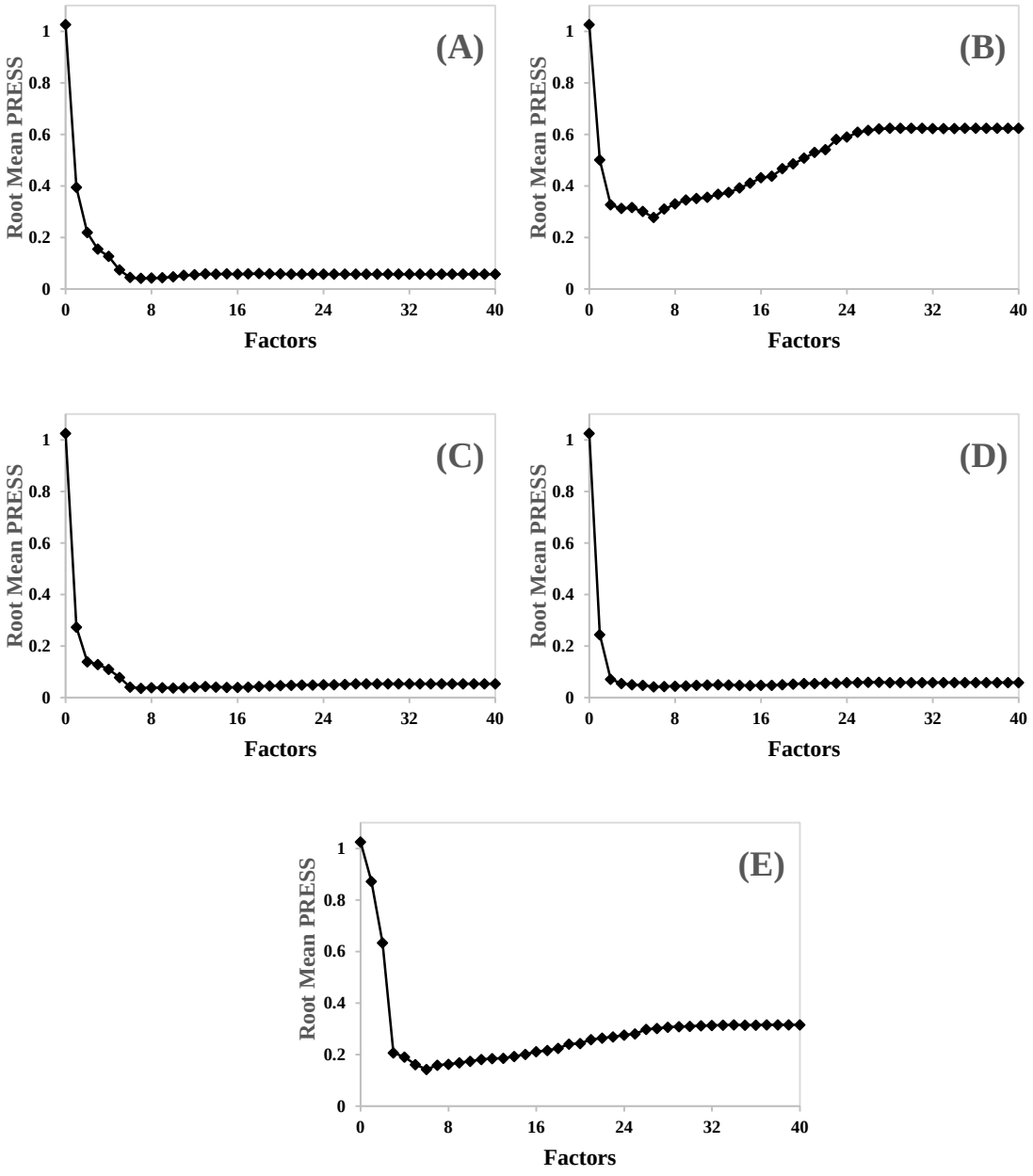


Figure 4-7: Plot of Root Mean PRESS against number of factors for forty CARV, DIAZ, CARB, and FURO mixtures for: (A) PLS-2 model, (B) PLS-1 model for CARV, (C) PLS-1 model for DIAZ, (D) PLS-1 model for CARB, and (E) PLS-1 model for FURO.

The calibration models for the two chemometric methods were confirmed with the forty mixtures of the train set. The predictive capabilities PLS-1 and PLS-2 were examined for simultaneous determination of FURO, DIAZ, CARB, and CARV in each mixtures. Moreover, the prediction error of a single component in the mixture contain the four drugs samples (N) and total prediction error of forty sample mixtures (M) were calculated as the standard error (R.S.E %) of the prediction concentrations[159] (Tables 4-4 and 4-5 for PLS-1 and PLS-2 models respectively). The results show excellent values of single relative standard error for CARV, CARB and FURO (1.7517, 1.4605, 2.0892 and 2.2913, 2.1228, 2.2763 for PLS-1 and PLS-2 respectively) and acceptable values for DIAZ (9.4830 and 9.6932 for PLS-1 and PLS-2 respectively). In addition, the total relative standard error are reasonable for such a system (4.4747 and 4.7132 for PLS-1 and PLS-2 respectively).

Table 4-4: Composition of training set and their predictions by PLS-1 model.

Mixture (µg.mL^{-1})				Prediction (µg.mL^{-1})				Recovery %			
CARV	***DIAZ***	***CARB***	***FURO***	***CARV***	***DIAZ***	***CARB***	***FURO***	***CARV***	***DIAZ***	***CARB***	***FURO***
0.0	6.7	13.3	0.0	----	6.6419	13.1618	----	----	99.1331	98.9605	----
0.0	6.7	0.0	13.3	----	6.9000	----	13.4363	----	102.9848	----	101.0247
13.3	0.0	6.7	0.0	13.3069	----	6.4619	----	100.0521	----	96.4458	----
0.0	16.7	0.0	3.3	----	16.2822	----	3.4805	----	97.4984	----	105.4688
13.3	0.0	0.0	6.7	13.3477	----	----	6.9738	100.3586	----	----	104.0866
3.3	6.7	3.3	6.7	3.3028	6.7083	3.2672	6.7259	100.0833	100.1236	99.0067	100.3870
3.3	6.7	10.0	0.0	3.3059	6.7353	9.8370	----	100.1791	100.5263	98.3704	----
6.7	0.0	0.0	13.3	6.8253	----	----	13.1020	101.8700	----	----	98.5110
0.0	0.0	6.7	13.3	----	----	6.5211	13.2477	----	----	97.3294	99.6065
0.0	3.3	6.7	10.0	----	3.5129	6.5778	9.8974	----	106.4524	98.1754	98.9742
10.0	3.3	3.3	3.3	9.7730	3.2490	3.3934	3.2979	97.7304	98.4536	102.8300	99.9364
10.0	10.0	0.0	0.0	9.8462	9.5650	----	----	98.4616	95.6500	----	----
3.3	0.0	16.7	0.0	3.4283	----	16.6501	----	103.8864	----	99.7009	----
3.3	3.3	6.7	6.7	3.3103	2.9279	6.9593	6.6225	100.3118	88.7255	103.8694	98.8437
16.7	0.0	0.0	3.3	16.4366	----	----	3.4159	98.4230	----	----	103.5121
10.0	3.3	6.7	0.0	10.2605	2.9712	6.6624	----	102.6046	90.0358	99.4381	----
1.0	6.7	6.7	0.0	1.0181	9.1676	6.7303	----	101.8110	136.8301	100.4527	----
6.7	0.0	10.0	3.3	6.7393	----	10.1692	3.1144	100.5864	----	101.6919	94.3767
3.3	10.0	6.7	0.0	3.2627	9.5974	6.6977	----	98.8703	95.9735	99.9660	----
0.0	0.0	0.0	20.0	----	----	----	19.8866	----	----	----	99.4332
0.0	6.7	6.7	6.7	----	6.6007	6.6449	6.3972	----	98.5175	99.1770	95.4801
20.0	0.0	0.0	0.0	19.9527	----	----	----	99.7634	----	----	----
6.7	3.3	10.0	0.0	6.8137	3.0709	10.1120	----	101.6966	93.0582	101.1198	----
3.3	6.7	0.0	10.0	3.3405	6.5973	----	9.8320	101.2279	98.4675	----	98.3202
16.7	0.0	3.3	0.0	16.3727	----	3.2995	----	98.0401	----	99.9861	----
0.0	6.7	3.3	10.0	----	4.1964	3.3130	10.2233	----	62.6330	100.3924	102.2326
0.0	10.0	6.7	3.3	----	10.2214	6.8243	3.4514	----	102.2139	101.8551	104.5873
0.0	13.3	0.0	6.7	----	13.7544	----	6.7057	----	103.4162	----	100.0851
0.0	0.0	16.7	3.3	----	----	16.7449	3.4961	----	----	100.2689	105.9430
3.3	0.0	0.0	16.7	3.3616	----	----	16.2811	101.8658	----	----	97.4917
6.7	3.3	6.7	3.3	6.5765	3.1704	6.8300	3.3204	98.1569	96.0730	101.9407	100.6185
6.7	10.0	0.0	3.3	6.7741	9.9082	----	3.3084	101.1061	99.0816	----	100.2530
0.0	13.3	3.3	3.3	----	13.1874	3.2853	3.2559	----	99.1536	99.5539	98.6624
0.0	3.3	13.3	3.3	----	3.6701	13.4403	3.1033	----	111.2155	101.0552	94.0400
13.3	6.7	0.0	0.0	13.7628	6.1754	----	----	103.4796	92.1701	----	----
3.3	0.0	6.7	10.0	3.3276	----	6.7099	10.2565	100.8370	----	100.1470	102.5652
10	6.7	0.0	3.3	10.2618	6.6092	----	3.2647	102.6182	98.6454	----	98.9315
3.3	0.0	10.0	6.7	3.2201	----	9.9697	6.7985	97.5776	----	99.6970	101.4703
6.7	0.0	6.7	6.7	6.6382	----	6.4753	6.5328	99.0776	----	96.6458	97.5037
0.0	0.0	3.3	16.7	----	----	3.4652	17.1639	----	----	105.0061	102.7779
Mean recovery								100.4106	98.6264	100.1185	100.1830
***R.S.E.(%) single**								1.7517	9.4830	1.4605	2.0892
$^{+}$R.S.E.(%) total								4.4747			

Table 4-5: Composition of training set and their predictions by PLS-2 model.

Mixture (µg.mL^{-1})				Prediction (µg.mL^{-1})				Recovery %			
CARV	*DIAZ*	*CARB*	*FURO*	*CARV*	*DIAZ*	*CARB*	*FURO*	*CARV*	*DIAZ*	*CARB*	*FURO*
0.0	6.7	13.3	0.0	----	6.6364	13.0694	----	----	99.0509	98.2666	----
0.0	6.7	0.0	13.3	----	6.8968	----	13.4447	----	102.9373	----	101.0880
13.3	0.0	6.7	0.0	13.0271	----	6.4965	----	97.9479	----	96.9630	----
0.0	16.7	0.0	3.3	----	16.29054	----	3.4674	----	97.5481	----	105.0730
13.3	0.0	0.0	6.7	13.3204	----	----	6.9608	100.1536	----	----	103.8924
3.3	6.7	3.3	6.7	3.3673	6.7066	3.2395	6.7191	102.0379	100.0984	98.1661	100.2855
3.3	6.7	10.0	0.0	3.2547	6.7181	9.8085	----	98.6261	100.2707	98.0842	----
6.7	0.0	0.0	13.3	6.8455	----	----	13.0410	102.1716	----	----	98.1202
0.0	0.0	6.7	13.3	----	----	6.4785	13.2628	----	----	96.6928	99.7203
0.0	3.3	6.7	10.0	----	3.5023	6.6165	9.8987	----	106.1312	98.7537	98.9872
10.0	3.3	3.3	3.3	9.9292	3.24664	3.3524	3.2893	99.2917	98.3830	101.5888	99.6748
10.0	10.0	0.0	0.0	9.9900	9.56859	----	----	99.9003	95.6859	----	----
3.3	0.0	16.7	0.0	3.5973	----	16.3693	----	109.0082	----	98.0195	----
3.3	3.3	6.7	6.7	3.4830	2.9402	6.9892	6.5460	105.5461	89.0970	104.3163	97.7019
16.7	0.0	0.0	3.3	16.4600	----	----	3.3786	98.5631	----	----	102.3806
10.0	3.3	6.7	0.0	10.3101	2.9691	6.7092	----	103.1011	89.9724	100.1370	----
1.0	6.7	6.7	0.0	1.0940	9.21631	6.7272	----	109.4030	137.5569	100.4063	----
6.7	0.0	10.0	3.3	6.7841	----	10.1220	3.05354	101.2558	----	101.2200	92.5315
3.3	10.0	6.7	0.0	3.39223	9.5978	6.7155	----	102.7948	95.9778	100.2313	----
0.0	0.0	0.0	20.0	----	----	----	19.9132	----	----	----	99.5659
0.0	6.7	6.7	6.7	----	6.62133	6.6276	6.33388	----	98.8258	98.9187	94.5355
20.0	0.0	0.0	0.0	19.7313	----	----	----	98.6566	----	----	----
6.7	3.3	10.0	0.0	6.7687	3.0324	10.3548	----	101.0260	91.8918	103.5484	----
3.3	6.7	0.0	10.0	3.3569	6.6559	----	9.8052	101.7236	99.3418	----	98.0519
16.7	0.0	3.3	0.0	16.2479	----	3.3753	----	97.2931	----	102.2803	----
0.0	6.7	3.3	10.0	----	4.1061	3.4276	10.287	----	61.2851	103.8652	102.8717
0.0	10.0	6.7	3.3	----	10.1532	6.8250	3.4807	----	101.5322	101.8661	105.4767
0.0	13.3	0.0	6.7	----	13.7240	----	6.7221	----	103.1881	----	100.3300
0.0	0.0	16.7	3.3	----	----	16.7222	3.5453	----	----	100.1328	107.4409
3.3	0.0	0.0	16.7	3.2589	----	----	16.3031	98.7530	----	----	97.6235
6.7	3.3	6.7	3.3	6.6819	3.1810	6.9110	3.3329	99.7300	96.3924	103.1485	100.9970
6.7	10.0	0.0	3.3	6.8030	9.9178	----	3.2850	101.5376	99.1776	----	99.5448
0.0	13.3	3.3	3.3	----	13.1989	3.0922	3.2673	----	99.2400	93.7018	99.0097
0.0	3.3	13.3	3.3	----	3.6932	13.6504	3.1044	----	111.9164	102.6347	94.0727
13.3	6.7	0.0	0.0	13.8944	6.1909	----	----	104.4689	92.4009	----	----
3.3	0.0	6.7	10.0	3.5464	----	6.7165	10.2375	107.4658	----	100.2463	102.3752
10.0	6.7	0.0	3.3	10.2101	6.61428	----	3.2214	102.1006	98.7206	----	97.6194
3.3	0.0	10.0	6.7	3.4271	----	10.0107	6.7862	103.8509	----	100.1066	101.2869
6.7	0.0	6.7	6.7	6.7741	----	6.4625	6.49546	101.1064	----	96.4552	96.9472
0.0	0.0	3.3	16.7	----	----	3.4553	17.2014	----	----	104.7052	103.0023
Mean recovery								101.8274	98.6093	100.1714	100.0074
***R.S.E.(%) single**								2.2913	9.6932	2.1228	2.2763
$^{+}$R.S.E.(%) total								4.7132			

Where *R.S.E. (%) single = $\sqrt{\sum_{i=1}^{N}(\hat{y}_i - y_i)^2 / \sum_{i=1}^{N}(y_i)^2} \times 100$, and

+R.S.E. (%) total = $\sqrt{\sum_{j=1}^{M}\sum_{i=1}^{N}(\hat{y}_{ji} - y_{ji})^2 / \sum_{j=1}^{M}\sum_{i=1}^{N}(y_{ji})^2} \times 100$

In order to check the goodness of the results obtained for the prediction of training mixtures containing the four drugs in terms of amounts added and found in, the accuracy and precision of the results were calculated. Table (4-6) shows the results obtained of almost four replicates analyses conducted in two consecutive days.

Table 4-6: Evaluation of accuracy and precision of the proposed method.

Drug	Taken (µg.mL^{-1})	Model	Found (µg.mL^{-1})				Mean	RE%	RSD %
CARV	3.3	PLS-1	3.3028	3.3059	3.4283	3.3103	3.3368	1.1159	1.8299
		PLS-2	3.3673	3.2547	3.5973	3.4830	3.4256	3.8053	4.3096
	10	PLS-1	9.7730	9.8462	10.2605	10.2618	10.0354	0.3538	2.6149
		PLS-2	9.9292	9.9900	10.3101	10.2101	10.1099	1.0985	1.7800
	13.3	PLS-1	13.3069	13.3477	13.7628	----	13.4725	1.2967	1.8724
		PLS-2	13.0271	13.3204	13.8944	----	13.4140	0.8569	3.2888
DIAZ	3.3	PLS-1	3.249	3.0709	3.1704	3.5129	3.2508	-1.4909	5.8238
		PLS-2	3.24664	3.0324	3.181	3.5023	3.2406	-1.8005	6.0529
	6.7	PLS-1	6.6419	6.9000	6.7083	6.7353	6.7464	0.6922	1.6257
		PLS-2	6.6364	6.8968	6.7066	6.7181	6.7395	0.5892	1.6459
	10	PLS-1	9.565	9.5974	10.2214	9.9082	9.8230	-1.7700	3.1292
		PLS-2	9.56859	9.5978	10.1532	9.9178	9.8093	-1.9065	2.8393
CARB	3.3	PLS-1	3.2672	3.3934	3.2995	3.3130	3.3183	0.5538	1.6166
		PLS-2	3.2395	3.3524	3.3753	3.4276	3.3487	1.4758	2.3684
	6.7	PLS-1	6.4619	6.5211	6.5778	6.9593	6.6300	-1.0444	3.3870
		PLS-2	6.4965	6.4785	6.6165	6.9892	6.6452	-0.8183	3.5723
	10	PLS-1	9.837	10.1692	10.112	9.9697	10.0220	0.2198	1.4881
		PLS-2	9.8085	10.122	10.3548	10.0107	10.0740	0.7400	2.2611
FURO	3.3	PLS-1	3.4805	3.2979	3.4159	3.1144	3.3272	0.8235	4.8311
		PLS-2	3.4674	3.2893	3.3786	3.0535	3.2972	-0.0845	5.3978
	10	PLS-1	9.8974	9.8320	10.2233	10.2565	10.0523	0.5230	2.1754
		PLS-2	9.8987	9.8052	10.2870	10.2375	10.0571	0.5710	2.3942
	13.3	PLS-1	13.4363	13.102	13.2477	----	13.2620	-0.2857	1.2638
		PLS-2	13.4447	13.041	13.2628	----	13.2495	-0.3797	1.5259

F-test was used to compare the obtained results by the two methods. Values obtained in Table (4-7) show that there were no significant differences between the results obtained in the determination of each drug in the presence of the others in different days.

Table 4-7: F-Test Two-Sample (PLS-1 and PLS-2) for Variances for the predicated results of training sets obtained by PLS-1 and PLS-2 models of the analysis of each drug in the presence of the others.

F-Test Two-Sample for Variances

	CARV		DIAZ		CARB		FURO	
	PLS-1	***PLS-2***	***PLS-1***	***PLS-2***	***PLS-1***	***PLS-2***	***PLS-1***	***PLS-2***
Mean	7.8679	7.9060	7.1212	7.1197	7.7001	7.7048	7.7354	7.7280
Variance	25.8514	25.1877	13.1772	13.1883	14.9372	14.8420	23.9120	24.0125
Observations	26	26	25	25	26	26	28	28
df	25	25	24	24	25	25	27	27
F	1.0264		1.0008		1.0064		1.0042	
P(F<=f) one-tail	0.4743		0.4992		0.4937		0.4957	
F Critical one-tail	1.9554		1.9838		1.9554		1.9048	

Figure 4-8, represents the plots of the predicted concentrations of CARV, DIAZ, CARB, and FURO obtained via PLS-1 and PLS-2 models. The plots show a large agreement in the results of both models.

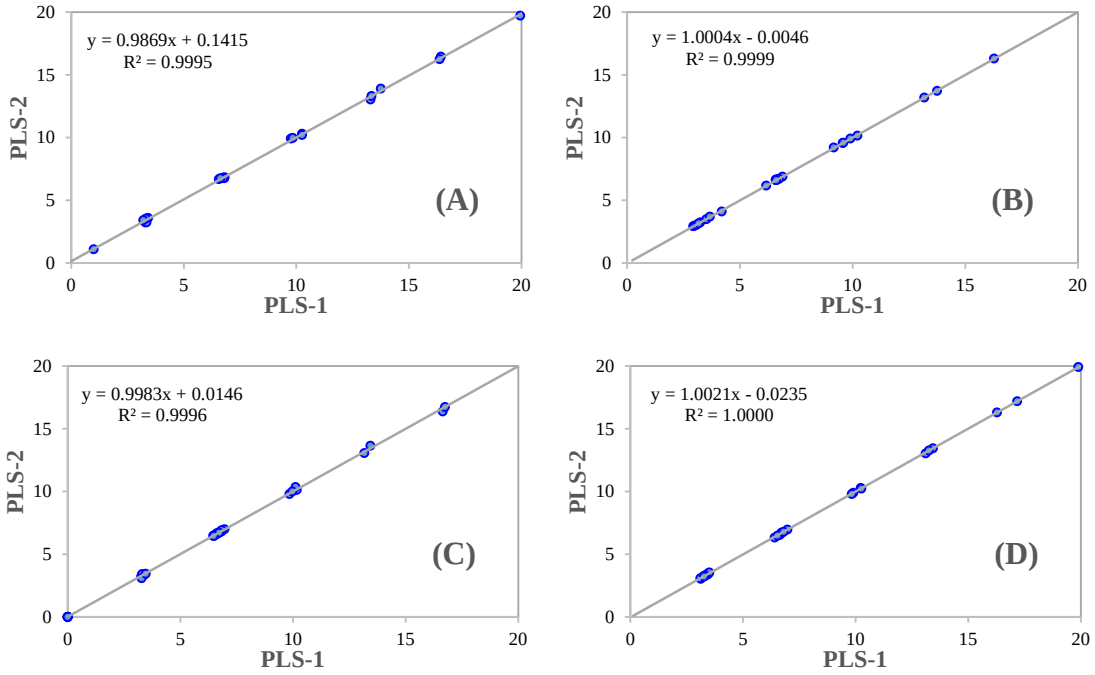


Figure 4-8: Plots of the predicated results of training sets obtained by PLS-1 against those obtained by PLS-2 model for the analyses of: **(A)** CARV, **(B)** DIAZ, **(C)** CARB and **(D)** FURO in their quaternary mixtures.

4-1-4-4 Application of the method

The established PLS-1 and PLS-2 models fail to give satisfactory and convincing results when used to predict the concentrations of the studied these drugs in real samples formulation. Therefore, a new calibrations were constructed for PLS-1 and PLS-2 models, using a eighty-four quaternary mixtures set, to be applied to the determination of CARV, DIAZ, CARB, and FURO in real matrix samples (i.e. in pharmaceutical formulations). The performance of the new constructed PLS models were studied. Figure (4-9) shows PRESS - number of factors plots for PLS-1 and PLS-2 models.

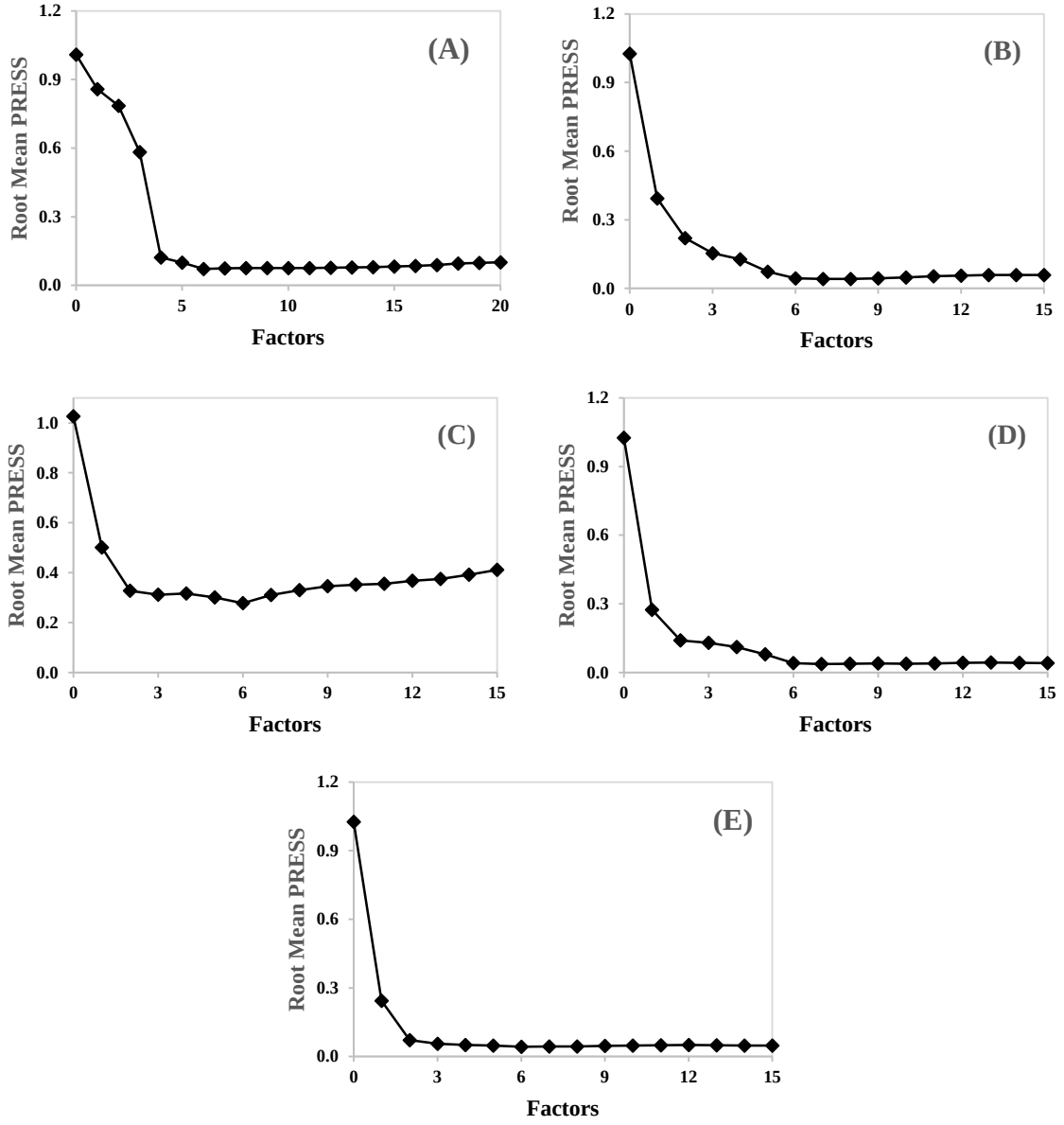


Figure 4-9: Plot of Root Mean PRESS against number of factors for eighty-four CARV, DIAZ, CARB, and FURO mixtures: (A) PLS-2 model, (B) PLS-1 model for CARV, (C) PLS-1 model for DIAZ, (D) PLS-1 model for CARB and (E) PLS-1 model for FURO

The developed PLS-1 and PLS-2 models were validated by testing their predictive abilities for simultaneous determination of CARV, DIAZ, CARB, and FURO in twenty quaternary synthetic mixtures set. Tables 4-8 and 4-9 show the predicted results and other statistical parameters.

Table 4-8: Composition of synthetic samples, their predictions by PLS-1 model and statistical parameters for the system.

Mixture (µg.mL^{-1})				Prediction (µg.mL^{-1})				Recovery %			
CARV	*DIAZ*	*CARB*	*FURO*	*CARV*	*DIAZ*	*CARB*	*FURO*	*CARV*	*DIAZ*	*CARB*	*FURO*
3.3	13.3	0.0	3.3	3.2026	13.1366	0.0760	3.1819	97.0485	98.7717	----	96.4197
10.0	0.0	0.0	10.0	10.1235	0.1217	0.0019	10.1581	101.2346	----	----	101.5812
6.7	0.0	13.3	0.0	6.6499	0.0801	13.3562	----	99.2518	----	100.4228	----
6.7	13.3	0.0	0.0	6.7282	12.9315	0.1959	----	100.4210	97.2295	----	----
3.3	0.0	13.3	3.3	3.3149	0.0451	13.3295	3.3450	100.4500	----	100.2219	101.3639
16.7	3.3	0.0	0.0	17.0041	3.1223	----	0.2181	101.8211	94.6158	----	----
13.3	3.3	0.0	3.3	13.3296	3.3349	0.0793	3.3538	100.2222	101.0579	----	101.6297
3.3	6.7	6.7	3.3	3.2162	6.5365	6.9274	3.2214	97.4615	97.5591	103.3946	97.6170
6.7	0.0	3.3	10.0	6.7898	0.0885	3.3884	10.0796	101.3397	----	102.6788	100.7961
0.0	10.0	10.0	0.0	----	9.6756	10.2171	----	----	96.7557	102.1714	----
0.0	3.3	3.3	13.3	0.0268	3.3183	3.4774	13.4510	----	100.5539	105.3767	101.1354
0.0	0.0	10.0	10.0	0.0742	----	9.9992	10.1223	----	----	99.9918	101.2232
0.0	13.3	6.7	0.0	----	13.1811	6.7627	----	----	99.1062	100.9351	----
0.0	0.0	20.0	0.0	----	0.1276	19.9646	----	----	----	99.8229	----
0.0	10.0	0.0	10.0	----	10.0680	0.0134	9.8024	----	100.6795	----	98.0244
0.0	20.0	0.0	0.0	----	19.8387	----	----	----	99.1935	----	----
10.0	0.0	10.0	0.0	10.3627	----	9.8465	----	103.6272	----	98.4650	----
0.0	0.0	13.3	6.7	0.0305	----	13.2890	6.8378	----	----	99.9165	102.0564
3.3	10.0	0.0	6.7	3.2859	9.8743	0.0476	6.6804	99.5715	98.7426	----	99.7069
0.0	6.7	10.0	3.3	----	6.5256	10.1708	3.3019	----	97.3975	101.7081	100.0564
Mean recovery								100.2227	98.4719	101.2588	100.1342
***R.S.E. (%) single**								2.4059	1.8988	1.3259	2.1000
+R.S.E. (%) total								1.8828			

Table 4-9: Composition of synthetic samples, their predictions by PLS-2 model and statistical parameters for the system.

Mixture (µg.mL^{-1})				Prediction (µg.mL^{-1})				Recovery %			
CARV	*DIAZ*	*CARB*	*FURO*	*CARV*	*DIAZ*	*CARB*	*FURO*	*CARV*	*DIAZ*	*CARB*	*FURO*
3.3	13.3	0.0	3.3	3.3481	13.1693	0.1820	3.1360	101.4576	99.0173	----	95.0300
10.0	0.0	0.0	10.0	10.2570	0.0835	0.0261	10.0956	102.5700	----	----	100.9560
6.7	0.0	13.3	0.0	6.5809	0.0699	13.3650	0.0068	98.2224	----	100.4887	----
6.7	13.3	0.0	0.0	6.7602	12.9805	0.1865	----	100.8985	97.5977	----	----
3.3	0.0	13.3	3.3	3.3314	0.0086	13.3641	3.3733	100.9515	----	100.4820	102.2206
16.7	3.3	0.0	0.0	17.0327	3.1270	0.1241	0.2419	101.9922	94.7576	----	----
13.3	3.3	0.0	3.3	13.3979	3.3460	0.0817	3.3495	100.7361	101.3939	----	101.5009
3.3	6.7	6.7	3.3	3.3528	6.5393	6.9267	3.1975	101.6000	97.6015	103.3836	96.8939
6.7	0.0	3.3	10.0	6.8766	0.0705	3.3952	10.0174	102.6358	----	102.8848	100.1743
0.0	10.0	10.0	0.0	----	9.7072	10.1334	----	----	97.0720	101.3340	----
0.0	3.3	3.3	13.3	0.0248	3.3474	3.4425	13.4353	----	101.4364	104.3182	101.0173
0.0	0.0	10.0	10.0	0.0861	----	10.0766	10.1162	----	----	100.7660	101.1617
0.0	13.3	6.7	0.0	----	13.1707	6.8976	----	----	99.0278	102.9493	----
0.0	0.0	20.0	0.0	----	0.1239	20.0435	----	----	----	100.2175	----
0.0	10.0	0.0	10.0	----	10.1378	0.0041	9.8108	----	101.3780	----	98.1082
0.0	20.0	0.0	0.0	----	19.9132	0.1409	----	----	99.5660	----	----
10.0	0.0	10.0	0.0	10.2569	----	9.9736	0.0262	102.5690	----	99.7360	----
0.0	0.0	13.3	6.7	0.0631	----	13.3380	6.8429	----	----	100.2857	102.1324
3.3	10.0	0.0	6.7	3.3726	9.8785	0.0820	6.6104	102.2000	98.7850	----	98.6624
0.0	6.7	10.0	3.3	0.0290	6.5577	10.1353	3.2597	----	97.8761	101.3530	98.7782
Mean recovery								101.4393	98.7924	101.5165	99.7197
***R.S.E. (%) single**								2.4413	1.7160	1.4215	2.1113
+R.S.E. (%) total								1.8620			

The mean squares error (**MSE** = $\frac{\Sigma(y_i-\hat{y}_i)^2}{N}$**),** the root mean squares error (**RMSE** = $\sqrt{\frac{\Sigma(y_i-\hat{y}_i)^2}{N}}$), which is an indication of the average error in the analysis, for each component, was determined for the calibration (**RMSEC**) [179]. Moreover, mean standard deviation of the entire calibrated mixture solutions and correlation coefficient (**r)** between the used and calculated concentration in the synthetic mixture are given in Tables 4-10 and 4-11 for PLS-1 and PLS-2 models respectively.

Table 4-10: Goodness of fit statistics for synthetic samples set analyses by PLS-1.

Parameter	Variable			
	CARV	**DIAZ**	**CARB**	**FURO**
Observations	11	12	12	12
Sum of weights	11	12	12	12
Correlation coefficient (r)	0.9997	0.9997	0.9998	0.9996
Std. deviation	0.1493	0.1314	0.1140	0.1141
MSE	0.0244	0.0349	0.0167	0.0127
RMSEC	0.1562	0.1868	0.1292	0.1128

Table 4-11: Goodness of fit statistics for synthetic samples set analyses by PLS-2.

Parameter	Variable			
	CARV	**DIAZ**	**CARB**	**FURO**
Observations	11	12	12	12
Sum of weights	11	12	12	12
Correlation coefficient (r)	0.9998	0.9997	0.9999	0.9995
Std. deviation	0.1291	0.1338	0.0712	0.1176
MSE	0.0284	0.0286	0.0145	0.0127
RMSEC	0.1686	0.1691	0.1205	0.1127

The predicted concentrations of synthetic mixture samples via PLS-1 and PLS2 models for the four drugs in their quaternary synthetic mixtures were plotted versus the true concentrations, Figures 4-10 and 4-11. The plots show a large agreement in the results.

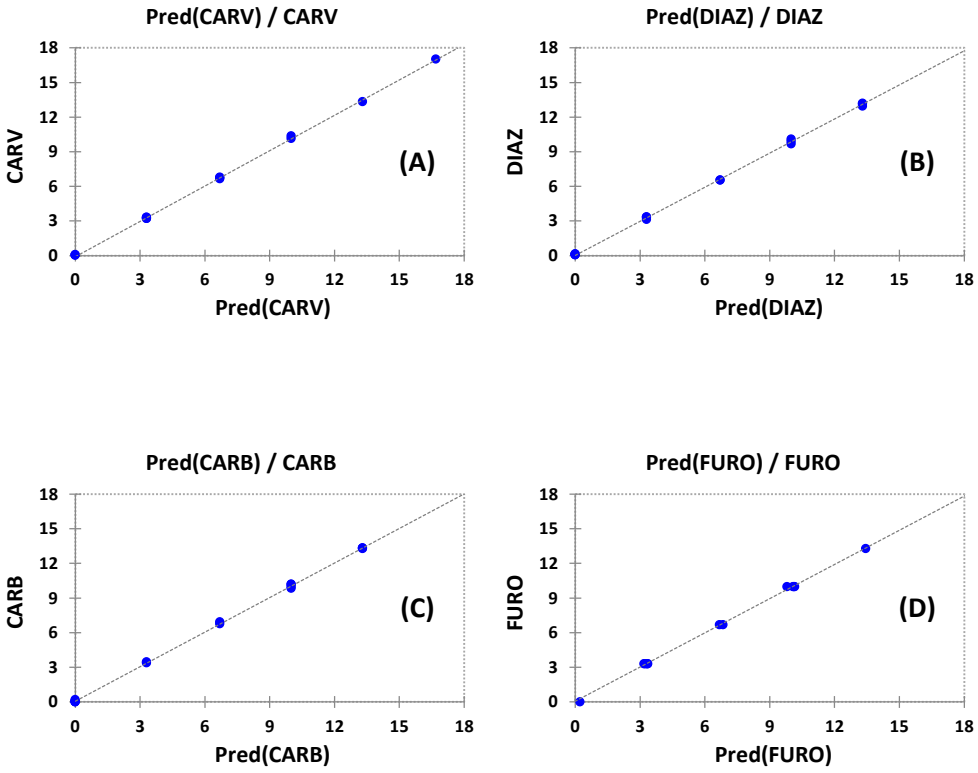


Figure 4-10: Plots of PLS-1 predicated vs true drugs concentrations in synthetic quaternary mixtures; (A) CARV, (B) DIAZ, (C) CARB, and (D) FURO.

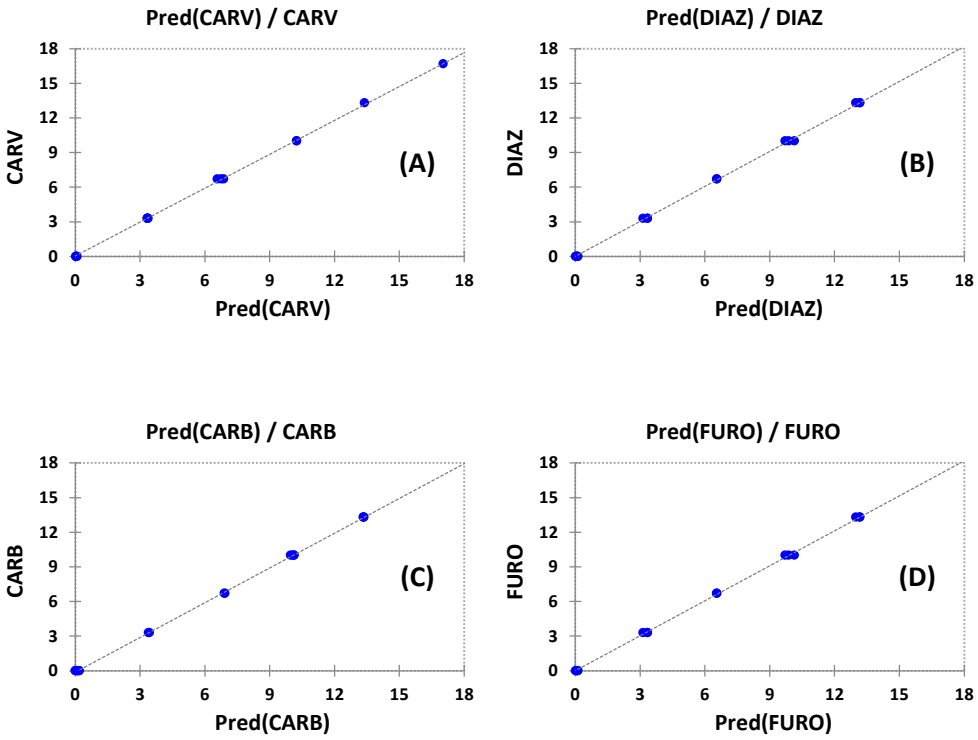


Figure 4-11: Plots of PLS-2 predicated vs true drugs concentrations in synthetic quaternary mixtures; (A) CARV, (B) DIAZ, (C) CARB, and (D) FURO.

Figures 4-12 and 4-13 represent the plots of the standardized concentration residuals vs the predicted concentrations of the tested mixtures. The residuals for CARV, DIAZ, CARB, and FURO in all samples appeared to be randomly distributed around zero.

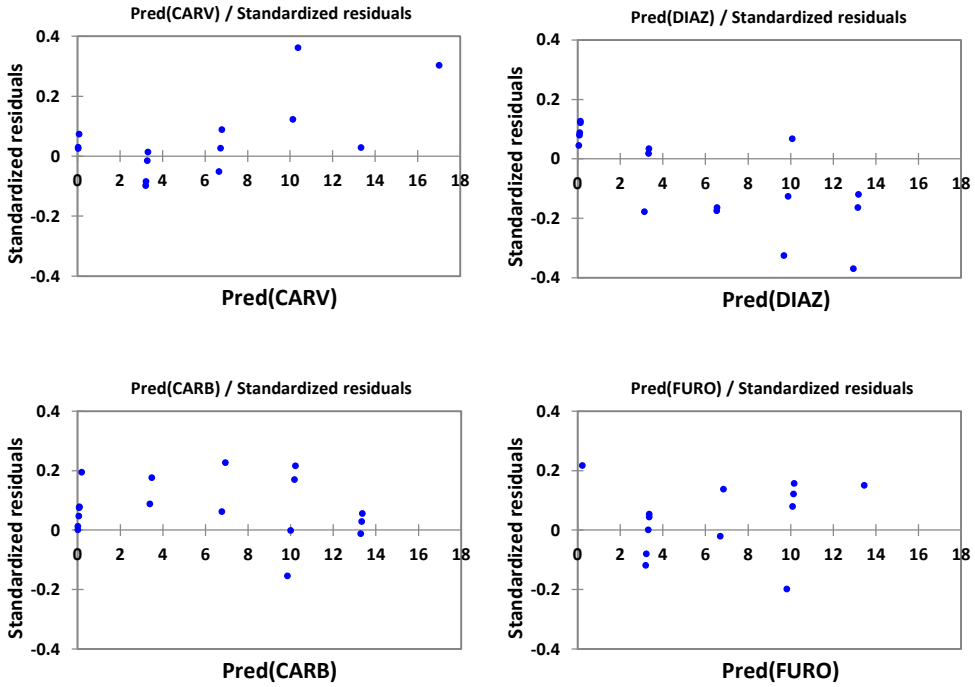


Figure 4-12: Plots of standardized concentration residuals vs predicted drugs concentrations for the quaternary synthetic mixture samples, by PLS-1 model.

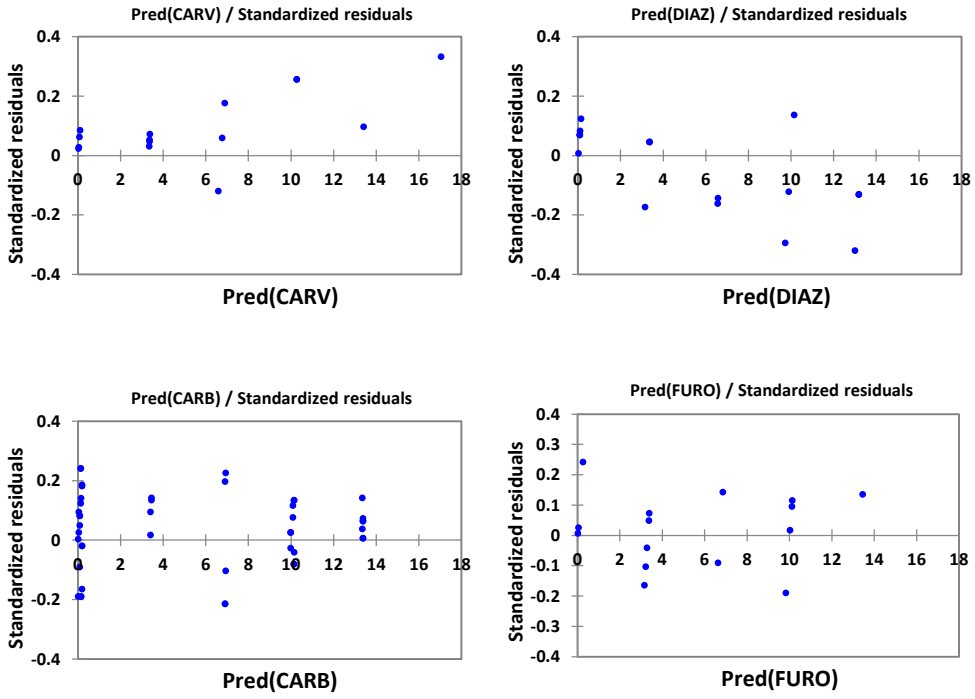


Figure 4-13: Plots of standardized concentration residuals vs predicted drugs concentrations for the quaternary synthetic mixture samples, by PLS-2 model.

4-1-4-5 Accuracy and Precision

Two levels of concentrations of each drug were used to check the accuracies and precisions of PLS-1 and PLS-2 models in the simultaneousl determination of CARV, DIAZ, CARB and FURO in quaternary synthetic mixtures containing amounts of analytes ranged between 3.3-16.7 $\mu g.mL^{-1}$. The accuracy and precision were checked by calculating the values of relative error percentage and relative standard deviation. Results are depicted in Table 4-12 indicated excellent accuracy and precision values of the method at each concentration level.

Table 4-12: Accuracy and precision of the proposed method.

Drug	Taken ($\mu g.mL^{-1}$)	Model	Found ($\mu g.mL^{-1}$)				Mean	RE %	RSD %
CARV	3.3	PLS-1	3.2026	3.3149	3.2162	3.2859	3.2549	-1.3671	1.6624
		PLS-2	3.3481	3.3314	3.3528	3.3726	3.3512	1.5523	0.5059
	6.7	PLS-1	6.6499	6.7282	6.7898	---	6.7226	0.3378	1.0430
		PLS-2	6.5809	6.7602	6.8766	---	6.7392	0.5856	2.2104
DIAZ	3.3	PLS-1	3.1223	3.3349	3.3183	---	3.2585	-1.2575	3.6284
		PLS-2	3.1270	3.3460	3.3474	---	3.2735	-0.8040	3.8750
	13.3	PLS-1	13.1366	12.9315	13.1811	---	13.0831	-1.6311	1.0176
		PLS-2	13.1693	12.9805	13.1707	---	13.1068	-1.4524	0.8348
CARB	10.0	PLS-1	10.2171	9.9992	9.8465	10.1708	10.0584	0.5841	1.6857
		PLS-2	10.1334	10.0766	9.9736	10.1353	10.0797	0.7972	0.7521
	13.3	PLS-1	13.3562	13.3295	13.2889	---	13.3249	0.1870	0.2545
		PLS-2	13.3650	13.3641	13.3380	---	13.3557	0.4188	0.1148
FURO	3.3	PLS-1	3.1819	3.3450	3.3538	3.2214	3.2755	-0.7424	2.6534
		PLS-2	3.1360	3.3733	3.3495	3.1975	3.2641	-1.0886	3.5405
	10.0	PLS-1	10.1581	10.0796	10.1223	9.8024	10.0406	0.4062	1.6134
		PLS-2	10.0956	10.0174	10.1162	9.8108	10.0100	0.1000	1.3930

The proposed methods were successfully applied to several real samples for determination of these drugs in tablet formulations, Tables 4-13 and 4-14. Five replicate measurements were made and the results are shown in Table 4-15. The results show that there is a respectable agreement between the calculated values and the label claims indicates the applicability of the proposed PLS models for the simultaneous determination of CARV, DIAZ, CARB, and FURO in real sample.

Table 4-13: Composition of analyzed pharmaceutical sample solutions with their predictions by PLS-1 model and statistical parameters for the system.

Mixture (µg.mL^{-1})				**Prediction (µg.mL^{-1})**				**Recovery %**			
CARV	***DIAZ***	***CARB***	***FURO***	***CARV***	***DIAZ***	***CARB***	***FURO***	***CARV***	***DIAZ***	***CARB***	***FURO***
3.0	3.0	3.0	3.0	3.1548	2.9504	2.7702	3.0323	105.1593	98.3467	92.3413	101.0767
5.0	5.0	5.0	5.0	5.2593	4.9099	4.8400	5.0372	105.1852	98.1974	96.8006	100.7440
10.0	10.0	10.0	10.0	10.4140	9.3494	9.4821	9.8078	104.1395	93.4943	94.8206	98.0781
15.0	15.0	15.0	15.0	15.4634	13.9666	14.2769	14.9109	103.0891	93.1103	95.1792	99.4059
20.0	20.0	20.0	20.0	20.3214	18.3593	19.5795	20.4232	101.6068	91.7963	97.8976	102.1159
Mean recovery								**103.8360**	**94.9890**	**95.4078**	**100.2841**
^{a}R.S.E.(%) single								**2.8461**	**7.4722**	**3.7105**	**1.7956**
^{b}R.S.E.(%) total								**4.4980**			

Table 4-14: Composition of analyzed pharmaceutical samples with their predictions by PLS-2 model and statistical parameters for the system.

Mixture (µg.mL^{-1})				Prediction (µg.mL^{-1})				Recovery %			
CARV	*DIAZ*	*CARB*	*FURO*	*CARV*	*DIAZ*	*CARB*	*FURO*	*CARV*	*DIAZ*	*CARB*	*FURO*
3.0	3.0	3.0	3.0	3.1397	2.9932	2.8257	3.1747	104.6557	99.7737	94.1890	105.8223
5.0	5.0	5.0	5.0	5.2630	5.0005	4.8830	5.1546	105.2608	100.0106	97.6594	103.0926
10.0	10.0	10.0	10.0	10.4493	9.4971	9.5350	9.9229	104.4927	94.9714	95.3503	99.2289
15.0	15.0	15.0	15.0	15.5454	14.3313	14.3238	14.9487	103.6361	95.5419	95.4921	99.6581
20.0	20.0	20.0	20.0	20.3342	18.8833	19.6715	19.8400	101.6710	94.4166	98.3574	99.1998
Mean recovery								**103.9433**	**96.9428**	**96.2096**	**101.4003**
***R.S.E.(%) single**								**3.0872**	**5.1421**	**3.3009**	**1.4381**
+R.S.E. (%) total								**3.4977**			

Tables 4-15: Application of the PLS methods to the CARV, DIAZ, CARB, and FURO concentration measurements in drugs tablet formulation samples.

Sample	Weight labeled (mg/tablet)	Weight found (mg/tablet)					Mean (mg/tablet)	Recovery %	C.V. %
PLS-1									
CARV (India) tablet 25mg	25	26.164	26.315	26.123	25.909	25.418	25.986	103.943	1.344
DIAZ (Iraq) tablet 2mg	2	1.995	2.000	1.899	1.911	1.888	1.939	96.943	2.809
CARB (Switzerland) tablet 200mg	200	188.682	195.633	191.006	191.290	197.030	192.728	96.364	1.804
FURO (France) tablet 40mg	40	42.397	41.303	39.755	39.927	39.744	40.625	101.563	2.918
PLS-2									
CARV (India) tablet 25mg	25	26.290	26.297	26.035	25.772	25.402	25.959	103.836	1.460
DIAZ Iraq tablet 2mg	2	1.967	1.964	1.870	1.862	1.836	1.900	94.989	3.225
CARB (Switzerland) tablet 200mg	200	184.976	193.910	189.946	190.664	196.109	191.121	95.560	2.220
FURO (France) tablet 40mg	40	40.495	40.362	39.294	39.826	40.912	40.178	100.445	1.563

4-2 Simultaneous Determination of Furosemide, Carbamazepine, Diazepam, and Carvedilol via Derivative Spectrophotometry

4-2-1 Introduction

The classic analysis problem in the field of UV-Vis spectrophotometry is the resolution of a number of components in mixture, especially when the absorbance bands of these components are strongly overlapped. The resolve of this problem is one of the derivative spectrophotometry applications [180].

As mentioned in section 1-2-1, derivative spectrophotometry is an analytical technique based on transformation of normal-mode spectra into n^{th}-order derivative spectra. For a single peak spectrum, first derivative spectrum is a plot of gradient of the absorbance curve ($dA/d\lambda$) against wavelength, and features a maximum and a minimum at the same λ_{max} of the parent peak. The second derivative is a plot of $d^2A/d\lambda^2$ against wavelength, and it has two maxima with a minimum between them at the same λ_{max} of the normal absorption band [37].

Derivative spectral analysis is often used in identifying weak absorption peaks obscured by large peaks, identifying closely adjacent absorption bands, and most importantly in performing quantitation assay of certain analytes in the presence of other absorbing compounds [181].

- ***Literature review for the determination of the titled drugs via derivative spectrophotometry***

Because of the lack of any published method for simultaneous determination of the titled drugs in their quaternary mixture, an attempt was carried out to study the possibility of the application of derivative spectrophotometry for quantitative determination of these drugs in their mixture or in pharmaceutical preparations. However, there are many articles

deals with the individual determination of the proposed drugs or in mixtures with other drugs using this technique; some of these are listed in Table (4-16).

Table 4-16: Analytical study of derivative methods for determination of FURO, CARB, DIAZ and CARV in pharmaceutical formulations and biological fluids.

Pharmaceutical compounds	λ_{max} (nm)	Derivative order and application remark	Ref.
Furosemide	350.0	**1st**, *combined tablet dosage form*	182
	277.0	**2nd**, *bulk drug and pharmaceutical formulations*	183
Carbamazepine	285.0, 287.0	**1st** & **2nd**, *pharmaceutical preparations (Tegretol, Karberol, and Karbalex tablets)*	184
	308.9	**1st**, *synthetic mixtures, urine*	185
Diazepam	313.0	**4th**, *Human plasma*	186
	248.0	**1st**, *combined tablet dosage form*	99
Carvedilol	245.9, 247.4	**3rd** & **4th**, *tablets, spiked human plasma*	187
	301.0	**1st**, *pharmaceutical dosage form*	188

4-2-2 Experimental

4-2-2-1 Instrumentation

All absorption spectra were recorded by Cecil CE7200 UV-Visible double beam spectrophotometer (Cambridge-England) on a range of 200-380 nm (the other instrumental parameters were described in section 4-1-4-1). The resulted absorption data were digitalized, plotted, and manipulated by Shimadzu 1800 software (UVProb 2.34) to obtain the first, second, third and fourth order derivatives.

4-2-2-2 Chemicals

All chemicals used in this study are described in Table (2-1), section (2-1).

4-2-2-3 Pharmaceutical Compounds

Table (2-2), section (2-2), illustrates the pharmaceutical compounds used and their manufacturers.

4-2-2-4 Standard Drugs Solutions

The procedures used throughout the preparation of the standard pharmaceutical solutions, which have been used throughout the investigation, are given in section (2-5-3).

4-2-2-5 Pharmaceutical Product Solution

The procedures used throughout the preparation of pharmaceutical formulation solutions, which has been used for the application study, are given in section (2-5-5).

4-2-2-6 General Recommended Procedures

- ***Assay procedure for individual determination of furosemide, carbamazepine, diazepam, and carvedilol***

1.0 mL aliquots, of each drug standard solution containing 5-100 μg were transferred to a series of 5 mL volumetric flask and diluted with methanol. The spectrum for each solution was recorded against the solvent blank. Zero-order spectrum was then manipulated for each to get its first (D1), second (D2), third (D3) and fourth (D4) derivative.

- ***Assay of laboratory prepared mixtures of furosemide, carbamazepine, diazepam, and carvedilol***

Mixtures containing different ratios of FURO, CARB, DIAZ and CARV, which were used in this study, are given in section (4-1-3-5) Table (4-3). The

absorption spectrum for each solution was recorded against solvent blank and manipulated to get its first, second, third, and fourth order derivatives.

4-2-3 Results and discussion

Although the derivative spectra are more complex than their corresponding original spectra, the intrinsic quantitative information is not increased [45]. The main advantage of the derivative methods is the chance of doing easy measurements in correspondence of peaks so it allows using the wavelength of the highest value of analytical signals (a maximum or a minimum) in addition to many other modes of measurements. Moreover, the presence of several maxima and minima is another advantage by the fact that these wavelengths give an opportunity for the estimation of active compound in the presence of foreign compounds that possibly interfere in the assay[189].

4-2-3-1 Absorption Spectra

Normal mode spectra of FURO, CARB, DIAZ, and CARV (Figure 4-1) show a significant spectral-overlap, which interfere with direct spectrophotometric determination of the studied drugs.

The absorption spectra of FURO, CARB, DIAZ, CARV and the spectrum of their mixture were recorded against methanol as a blank (Figure 4-14). The spectra of CARV and FURO are characterized by the presence of three peaks descending in their absorbance values at 242.0, 285.5, 332.5 nm and at 229.0, 275.0, 334.0 nm respectively. On the other hand, the spectrum of CARB shows two peaks with a shoulder between them at 214.5, 286.0, 237.0 nm respectively, while DIAZ has three peaks with a shoulder between them at 205.0, 229.0, 257.0 and 316.0 nm respectively. The spectrum of the four drugs mixture is characterized by the presence of two peaks, the first with the

higher intensity at 227.0 nm resulted from the contribution of the absorption maxima of the four compounds and the second at 278.5 nm.

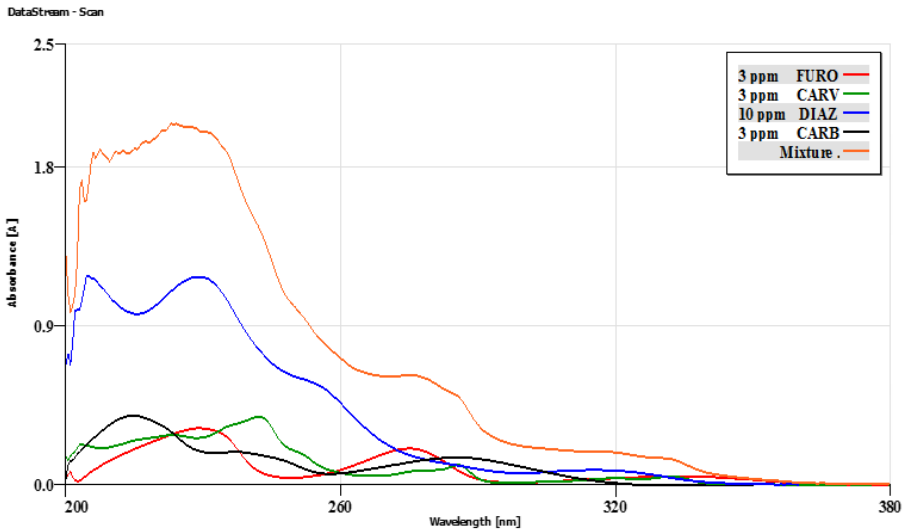


Figure 4-14: Zero-order absorption spectra of 3 µg.mL^{-1} **CARV**, 10 µg.mL^{-1} **DIAZ**, 3 µg.mL^{-1} **CARB**, 3 µg.mL^{-1} **FURO**, and their 3.3 µg.mL^{-1} **quaternary mixture** for CARV, CARB, FURO and 10 µg.mL^{-1} for DIAZ against methanol as a blank.

4-2-3-2 First, second, third and fourth derivative modes

The simultaneous analysis of the titled drugs in their mixtures by common spectrophotometric methods is hindered by spectral overlap, so derivative spectrophotometry was suggested for this purpose. To select the derivative order, standard solutions of FURO, CARB, DIAZ and CARV were scanned separately in the UV range, and the obtained normal mode spectra were then processed to get their corresponding first, second, third, and fourth derivative spectra.

The investigation reveals that FURO and CARV could be determined by all modes of derivative (i.e. first to fourth order); CARB could be determined by first, second, and third order; while only first and second modes of derivative could be used in the determination of DIAZ in presence of other

investigated drugs. The overlaid spectra of the first, second, third, and fourth order derivatives of each of the cited drugs are shown in Figures (4-15), (4-16), (4-17) and (4-18) respectively.

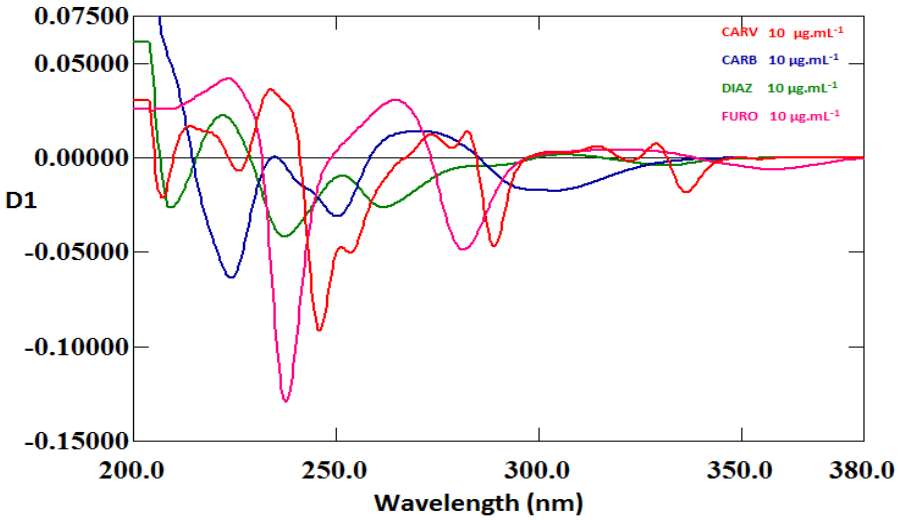


Figure 4-15: Overlaid first order derivative spectra of 10 µg.mL^{-1} of FURO, CARB, DIAZ and CARV in methanol.

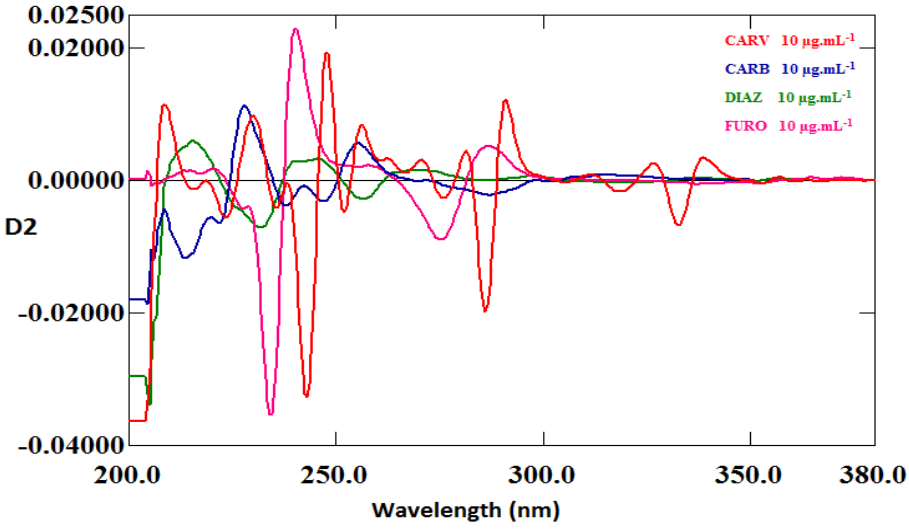


Figure 4-16: Overlaid second derivative spectra of 10 µg.mL^{-1} of FURO, CARB, DIAZ and CARV in methanol.

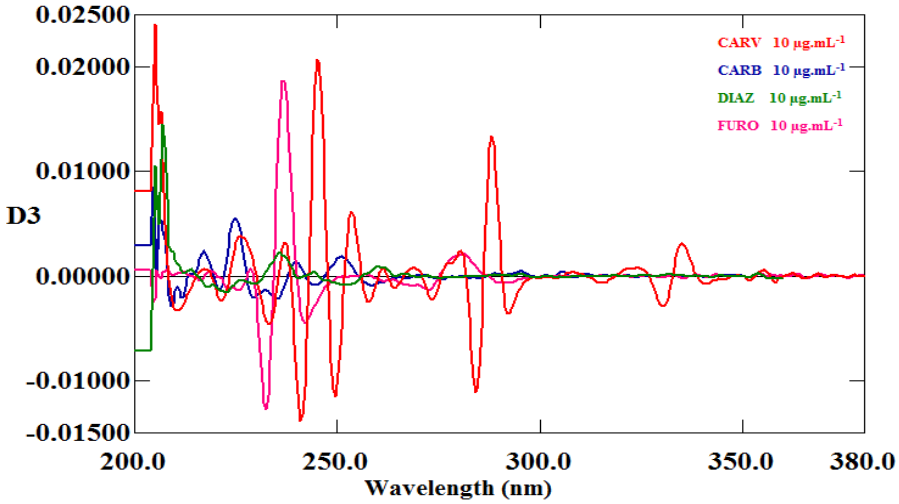


Figure 4-17: Overlaid third derivative spectra of 10 µg.mL^{-1} of FURO, CARB, DIAZ, and CARV in methanol.

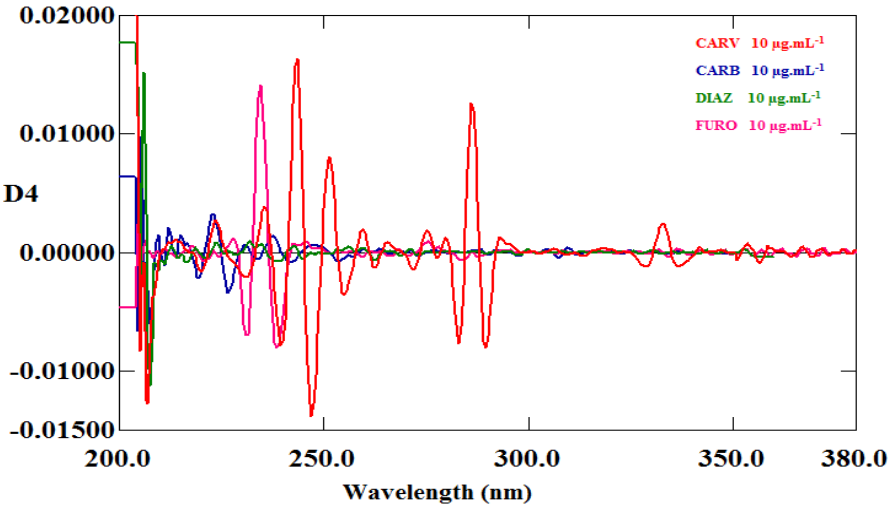


Figure 4-18: Overlaid fourth derivative spectra of 10 µg.mL^{-1} of FURO, CARB, DIAZ, and CARV in methanol.

4-2-3-3 Individual Calibration of Each Drug

The absorption spectra of the standard FURO, CARB, DIAZ, and CARV solutions in methanol were recorded in wavelength range 200–380 nm against methanol, using 1.0 cm quartz cells. Calibration graph for each drug was constructed in the concentration range of (1 – 20 µg.mL^{-1}) at 233.0 nm, 215.0 nm, 228.5 nm and 242.5 nm for FURO, CARB, DIAZ, and CARV

respectively. Linear calibration relations were obtained in studied concentration ranges.

4-2-3-4 Simultaneous Calibration of Quaternary Mixture via Derivative Mode

Quaternary mixtures for the cited drugs with different ratios were prepared depending on the results obtained in the individual calibration so that to keep the upper absorption range of the prepared mixtures to be less than 3.0.

The first step in the calibration is to select the more convenient analytical wavelengths at which the multi-component system is analyzed by derivative spectrophotometry[190]. In practice, the selected wavelength exhibited the best linear response, giving a zero or nearby zero intercept with the ordinate of the calibration curve, and not affected by the presence of any other component[191].

The analysis of the derivative spectra is done through the determination of the derivative values, which is carried out by one of the four graphical methods, which included area under peak, baseline-to-peak (peak height), peak-to- peak and zero crossing techniques.

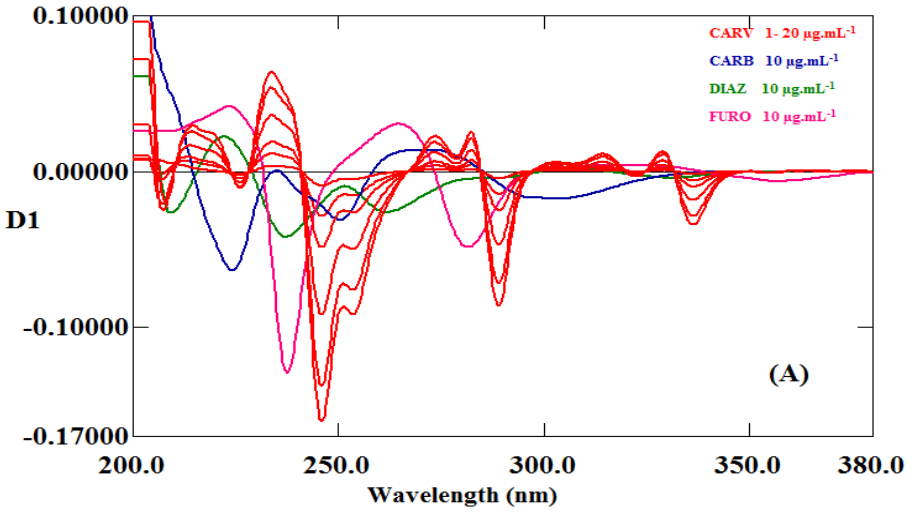
In area under peak technique, the area of a peak or a valley is measured. In the baseline to peak technique, the span of the peak is measured from a maximum to the zero line or from a minimum to the zero line. In the peak-to-peak technique, the determination is carried out by measuring the amplitude from a maximum to a minimum of the curve. In zero crossing technique, measurement of the derivative value at a wavelength, at which the derivative of the interfering species accepts value zero-crosses the zero line. This technique should be determined for at least two concentrations [38].

In the present study, several attempts have been done for the determination of the titled drugs via derivative spectrophotometry through applying the

mentioned four techniques, but only zero crossing method proved the utility for simultaneous analysis of the cited drugs in their quaternary mixtures. Calibration curves were constructed by plotting the value of derivative of the 1st, 2nd, 3rd and 4th order derivatives spectra against the corresponding concentrations of the examined drugs.

– ***Carvedilol***

Figure (4-19A) depicts the overlaid first derivative spectra of 10 µg.mL^{-1} solutions of CARB, FURO and DIAZ and for different concentrations (1-20 µg.mL^{-1}) of CARV. By careful eye inspection of the figure, several zero crossing points were found. All of these points were proven to be un useful and were therefor, discarded except the zero–cross point at 336.5 nm. The point at 336.5 nm is located almost in the center of the peak in the 1st derivative spectra of CARV (Figure (4-19 B)).



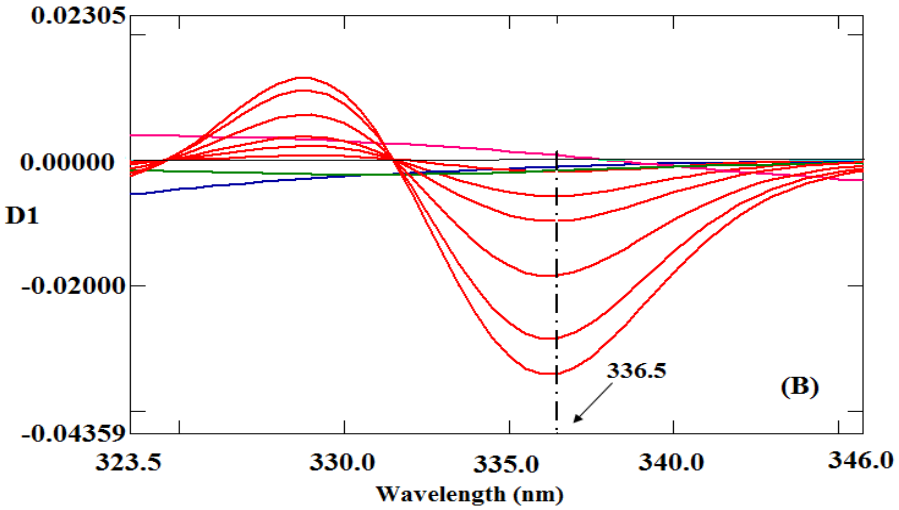


Figure 4-19: First derivative spectra of (1-20 µg.mL^{-1}) carvedilol and 10 µg.mL^{-1} for FURO, CARB, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 323.5- 346.0 nm**.**

Therefore, calibration plot was constructed for the assay of CARV in the presence of CARB, FURO and DIAZ at 336.5 nm by plotting the measured values of derivative D1 (as signals) versus the concentration of CARV. A linear relation was obtained (Figure 4-20), from which the regression equation and coefficient of determination (R^2) were calculated.

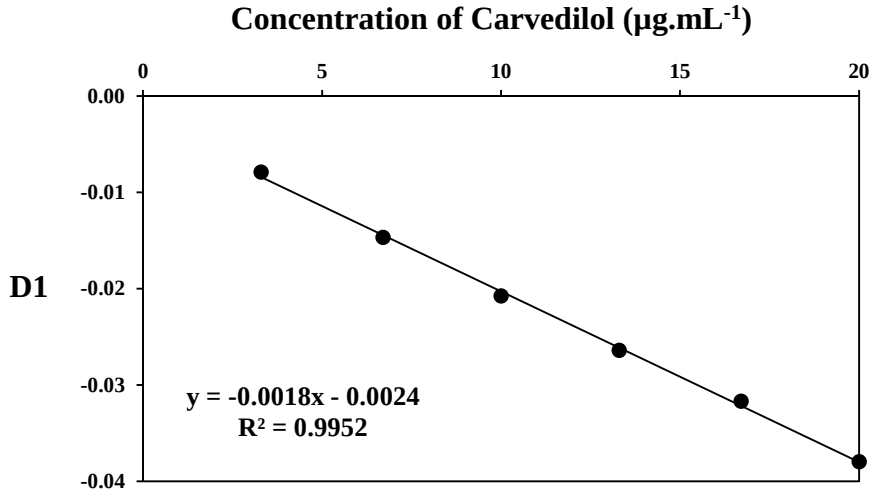


Figure 4-20: Calibration curve obtained via first mode derivative of carvedilol for height measurements at zero cross at 336.5 nm.

For the second derivative technique, the spectra were recorded for the same previous solutions of CARV, CARB, FURO and DIAZ. It is obvious from Figure (4-21), that only CARV shows second derivative signal in the spectral region above 323.0 nm at which the other three drugs have no any signal. After careful inspection, a wavelength of 332.5 nm was selected to carry out peak height measurements for CARV, since at this wavelength, there is no possibility for spectral overlapping and therefore, it is expected to obtain more accurate results. Fig (4-22) represents the obtained calibration curve.

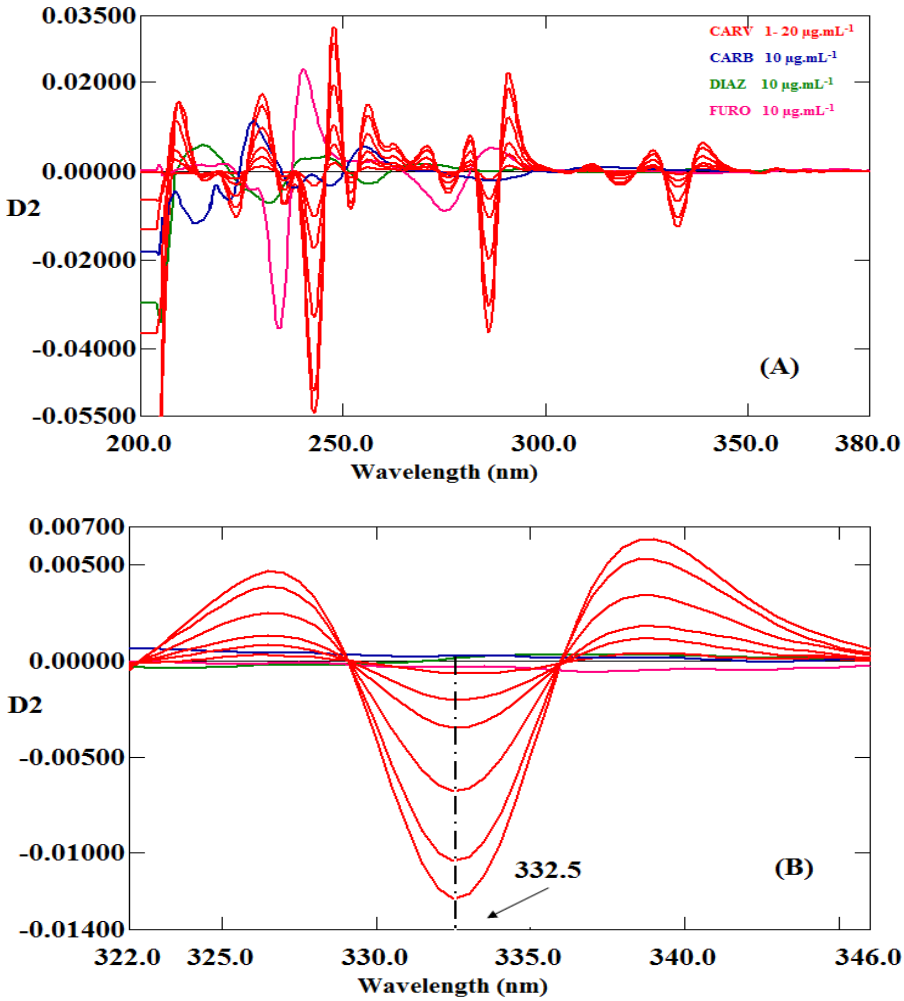


Figure 4-21: Second derivative spectra of (1-20 µg.mL^{-1}) carvedilol and 10 µg.mL^{-1} for FURO, CARB, and DIAZ in methanol for the range: **(A)** 200-380 nm, **(B)** 322.0- 346.0 nm.

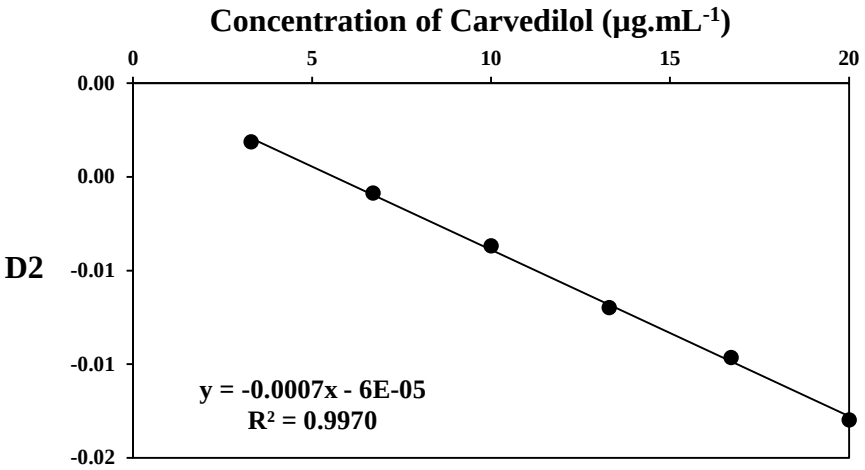


Figure 4-22: Calibration curve obtained via peak height measurements at 332.5 nm in second mode derivative of carvedilol.

The third derivative spectra of methanolic solutions of CARV, FURO, CARB and DIAZ were investigated in order to select the appropriate analytical wavelength (s) for the estimation of CARV. The derivative signal peaks of CARV were isolated in wavelength range of 275-350 nm (Figure (4-23). A wavelength of 288.0 nm was selected for CARV determination since, at this wavelength, accurate and reproducible readings for peak height were obtained. Figure (4-24) displays the calibration curve of CARV via peak height measurements at 288.0 nm.

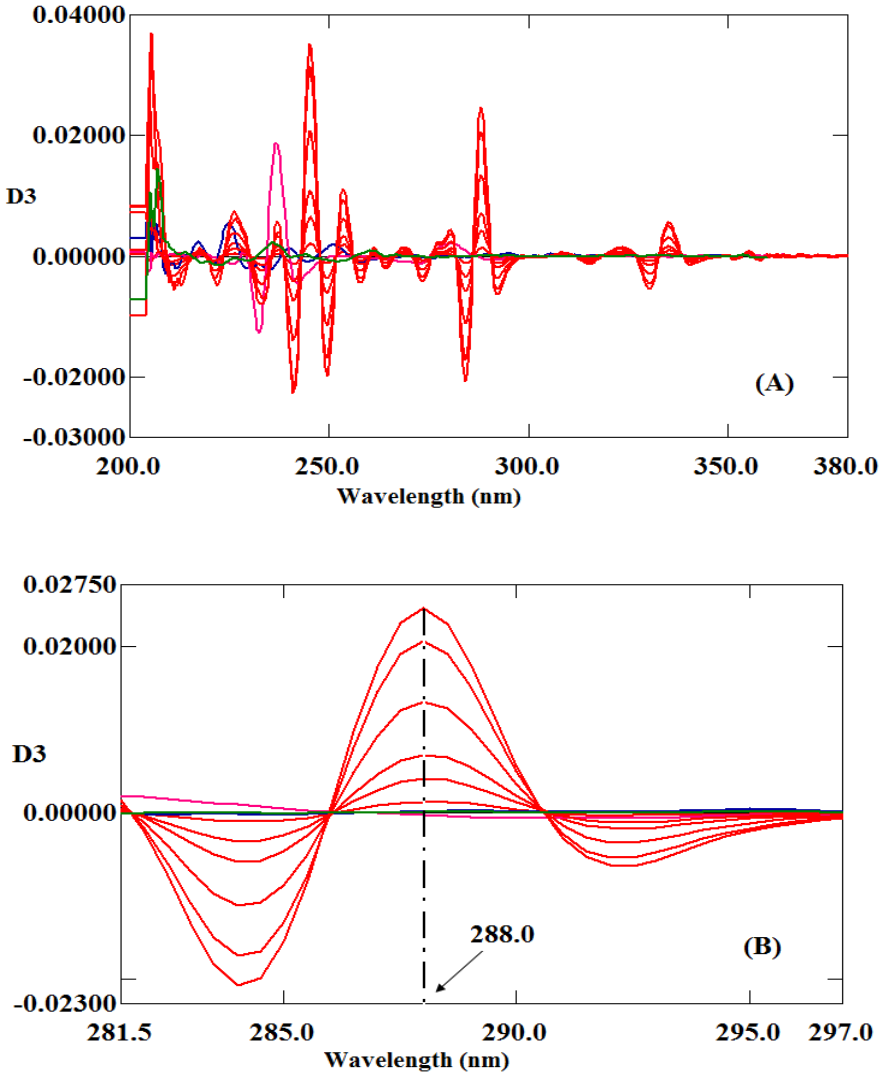


Figure 4-23: Third derivative spectra of (1-20 µg.mL^{-1}) carvedilol and 10 µg.mL^{-1} for FURO, CARB, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 281.5-297.0 nm**.**

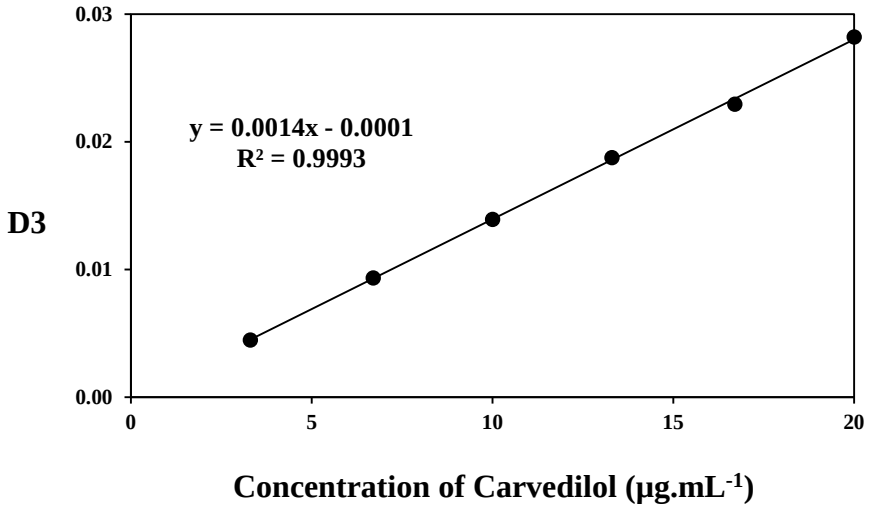
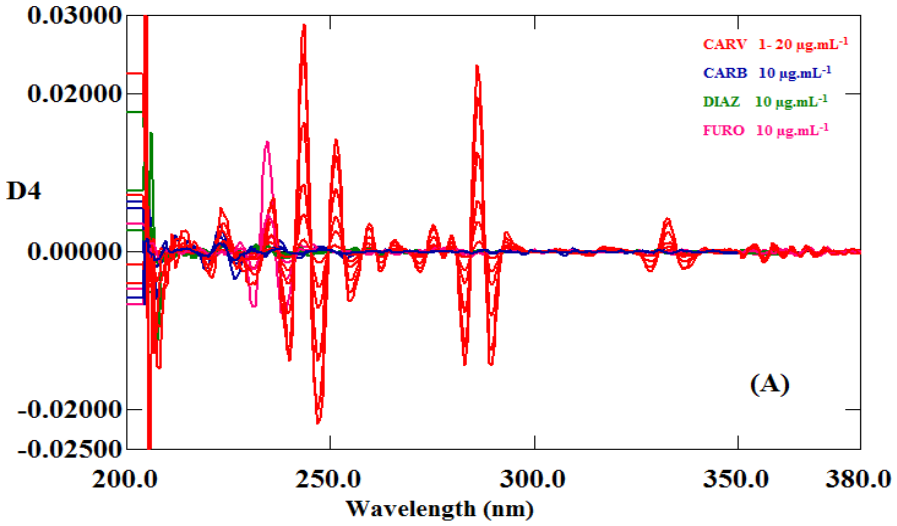


Figure 4-24: Calibration curve obtained via third mode derivative of carvedilol for peak height at 288.0 nm.

Finally, the fourth derivative spectra for the mentioned solutions were recorded, and peak height measurement at the specified analytical wavelength (286.0 nm) was used to determine the exact concentration of CARV in quaternary mixtures. Figure 4-25 shows the recorded fourth derivative spectra of CARV, DIAZ, FURO and CARB, and Figure 4-26 depicts the resulted calibration curve.



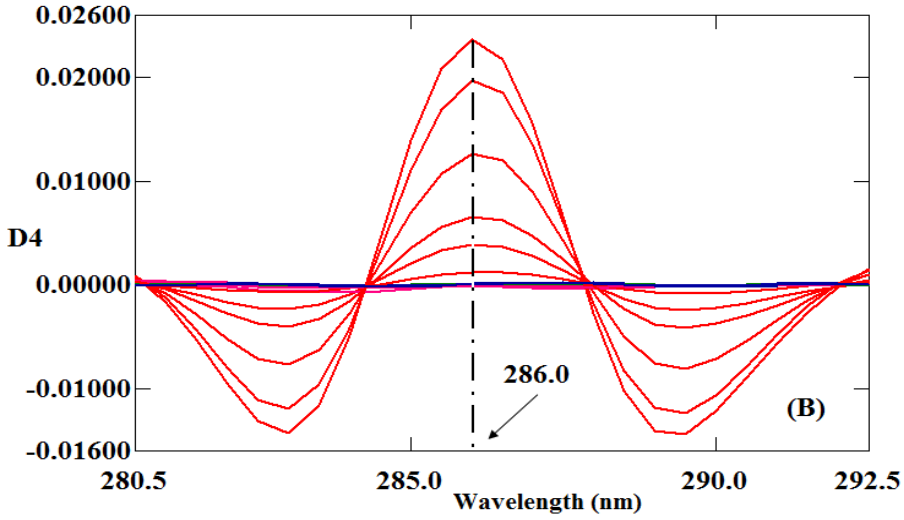


Figure 4-25: Fourth derivative spectra of (1-20 µg.mL^{-1}) carvedilol and 10 µg.mL^{-1} for FURO, CARB, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 280.5-292.5 nm**.**

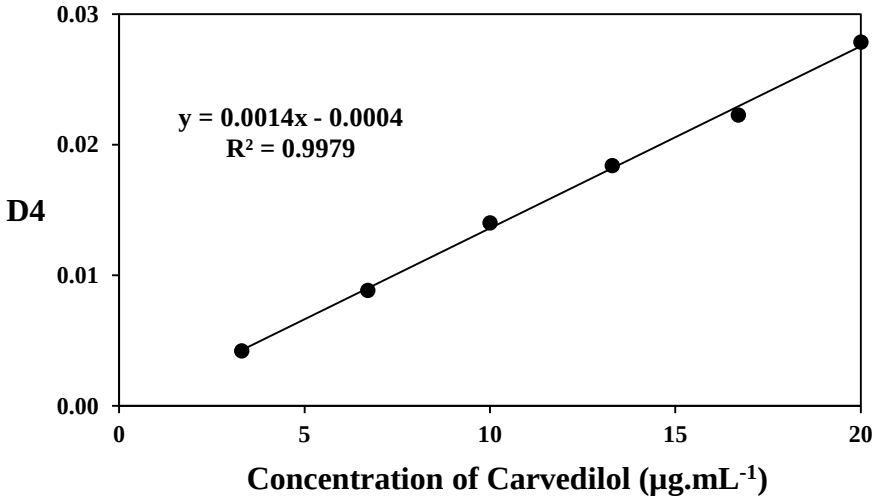


Figure 4-26: Calibration curve obtained via peak height measurement at 286.0 nm in fourth mode derivative of carvedilol.

Some analytical characteristics, namely Beer's law limit, detection limit, slope, intercept and correlation coefficient for the determination of carvedilol, in each derivative mode, were calculated and the results are reported in Table 4-17.

Table 4-17: Analytical parameters for the determination of carvedilol using first, second, third and fourth derivative spectrophotometric techniques.

Taken range ($\mu g.mL^{-1}$)	Derivative Mode	λ(nm)	Regression Equation	R^2	Detection limit* ($\mu g.mL^{-1}$)
3.3 - 20.0	**D1** (*zero cross*)	336.5	y = -0.0018x - 0.0024	0.9952	0.30474
	D2 (*peak height*)	332.5	y = -0.0007x - 0.00006	0.9970	0.39386
	D3 (*peak height*)	288.0	y = 0.0014x - 0.0001	0.9993	0.07565
	D4 (*peak height*)	286.0	y = 0.0014x - 0.0004	0.9979	0.06147

***Detection limit = 3.3 (SD / slope), n = 5 measurements.**

– ***Furosemide, Carbamazepine and Diazepam***

Taking into account the behavior of the derivative spectra of FURO, CARB and DIAZ, the best analytical wavelengths were chosen by the same mentioned manner used in the determination of CARV.

The investigation reveals that first, second, third and fourth derivative order could be applied for the determination of furosemide. The derivative spectra of FURO (Figures 4-27, 29, 31 and 33) show that the heights at the selected analytical wavelengths were not affected by the presence of the three other drugs over the range of studied concentrations. The optical and regression characteristics for the mentioned derivative orders were calculated, and the results show excellent values of correlation coefficient and detection limit for such a system.

Determination of carbamazepine was done by using first, second and third derivative modes. It is obvious in Figures 4-35, 37 and 39 that there is sever spectral overlapping. Consequently, the useful analytical wavelengths were limited and by careful eye inspection of the figures, three points were found

to be applicable for this study. Calibration curves at these points were linearly proportional to CARB concentration.

Due to the spectral behavior of diazepam, the selection of appropriate wavelength and derivative mode was difficult. This is because of the limited number of peaks in the normal spectrum of DIAZ in comparison with the spectra of the other drugs. As a result, attempts to use third and fourth modes of derivative were failed to determine DIAZ due to the absence of adequate zero crossing points, while a zero-cross point could be identified for this purpose in first and second modes of derivative. Table 4-18 summarizes the analytical parameters for the selected methods that have been used in the determination of FURO, CARB and DIAZ.

Table 4-18: Summary of the selected methods for the determination of FURO, CARB and DIAZ and their analytical parameters.

Drug	Taken range ($\mu g.mL^{-1}$)	Derivative Mode	λ(nm)	Regression Equation	R^2	Detection limit* ($\mu g.mL^{-1}$)	Figure No.
FURO	3.3 - 20.0	**D1** (*zero cross*)	358.5	y = -0.0007x + 0.0009	0.9925	0.52135	4-27 & 28
		D2 (*zero cross*)	273.5	y = -0.0010x + 0.0012	0.9966	0.34104	4-29 & 30
		D3 (*zero cross*)	237.5	y = 0.0024x - 0.0046	0.9982	0.57262	4-31 & 32
		D4 (*zero cross*)	234.0	y = 0.0021x - 0.0058	0.9897	0.49775	4-33 & 34
CARB		**D1** (*zero cross*)	306.0	y = -0.002x + 0.0049	0.9996	0.10539	4-35 & 36
		D2 (*zero cross*)	226.0	y = 0.0009x - 0.0014	0.9933	0.59388**	4-37 & 38
		D3 (*zero cross*)	224.0	y = 0.0007x - 0.0021	0.9957	0.49068**	4-39 & 40
DIAZ		**D1** (*zero cross*)	331.5	$y = -0.0004x + 8 \times 10^{-5}$	0.9970	0.86439**	4-41 & 42
		D2 (*zero cross*)	218.5	y = 0.0006x - 0.0013	0.9846	0.93702**	4-43 & 44

***Detection limit = 3.3 (SD / slope), n = 5 measurements, ** n = 3 measurements.**

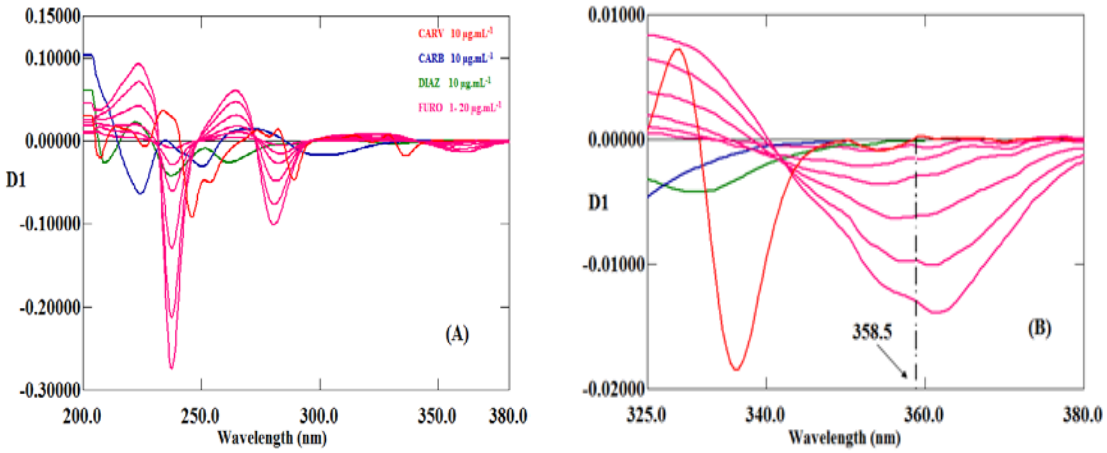


Figure 4-27: First derivative spectra of (1-20 µg.mL^{-1}) furosemide and 10 µg.mL^{-1} for CARV, CARB, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 325.0-380 nm**.**

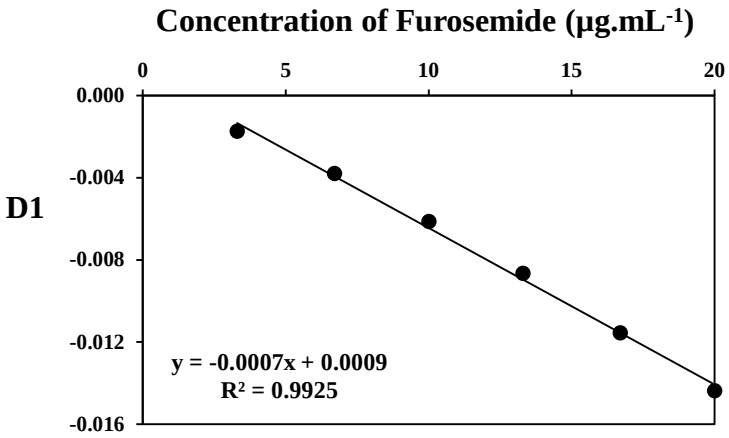


Figure 4-28: Calibration curve obtained via first derivative spectra of furosemide for height at zero cross at 358.5 nm.

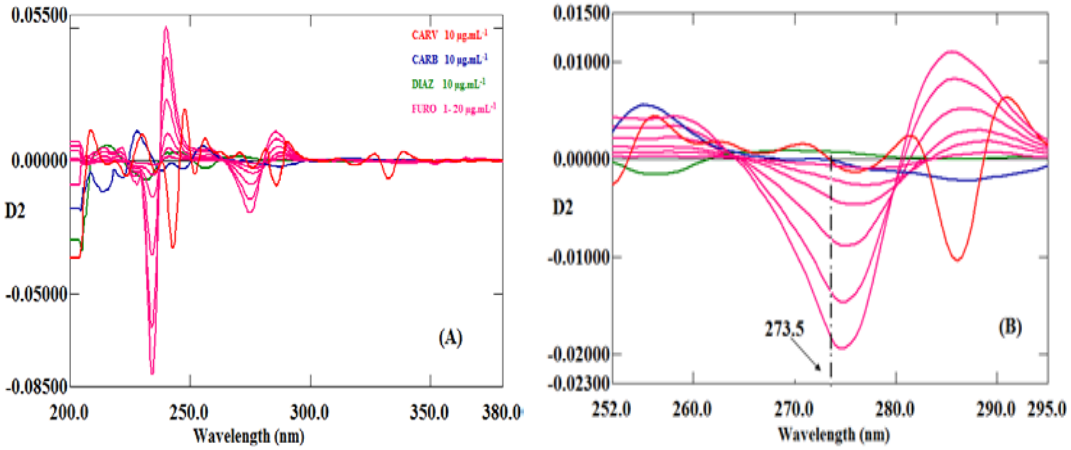


Figure 4-29: Second derivative spectra of (1-20 µg.mL^{-1}) furosemide and 10 µg.mL^{-1} for CARV, CARB, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 252.0-295.0 nm**.**

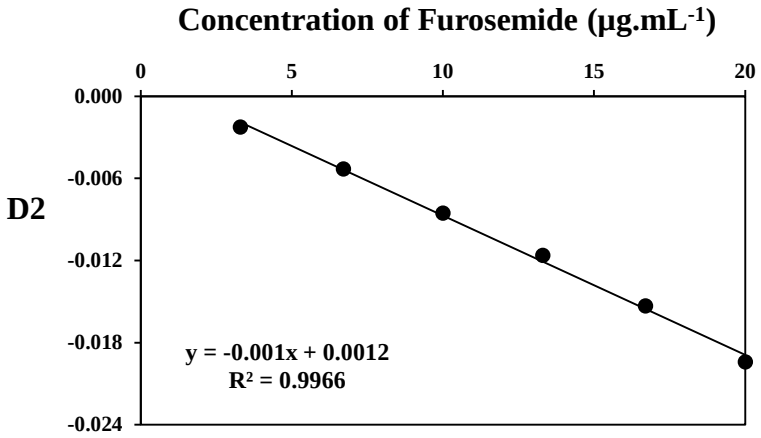


Figure 4-30: Calibration curve obtained via second derivative spectra of furosemide for height at zero cross at 273.5 nm.

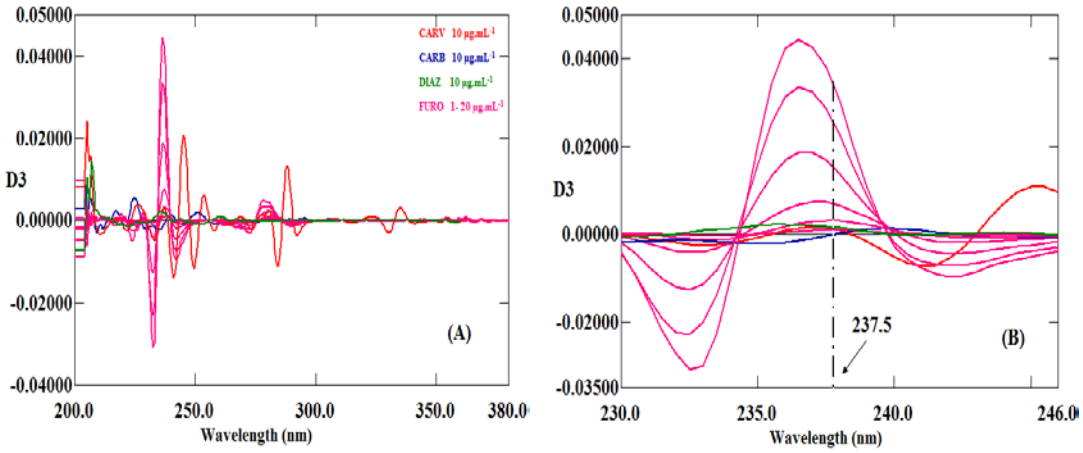


Figure 4-31: Third derivative spectra of (1-20 µg.mL-1) furosemide and 10 µg.mL-1 for CARV, CARB, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 230.0-246.0 nm**.**

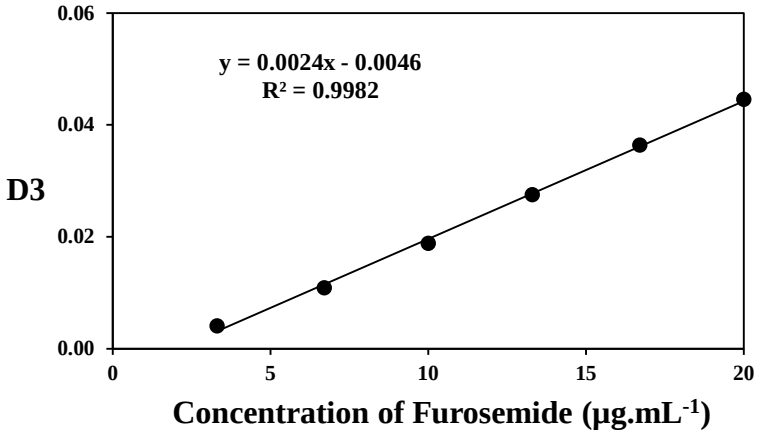


Figure 4-32: Calibration curve obtained via third derivative spectra of furosemide for height at zero cross at 237.5 nm.

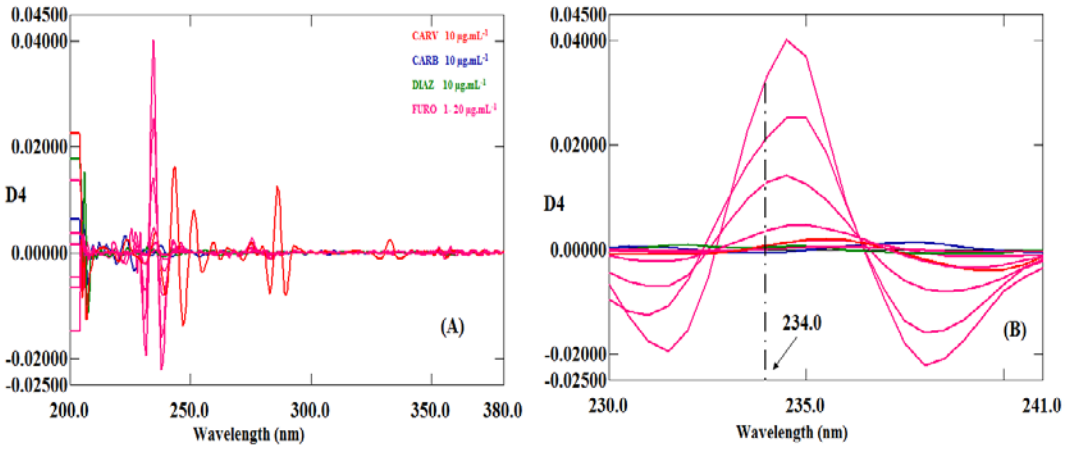


Figure 4-33: Fourth derivative spectra of (1-20 µg.mL^{-1}) furosemide and 10 µg.mL^{-1} for CARV, CARB, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 230.0-241.0 nm**.**

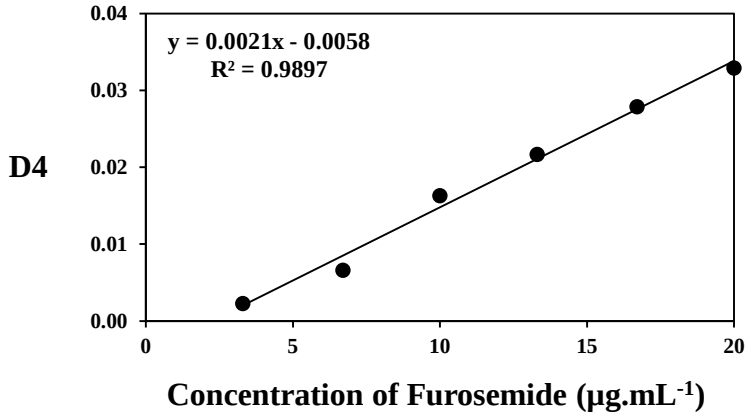


Figure 4-34: Calibration curve obtained via fourth derivative spectra of furosemide for height at zero cross at 234.0 nm.

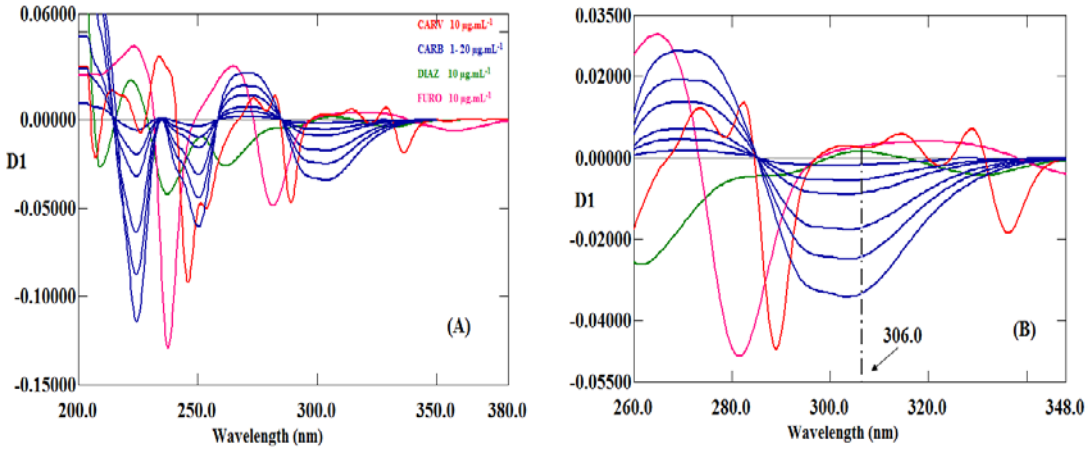


Figure 4-35: First derivative spectra of (1-20 µg.mL^{-1}) carbamazepine and 10 µg.mL^{-1} for CARV, FURO, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 260.0-348.0 nm**.**

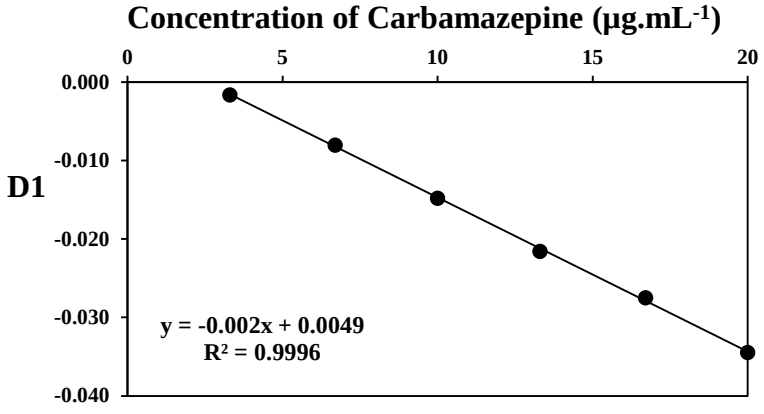


Figure 4-36: Calibration curve obtained via first derivative spectra of carbamazepine for height at zero cross at 306.0 nm.

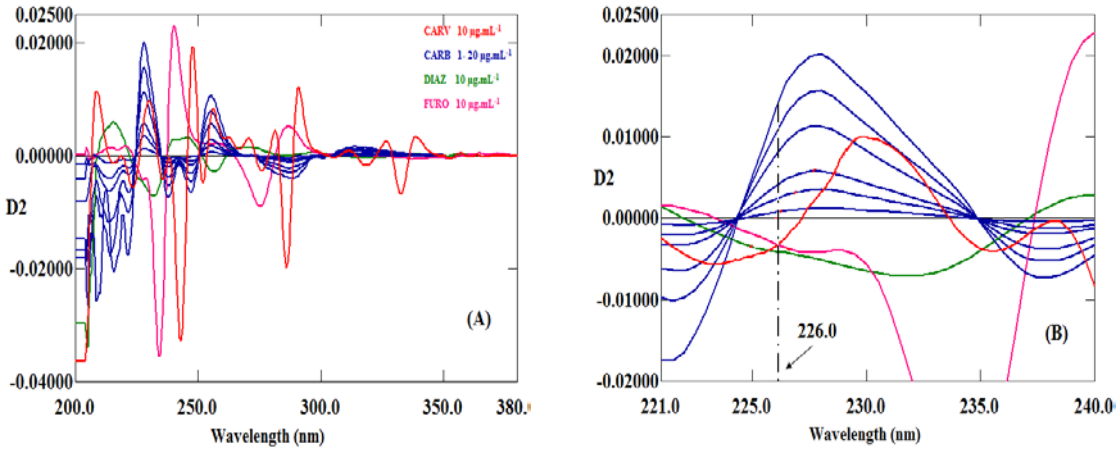


Figure 4-37: Second derivative spectra of (1-20 µg.mL^{-1}) carbamazepine and 10 µg.mL^{-1} for CARV, FURO, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 221.0-240.0 nm**.**

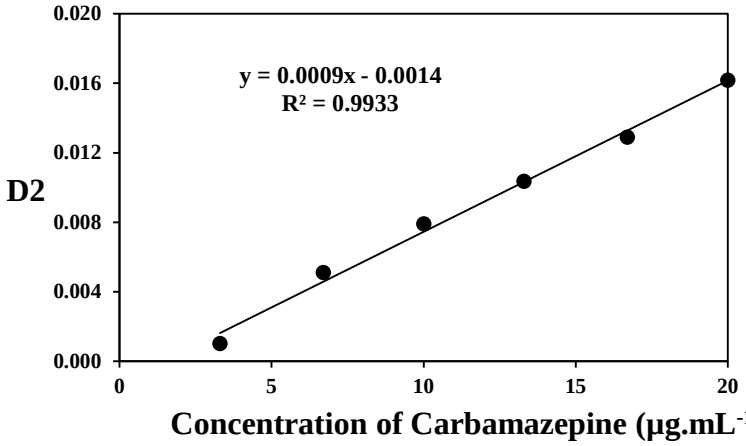


Figure 4-38: Calibration curve obtained via second derivative spectra of carbamazepine for height at zero cross at 226.0 nm.

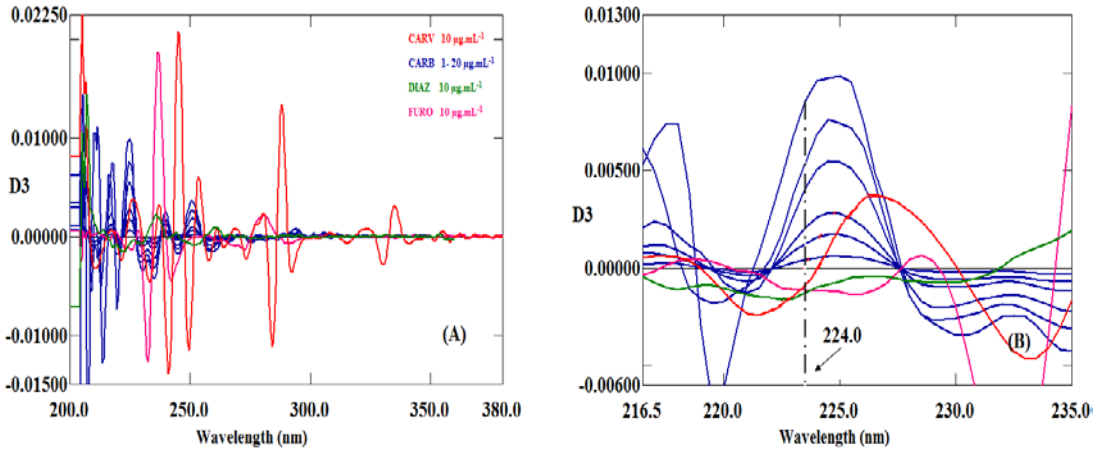


Figure 4-39: Third derivative spectra of (1-20 µg.mL^{-1}) carbamazepine and 10 µg.mL^{-1} for CARV, FURO, and DIAZ in methanol for the range **(A)** 200-380 nm, **(B)** 216.5-235.0 nm**.**

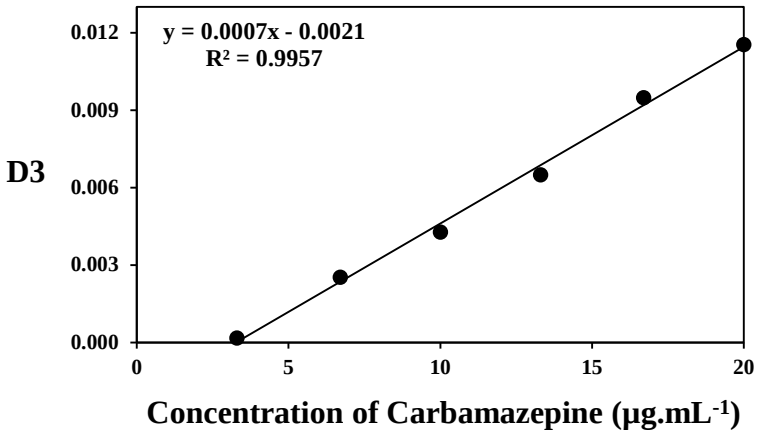


Figure 4-40: Calibration curve obtained via third derivative spectra of carbamazepine for height at zero cross at 224.0 nm.

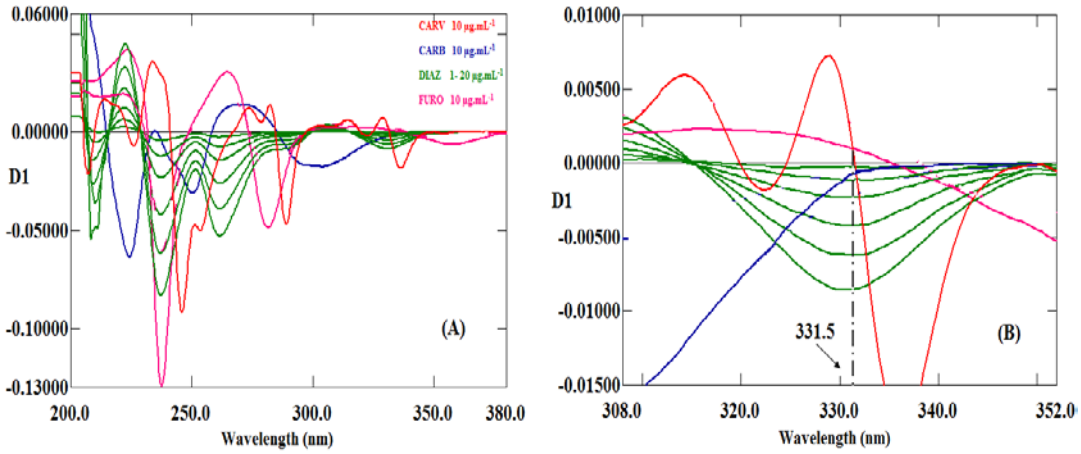


Figure 4-41: First derivative spectra of (1-20 µg.mL^{-1}) diazepam and 10 µg.mL^{-1} for CARV, FURO, and CARB in methanol for the range **(A)** 200-380 nm, **(B)** 308.0-352.0 nm**.**

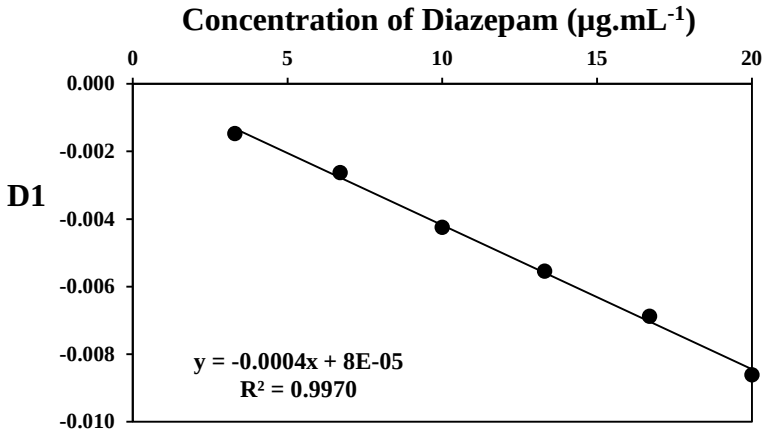


Figure 4-42: Calibration curve obtained via first derivative spectra of diazepam for height at zero cross at 331.5 nm.

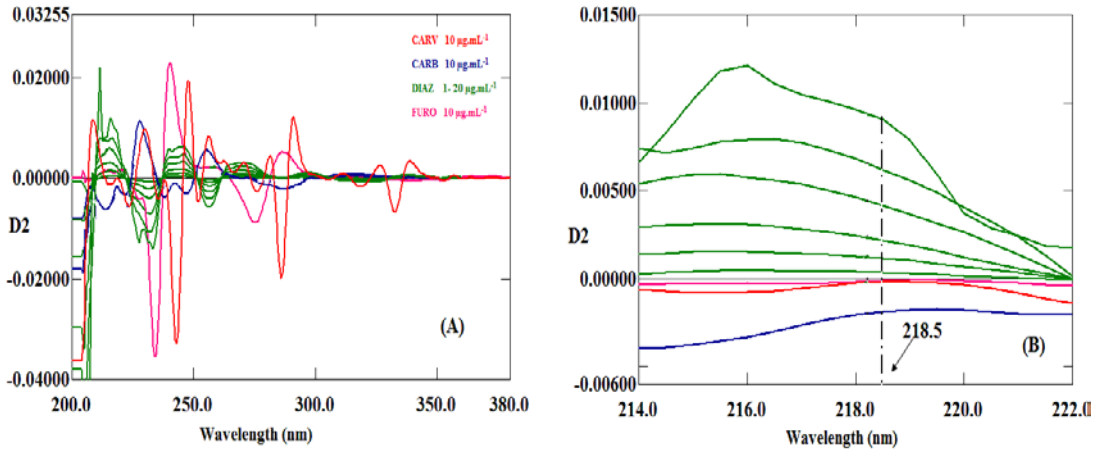


Figure 4-43: Second derivative spectra of (1-20 µg.mL^{-1}) diazepam and 10 µg.mL^{-1} for CARV, FURO, and CARB in methanol for the range **(A)** 200-380 nm, **(B)** 214.0-222.0 nm**.**

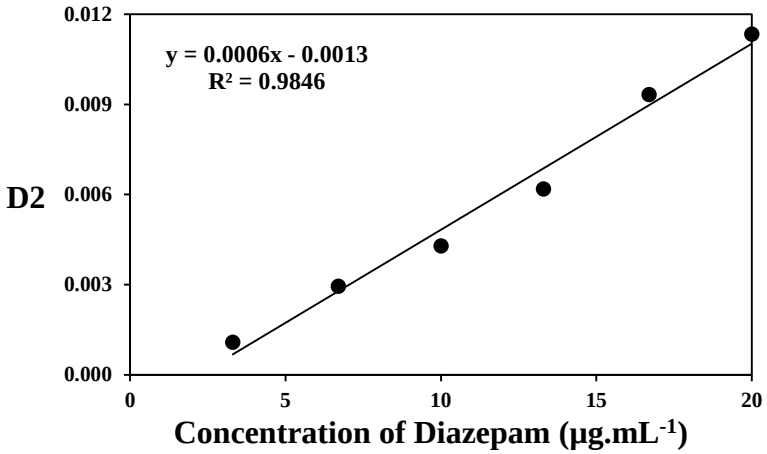


Figure 4-44: Calibration curve obtained via second derivative spectra of diazepam for height at zero cross at 218.5 nm.

4-2-3-5 Accuracy and precision

The accuracy and precision of the determination of CARV, FURO, CARB and DIAZ via the proposed methods which were established by calculating the values of percentage of the relative error (RE %) and relative standard deviation percent (RSD %), for three replicate analyses at two different levels of analytes concentrations in the range of 3.3-16.7 µg.mL^{-1} at the same day.

The calculated analytical results show good accuracy with reasonable precision of the proposed methods as reported in Table 4-19.

Table 4-19: Evaluation of accuracy and precision for the determination of CARV, FURO, CARB and DIAZ via derivative spectrophotometry.

Drug	Derivative Mode	Taken ($\mu g.mL^{-1}$)	Found ($\mu g.mL^{-1}$)			Mean ($\mu g.mL^{-1}$)	RE%	RSD%
CARV	D1	3.3	3.2780	3.0830	3.1670	3.1760	-3.7576	3.0797
		13.3	13.1560	13.5220	13.6890	13.4557	1.1704	2.0261
	D2	6.7	6.5000	6.7140	6.4860	6.5667	-1.9900	1.9460
		10	10.0000	9.6860	10.2860	9.9907	-0.0933	3.0039
	D3	6.7	6.6930	6.7210	6.8070	6.7403	0.6020	0.8814
		13.3	13.4640	13.5290	13.3860	13.4597	1.2005	0.5319
	D4	3.3	3.3360	3.3070	3.2930	3.3120	0.3636	0.6622
		10	10.1640	10.3000	10.2640	10.2427	2.4267	0.6880
FURO	D1	3.3	3.0860	3.1290	3.2710	3.1620	-4.1818	3.0618
		6.7	6.5286	6.8000	6.8714	6.7333	0.4975	2.6861
	D2	3.3	3.2400	3.2300	3.3500	3.2733	-0.8081	2.0341
		10	9.5000	9.5200	10.2000	9.7400	-2.6000	4.0913
	D3	6.7	6.8630	6.8750	6.7500	6.8293	1.9303	1.0099
		10	9.9250	9.8130	10.3300	10.0227	0.2267	2.7137
	D4	3.3	3.3480	3.5240	3.2667	3.3796	2.4111	3.8917
		13.3	12.9330	13.1860	13.1330	13.0840	-1.6241	1.0198
CARB	D1	10	10.0600	10.0800	9.9600	10.0333	0.3333	0.6408
		13.3	13.2200	13.5550	13.4300	13.4017	0.7644	1.2632
	D2	10	10.1330	10.5670	9.6000	10.1000	1.0000	4.7955
		16.7	16.0780	15.9330	16.6667	16.2259	-2.8390	2.3946
	D3	6.7	6.8286	6.6000	6.5714	6.6667	-0.4975	2.1145
		13.3	12.8857	12.6286	13.1286	12.8810	-3.1506	1.9411
DIAZ	D1	6.7	6.8250	6.8250	6.1250	6.5917	-1.6169	6.1312
		10	10.0300	10.3500	9.7000	10.0267	0.2667	3.2415
	D2	10	9.3170	9.7000	10.5200	9.8457	-1.5433	6.2422
		13.3	13.6700	12.5500	12.7500	12.9900	-2.3308	4.5984

4-2-3-6 Application

Commercially available tablets of CARV, FURO, CARB and DIAZ (Carvedilol®, Lasix®, Tegretol® and Valiapam® respectively) were subjected to analysis by the proposed derivative procedures. The quantitative determination of four concentration levels of CARV, FURO, CARB and DIAZ was carried out via derivative spectrophotometry. Table 4-20 shows the values of recovery percentage obtained for the analyzed samples by the application of the mentioned derivative techniques on the pharmaceutical formulations. The results of the analysis were satisfactory, i.e. precise and accurate, as indicated by the excellent recovery percent. In addition, the results are in respectable agreement with the label claims and this indicates the applicability of the proposed methods for the simultaneous estimation of the cited drugs in real samples (Table 4-21).

Moreover, standard addition method was applied to analyze CARV, FURO, CARB and DIAZ in their pharmaceutical preparations to verify the efficiency of the proposed procedures. This study was performed by adding known amounts of pure drug to a given concentration of the commercial pharmaceutical solution. The resulting mixtures were analyzed by following the recommended procedures, and the total found amounts[192] were calculated from the corresponding regression equation of each drug (Table 4-22). The evaluation of the application of the proposed methods were checked by calculating the values of percentage of the recovery and coefficient of variation percent (C.V %). Figures (4-45, 4-46, 4-47, 4-48) represents the obtained derivative spectra by applying standard addition method for CARV, FURO, CARB and DIAZ respectively.

Table 4-20: Application of the derivative spectrophotometry to the CARV, FURO, CARB and DIAZ concentration measurements in their pharmaceutical formulations.

Derivative Mode	Mixture (µg.mL^{-1})				Found (µg.mL^{-1})				Recovery %			
	CARV	*FURO*	*CARB*	*DIAZ*	*CARV*	*FURO*	*CARB*	*DIAZ*	*CARV*	*FURO*	*CARB*	*DIAZ*
D1	5.0	5.0	5.0	5.0	4.7222	5.0857	5.5950	5.4250	94.4444	101.7143	111.9000	108.5000
	10.0	10.0	10.0	10.0	9.5667	10.2000	10.7350	10.0500	95.6667	102.0000	107.3500	100.5000
	15.0	15.0	15.0	15.0	15.0944	15.4857	14.8850	15.1500	100.6296	103.2381	99.2333	101.0000
	20.0	20.0	20.0	20.0	18.1111	20.1571	19.4050	20.4000	90.5556	100.7857	97.0250	102.0000
Mean recovery									95.3241	101.9345	103.8771	103.0000
D2	5.0	5.0	5.0	5.0	5.0571	5.2100	5.6110	5.8667	101.1429	104.2000	112.222	117.3333
	10.0	10.0	10.0	10.0	10.2286	10.0900	9.5889	9.7000	102.2857	100.9000	95.8889	97.0000
	15.0	15.0	15.0	15.0	15.4857	15.4100	13.7778	14.0833	103.2381	102.7333	91.8519	93.8889
	20.0	20.0	20.0	20.0	20.6143	20.6900	17.0000	18.3000	103.0714	103.4500	85.0000	91.5000
Mean recovery									102.4345	102.8208	96.2408	99.9306
D3	5.0	5.0	5.0	5.0	5.1357	5.3500	5.7143	——	102.7143	107.0000	114.2857	——
	10.0	10.0	10.0	10.0	10.2357	10.7083	10.1714	——	102.3571	107.0833	101.7143	——
	15.0	15.0	15.0	15.0	15.4643	15.9042	14.3714	——	103.0952	106.0278	95.8095	——
	20.0	20.0	20.0	20.0	20.4500	21.3458	18.5286	——	102.2500	106.7292	92.6429	——
Mean recovery									102.6042	106.7101	101.1131	——
D4	5.0	5.0	5.0	5.0	5.1857	5.1429	——	——	103.7143	102.8571	——	——
	10.0	10.0	10.0	10.0	10.3214	10.1143	——	——	103.2143	101.1429	——	——
	15.0	15.0	15.0	15.0	15.8857	16.0286	——	——	105.9048	106.8571	——	——
	20.0	20.0	20.0	20.0	21.0357	18.4429	——	——	105.1786	92.2143	——	——
Mean recovery									104.5030	100.7679	——	——

Table 4-21: Statistical validation data for quantitative assessment of commercial tablet formulation for CARV, DIAZ, CARB, and FURO.

Sample	Weight labeled (mg/tablet)	Weight found (mg/tablet)				Mean (mg/tablet)	Recovery %	C.V. %
D1								
CARV (India) tablet 25mg	25	23.611	23.917	25.157	22.639	23.831	95.324	4.358
DIAZ (Iraq) tablet 2mg	2	2.170	2.010	2.020	2.040	2.060	103.000	3.611
CARB (Switzerland) tablet200mg	200	223.800	214.700	198.467	194.050	207.754	103.877	6.691
FURO (France) tablet 40mg	40	40.686	40.800	41.295	40.314	40.774	101.934	0.993
D2								
CARV (India) tablet 25mg	25	25.286	25.572	25.810	25.768	25.609	102.434	0.934
DIAZ (Iraq) tablet 2mg	2	2.347	1.940	1.878	1.830	1.999	99.931	11.827
CARB (Switzerland) tablet 200mg	200	224.440	191.778	183.704	170.000	192.481	96.240	12.014
FURO (France) tablet 40mg	40	41.680	40.360	41.093	41.380	41.128	102.821	1.375
D3								
CARV (India) tablet 25mg	25	25.679	25.589	25.774	25.563	25.651	102.604	0.373
CARB (Switzerland) tablet 200mg	200	228.572	203.428	191.619	185.286	202.226	101.113	9.447
FURO (France) tablet 40mg	40	41.680	40.360	41.093	41.380	41.128	102.821	1.375
D4								
CARV (India) tablet 25mg	25	25.929	25.804	26.476	26.295	26.126	104.503	1.198
FURO (France) tablet 40mg	40	41.680	40.360	41.093	41.380	41.128	102.821	1.375

Table 4-22: Application of standard addition method to the analysis of CARV, FURO, CARB and DIAZ using derivative technique.

Drug	Derivative Mode	Amount of sample taken ($\mu g.mL^{-1}$)	Amount of standard added ($\mu g.mL^{-1}$)	Total amount found ($\mu g.mL^{-1}$)	Recovery %
CARV	D1	5	3	7.683	96.042
			5	9.611	96.111
			8	12.983	99.872
	D2	5	3	8.457	105.714
			5	10.343	103.429
			8	13.543	104.176
	D3	5	3	8.550	106.875
			5	10.286	102.857
			8	13.536	104.121
	D4	5	3	8.750	109.375
			5	10.386	103.857
			8	13.829	106.374
FURO	D1	5	3	8.329	104.107
			5	9.957	99.571
			8	13.171	101.319
	D2	5	3	8.130	101.625
			5	10.090	100.900
			8	12.850	98.846
	D3	5	3	8.575	107.188
			5	10.467	104.667
			8	13.813	106.250
	D4	5	3	7.638	95.476
			5	9.571	95.714
			8	12.038	92.601
CARB	D1	5	3	8.795	109.938
			5	10.470	104.700
			8	13.675	105.192
	D2	5	3	8.267	103.333
			5	10.200	102.000
			8	12.644	97.265
	D3	5	3	9.071	113.393
			5	10.714	107.143
			8	12.400	95.385
DIAZ	D1	5	3	8.750	109.375
			5	11.050	110.500
			8	13.850	106.538
	D2	5	3	8.150	101.875
			5	10.067	100.667
			8	12.333	94.872

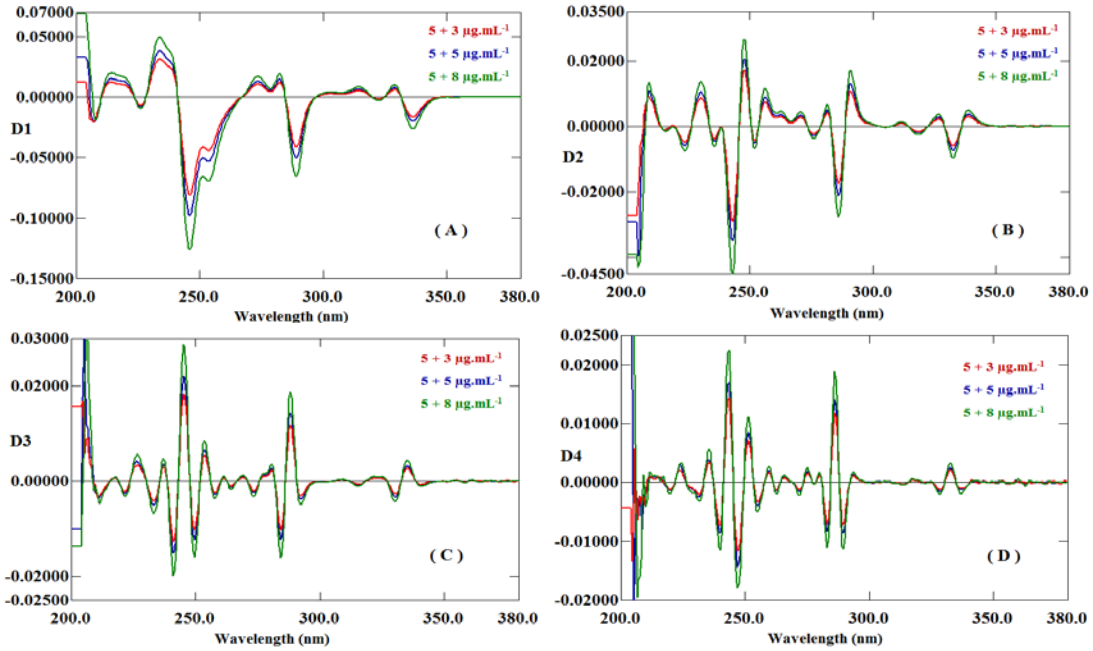


Figure 4-45: Derivative spectra of pure CARV (3, 5 and 8 µg.mL^{-1}) and commercial tablet (5 µg.mL^{-1}) **(A)** 1st derivative, **(B)** 2nd derivative, **(C)** 3rd derivative and **(D)** 4th derivative.

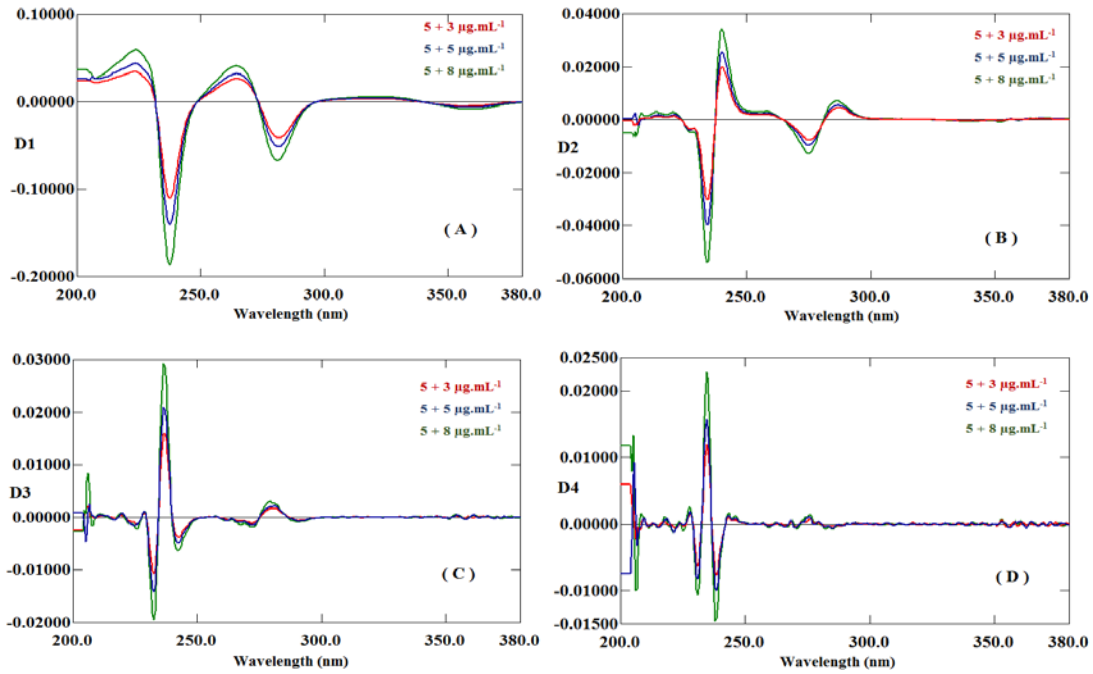


Figure 4-46: Derivative spectra of pure FURO (3, 5 and 8 µg.mL^{-1}) and commercial tablet (5 µg.mL^{-1}) **(A)** 1st derivative, **(B)** 2nd derivative, **(C)** 3rd derivative and **(D)** 4th derivative.

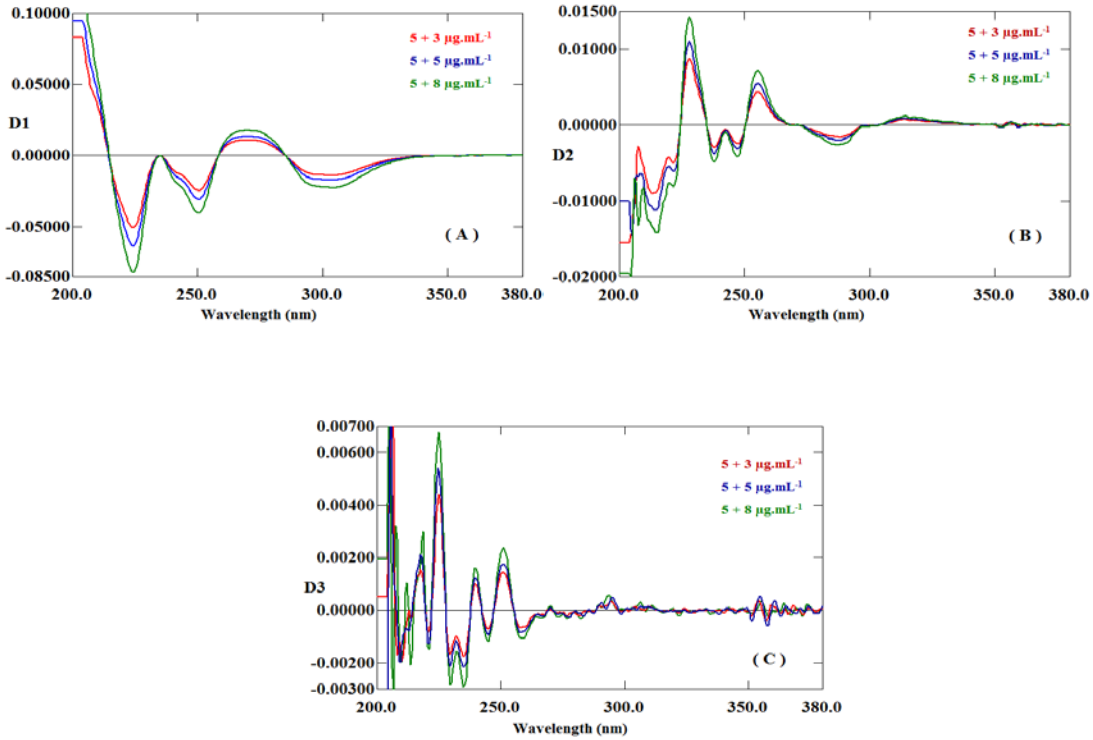


Figure 4-47: Derivative spectra of pure CARB (3, 5 and 8 µg.mL^{-1}) and commercial tablet (5 µg.mL^{-1}) **(A)** 1st derivative, **(B)** 2nd derivative and **(C)** 3rd derivative.

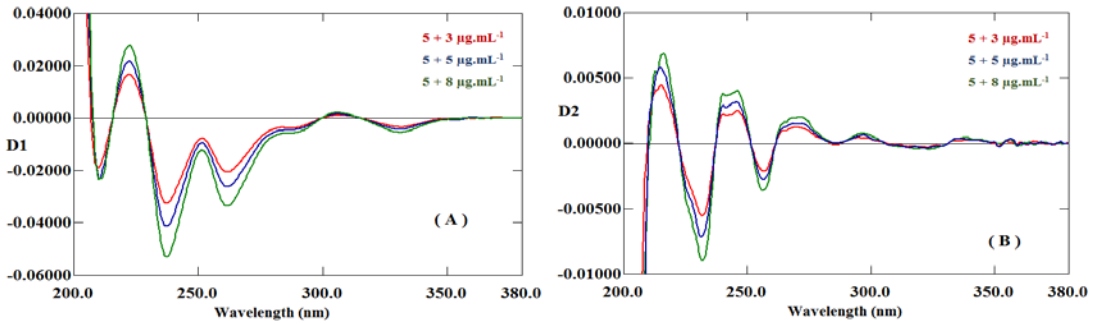


Figure 4-48: Derivative spectra of pure DIAZ (3, 5 and 8 µg.mL^{-1}) and commercial tablet (5 µg.mL^{-1}) **(A)** 1st derivative and **(B)** 2nd derivative.

4-3 Statistical evaluation

Figure 4-49 represents the box-plots for the recovery % of each drug analyzed by the recommended methods. The plots show the distributional characteristics of the results in each case as well as the level of the results.

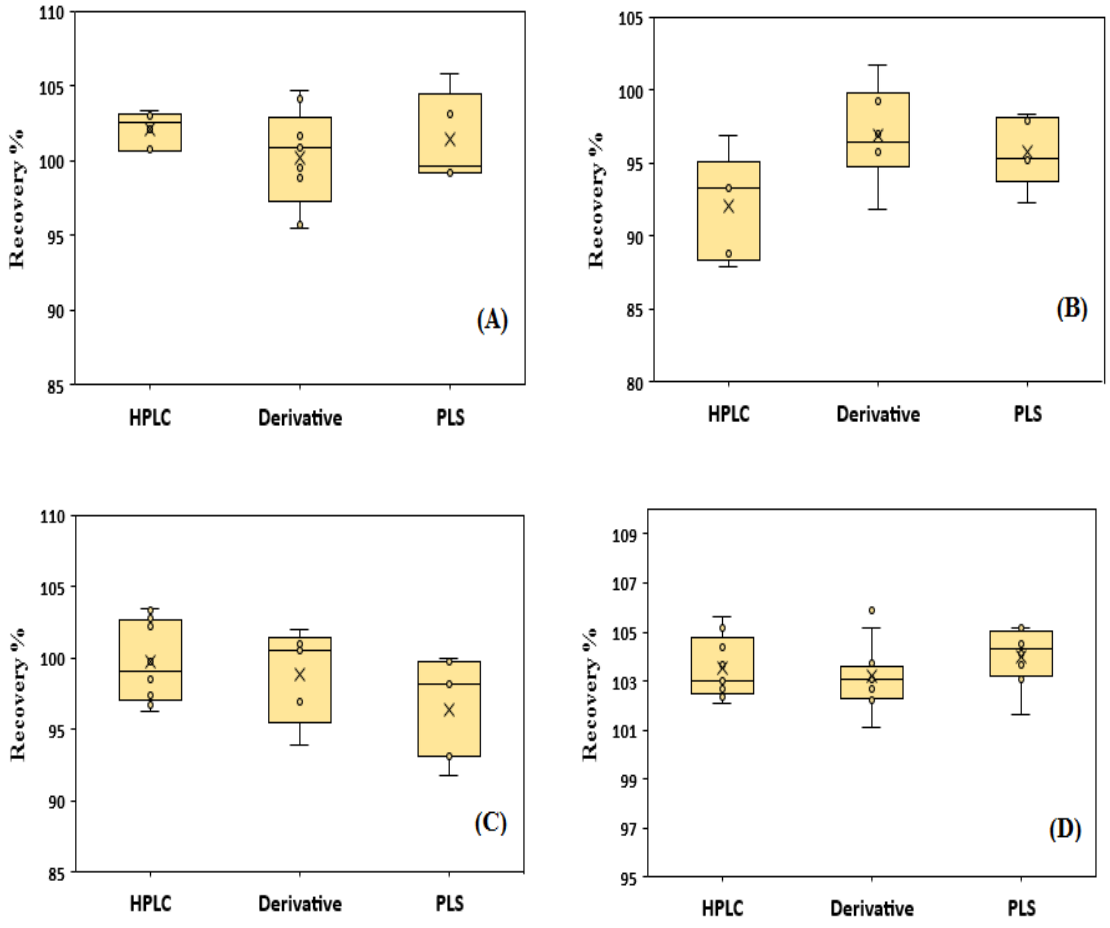


Figure 4-49: Box plot for the obtained recoveries for (A) FURO, (B) CARB, (C) DIAZ, and (D) CARV via the proposed methods.

Since there is no reported standard method for the simultaneous analysis of the quaternary mixtures of the mentioned drugs, the results of the proposed PLS and derivative spectrophotometry methods (Tables 4-13, 4-14 and 4-20 respectively) were compared statistically with those of the developed RP-HPLC method (Table 3-9). The comparison was performed by using student's t-test and F-test at 95 % confidence level with regards to recovery percentage. As shown in Table 4-23, the statistic t value and calculated F value for the studied drugs are smaller than the critical ones, which indicates that there is no significant difference between the results of the proposed methods. Moreover, one-way ANOVA (or Anova: Single Factor), was applied further for variance comparison of the obtained results via the proposed methods for each drug. The results revealed that there is no significant difference between the three methods as the critical F-value is higher than the calculated one (Table 4-24).

Table 4-23: Statistical comparison of the results obtained by the three proposed methods.

Furosemide

t-Test: Two-Sample with Equal Variances				F-Test Two Sample for Variances			
	Derivative	*HPLC*	*PLS*		*Derivative*	*HPLC*	*PLS*
Mean	101.5764	102.1806	100.2949	Mean	101.5764	102.18058	100.2949
Variance	4.6598	1.5103	3.5227	Variance	4.6598	1.51034	3.5227
Observations	7	6	4	Observations	7	6	4
df	11	8		df	6	5	3
| t Stat |	0.6044	1.9411		F	3.0852	2.3324	
P(T<=t) two-tail	0.2789	0.04410		P(F<=f) one-tail	0.1185	0.1911	
t Critical two-tail	2.2010	2.306		F Critical one-tail	4.9503	5.4095	

Carbamazepine

t-Test: Two-Sample with Equal Variances				F-Test Two Sample for Variances			
	Derivative	*HPLC*	*PLS*		*Derivative*	*HPLC*	*PLS*
Mean	96.9205	92.6575	95.8252	Mean	96.9205	92.0362	95.8252
Variance	11.26595	10.4970	5.8718	Variance	11.2659	13.6244	5.8718
Observations	6	5	5	Observations	6	5	5
df	9	8		df	5	4	4
| t Stat |	2.1300	1.7507		F	1.20934	2.3203	
P(T<=t) two-tail	0.0620	0.1181		P(F<=f) one-tail	0.4106	0.2175	
t Critical two-tail	2.2622	2.3060		F Critical one-tail	5.1922	6.3882	

Diazepam

t-Test: Two-Sample with Equal Variances				F-Test Two Sample for Variances			
	Derivative	*HPLC*	*PLS*		*Derivative*	*HPLC*	*PLS*
Mean	98.87778	99.7321	97.6249	Mean	98.8778	99.7321	96.3899
Variance	11.32466	7.3516	11.2826	Variance	11.3247	7.3516	11.9829
Observations	5	16	5	Observations	5	16	7
df	19	19		df	4	15	6
| t Stat |	0.5828	1.4381		F	1.5404	1.6300	
P(T<=t) two-tail	0.5669	0.1667		P(F<=f) one-tail	0.2410	0.2068	
t Critical two-tail	2.0930	2.0930		F Critical one-tail	3.0556	2.7905	

Carvedilol

t-Test: Two-Sample with Equal Variances				F-Test Two Sample for Variances			
	Derivative	*HPLC*	*PLS*		*Derivative*	*HPLC*	*PLS*
Mean	103.1806	103.5544	103.9956	Mean	103.1806	103.5544	103.9956
Variance	1.67606	1.5674	1.4472	Variance	1.6760	1.5674	1.4472
Observations	12	9	8	Observations	12	9	8
df	19	15		df	11	8	7
| t Stat |	0.6640	0.73851		F	1.0693	1.0831	
P(T<=t) two-tail	0.5147	0.4716		P(F<=f) one-tail	0.4745	0.46461	
t Critical two-tail	2.0930	2.1314		F Critical one-tail	3.3130	3.72571	

Table 4-24: One-way ANOVA of the results obtained from the analyses of drugs mixtures by the proposed methods.

Summary Statistics of **FURO** *determination*						
Method	***N***		***Sum***	***Mean***		***SD***
HPLC	6		613.0835	102.1806		1.2290
Derivative	9		902.225	100.2472		3.2334
PLS	5		507.0017	101.4003		2.9585
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	13.9972	2	6.9986	0.9427	0.4090	3.5915
Within Groups	126.2029	17	7.4237			
Summary Statistics of **CARB** *determination*						
Method	***N***		***Sum***	***Mean***		***SD***
HPLC	5		460.1809	92.0362		3.6911
Derivative	6		581.5229	96.9205		3.3565
PLS	5		479.1258	95.8252		2.4232
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	69.4122	2	34.7061	3.3591	0.0667	3.8056
Within Groups	134.3146	13	10.3319			
Summary Statistics of **DIAZ** *determination*						
Method	***N***		***Sum***	***Mean***		***SD***
HPLC	16		1595.7143	99.7321		2.7114
Derivative	5		494.3889	98.8778		3.3652
PLS	7		674.7293	96.3899		3.4616
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	54.5049	2	27.2525	2.9952	0.0682	3.3852
Within Groups	227.4691	25	9.0988			
Summary Statistics of **CARV** *determination*						
Method	***N***		***Sum***	***Mean***		***SD***
HPLC	9		931.9896	103.5544		1.2520
Derivative	12		1238.1667	103.1806		1.2946
PLS	8		831.9644	103.9956		1.2030
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	3.2024	2	1.6012	1.0128	0.3771	3.3690
Within Groups	41.1059	26	1.5810			

- *Null Hypothesis: The means of all selected datasets are equal.*
- *Alternative Hypothesis: The means of one or more selected datasets are different.*
- *At the 0.05 level, the population means are not significantly different.*

4-4 Conclusion:

Simultaneous determination of furosemide, carbamazepine, diazepam and carvedilol using simple isocratic RP-HPLC, PLS as a chemometric tool, and derivative spectrophotometry are proposed in this work. Although the developed RP-HPLC method is fast, automated and the obtained results are accurate, but its complexity, costly and the low sensitivity to certain analytes restricts this method. The recommended derivative spectrophotometric method is suitable for the simultaneous analysis of multicomponent mixture due to its simplicity, low cost and short analysis time, nevertheless the effects of several instrument parameters on the derivative spectra causes a limitation in its application. As a green chemistry tool, the suggested PLS model has been applied since there is no chemical reaction involved as well as the simultaneous determination of the studied drugs were performed without preliminary separation step.

The three proposed methods are simple, fast, inexpensive, and non-destructive and show good linearity and sensitivity. The recommended methods enable the estimation of the cited drugs either in laboratory prepared mixtures or in pharmaceutical formulations without prior separation and other previous sample treatments.

4-5 Suggestions:

1. Extending this work for the analysis of other types of drugs.
2. Application of the proposed methods for the determination of furosemide, carbamazepine, diazepam and carvedilol in biological samples i.e. urine and serum.
3. Investigation of the possibility of using other chemometrics methods such as artificial neural network for simultaneous determination of quaternary mixture of the cited drugs.

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APPENDIX

***Capacity (retention) factor (ḱ)*:**

$$\acute{k} = t_R' / t_0 = (t_R - t_0) / t_0$$

***Selectivity (separation) factor (α)*:**

$$\alpha = t_R'(X) / t_R'(Y) \quad (\text{where } t_R'(X) > t_R'(Y))$$

***Theoretical plate number (N)*:**

$$N = 2\pi (t_R\ h / A)^2$$

***Resolution (Rs)*:**

$$Rs = \sqrt{N}/4\ (\alpha - 1 / \alpha)\ (\acute{k}_2 / \acute{k}_2 + 1)$$

الخلاصة

ان العمل المدرج في الاطروحة هو محاولة لتطوير طرائق تحليلية جديدة في تقدير اربعة عقاقير تنتمي الى فئات علاجية مختلفة وهي: الفيوروسمايد، الكاربامازابين، الدايازيبام والكارفيديلول. الدراسة التي تتضمن تقدير العقاقير المدروسة بصيغها النقية ومستحضراتها الصيدلانية (حبوب – امبول – معلق فموي) قد تم وصفها في أربعة فصول كما يأتي:

الفصل الأول: يتضمن المباديء الأساسية ، التصنيفات والأساس النظري للكروموتوغرافيا، وكذلك المباديء الأساسية ، الصيغ أو الأنواع والأجهزة لكروموتوغرافيا السائل ذات الأداء العالي. كما تم وصف طريقة المشتقة الطيفية وطريقة المربعات الصغرى الجزئية من حيث المباديء الأساسية والتطبيقات في مجال التحليلات الصيدلانية. علاوة على ذلك، تضمن هذا الفصل وصف موجز للتسمية النظامية ، التركيب الكيميائي والأدبيات المنشورة للطرائق التحليلية المختلفة في تقدير العقاقير قيد الدراسة.

الفصل الثاني: يصف هذا الفصل الجزء العملي للدراسة والمتضمن المواد الكيميائية ، المذيبات والأجهزة المستخدمة وكذلك تحضير المحاليل القياسية للعقاقير وأيضا المحاليل المنظمة المستخدمة.

الفصل الثالث: تناول تطوير وتقييم طريقة بسيطة في كروموتوغرافيا السائل عالي الأداء ذي الطور العكوس من أجل التحليل الآني (فصل وتقدير) الفيوروسمايد، الكاربامازابين، الدايازيبام والكارفيديلول. كما تضمن دراسة تأثير العوامل المختلفة مثل مكونات الطور المتحرك ، معدل سرعة الجريان ، نسبة المحوِّر العضوي والنسبة المئوية للمحلول المنظم بالإضافة إلى الأس الهيدروجيني للمحلول المنظم ، درجة الحرارة وحجم الحقن في الأداء الكروموتوغرافي للعقاقير المقاسة. وتحت الظروف الفضلى تم إجراء الفصل الكروموتوغرافي بإستخدام عمود من نوع RP-NUCLEODUR® 100-5 C18ec بأبعاد (4.6 x250 ملمتر) وحجم جسيمات 5 مايكرومتر بإستخدام نظام الإسترداد الآيزوكراتك ومعدل جريان ثابت مقداره 1.5مللتر.ثا$^{-1}$. أما الطور المتحرك فيتألف من الأسيتونتريل: الماء منزوع الأيونات والمحمض بحامض الخليك (الأس الهيدروجيني = 3.6) بنسبة 50:50حجم/حجم، درجة حرارة العمود 40 درجة مئوية وحجم حقن 10 مايكرولتر. أجري الكشف عند الطول الموجي 226 نانومتر بإستخدام مكشاف الأشعة فوق البنفسجية وكانت ازمان الإحتجاز لكل من الفيوروسمايد، الكاربامازابين، الدايازيبام والكارفيديلول 1.9 دقيقة ، 2.79 دقيقة ، 5.39 دقيقة و 9.56 دقيقة على التوالي. وقد بنيت منحنيات المعايرة القياسية لكل عقار وذلك برسم العلاقة بين مساحة القمة مقابل التركيز وكذلك إرتفاع القمة مقابل التركيز بمديات تركيز تراوحت بين 1.0 -100

مايكروغرام.مللتر$^{-1}$ للفيوروسمايد ، 2.5-100 مايكروغرام.مللتر$^{-1}$ للكارباميازابين و الدايازيبام و5.0-100 مايكروغرام.مللتر$^{-1}$ للكارفيديلول. لقد تم حساب توافق النتائج بدلالة النسبة المئوية للانحراف القياسي النسبي ووجد ان القيم لم تتجاوز 4% ، بينما لم تتجاوز قيم الدقة المحسوبة بدلالة النسبة المئوية للخطأ النسبي 5%. طبقت الطريقة المقترحة وبنجاح في تقدير العقاقير المدروسة في بعض مستحضراتها الصيدلانية ووجد ان معامل الاسترجاع المئوي تراوحت بين 97.2-104.1 % للفيوروسمايد، 88.8-92.4 % للكارباميازابين، 98.4-102.9 % للدايازيبام و94.0-106.7 % للكارفيديلول.

الفصل الرابع: يعنى هذا الفصل بالتقدير الآني للعقاقير الأربعة في مزيجاتها الرباعية باتباع طريقين هما: طريقة المربعات الصغرى الجزئية وطريقة المشتقة الطيفية. لقد تم اتباع طريقة المربعات الصغرى الجزئية الكيمومترية للتقدير الطيفي الآني للعقاقير CARV،FURO, CARB, DIAZ في مزيجاتها الرباعية، حيث بنيت منحنيات المعايرة القياسية لكل عقار بمدى تركيز تراوح بين 1.0 -20.0 مايكروغرام.مللتر$^{-1}$ عند الاطوال الموجية 233.0 نانومتر ، 215.0 نانومتر ، 238.5 نانومتر و 242.5 نانومتر لكل من الفيوروسمايد، الكارباميازابين، الدايازيبام والكارفيديلول على التوالي.

تضمنت الخطوة الاولى لطريقة المربعات الصغرى الجزئية اعداد مجوعة التدريب بالاعتماد على النتائج الطيفية التي تم تسجيلها بعناية لمجموعة من النماذج المكونة من 84 مزيج والتي تم إختيارها بتصميم Simplex Lattice Mixture Design. تم الحصول على مصفوفة المعايرة بعمل مسح لطيف الامتصاص للمحاليل الاربعة وثمانين بمدى 200-350 نانومتر، مسح بسرعة 10 نانومتر.ثا$^{-1}$ ، عرض شق 1.8 نانومتر وبفاصل 0.5 نانومتر مقابل محلول الخلب. تم استخدام برنامج OriginPro software version 2015 لبناء طراز انحدار المربعات الصغرى الجزئية 1 و 2 ومن خلالهما تم التنبؤ بتراكيز العقاقير الأربعة. نتائج النسب المئوية للانحراف القياسي النسبي وللخطأ النسبي كانت تتراوح ضمن المديات (1.2638-6.0529) و(-0.0845- 3.8053) على التوالي، وقد أمكن تطبيق الطريقة المقترحة وبنجاح في تقدير العقاقير الأربعة في بعض المستحضرات الصيدلانية وفي نماذجهم المصنعة.

طورت تقنيات المشتقة الطيفية (المشتقة الأولى، الثانية، الثالثة والرابعة) للتقدير الآني للعقاقير الأربعة في مزيجاتها الرباعية وقد أمكن تقديركل من الفيوروسمايد والكارفيديلول بواسطة المشتقة الأولى، الثانية، الثالثة والرابعة ، والكارباميازابين تم تقديره بواسطة المشتقة الأولى، الثانية والثالثة، أما تقديرالدايازيبام فقد تم بواسطة المشتقة الأولى والثانية فقط.

طبقت طريقة التقاطع الصفري في قياسات المشتقة الأولى ووجد أن ارتفاع القمة نسبة الى خط الأساس عند التقاطع الصفري تتناسب طرديا مع تركيزالعقار، لذلك استخدمت الأطوال الموجية 336.5 نانومتر، 358.5 نانومتر، 306.0 نانومتر و331.5 نانومتر لتقدير الكارفيديلول، الفيوروسمايد، الكاربامازابين و الدايازيبام على التوالي. ولنفس الخلائط أمكن استعمال الأطوال الموجية 272.5 نانومتر، 226.0 نانومتر و 218.5 نانومتر (ارتفاع القمة نسبة الى خط الأساس عند التقاطع الصفري) للتقدير الكمي لكل من الفيوروسمايد، الكاربامازابين و الدايازيبام على التوالي والطول الموجي 332.5 نانومتر (ارتفاع القمة نسبة الى خط الأساس) لتقدير الكارفيديلول وبالاستفادة من قيم المشتقة الثانية للأطياف المحسوبة. في حين اعتمدت الأطوال الموجية 237.5 نانومتر و 224.0 نانومتر (ارتفاع القمة نسبة الى خط الأساس عند التقاطع الصفري) للتقدير الكمي لكل من الفيوروسمايد والكاربامازابين على التوالي والطول الموجي 288.0 نانومتر (ارتفاع القمة نسبة الى خط الأساس) لتقدير الكارفيديلول وبالاستفادة من قيم المشتقة الثالثة للأطياف المحسوبة. عند تفحص اطياف المشتقة الرابعة لنفس الخلائط وبدقة وجد أن ارتفاع القمة نسبة الى خط الأساس عند التقاطع الصفري تتناسب طرديا مع تركيز الفيوروسمايد عند الطوال الموجي 234.0 نانومتر وارتفاع القمة نسبة الى خط الأساس عند الطول الموجي 288.0 نانومتر تتناسب طرديا مع تركيز الكارفيديلول.

أظهرت النتائج عدم وجود أي تأثير للمضافات في التقدير الكمي للعقاقير الأربعة عند تطبيق الطرق الثلاثة المقترحة (طريقة كروموتوغرافيا السائل عالي الأداء ذي الطور العكوس، طريقة انحدار المربعات الصغرى الجزئية 1 و 2 وطريقة مشتقة الطيف)، لذلك تم اعتمادها في تقدير العقاقير قيد الدراسة في بعض مستحضراتها الصيدلانية.

بِسْمِ اللَّهِ الرَّحْمنِ الرَّحِيمِ

وَمَن يَتَوَكَّلْ عَلَى اللَّهِ فَهُوَ حَسْبُهُ

إِنَّ اللَّهَ بَالِغُ أَمْرِهِ قَدْ جَعَلَ اللَّهُ

لِكُلِّ شَيْءٍ قَدْرًا

صدَقَ اللهُ العَظِيم

سورة الطلاق الآية (٣)

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بغداد
كلية التربية للعلوم الصرفة / ابن الهيثم
قسم الكيمياء

تطبيق الكيمومترية في تقدير بعض العقاقير بطرائق تحليلية مختلفة

رسالة مقدمة الى
مجلس كلية التربية للعلوم الصرفة / إبن الهيثم جامعة بغداد
وهي جزء من متطلبات نيل درجة الدكتوراه في علوم الكيمياء

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