"POLYMORPH SCREENING, DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR ESTIMATION OF SELECTED ANTIHYPERTENSIVE DRUGS BY AQBD APPROACH"

Thesis submitted to

THE KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

(KLE DEEMED UNIVERSITY)

[Declared as Deemed-to-be-University u/s 3 &12 B of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A^{+}' Grade by NAAC) (3rd Cycle) [Placed in Category 'A' by MoE (GoI)]

KLE
EMPOWERING PROFESSIONALS

For the award of the degree of

Doctor of Philosophy

In the Faculty of

Pharmacy

By

Mr. KRANTISINHA HANUMANT RANDIVE M. Pharm

(Registration No: KLEU/Ph.D./2020-21/ DO1220040)

Under the Guidance of

(Prof.) Dr. MEENAXI M. MASTE M. Pharm, Ph. D. PGDHPE

Department of Pharmaceutical Chemistry,

KLE College of Pharmacy, Belagavi,

KLE Academy of Higher Education and Research,

Belagavi-590010

FEBRUARY 2025

UNDERTAKING

I, **Mr. Krantisinha Hanumant Randive** hereby declare that the information and the data mentioned in my thesis entitled **"Polymorph Screening, Development and Validation of Analytical Method for Estimation of Selected Antihypertensive Drugs by AQbD Approach"** belongs to me and is original.

I am aware of definition of plagiarism as detailed below:

- An act or instance of using or closely imitating the language and thoughts of another author without authorization and the representation of that author's work as one's own, as by not crediting the original author.
- A piece of writing or other work reflecting such unauthorized use or imitation.
- The deliberate or reckless representation of another's words, thoughts or ideas as one's own without attribution in connection with submission of academic work, whether graded or otherwise.

I hereby declare that the thesis prepared by me is original-one and does not involve plagiarism anywhere. In case at a later stage it is found that I have indulged in plagiarism, then I am solely responsible for the same and the Institution is at liberty to take any disciplinary action against me including cancellation of dissertation or any other penalties imposed by the University.



Mr. Krantisinha Hanumant Randive
Part-Time Ph. D Research Scholar
KLE College of Pharmacy, Belagavi,
KAHER, Belagavi, Karnataka.

Place: Belagavi
Date: 27/05/2025

PLAGIARISM REPORT

Ref. No. KAHER/AA/24-25/D-654

24th January 2025

Sir,

The soft copy of Ph.D. research thesis of **Mr. Krantisinha H. Randive, Faculty of Pharmacy, KAHER, Belagavi** has been submitted for anti-plagiarism check at the office of the undersigned through "Turn-it-in" package. The scan has been carried out and the scanned output reveals a match percentage of **9%** which is within the acceptable limit of 10%.

To obtain the comprehensive report of the plagiarism test, research scholar can send a mail to **diracademic@kledeemeduniversity.edu.in** along with the Registration Number, Name of the Scholar, Name of Guide/Co-guide and title of the thesis.

DIRECTOR ACADEMIC AFFAIRS Deemed-to-be University

Dr.(Mrs.) Jyoti M. Nagamoti
Director, Academic Affairs

To,

Mr. Krantisinha H. Randive
Part-time Ph.D. Research Scholar, 2020-21 Batch,
KLE College of Pharmacy,
Faculty of Pharmacy, KAHER
Belagavi.

Cc to :

1) The Principal, KLE College of Pharmacy, Belagavi.
2) Dr. Meenaxi M Maste, Professor of Pharmaceutical Chemistry, KLE College of Pharmacy, KAHER, Belagavi. – **Guide**

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

(KLE DEEMED UNIVERSITY)

[Declared as Deemed-to-be-University u/s 3 & 12 B of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A+' Grade by NAAC) (3rd Cycle) [Placed in Category 'A' by MoE (GoI)}

KLE
EMPOWERING PROFESSIONALS

COPYRIGHT DECLARATION

We hereby declare that **KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI, KARNATAKA,** *shall have the rights to preserve, use and disseminate this thesis in print or electronic format for academic/research purpose.*

Mr. Krantisinha Hanumant Randive
Part-Time Ph. D Research Scholar
KLE College of Pharmacy, Belagavi,
KAHER, Belagavi, Karnataka.

Dr. Meenaxi M. Maste
Professor and Head
Department of Pharmaceutical Chemistry,
KLE College of Pharmacy, Belagavi,
KAHER, Belagavi, Karnataka

Place: Belagavi
Date: 27/05/2025

© KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

(KLE DEEMED UNIVERSITY)

[Declared as Deemed-to-be-University u/s 3 & 12 B of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A+' Grade by NAAC) (3rd Cycle) [Placed in Category 'A' by MoE (GoI)}

KLE
EMPOWERING PROFESSIONALS

DECLARATION

I hereby declare that the thesis entitled **"Polymorph Screening, Development and Validation of Analytical Method for Estimation of Selected Antihypertensive Drugs by AQbD Approach"** *is a bonafide and original research carried out by me under the guidance of* **Dr. Meenaxi M. Maste,** *Professor and Head, Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi. The thesis or any part thereof has not formed the basis for the award of any degree/fellowship or similar title to any candidate of any University.*

Mr. Krantisinha Hanumant Randive
Part-Time Ph. D Research Scholar
KLE College of Pharmacy, Belagavi,
KAHER, Belagavi, Karnataka.

Place: Belagavi
Date: 27/05/2025

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

(KLE DEEMED UNIVERSITY)

[Declared as Deemed-to-be-University u/s 3 & 12 B of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A+' Grade by NAAC) (3rd Cycle) [Placed in Category 'A' by MoE (GoI)}

KLE
EMPOWERING PROFESSIONALS

CERTIFICATE

This is to certify that the thesis entitled **"Polymorph Screening, Development and Validation of Analytical Method for Estimation of Selected Antihypertensive Drugs by AQbD Approach"** *is a bonafide and genuine research carried out by* **Mr. Krantisinha Hanumant Randive** *under the guidance of* **Dr. Meenaxi M. Maste,** *Professor and Head Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi.*

Prof (Dr). Vinayak Mastiholimath
Dean, Faculty of Pharmacy
KAHER, Belagavi- 590010

Place: Belagavi
Date: 27/05/2025

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

(KLE DEEMED UNIVERSITY)

[Declared as Deemed-to-be-University u/s 3 & 12 B of the UGC Act, 1956
vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]
(Accredited 'A+' Grade by NAAC) (3rd Cycle) [Placed in Category 'A' by MoE (GoI)}

KLE
EMPOWERING PROFESSIONALS

CERTIFICATE

This is to certify that the thesis entitled **"Polymorph Screening, Development and Validation of Analytical Method for Estimation of Selected Antihypertensive Drugs by AQbD Approach"** *is a bonafide and genuine research carried out by* **Mr. Krantisinha Hanumant Randive** *under the guidance of* **Dr. Meenaxi M. Maste,** *Professor and Head, Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi.*

Dr. Sunil S. Jalalpure
Principal,
KLE College of Pharmacy, Belagavi,
KAHER, Belagavi, Karnataka.

Place: Belagavi
Date: 27/05/2015

COLLEGE OF PHARMACY BELAGAVI

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH,

BELAGAVI

(KLE DEEMED UNIVERSITY)

[Declared as Deemed-to-be-University u/s 3 & 12 B of the UGC Act, 1956
vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]
(Accredited 'A+' Grade by NAAC) (3rd Cycle) [Placed in Category 'A' by MoE (GoI)}

KLE
EMPOWERING PROFESSIONALS

CERTIFICATE

This is to certify that the thesis entitled **"Polymorph Screening, Development and Validation of Analytical Method for Estimation of Selected Antihypertensive Drugs by AQbD Approach"** *is a bonafide record of original research carried out by* **Mr. Krantisinha Hanumant Randive** *for the award of degree of DOCTOR OF PHILOSOPHY IN FACULTY OF Pharmacy under my supervision and guidance.*

Dr. Meenaxi M. Maste
Professor and Head
Department of Pharmaceutical Chemistry,
KLE College of Pharmacy, Belagavi,
KAHER, Belagavi, Karnataka

Place: Belagavi
Date: 27/05/2025

ACKNOWLEDGEMENT

I wholeheartedly dedicate this thesis to my respected parents, whose unwavering love, encouragement, and sacrifices have been the foundation of my academic journey.

I humbly express my heartfelt gratitude to my family, ***Godde Jyotiba,*** *and* ***Shree Sai Baba,*** *whose presence has been a guiding force behind my academic endeavours. Their blessings and unwavering support have provided me with the strength to sustain myself throughout my research journey.*

First and foremost, I express my heartfelt gratitude to my esteemed guide, ***Dr. Meenaxi M Maste****, Professor and Head, Department of Pharmaceutical Chemistry for their invaluable mentorship, constant support, and thoughtful advice throughout my Ph. D. research. Their knowledge, encouragement, and constructive feedback have played a key role in successful completion.*

I sincerely thank KLE Academy of Higher Education and Research (KAHER), Belagavi, for providing the platform and resources to pursue this research. ***Dr. Nitin Gangane****, Vice-Chancellor,* ***Dr. M S Ganachari,*** *Registrar, for their visionary leadership and unwavering support. I am thankful to* ***Dr. (Mrs) Jyoti M Nagamoti,*** *Director of Academic Affairs,* ***Dr. Chandra Metgud,*** *CoE, KAHER, Belagavi and* ***Prof. (Dr.) Vinayak Mastiholimath,*** *Dean, Faculty of Pharmacy, KAHER, Belagavi, for their guidance and cooperation.*

I am deeply grateful to the ***Prof****. (****Dr.) Sunil S Jalalpure, Principal****, KLE College of Pharmacy, Belagavi for their leadership and vision in fostering a supportive academic environment. Their commitment to excellence in education has not only contributed to my growth but has also created opportunities for students like me to thrive. The principal support and encouragement throughout my academic journey*

have been a constant source of motivation. I am also thankful to ***Prof. (Dr.) M B Patil, Vice Principal****, KLE College of Pharmacy, Belagavi for their timely help.*

*My sincere appreciation goes to****, Prof. (Dr.) Meenaxi M Maste, Head of the Department*** *for their continuous encouragement and guidance, which has been invaluable during my research. Also thankful to* ***Dr. Mahesh S Palled, Dr. Shankar G Alegaon, Dr. Shailendra Suryawanshi, Mr. Rohan Shingadi*** *and* ***Mr. Nikhil Gawas*** *from Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi*

I would like to extend a special thanks to ***Prof. (Dr.) Ravindra D Hiremath*** *former principal and* ***Prof. (Dr.) Amolkumar A Kempawade,*** *KLE college of Pharmacy, Nipani for their helpful guidance, timely suggestions, and continuous support. Their advice has been invaluable in improving the quality of this thesis, and I am fortunate to have had their expertise to rely on.*

I want to express my gratitude to my Principal ***Dr. Pramod C Gadad****, and my colleagues from KLES's College of Pharmacy, Nipani. everyone who contributed, directly or indirectly, to the completion of this work.*

I also want to thanks my Ph. D. colleagues ***Rupesh Kulkarni, Anand Ammanage, Mahantesh Kunchanur*** *and* ***Umeshkumar Patel*** *for their support.*

I extend my sincere gratitude to ***4care Life Science PVT. Ltd. Bagdol, Gujrat*** *for providing the gift samples.*

Finally, I want to express my gratitude to my respected ***parents*** *for their love, sacrifices and support that have been fundamental to my success. My heartfelt thanks to my father,* ***Mr Hanumant Randive and my mother, Mrs Parvati Randive, for their blessings.*** *I am extremely thankful to my loving* ***Wife****,* ***Anagha*** *and my* ***daughters Swara*** *and* ***Abhidnya****, your patience, understanding, and support throughout this journey.*

Mr. Krantisinha Hanumant Randive

TABLE OF CONTENTS

Sl. No.	Particulars	Page No.
1.	**INTRODUCTION**	**1-15**
	1.1 Background	1-11
	1.2 Need for the study	12-14
	1.3 Aim and Objectives	15
2.	**REVIEW OF LITERATURE**	**16-33**
	2.1 Drug profile	16-18
	2.2 QbD analysis of drugs and pharmaceuticals	19-23
	2.3 Analytical method development and estimation of selected drugs	24-31
	2.4 Polymorph screening of selected drugs	31-33
3.	**MATERIALS AND METHODS**	**34-54**
	3.1 Development and validation of a RP-HPLC method for Carvedilol phosphate in bulk and formulation	36-42
	3.2 Development and validation of a RP-HPLC method for Nebivolol hydrochloride in bulk and formulation	43-50
	3.3 Polymorphism study	51-54
4.	**RESULTS AND DISCUSSION**	**55-124**
	4.1 Development and validation of a RP-HPLC method for Carvedilol phosphate in bulk and formulation	55-82
	4.2 Development and validation of a RP-HPLC method for Nebivolol hydrochloride in bulk and formulation	83-106
	4.3 Polymorphism study	107-124
5.	**SUMMARY**	**125-128**
6.	**CONCLUSION**	**129-130**
7.	**BIBLIOGRAPHY**	**131-141**
8.	**ANNEXURE**	**142-169**

ABBREVIATIONS

%	*Percentage*
%CV	Percentage Coefficient of Variance
%RSD	Percentage Residual Standard Deviation
2D	Two Dimensional
3D	Three Dimensional
ACN	Acetonitrile
FA	Formic acid
ANOVA	Analysis of Variance
AQbD	Analytical Quality by Design
ATP	Analytical Target Profile
AUC	Area Under Curve
BBD	Box-Behnken design
CAAs	Critical Analytical Attributes
CQAs	Critical Quality Attributes
CV	Coefficient of Variance
DoE	Design of Experiment
DW	Distilled Water
CAV	Carvedilol
F	Value of F-Distribution
g	Gram
HCL	Hydrochloride
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization

IFB	Ishikawa Fishbone
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MeOH	Methanol
min	Minute
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
ml	Milliliter
mM	Millimolar
MW	Milli-Q-Water
nm	nano meter
OPA	Ortho Phosphoric Acid
p value	Probability value
PA	Peak Area
PDA	Photo Diode Array
QbD	Quality by Design
QRM	Quality Risk Management
r2	Correlation Coefficient
RA	Risk Assessment
RSD	Relative Standard Deviation
RT	Retention Time
SD	Standard Deviation
TF	Tailing Factor
NTP	Number Theoretical Plates
USFDA	United States Food and Drug Administration

v/v	Volume by Volume
WHO	World Health Organization
µ	Micron
µg	Microgram
µg/ml	Micro Gram Per Milli Liter
µl	Microliter
σ	Standard Deviation of y-intercept of calibration curve
SEM	*Scanning Electron Microscopy*
CAV	*Carvedilol Phosphate*
NBV	*Nebivolol Hydrochloride*

LIST OF TABLES

SL. NO.	PARTICULARS	PAGE NO.
1	List of antihypertensive drugs selected for proposed research	**16**
2	Carvedilol phosphate drug profile	**17**
3	Nebivolol hydrochloride drug profile	**18**
4	Taguchi design matrix for screening	**38**
5	3^2 Box-Behnken Design (Variables and their levels used)	**39**
6	QTPP, CQAs and Process Parameters of Proposed RP-HPLC Method	**45**
7	BBD (variables and their levels used)	**48**
8	Details of stoichiometric ratio of Carvedilol Phosphate (CAV) API and co-formers	**53**
9	Details of stoichiometric ratio of Nebivolol hydrochloride (NBV) API and co-formers	**53**
10	3^2 Box-Behnken design along with the observed values for optimization of RP-HPLC method for Carvedilol Phosphate	**62**
11	Regression analysis for the best fitting of different polynomial models and their responses (Y_1-Retention time, Y_2-Peak area, Y_3-Tailing factor, Y_4-Number of theoretical plates)	**63**
12	Results of Analysis of Variance (ANOVA) and adequate precision for the various responses	**64**
13	System suitability test parameters (n=6) at concentration 15µg/mL	**72**
14	Table 14: Intra-day and inter-day precision of Carvedilol Phosphate by RP-HPLC method	**76**

15	Recovery studies of Carvedilol Phosphate by RP-HPLC (n = 3)	**77**
16	Results of robustness study (n=6) by RP-HPLC method	**79**
17	Assay result of commercial tablets of Carvedilol Phosphate by RP-HPLC method	**82**
18	Results for Preliminary trial Runs	**84**
19	Box-Behnken design (BBD) based independent variable and their effect on dependent variables	**87**
20	Regression analysis for responses Retention time; Peak area, Tailing factor for fitting to different polynomial models	**88**
21	Analysis of variance (ANOVA) for the responses (Y1: Retention time; Y2: Peak area, Y3: Tailing factor)	**89**
22	System suitability test parameters (n=6) at Concentration 30 µg/mL	**97**
23	Intra-day and inter-day precision of nebivolol by RP-HPLC method	**99**
24	Recovery studies of Nebivolol Hydrochloride (n = 3)	**100**
25	Robustness study of the nebivolol by RP-HPLC method	**102**
26	Ruggedness study of the Nebivolol hydrochloride by RP-HPLC method	**105**
27	Characteristic diffraction peaks of Carvedilol phosphate and its cocrystals	**112**
28	Characteristic diffraction peaks of Nebivolol hydrochloride and its cocrystals	**122**

LIST OF FIGURES

SL. NO.	PARTICULARS	PAGE NO.
1	Traditional VS AQbD Approach for Analytical Method Development	**6**
2	Overview of method development of HPLC by AQbD Approach	**35**
3	Ishikawa fish bone diagram	**46**
4	Chemical structure of drug and conformers	**51**
5	Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (MeOH:Water)	**56**
6	Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (ACN:Water)	**57**
7	Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (MeOH:0.1%FA)	**58**
8	Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (ACN:0.1%FA)	**58**
9	Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (MeOH:OPA)	**59**
10	Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (MeOH:0.1%OPA and ACN:0.1%OPA)	**60**
11	A-D Half-normal plots and Pareto charts indicating the factor effects of the responses, i.e., retention time, peak area, tailing factor and number of theoretical plates.	**65**

12	3D-Response surface (A-D) and 2D-contour (E-H) plots showing the effect of %V/V of Methanol in mobile phase and injection volume on retention time, peak area, tailing factor and number of theoretical plates. X1= A: Methanol in the mobile phase and X2= B: Injection volume	**66**
13	Perturbation plots (A-D) showing the effect of %V/V of Methanol in mobile phase and injection volume on retention time, peak area, tailing factor and number of theoretical plates. A: Methanol in the mobile phase and B: Injection volume	**67**
14	The overlay plot showing the optimized chromatographic conditions and their predicted responses	**67**
15	Chromatogram of Carvedilol phosphate in system suitability test	**73**
16	Calibration curve of Carvedilol Phosphate	**73**
17a	Chromatogram of Carvedilol Phosphate (5 µg/mL)	**74**
17b	Chromatogram of Carvedilol Phosphate (10 µg/mL)	**74**
17c	Chromatogram of Carvedilol Phosphate (15 µg/mL)	**74**
17d	Chromatogram of Carvedilol Phosphate (20 µg/mL)	**75**
17e	Chromatogram of Carvedilol Phosphate (25 µg/mL)	**75**
17f	Chromatogram of Carvedilol Phosphate (30 µg/mL)	**75**
18	Precision Chromatogram of Carvedilol Phosphate (15 µg/mL)	**77**
19a	Chromatogram of Carvedilol Phosphate (Accuracy 50%)	**78**
19b	Chromatogram of Carvedilol Phosphate (Accuracy 100%)	**78**
19c	Chromatogram of Carvedilol Phosphate (Accuracy 150%)	**78**

20a	Chromatogram of Carvedilol phosphate effect of change in flow rate of 0.9 mL/min	**80**
20b	Chromatogram of Carvedilol phosphate effect of change in flow rate of 1.1 mL/min	**80**
20c	Chromatogram of Carvedilol phosphate effect of change in concentration of mobile phase (MeOH:0.1%OPA,64:36)	**80**
20d	Chromatogram of Carvedilol phosphate effect of change in concentration of mobile phase (MeOH:0.1%OPA,66:34)	**81**
20e	Chromatogram of Carvedilol phosphate effect of change in column temperature (29°C)	**81**
20f	Chromatogram of Carvedilol phosphate effect of change in column temperature (31°C)	**81**
21	Assay chromatogram of Carvedilol Phosphate by RP-HPLC method	**82**
22	A-F: Preliminary trials to optimize chromatographic conditions for Nebivolol hydrochloride method development	**83**
23	Preliminary trials to optimize chromatographic conditions for Nebivolol method development MeOH:0.1%FA	**85**
24	Plot (a) showing true versus predicted values, with residual plot (b) for retention time	**91**
25	Figure 25: Relation plot (A1-A3 & B1-B3) 2D contour plots and 3D response surface plots showing the influence of CMP's i.e., (a) Solvent concentration in mobile phase (% v/v), (b) Flow rate (mL/min), (c)Wavelength (nm) on retention time (RT) as a CAA	**91**

26	Plot (a) showing true versus predicted values, with residual plot (b) for peak area	**93**
27	Relation plot (C1-C3 & D1-D3) 2D counter plots and 3D response surface plots showing the influence of CMP's i.e., (a) Solvent concentration in mobile phase (% v/v), (b) Flow rate (ml/min), (c)Wavelength (nm) on Peak area (PA) as a CAA	**93**
28	Plot (a) showing true versus predicted values, with residual plot (b) for tailing factor	**95**
29	Relation plot (E1-E3 & F1-F3) 2D contour plots and 3D response surface plots showing the influence of CMP's i.e., (a) Solvent concentration in mobile phase (% v/v), (b) Flow rate (mL/min), (c)Wavelength (nm) on Tailing factor (TF) as a CAA	**95**
30	a. Overlay plot showing the optimized chromatographic conditions and their predicted responses, b. Graphs showing numerical optimization with highest desirability	**96**
31	System suitability parameter of Nebivolol hydrochloride	**98**
32	Calibration curve of Nebivolol hydrochloride by RP-HPLC method	**98**
33	Chromatogram of Nebivolol hydrochloride (Accuracy 80%, 100% & 120%)	**101**
34a	Chromatogram of Nebivolol hydrochloride effect of change in flow rate of 0.7 mL/min	**103**
34b	Chromatogram of Nebivolol hydrochloride effect of change in flow rate of 0.9 mL/min	**103**
34c	Chromatogram of Nebivolol hydrochloride effect of change in concentration of mobile phase (70:30)	**103**

34d	Chromatogram of Nebivolol hydrochloride effect of change in concentration of mobile phase (72:28)	**104**
34e	Chromatogram of Nebivolol hydrochloride effect of change of wavelength (281nm, 283nm)	**104**
35	Ruggedness chromatogram of Nebivolol hydrochloride (20 µg/mL)	**105**
36	Assay chromatogram of Nebivolol hydrochloride	**106**
37	DSC thermogram of Carvedilol phosphate (API)	**107**
38	DSC thermogram of Carvedilol - nicotinamide (1:1)	**108**
39	Overlay of DSC thermogram of Carvedilol Phosphate and Carvedilol-nicotinamide	**108**
40	DSC thermogram of Carvedilol - oxalic acid (1:1)	**108**
41	Overlay of DSC thermogram of Carvedilol and Carvedilol-oxalic acid	**109**
42	FT-IR spectra of Carvedilol Phosphate	**110**
43	FTIR spectra of Carvedilol-nicotinamide cocrystals (Z1, 1:1)	**110**
44	FTIR of Carvedilol-oxalic acid (W1, 1:1)	**111**
45	P-XRD diffraction patterns of Carvedilol phosphate, Carvedilol-nicotinamide (Z1, 1:1) and Carvedilol- oxalic acid (W1, 1:1) cocrystals	**113**
46	SEM micrographs of A-Carvedilol pure drug, B- Carvedilol-oxalic acid (1:1) and C- Carvedilol-nicotinamide (1:1) co-crystal	**114**

47	DSC thermogram of Nebivolol hydrochloride (API)	**115**
48	DSC thermogram of Nebivolol - salicylic acid (1:1)	**116**
49	Overlay of DSC thermogram of Nebivolol - salicylic acid	**116**
50	DSC thermogram of Nebivolol - nicotinamide (1:1)	**116**
51	DSC thermogram of Nebivolol - nicotinamide (1:2)	**117**
52	DSC thermogram of Nebivolol - nicotinamide (1:3)	**117**
53	Overlay of DSC thermogram of nebivolol - nicotinamide	**117**
54	FTIR of Nebivolol hydrochloride	**119**
55	FTIR of Nebivolol - salicylic acid (1:1)	**119**
56	FTIR of Nebivolol - nicotinamide (1:1)	**120**
57	FTIR of Nebivolol - nicotinamide (1:2)	**120**
58	FTIR of Nebivolol - nicotinamide (1:3)	**121**
59	P-XRD diffraction patterns of Nebivolol hydrochloride (NBV), Nebivolol-salicylic acid (B1), Nebivolol-nicotinamide (D1)	**123**
60	SEM micrographs of A-Nebivolol pure drug, B- Nebivolol-Salicylic acid and C- Nebivolol-Nicotinamide	**124**

ABSTRACT

Background: Quality by Design (QbD) is a systematic approach to pharmaceutical development that focusses on process understanding and quality risk management, ensures reliable and high-quality analytical procedures. Antihypertensive drugs require precise quantification and employing QbD principles improves accuracy, sensitivity, and repeatability. DoE and optimization algorithms help in identifying critical factors that influence method performance, while validation as per ICH guidelines provides specificity, accuracy, and robustness. QbD simplifies regulatory compliance and improves pharmaceutical quality control. Further, polymorphic screening is an important aspect of pre-formulation studies during formulation development. Polymorphic from plays an important role in therapeutic efficacy.

Objectives The main objective of this research is to develop, optimize, and validate QbD assisted analytical methods for the estimation of selected antihypertensive drugs, ensuring accuracy, precision, sensitivity, robustness, and regulatory compliance for routine quality control. Also, this research is aimed to prepare and characterize multicomponent polymorphs (Cocrystals) using advanced formulation techniques.

Methodology: In the present study, Carvedilol Phosphate and Nebivolol Hydrochloride were analyzed using a validated RP-HPLC method, ensuring specificity, sensitivity, precision, robustness and accuracy asper ICH guidelines. QbD principles guided method development and marketed formulations were evaluated for feasibility. Simultaneously, multicomponent polymorphs formation was done by the interaction of API with different conformers, using Ultrasound-assisted Co-crystallization technique (VC750 Sonicator) at 15-20°C in methanol.

Results Quality by Design based chromatographic method was developed for estimation of Carvedilol phosphate and Nebivolol hydrochloride using Methanol, Millipore water, O-phosphoric acid and acetic acid. Shim-pack GIST C18 column (250 x 4.5 mm i.d., 5µm,) and Agilent C18 column as a stationary Phase. Box-Behnken Design was found effective for optimization of suggested chromatographic method. The validation findings revealed that the developed methods are specific, with linearity (r^2 = 0.999) and sensitive. The developed methods were reliable, precise and robust making a valuable tool for the estimation of Carvedilol phosphate and Nebivolol hydrochloride in pharmaceutical formulations. Further, multicomponent polymorphs were successfully identified and characterized by DSC, IR, SEM and P-XRD techniques.

Conclusion The developed and validated HPLC methods, designed using the Analytical Quality by Design approach, were proven to be simple, precise, and accurate for the estimation of selected antihypertensive drugs. These methods are suitable for routine quality control analysis in the pharmaceutical industry. Also, obtained multicomponent polymorphs of Carvedilol phosphate and Nebivolol hydrochloride provides scope for development of new formulations with enhanced therapeutic performance.

Keywords: Analytical Quality by Design, Box-Behnken Design, Carvedilol Phosphate, HPLC, Nebivolol Hydrochloride, Polymorphs

1. INTRODUCTION

1.1 BACKGROUND

A medication is any biologically active chemical substance that can be obtained from variety of sources and administered internally or externally in the appropriate dosage forms to treat, prevent, ameliorate, diagnose, or cure diseases in both humans and animals. Pharmaceutical companies manufacture a variety of pharmaceuticals, and each one is put through a rigorous quality testing process before being put on the market. Titrimetric, spectrophotometric, chromatographic, and biological approaches are only a few of the methods used in research and development and quality control departments to carry out quality control of medicines in both bulk and finished forms.[1]

Pharmaceutical development has greatly improved human health, but the efficacy of these drugs depends on their purity and precise dosage. Numerous chemical and instrumental techniques for drug analysis have been developed to guarantee that medications fulfill their intended function. During the development, transit, and storage phases of a pharmaceutical's lifespan, contaminants may appear. Maintaining the safety and purity of drugs depends on the detection and measurement of these impurities. Every step of the process, from drug development to clinical research and post-market testing, depends on analytical tools and techniques. Some conventional procedures have been in use for more than a century, despite the ongoing introduction of new analytical techniques.[2]

Analytical chemistry focuses on the application of various procedures and techniques to gather and evaluate qualitative, quantitative, and structural data regarding the nature of matter. It can be broadly divided into three categories.

- Identification of the components, species, and/or chemicals included in a sample is known as qualitative analysis.
- Quantitative analysis is the process of figuring out how much of a species or molecule is present in a sample, either absolutely or relatively.
- Determining the spatial arrangement of atoms in an element or molecule or identifying distinctive functional groups of atoms are examples of structural analysis.[3, 4]

Drug analysis is vital in the pharmaceutical industry, focusing on qualitative and quantitative aspects for quality control. Ensuring the quality control of drugs from various sources relies on advanced analytical techniques and modern technology. These procedures include optical or spectral methods, chromatographic techniques, electrochemical methods, physical or thermoanalytical techniques, biological and microbiological assays, radioactive methods, titration techniques, and other specialized methods.

Spectral methods examine light absorbed or emitted by pharmaceuticals substances. These methods rely on several kinds of radiation for the analysis. Spectral analysis is a key technique for determining a drug's chemical structure, enabling accurate qualitative and quantitative drug analysis.[1]

UV Spectrophotometry is a key analytical tool in the pharmaceutical industry. The UV and visible spectrums are distinctive spectra produced when a molecular solution or analyte is subjected to electromagnetic radiation. In this range, electromagnetic radiation has wavelengths between 200 and 800 nm, the visible spectrum between 400 and 800 nm, and the ultraviolet spectrum between 200 and 400 nm. When a solution absorbs light at particular wavelengths, the analyte's valence

electrons absorb the light and become excited, giving the medication or analyte its own UV or visible spectrum.[5]

This technique uses a stationary and mobile phase to separate a mixture into its constituent parts. Chromatography is essential to the pharmaceutical industry for identifying and evaluating trace amounts of drugs and other compounds in samples. It is also a crucial stage in the process of finding and creating novel medications.[6,7]

For quantitative analysis, chromatographic techniques such as HPLC are quite useful, especially when it comes to isolating and separating complex mixtures of molecules from natural, biological, or chemical sources. HPLC is frequently used in quality control and pharmaceutical and natural product analysis because of its great precision, accuracy, and specificity. HPLC is one of utmost extensively used chromatographic techniques in these domains, according to a substantial body of research.[1,8]

The above said applications are applicable only after development and validation of consistent and precise analytical method. The development of efficient analytical method is a very tedious job as it involves the consideration of various variable factors related to method. Traditionally the analytical scientist used to practice One Factor at a time (OFAT) approach for development of method. This approach has various limitations like it requires more experiments which is time consuming as well as economically not affordable as large amount of chemicals are wasted. Further these approaches do not allow the investigator to study the interactions between analytical parameters. Thus, this approach may lead to a method with certain limitations.

The scientific and technological advances in the field of analysis have introduced various high-end instruments as well as approaches to tackle the earlier limitations in development and validation of analytical techniques. Nowadays most widely practiced approach for process development and validation is Quality by Design (QbD).[9,10]

According to ICHQ8(R2), the quality guideline of the International Conference on Harmonisation, QbD is defined as a "systematic approach to development that starts with established goals and emphasizes both product understanding and process control, based on sound science and quality risk management."[11]

For developing analytical methods supported by solid scientific evidence, Analytical Quality by Design (AQbD) is a systematic approach. With this strategy, we can create analytical tools that are effective, compliant with regulations, affordable, and easy to use. Due to the large number of variables that could affect the accuracy of analytical methods developed using state-of-the-art equipment like HPLC, it is reasonable to employ AQbD approach in this context. This approach helps us to simultaneously investigate the effect of various variables on outcome of the method. Thus simple, robust and precise analytical method can be developed in small span of time without wastage of chemicals on large number of trials. The regulatory authorities are also showing more orientation and reliance towards QbD approaches. Thus AQbD is currently playing a key role in achieving the desired performance in analytical field.[9,12]

Implementing QbD concepts is essential for creating organized and effective procedures, particularly when it comes to reducing risks in analytical techniques. This method is known as AQbD when it is used for analytical development. With an

emphasis on measurement excellence, AQbD aims to develop strong, dependable techniques that continuously deliver on their promises over the course of their lifecycle. Creating analytical methods based on QbD concepts and successfully validating them are among its primary goals.[13]

AQbD is more important since it can operate across a wider experimental range and produces fewer out-of-trend or out-of-specification outcomes because it understands processes early. As AQbD principles are now included into both the US and European Pharmacopoeias, method modifications can be made without revalidation. The main idea is to include quality into the analytical process as it is being developed, which calls for a systematic, goal-oriented approach.[14,15]

There are many advantages to applying QbD concepts to analytical processes since they help to detect and reduce variability and guarantee reliable techniques that continuously satisfy performance standards over the course of their lifecycle. Improved decision-making flexibility, increased lifecycle approval chances, decreased batch errors, and a better comprehension of procedures are some of the main benefits. Additionally, by lowering the variability in analytical qualities, it increases method robustness, minimizes deviations and expensive investigations, enhances the quality of reviews, and promotes continuous improvement during method development, all of which have a favourable impact on process and product development.[16]

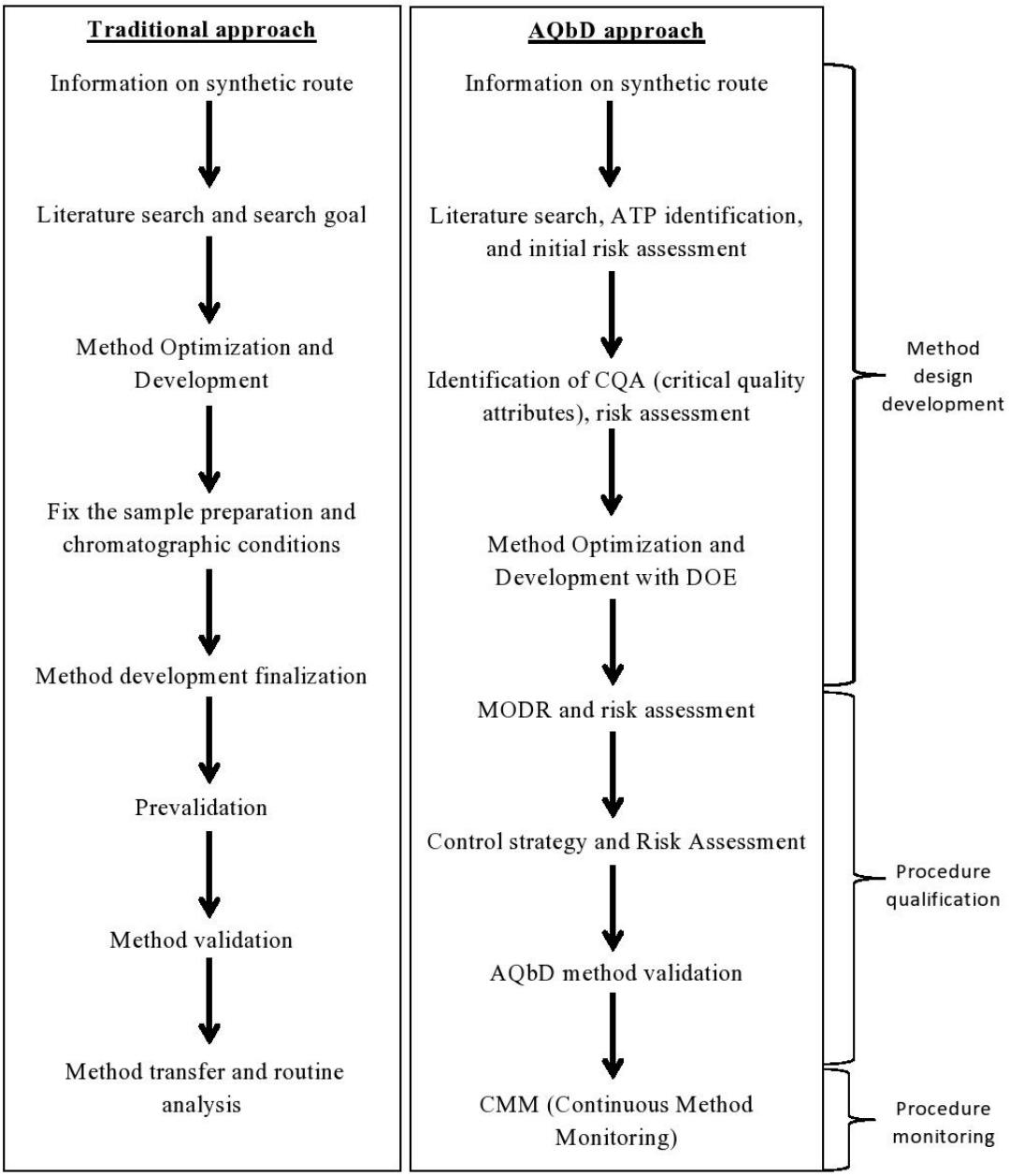


Figure 1: Traditional *VS* AQbD Approach for Analytical Method Development[17]

Polymorphism screening

Polymorphism is a property of a substance wherein it exists in more than one crystalline form. Each form of substance is called a polymorph. During the synthesis, various molecules get arranged in different pattern to form crystal lattice which results in formation of various polymorphs having same molecular formula but different molecular arrangement. Thus, in brief, polymorphs are set of crystals having same

chemical composition with similar molecular connectivity but having altered confirmations by rotation around single bonds. Almost 85% of the active pharmaceutical ingredients exhibit polymorphic forms.

As the polymorphic forms of the same molecule or substance have different crystalline lattices, these vary in their physicochemical properties further their chemical stability also varies. Example: Chloramphenicol Palmitate.

Chloramphenicol Palmitate, an antibiotic appears in three polymorphic forms *Viz* Form A, B and C. The Form A is stable but is biologically inactive while Form C unstable. Form B is metastable form which is biologically active. There are many such examples of polymorphs in pharmaceutical field like Phenobarbitone (Form I, Form II and Form III), Spironolactone (Form I is needle shaped, Form II is prism shaped), Riboflavin Form I, II, III (Aqueous solubility 60, 80, 120 mg/mL respectively) etc. These polymorphs differ in their physicochemical properties. Thus, selection of proper polymorph is prerequisite for the therapeutic efficacy of the product.

In this context pharmaceutical industries are now engaged with polymorphic screening studies in the initial stages of formulation development. During these studies various polymorphic forms are identified and their therapeutic potential is determined.[18-20]

Recent advancement in polymorphic sciences have utilized the mechanochemistry based methods to develop multicomponent polymorphs (cocrystals) of flouroquinolone like ciprofloxacin and maleic acid. The approach resulted into three different polymorphic forms of ciprofloxacin with distinct physicochemical properties. Thus, mechanochemistry based multicomponent systems

(cocrystals) could pave a new path for altering the physicochemical properties of active API.[21]

Formation of Co-crystal

Approximately 50% of all Active Pharmaceutical Ingredients (APIs) used today are salts. The formation of salts requires the presence of ionizable groups within the molecule, which is a key prerequisite for their formation. Approximately half of all medicines sold in the market are given as salts, making salt production a popular solid-state method for altering the physical properties of APIs. Improving solubility and other characteristics is where this method really shines. A key drawback of this approach is that salt production can only take place in APIs that have the correct ionisable group, which can be acidic or basic.

In contrast, co-crystals present an alternative method for enhancing the properties of APIs. Unlike salts, co-crystals do not require an ionizable group. Any API, whether acidic, basic, or neutral, can potentially be co-crystallized with suitable co-formers. This makes co-crystallization a more versatile approach, as it opens up possibilities for a wider range of molecules.

While salts are often more desirable due to their generally higher solubility in water, owing to their ionic nature, there are cases where co-crystals might be more advantageous. When the dissolution rate is more important than the equilibrium solubility (the maximum amount of the API that can dissolve), co-crystals may outperform salts. This is because co-crystals can be designed to enhance the dissolution rate without the solubility limitations that salts might have in certain formulations. Thus, the choice between salts and co-crystals depends on the specific needs of the API in terms of solubility and dissolution characteristics.[22-24]

Methods of analysis for polymorph characterization:

Thermal techniques

The relative stability of polymorphic modifications, the energies required for phase transitions between them, and whether such transitions are monotropic or enantiotropic are all quantitatively determined by thermal analysis.

Differential Scanning Calorimetry (DSC)

A number of thermal analysis methods are developed to measure difference in heat flow among sample being studied and an inert reference in order to determine the enthalpy associated with the changes. All of these techniques are now frequently referred to as DSC.

The sample mass, particle size, presence of contaminants, crystalline particle shape, and presence of nuclei or seeds of different polymorphs are all factors that affects quality of DSC measurement and the information that can be gleaned from it. DSC measurements provide the quantitative data needed to help prepare the free-energy temperature for the study of solvates (pseudopolymorphism).

Thermogravimetric Analysis (TGA)

TGA involves monitoring the mass of a sample as its temperature changes. This method is particularly useful for detecting volatile substances, such as solvents or water, which contribute to the formation of solvates or hydrates. It also aids in analyzing thermal events like decomposition and sublimation.

X-ray Crystallography

Polymorphs are frequently identified and described using X-ray crystallography techniques, which emphasize differences in crystal structures. X-ray crystallographic techniques distinguish polymorphs based on differences in crystal structure. These methods are categorized into two main approaches:

Powder X-ray Diffraction (PXRD): Used for qualitative identification of distinct polymorphic phases or their mixtures.

Single-Crystal X-ray Diffraction: Provides detailed molecular and crystal structure information.

Infrared Spectroscopy (IR)

The vibrational modes of bound atoms are measured by infrared spectroscopy; these modes are usually within the absorption range of 400 - 4000 cm^{-1}. It is among the most often utilized methods for studying a material's propensity to take on polymorphic forms.

Scanning Electron Microscopy (SEM)

SEM reveals surface morphological details of polymorphs, which aids in determining physical property variations.

Nuclear Magnetic Resonance (NMR)

NMR spectroscopy is main tool used in nuclear magnetic resonance crystallography to examine the atomic-scale structure of solid materials. This method is especially useful for investigating amorphous and microcrystalline materials that are difficult to investigate with X-ray, neutron, or electron diffraction. Its capacity to

measure short-range interactions makes it useful. Additionally, changes in atomic environments brought about by variations in molecular conformation among polymorphs are recorded by solid-state NMR spectroscopy. [25-32]

An overwhelming majority of the world's hypertensive patients about 1.28 billion people are located in low and middle-income nations, according to recent worldwide studies. But nearly half of hypertensive patients don't know they have it, and even fewer get the treatment they need; even fewer manage their condition well. This shows over 10 million deaths annually, primarily due to cardiovascular complications such as heart attacks, strokes, and kidney disease. WHO aims to decrease hypertension occurrence by 33% by 2030, stressing need for improved diagnosis, treatment access, and public health initiatives, particularly in under-resourced regions.

Antihypertensive Drugs are active substances used to manage high blood pressure (hypertension). Antihypertensive medications are divided into several categories, each targeting different mechanisms involved in regulating blood pressure. Antihypertensive drugs are essential for managing hypertension, often used in combination to achieve optimal control. While diuretics and beta-blockers are commonly used as first-line treatments, the choice of medication depends on the individual's overall health, comorbid conditions, and the type of hypertension. These drugs aim to lower blood pressure to reduce the risk of cardiovascular events and organ damage, improving long-term health outcomes.[33,34]

1.2 NEED FOR THE STUDY

Quality, safety and efficacy are the three basic criteria required for any chemical entity to get approved as drug. The quality of drug product refers to its suitability for intended use and assured by various analytical methods. Thus, analytical methods play very vital role in each stage of product life cycle. Accurate, precise, robust analytical technique not only assures quality of product but also it helps to measure the purity of product at each stage. Nowadays analytical method failures at method transfer stage are becoming a headache for pharmaceutical companies. Further, use of very costly chemicals and time-consuming procedures are adding burden on analysis cost. Within this framework, pharmaceutical companies are actively seeking for alternative policies or components to the current quality management system.

The pharmaceutical industry now considers QbD to be a crucial norm, due to its implementation by the ICH/USFDA. Joseph M. Juran was the first to describe the idea of QbD. He claims that QbD is an organized method of development that places an emphasis on comprehending products and processes as well as controlling those processes. It starts with predetermined objectives.

The term "AQbD" has been coined to describe use of QbD principles to creation of analytical approaches. By using the AQbD methodology, analysts have the tools they need to effectively regulate their methods, which in turn increases their efficiency and decreases the likelihood of method failure during the transfer stage.

One of chief reasons of avoidable mortality and illness in India is hypertension. Between 2010 and 2013, cardiovascular disease was responsible for 23% of all fatalities and 32% of deaths among adults. It is a major risk factor for this

condition. A reduction of one-third in premature mortality from non-communicable diseases by 2030 is one of Sustainable Development Goals. India has committed to implementing multiple strategies to accomplish this objective. A number of serious health problems, including those influencing kidneys, heart, brain, and other organs, are increased risks in people with hypertension, or high blood pressure. Worldwide, around 1.13 billion people suffer from hypertension, often known as high blood pressure, as reported by WHO. One in five women and one in four men had hypertension in 2015, according to the WHO. According to research conducted in 2020, the incidence of hypertension is on the rise and it is the top modifiable risk factor for cardiovascular disease and early mortality globally. A worldwide goal for NCDs is to lower the incidence of hypertension by 25% by the year 2025.

Antihypertensive drugs are important for the use of effective treatment of high blood pressure and consumed by large number of populations. Before launch of products from industry into the pharmaceutical market, they have to be analyzed for its drug contents and hence quality control of these product play an important role.

According to literature review spectrophotometric and chromatographic methods were reported for approximation of selected antihypertensive drugs (Carvedilol Phosphate and Nebivolol Hydrochloride) in bulk and marketed dosage forms in single and combined dosage forms.

As per existing requirements of regulatory bodies analytical approaches have to be developed using new concept of AQbD approach. Literature study revealed that no analytical approaches were documented for assessment of selected antihypertensive medicines using AQbD approach, such as Carvedilol Phosphate, Nebivolol Hydrochloride etc.

Thus, analytical methodologies utilizing AQbD approach for quantification of specific antihypertensive medicines in bulk & pharmaceutical dosage forms must be developed and validated.

Literature study revealed that only few analytical approaches have been documented for assessment of selected antihypertensive drugs. Further the developed methods have been devoid of utilization of AQbD approach.

The pharmaceutical industry is constantly seeking innovative approaches to improve physicochemical properties of APIs. Multicomponent polymorphs (cocrystals) are one of strategy that has gained significant attention. These properties are playing important role in the therapeutic efficacy of the drug. Due to variation in these properties various polymorphic forms of the same drug have different therapeutic efficacy. Thus, selection of proper polymorphic forms during formulation development stage is essential. The study of different polymorphic forms of drug has become a preliminary task for chemical and formulation scientists. Further, its importance is more concerned with respect to the life-threatening diseases like hypertension. Thus, in the proposed study the polymorphic screening of the selected antihypertensive drugs will be carried out for their probable variations in physicochemical properties by using various analytical techniques. Further "AQbD" based chromatographic methods of analysis will be developed and validated as per current regulatory for selected antihypertensive drugs.

1.3 AIM AND OBJECTIVES

1. To identify and characterize the polymorphs of selected antihypertensive drugs if exhibits.

2. To develop an analytical method and validate it using AQbD approach to estimate selected antihypertensive drugs in bulk and dosage forms.

2. REVIEW OF LITERATURE

2.1 Drug profile

2.2 QbD Analysis of drugs and pharmaceuticals

2.3 Analytical method development and estimation of selected drugs

2.4 Polymorph screening of selected drugs

2.1 Drug Profile

The list of antihypertensive drugs selected for the proposed research work is given in Table 1.

The profile of the selected drugs is detailed in Table 1:

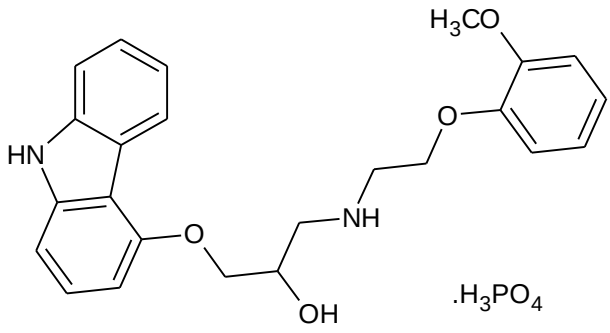
Table 1: List of antihypertensive drugs selected for proposed research

Sr. No.	Drug	Category	Mechanism of action (MOA)
51	Carvedilol phosphate	Non selective β-blocker	Non-selective β and α-adrenergic receptor blocking activities
2	Nebivolol hydrochloride	Highly selective β-blocker	Highly selective β-adrenergic receptor antagonist

Carvedilol Phosphate [35-37]

The drug profile of Carvedilol phosphate is shown in Table 2. It is non-selective β-adrenergic blocker with α_1-receptor blocking properties, is employed clinically as a racemic mixture. Carvedilol used to treat mild to moderate hypertension, angina pectoris, myocardial infarction, and mild to severe heart failure. It is an effective antihypertensive drug with a dual MOA. At lower doses, it acts as a β-adrenoceptor antagonist and a vasodilator. At higher concentration, it also inhibits calcium channels. It appears as white to almost white solid. Its administration, either alone or in combination with other drugs, makes it effective in improving survival in chronic heart failure patients and treating cardiovascular problems.

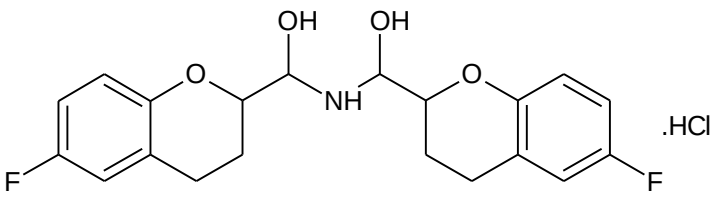
Table 2: Carvedilol phosphate drug profile

Chemical Name	1-(9H-carbazol-4-yloxy)-3-[2-(2-methoxyphenoxy) ethyl-amino] propan-2-ol;phosphoric acid
Molecular Formula	$C_{24}H_{29}N_2O_8P$
Molecular Weight	504.5 g/mol
Solubility	It is practically insoluble in aqueous medium and freely soluble in organic solvents such as methanol and 0.1N HCl
Appearance	It is white to almost white solid
Structure	

Nebivolol hydrochloride [38-41]

The drug profile of Nebivolol hydrochloride is shown in Table 3. It is an antihypertensive drug classified under β-blocker class, distinguished by its unique mechanism of action. It is primarily used for managing mild to moderate essential hypertension and chronic heart failure. Nebivolol selectively blocks β1 receptors in the heart, which slows the rate of heart and decrease the force of contractions, therefore lowering the blood pressure. Main feature of nebivolol is its ability to increase nitric oxide production, which relaxes blood vessels and increase vasodilation. This dual action of β1 receptor blockade with nitric oxide potentiation improves blood vessel functions and results in a more pronounced blood pressure lowering effect. This unique mode of action makes nebivolol an important therapeutic option for enhancing cardiovascular health.

Table 3: Nebivolol hydrochloride drug profile

Chemical Name	1-(6-fluorochroman-2-yl)-[2-(6-fluorochroman-2-yl)-2-hydroxyethyl) amino] ethanol hydrochloride
Molecular Formula	$C_{22}H_{26}ClF_2NO_4$
Molecular Weight	441.9 g/mol
Solubility	It is practically insoluble in aqueous medium and soluble in organic solvents such as methanol
Appearance	It is white crystalline powder
Structure	

2.2 AQbD analysis of drugs and pharmaceuticals

In recent years, QbD methodology has gained significant attention in pharmaceutical industry for optimizing and validating analytical methods used in drug estimation. This approach emphasizes a systematic and science-based framework to ensure robust and reliable analytical techniques. This section summarizes previous research that has focused on using QbD methodology to assess and analyze pharmaceutical products.

1. **Deshmukh SA *et al* 2020,** highlighted that RP- HPLC technique for Azilsartan was developed using QbD methodology and optimized using BBB. Concentration of acetonitrile in mobile phase, pH of aqueous phase, and flow rate were identified as CQAs. A mathematical model was developed to examine their outcome on reactions such as NTP, RT, and TF. Importance of these parameters was confirmed by ANOVA ($p < 0.05$). [42]
2. **Beg S *et al* 2015** developed a QbD-based stability indicating RP-HPLC method for quantitative analysis of olmesartan medoxomil. The chromatographic separation was achieved using a C18 column that was equipped with UV detection at 243 nm. The mobile phase consisted of acetonitrile:water (40:60 v/v) with 0.1% orthophosphoric acid (pH 3.5) and a flow rate of 1.0 mL/min. A face-centered cubic design was utilized to assess peak tailing, retention time, theoretical plates and peak area by optimizing CMPs such as flow rate and mobile phase ratio. This QbD methodology improved the method's sensitivity, reliability, and performance. [43]
3. **Patel KY *et al* 2021** reported HPLC method for Ceftriaxone sodium was developed and validated using DOE software. For the chromatography, a Phenomenex ODS C18 column (250 mm × 4.6 mm, 5.0 µ) and a mobile phase

composed of acetonitrile and water were used. At 270 nm, the detection was carried out with a flow rate of 1 ml/min and a retention time of 4.15 minutes. Indicators of system appropriateness included NTP (5236) and tailing factor (1.49), and the method showed linearity (r^2 = 0.991) using a concentration range of 10-200 μg/mL. The assay included a size of 99.73 ± 0.61%, a robustness of less than 2%, and percentage RSDs of 0.70-0.94 for intraday and 0.55-0.95 for interday, respectively. Peak purity verified that there were no co-eluting peaks, and the validation complied with ICH regulations. [44]

4. **Prajapati PB *et al* 2021** used risk-based and DoE-based DMAIC concepts in accordance with ICH Q9 to design and evaluate environment friendly HPLC and HPTLC methods for telmisartan analysis. On a Shimpack ODS C18 column, chromatographic separation was accomplished using acetonitrile:0.1% triethylamine (pH 6.5) mobile phase, flow rate of 1.0 mL/min. Method was used to analyze seven anti-hypertensive drugs and was validated in accordance with ICH Q2 (R1). It provides a cost-effective, versatile RPLC solution for QC in pharmaceutical dosage forms. [45]

5. **Palakurthi AK *et al* 2020** developed a QbD-based HPLC method to identify impurities in tablet form of telmisartan and hydrochlorothiazide simultaneously. To accomplish separation, a mobile phase consisting of 0.02M potassium dihydrogen phosphate and a water-acetonitrile mixture (100:900 v/v) was fed through an Inertsil ODS-3V column (150 × 4.6 mm, 3.5 μm) at a flow rate of 1.0 mL/min. With a column temperature of 40°C, detection occurred at 230 nm. Method was determined to be robust, stability-indicating, specific, and selective. It was also able to quantify impurities without interference, according to the experimental design. In six pill batches, the impurities were ≦ 0.1% for DSA, TEL2, and TMS-A and ≦ 0.04% (LOQ) for unidentified contaminants. [46]

6. **Suryawanshi SS *et al* 2022** In order to optimize and validate an RP-HPLC procedure for MET analysis, this study utilized AQbD and Box-Behnken Design (BBD) approaches. The chromatographic separation using an Agilent C18 column (5 µm, 250 × 4.6 mm) was carried out at a pH of 2.8, with a flow rate of 0.7 mL/min, a UV detector set at 231 nm, and a mobile phase consisting of methanol and 0.1% ortho-phosphoric acid (4:96 v/v). Based on the guidelines provided by ICH Q2 (R1), the method proved to be linear (5-25 µg/mL, r^2 = 0.999), quantitative (0.93 µg/mL) and having detection limits (0.30 µg/mL), as well as having precision, robustness, and accuracy (99.22%-100.25%). The methodology effectively quantified MET in bulk with a precision and robustness of less than 2% RSD. [47]

7. **Haque MA *et al* 2021** used a QbD methodology in line with ICH Q8(R2) to develop spectrophotometric techniques for dolutegravir estimation. The key parameters were identified using principal component analysis and an Ishikawa diagram. The study improved a UV technique for dolutegravir analysis in pharmaceuticals that is sensitive, precise, and economical. [48]

8. **Alruwaili NK 2021** reported a Box–Behnken statistical design, the AQbD methodology was used to enhance RP-HPLC technique for atorvastatin analysis. Chromatogram area, retention time (2.43 min), and tailing factor were all impacted by the mobile phase, flow rate, and UV wavelength. Technique demonstrated accuracy (%RSD < 5%), precision, and linearity (5–30 µg/mL, r^2 = 0.999). Under 0.1 M basic conditions, atorvastatin remained stable, but under UV light, it became unstable. This improved technique works well for pharmacokinetic research and atorvastatin quantification in formulations. [49]

9. **Thakur D *et al* 2017** used a fractional factorial design (FFD) to create a sensitive, stability-indicating HPLC technique for measuring gliclazide and metformin hydrochloride in tablets simultaneously. Critical parameters were found using Taguchi screening, and 16 experimental runs of FFD optimized drug resolution. The ideal parameters were a C18 Nucleosil ODS column at 227 nm, pH 7.5, column temperature 25°C, flow rate 1.0 mL/min, and mobile phase 60:20:20:0.5. The technique demonstrated great linearity (r^2 = 0.997 and 0.996), specificity, and accuracy when verified in accordance with ICH criteria. It is a trustworthy instrument for drug dose analysis because the LOD & LOQ for metformin were 0.40 and 0.55 µg/mL and gliclazide were 0.035 and 0.080 µg/mL. [50]

10. **Ganorkar SB *et al* 2017** Through development & validation of disclosed RP-HPLC-PDA technology, the rapid and accurate analysis of zileuton racemate in both tablet and bulk forms was accomplished. Hydrolytic, oxidative, photolytic, solid-state, and thermal variables were included in forced degradation tests. A mobile phase consisting of methanol and 0.2% orthophosphoric acid (80:20, v/v) was used in the procedure, flowing at a rate of 1.0 mL/min. An LC-GC Qualisil BDS C18 column with detection at 260 nm was also employed. Instead of the more conventional OFAT approach. DOE with CCD and RSM was employed to improve the process. Orthophosphoric acid concentration, flow rate, and methanol content variations were measured, and multiple linear regression was used to test robustness. [51]

11. **Patil TS and Deshpande AS 2019** developed a rapid and stable HPLC technique for examination of Clofazimine (CFZ), which was designed according to the "QbD" principles. An Ishikawa fish-bone (IFB) diagram was used to assess the risks and identified essential quality attributes. Important

parameters were optimized using Taguchi and BBB. The validated technique, conforming to ICH guidelines, using a UV detector at 284 nm and a mobile phase consisting of 75:25% methanol and ammonium acetate, at a Flow rate of 1.0 mL/min. It shows high sensitivity (linearity 2–10 µg/mL, r^2 = 0.9995), accuracy (99.68–100.44%), and low detection (0.0066 µg/mL) and quantitation limits (0.0199 µg/mL). Force degradation studies revealed Clofazimine's vulnerability to alkaline stress. Method is appropriate for Clofazimine analysis in formulations and biological samples. [52]

12. **Pareman R *et al* 2015** revealed that number of national drug applications have been recently accepted by the FDA as a part of regulatory flexibility strategy centered on AQbD. AQbD allows, analytical techniques to operate inside a predefined Method Operable Design Region (MODR), enhances method robustness and decreases out of trend and out of specification results. Pharma companies are gradually but surely implementing AQbD as part of their risk management, drug development, and ICH Q10. This study investigates AQbD role in the pharmaceutical excellence, specifically focusing on its connections to process analytical technology (PAT) and Product Quality by Design. [14]

13. **Mandpe SR *et al* 2022** stated RP-HPLC technique which was developed and validated for analyzing furbiprofen (FBP) in nanoparticle formulations using a design of experiment approach. Optimal conditions (25°C, 1 mL/min, 85% methanol in 0.05% OPA at pH 2.75) resulted in a RT of 4.75 min and a linear calibration (r^2 = 0.9997, 10-50 µg/mL). The method showed high precision, with an LOD of 0.14 µg/mL, and demonstrated stability under various stress conditions. Nanoparticle and regular pharmaceutical formulation FBP analysis can be done using this proven approach. [53]

2.3 Analytical method development and estimation of selected drugs

The existing literature documents inclusive range of analytical approaches for quantifying selected drugs in pharmaceutical preparations. These methods include spectrophotometric, chromatographic, and electrochemical techniques for both single and combined formulations. However, the reported quantification procedures present several limitations and challenges, such as:

- Dependence on traditional development approaches.
- Limited application of the QbD-based approach for estimating selected drugs using analytical methods
- Use of complex solvent systems
- Prolonged retention times in chromatographic techniques.
- Employment of expensive solvents, leading to increased operational costs.
- Non-robust development of analytical methods

These limitations highlight the need for advancements in analytical methodologies, with a focus on adopting QbD principles, simplifying solvent systems, minimizing retention times, and employing cost-effective practices to improve the efficacy and efficiency of drug quantification processes. A comprehensive review of the literature on selected drugs is presented in detail below.

14. **Patel BA *et al,* 2012** reported a validated RP-HPLC-fluorescence technique for Carvedilol Phosphate in bulk and dosage forms. A Brownlee C18 column was utilized for the separation, with a utilization of isocratic mobile phase (Acetonitrile: methanol: buffer, 70:20:10v/v, 5mM KH_2PO_4, 3.5 pH adjusted with H_3PO_4) flowing at 1.0 mL/min and detection was performed at 280 nm excitation and 340 nm emission, detection was made. 2.20 minutes was the

retention time. Linearity was shown with calibration range (10–60 mg/mL), yielding correlation coefficient r^2 = 0.990. The technique was used on commercial formulations after meeting ICH validation.[54]

15. **Adib N and Shekarchi** M **in 2015** tested carvedilol in human plasma using an isocratic RP-HPLC technique that is simple, fast, and sensitive. The analysis was carried out on a μ-Bondapak C18 column with a flow rate of 1 mL/min. The extraction solvent was methanol and mobile phase was acetonitrile-potassium dihydrogen phosphate (30:70 v/v, pH 2). A method achieved 0.05 mg/mL LOD and a 0.2 mg/mL LOQ. Recovery was 99.48±8.5%, and the precision (CV%) ranging from 2.45 to 7.92%. A bioequivalence study successfully used this technique. [36]
16. **Rao VU *et al* 2017** have reported developed and validated, stability-indicating RP-HPLC method for determining carvedilol content of tablets. The optimal chromatographic conditions were achieved by using a Waters C18 column in conjunction by mobile phase (40:60) phosphate buffer (pH 6.0) to acetonitrile. Carvedilol remained stable under photolytic and oxidative conditions, while forced degradation experiments revealed considerable deterioration under acidic and basic conditions. The approach is appropriate for routine pharmaceutical analysis since it is robust, precise, and accurate while adhering to ICH criteria. [37]
17. **Rahman A *et al* 2019,** analyzed a novel chiral HPLC technique for Carvedilol enantiomer separation. The technique was validated according to ICH standards for accuracy, precision, and robustness, and it produced excellent enantio-separation (resolution = 7.9) using a Chiral peak IC column and a mobile phase consisting of hexane, isopropyl alcohol, diethyl-amine, and acetic acid. The method with runtime of 17 minutes, outperformed earlier approaches and quickly

detected (S)- and (R)-Carvedilol. It is potentially useful for regular analysis and testing for enantiomeric purity in pharmaceutical formulations. [55]

18. **Yıldırım S *et al* 2022** Carvedilol and Nebivolol are β-blockers analyzed using advanced methods like HPLC with UV or DAD for pharmaceuticals and biological fluids. Carvedilol employs SPE (solid phase extraction) and magnetic SPE for plasma and serum, using mobile phases like Acetonitrile (ACN) and pH-adjusted buffers, while Nebivolol analysis in tablets and dissolution media uses ACN:water with trifluoroacetic acid. Stability testing for Carvedilol includes stress conditions (oxidation, photolysis, pH). For environmental samples, both drugs utilize SPE or DSPE with MIPs for selective extraction, ensuring accurate quality control and residue analysis. [56]
19. **Fadhil AK *et al* 2021** review highlights HPLC as a preferred method for carvedilol analysis, utilizing C18 and RP-18e columns with phosphate buffer-acetonitrile mobile phases (pH 2.5–3.5) and UV detection at 240–285 nm. These methods, validated for bulk drugs, formulations, and plasma, show high accuracy (99–102% recovery) and low RSD (<1.4%), ensuring precise quality control in carvedilol applications. [57]
20. **Raju TR *et al* 2015** presented the results of their work on RP-HPLC method that indicates stability for carvedilol and its contaminants in active pharmaceutical ingredients (APIs) and pharmaceutical formulations. The experiment employed a gradient elution method with UV detection at 240 nm on an Inertsil C8-3 column. The approach successfully separated carvedilol from its impurities and degradation products under stress conditions. The sensitivity, robustness, accuracy, and precision were validated according to ICH requirements. method is ideal for carvedilol impurity profiling and stability analysis. [58]

21. **Bechara V *et al* 2015** reported two spectrophotometric methods for estimating Carvedilol in bulk and marketed formulations. Method A involves oxidation with ferric ammonium sulfate and complexation with potassium ferricyanide, producing a bluish-green chromogen at 582 nm. Method B uses 2,4-dinitrophenylhydrazine oxidation, forming a brown chromogen at 500.8nm. Method A yielded a high correlation coefficient of 0.997 while Method B showed a linearity of 5-30 µg/mL. Both techniques were validated according to ICH criteria. The dependability of the methods for routine Carvedilol estimation is ensured by their sensitivity, as demonstrated by Method-A LOD and LOQ values of 0.0840 and 0.2545 µg/mL and Method-B 0.0667 and 0.2021 µg/mL. [59]
22. **Awotwe-Otoo D *et al* 2012** have detailed a QbD based chromatographic technique for protamine sulphate analysis that has been validated. Finding a reliable reverse phase-HPLC technique to separate hydrolyzed protamine sulphate peptides was the driving force behind this research. The findings shown that a protein analysis HPLC chromatographic method could be optimized using the Quality by Design principle with the least possible number of experimental runs. [60]
23. **Shah DA *et al* 2008** reported a new method for quantifying the tablet form of nebivolol hydrochloride using a validated RP-HPLC approach with a pH of 4.0 and a mobile phase made of methanol, acetonitrile, and 0.02 M potassium dihydrogen phosphate in a ratio of 60:30:10, v/v/v, a Phenomenex Gemini C-18 column was used. Method exhibited good linearity, recovery, and sensitivity. Forced degradation studies demonstrated the method's stability-indicating nature.[61]

24. **Sahoo MK et al 2009** reported a RP-HPLC method to determine the amount of nebivolol in tablets. A Hypersil ODS C18 column, a methanol-water mobile phase, & UV detection at 282 nm were employed in the analysis. The reference material utilized was chlorzoxazone. The technique demonstrated great sensitivity, outstanding accuracy (assay 99.974%), and precision. Nebivolol in tablet and bulk dose forms can be regularly quality controlled with this technology. [62]
25. **Patel LJ *et al* 2007** reported two analytical methods (RP-HPLC and HPTLC) to quantify nebivolol hydrochloride in tablets. With a retention time of 3.76 minutes, nebivolol hydrochloride was observed. An ethyl acetate, toluene, methanol, and ammonium hydroxide mobile phase were used in the HPTLC technique, which was carried out on silica gel plates. Both methods demonstrated excellent precision, linearity, accuracy, and sensitivity, making them suitable for routine QA of nebivolol hydrochloride in pharmaceutical preparations. [63]
26. **Szabo ZI et al 2014** reported a HPLC method to quantify Nebivolol in tablets. The method used UV detection at 281 nm and a BDS Hypersil C18 column with a mobile phase consisting of 0.1% trifluoroacetic acid in a water:acetonitrile (60:40, v/v) mixture. It was determined to be appropriate for regular nebivolol quality control in tablet. [64]
27. **Yilmaz B 2010 reported** a validated HPLC method to measure nebivolol in pharmaceutical products. This procedure made use of a 282 nm UV detector, a methanol-water mobile phase, and an Ace C18 column. Linearity, sensitivity, precision, and accuracy were all highly demonstrated by the approach. Regular quality control and stability investigations can benefit from its under-6-minute analysis time. [39]

28. **Kachhadia P et al 2008** have developed and validated simple HPLC method for the purpose of measuring nebivolol in solid dosage forms; this method is accurate, precise, and user-friendly. The analysis was conducted using a Phenomenex Luna C8 (2) column with UV detection set at 280 nm, using a mobile phase consisting of acetonitrile and phosphate buffer at a ratio of 35:65, v/v. This method was validated to make sure that it works in solutions and is specific, linear, accurate, precise, robust, and stable. In the concentration range of 40-160 µg/mL, the method showed a linear relationship that was both exact and accurate. Stress degradation studies confirmed the method's suitability as a stability-indicating tool. [65]
29. **Reddy CA *et al* 2022** reported a new, effective RP-HPLC technique to measure both Nebivolol and Valsartan in pharmaceutical formulations. This technique makes use of a Cosmosil C18 column and a methanol-water mobile phase. The optimal retention periods for Nebivolol and Valsartan were 5.04 minutes and 9.71 minutes, respectively. The method's dependability was established by rigorous validation, which followed ICH requirements. Linearity (r^2 = 0.999), accuracy, precision, robustness, and sensitivity were all proved. This technology offers a reliable option for routine quality monitoring in the pharmaceutical industry. [66]
30. **Kokil SU and Bhatia MS 2009** described RP-HPLC methods, utilizing C18 columns and methanol-water mobile phases with ion-pairing reagents. The developed methods were found to be accurate and precise for quantification of Nebivolol and Valsartan in pharmaceutical preparations. Rigorous validation, adhering to ICH guidelines, ensures method reliability through assessments of specificity, linearity, accuracy, precision, LOD, LOQ, and robustness. These validated methods provide reliable and efficient tools for routine quality control,

contributing to the overall quality assurance of these critical cardiovascular medications. [40]

31. **Nekkala K *et al* 2014** outlined developed and validated HPLC method that is easy to use, precise, and robust for measuring Valsartan and Nebivolol in combination and individual dosage forms. Using a C-18 column with a mobile phase consisting of acetonitrile, methanol, and 0.02M potassium hydrogen phosphate buffer (pH 4.0) in a ratio of 50:20:30 v/v, isocratic separations were performed at a flow rate of 1.0 mL/min. Nebivolol and Valsartan had 2.5 and 4.3minute retention times, respectively, and 210 nm was used for detection. [67]
32. **Ambhore J *et al* 2018** have developed RP-HPLC method and proven to be sensitive enough to measure both Nebivolol and Hydrochlorothiazide in tablet form simultaneously. By effectively isolating the medications from their degradation byproducts under a range of stress conditions, the method demonstrated its stability-indicating characteristics. A C-18 column was used for the chromatographic separation, and a mobile phase consisting of methanol and 0.05% orthophosphoric acid was introduced at a flow rate of 0.7 mL/min. At 281 nm, the detection was carried out. Strict validation in line with ICH guidelines ensured the method's specificity, linearity, accuracy, precision, LOD, LOQ, and robustness. [68]
33. **Annadi AM *et al* 2020** For solid dosage forms of Valsartan in conjunction with Nebivolol or Amlodipine, this study followed ICH criteria to design and evaluate RP-HPLC and RP-UPLC techniques. Operating at a flow rate of 1.0 mL/min, the RP-HPLC run made use of a mobile phase consisting of a buffer:acetonitrile:methanol (30:20:50, v/v) mixture. detecting wavelength used was 281 nm. Utilizing RP-UPLC, a mobile phase composed of a phosphate buffer:acetonitrile (55:45, v/v) was applied to an Eclipse Plus C18 column. The

flow rate was maintained at 0.3 mL/min, and the detection wavelength was set at 240 nm. A linear calibration was confirmed by an in-vitro dissolution investigation that showed an 85% release of Valsartan and Amlodipine within 15 minutes and Nebivolol after 20 minutes. [69]

34. **Cem ON *et al* 2021** established a reliable and validated HPLC technique for Nebivolol, Amlodipine and related chemicals. C18 column with an acetate buffer-acetonitrile mobile phase, 1.3 mL/min gradient elution, and 265 nm detection was used to achieve separation, with recovery rates ranging from 98.00 % to 101.50 % and precision (%RSD) less than 2%, the detection limits for Nebivolol were 0.2-10.0 µg/mL and for Amlodipine, 0.25-10.0 µg/mL. For pharmaceutical product quality assurance, the technique works as expected. [41]

2.4 Polymorph screening of selected drugs

35. **Surov AO *et al* 2019** reported the crystallization of ciprofloxacin with salicylic acid yielded a diverse array of solid forms, including an anhydrous salt, two polymorphic monohydrates, methanol and acetonitrile solvates, and a salt-cocrystal hydrate. These forms were characterized using techniques such as X-ray diffraction, thermal analysis, and spectroscopy. Real-time Raman spectroscopy was employed to study the mechanochemical formation pathways of these polymorphs, revealing that the grinding process's kinetics and pathways depend on the initial material and reaction conditions. Thermal analysis of the hydrated salts indicated a two-step dehydration process involving distinct intermediate crystalline products. [70]

36. **Fernandes GJ *et al* 2018** discussed various techniques for solubility enhancement of Carvedilol a BCS class II drug. Emerging techniques like co-crystallization and co-amorphous systems, particularly when combined with advanced manufacturing methods such as hot melt extrusion and sonocrystallization, show promise in overcoming challenges of solubility and improving the therapeutic efficacy of carvedilol. [71]
37. **Barikah KZ 2018** reviewed various methods for cocrystal formation. While traditional methods like solvent evaporation, grinding, and slurry techniques have been used, they often face limitations. These techniques offer simpler, more continuous processes for cocrystal production, making them attractive for pharmaceutical applications. [72]
38. **Seevakan K *et al* 2018** discussed various methods of crystal growth and their types. The authors emphasize that achieving supersaturation is a fundamental requirement for crystal growth, followed by nucleation. Understanding supersaturation and nucleation is crucial for crystal formation. The attainment of a supersaturated state is vital for any crystallization process, and the degree of supersaturation, or the deviation from equilibrium saturation, plays a key role in controlling the deposition process. [73]
39. **Hiendrawan S *et al* 2016** have prepared various new carvedilol pharmaceutical salts containing oxalic acid, fumaric acid, benzoic acid, and mandelic acid. Several methods, such as PXRD, DSC, TGA, FTIR, and SEM, were used to characterize these salts that were produced via solvent evaporation. They showed improved stability under accelerated conditions and were more soluble in a pH 6.8 phosphate buffer than the parent medication. Salt is emphasised by these results, as a promising approach to improve the solubility of carvedilol. [74]

40. **Aher S *et al* 2010** have prepared caffeine-maleic acid cocrystals in methanol using ultrasound-assisted solution cocrystallization (USSC). While other methods created mixtures, USSC produced pure 2:1 cocrystals by altering the molar ratio of caffeine to maleic acid (1:0.5 to 1:3.5). The efficiency of USSC is attributed to simultaneous supersaturation caused by ultrasound and the crucial function of the molar ratio in solution. [75]

3. MATERIAL AND METHODS

The experimental requirements for this study include analytical instruments, reagents/chemicals, active pharmaceutical ingredients (APIs) and formulations as given below.

API: Carvedilol phosphate and Nebivolol hydrochloride were selected as antihypertensive category of drug for the proposed study. In order to develop and validate the analytical approaches, APIs have been used. Teva Pharmaceutical Pvt ltd. of India provided the Carvedilol phosphate as an API sample. The Bagdol, Gujarat-based company 4care Life Science PVT. Ltd. offered Nebivolol hydrochloride as an API sample.

Chemicals and reagents: All the chemicals utilized for the research work were analytical grade and acquired from college chemical store. Methanol, acetonitrile, formic acid and o-phosphoric acid of analytical grade were obtained from mark India Ltd.

Marketed formulations: To verify the suitability of the developed method, marketed formulations of selected antihypertensive drugs were purchased from local market of Belagavi. Carvedilol phosphate (Cardivas - 3.125 mg Manufactured by: Sun Pharma Laboratories Ltd) Nebivolol hydrochloride (Nebicia hydrochloride-5mg Mfg. by Daxia Healthcare) were used as study samples.

Analytical instruments and equipments: An electronic analytical weighing machine of sartorius was used to weigh the analyte, an Ultrasonic bath Sonicator-Ultrasonic cleaner was used to sonicate the solutions and Millipore Water was collected for

analysis using a Direct Q UV aquatic distillation system. Instruments such as the Agilent 1100 system, and Lab solutions, as well as Chemstation software version 10.01. The software used was Version 5.97 SP1.

General steps followed in analytical method development and validation by AQbD approach

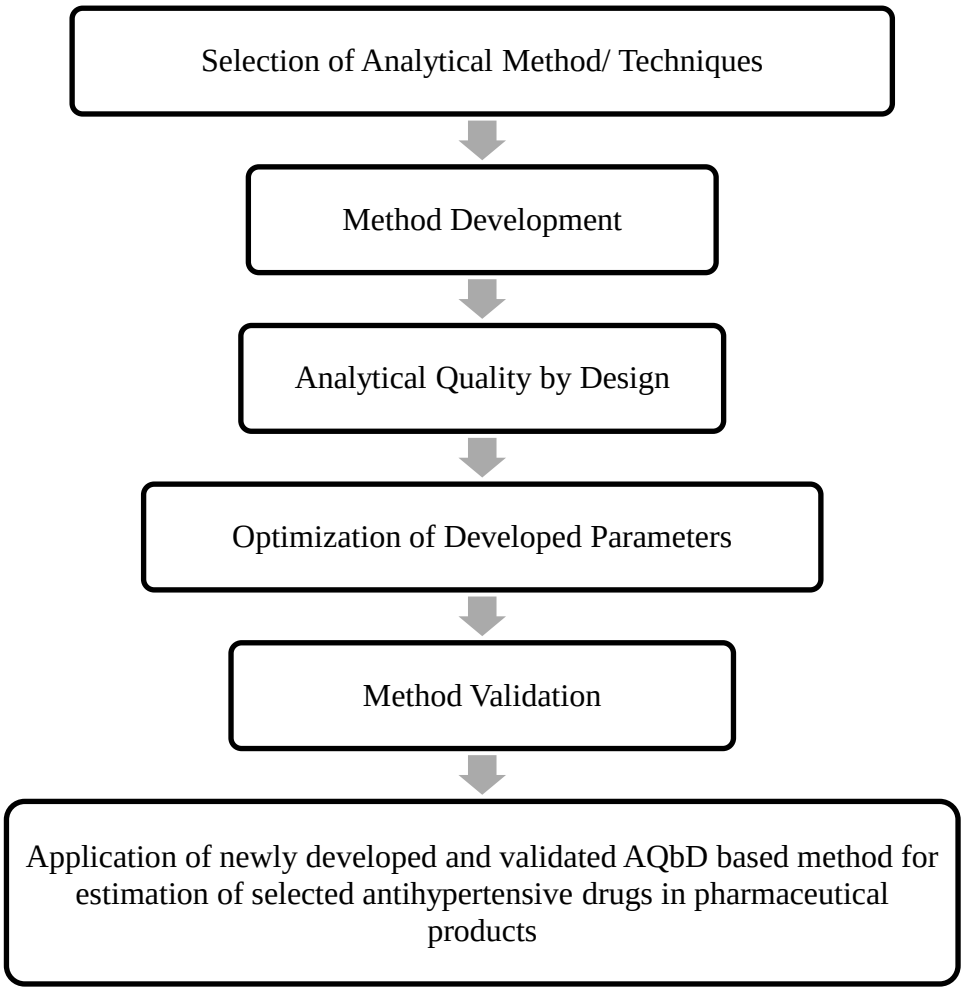


Figure 2: Overview of method development of HPLC by AQbD Approach

3.1 DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR CARVEDILOL PHOSPHATE IN BULK AND FORMULATION

Chromatographic conditions

Shimadzu Prominence - i series LC-2030c 3D plus system was equipped with Shim-pack GIST C18 column (250 x 4.5 mm i.d., 5µm,) having guard column C18 (ODS;4x3.0 mm i.d., Phenomenex, USA) was used to carry out the chromatographic (RP-HPLC) estimation. Lab solutions software (Version 5.97 SP1 software) was used for data collecting and processing. In the mobile phase, methanol (MeOH) and 0.1% Ortho Phosphoric acid (OPA) were mixed in a ratio of 35:65 (v/v), by maintaining the column temperature at 30°C and injected the mobile phase at a flow rate of 1.0 ml/min. The detection was carried out at 240 nm wavelength. A 10µL sample was injected for each analysis and the chromatographic run time was 10min.

Preparation of Sample solution

To prepare Carvedilol Phosphate stock solution, 10 mg of Carvedilol Phosphate was dissolved in 10 mL of methanol. To achieve a final concentration of 10 µg/mL the stock solution was serially diluted with mobile phase to obtain a working sample solution.

DEVELOPMENT OF AN AQbD-ENABLED RP-HPLC TECHNIQUE

ATP (Analytical Target Profile) and CAA (Critical Analytical Attributes) [76,77]

A critical first step in QbD methodology is defining the ATP. For the purpose of developing high-quality and accurate analytical procedures, ATP specifies the qualities that should be present in such method. The determination of ATP is affected by factors such as the method's application, the instrument's characteristics, the drug

characteristics, and the sample preparation. Meeting the ATP requires careful consideration when selecting CAA, which are quantifiable features of the chromatogram that correspond with the quality of analytical method. Some examples of CAA include Tailing factor (Tf), Retention time (Rt), Peak area (PA), and resolution between multiple peaks. Other examples include number of theoretical plates (NTPs).

Risk assessment studies and Factor screening analysis through Taguchi design [78]

Assessment of risks and possibilities of failure during method development is a critical part of QbD, which was done by risk assessment study. This risk assessment one was put together by utilizing a structured approach, such a Design of Experiment (DoE) ® research. The risk assessment was, conducted using Taguchi orthogonal array design including seven components at two levels, was utilized to study cause-effect association between Critical Process Parameters (CPPs) and Critical Material Attributes (CMAs). DoE® software (7.0.0 version, Stat-Ease Inc., Minneapolis, MN, USA) was used for the study.

Seven critical method variables were considered in study: injection volume, flow rate, temperature, concentration of organic modifiers, type of organic modifiers, type of solvent and solvent % by volume. Table 4 and 5 provide a summary of all of these elements, along with their respective levels.

Table 4: Taguchi design matrix for screening

Std. trial no.	Type of solvent	% v/v of solvent	Type of organic modifier	Conc. of organic modifier	Temp. (°C)	Flow rate (ml/min)	Injection volume
1	MeOH	65.00	OPA	0.20	30.00	0.80	20.00
2	ACN	65.00	OPA	0.05	30.00	1.00	20.00
3	ACN	60.00	FA	0.20	40.00	1.00	20.00
4	ACN	60.00	FA	0.05	30.00	0.80	10.00
5	MeOH	65.00	FA	0.05	40.00	1.00	10.00
6	ACN	65.00	OPA	0.20	40.00	0.80	10.00
7	MeOH	60.00	OPA	0.20	30.00	1.00	10.00
8	MeOH	60.00	OPA	0.05	40.00	0.80	20.00

Factors selected for screening studies	Levels of factors	
	Low (-1)	High (+)
Type of solvent	ACN	MeOH
% v/v of solvent	60	65
Type of organic modifier	FA	OPA
Conc. organic modifier	0.05	0.2
Temperature	30	40
Flow rate	0.8	1.0
Injection volume	10	20

Table 5: 3^2 Box-Behnken Design (Variables and their levels used)

Factor	Levels used, actual (coded)		
	Low (-1)	Medium (0)	High (+1)
Independent variables			
X_1: Solvent conc. in mobile phase (% v/v)	55 (-1)	60 (0)	65 (+1)
X_2: Injection volume	10 (-1)	15 (0)	20 (+1)
Dependent variables			
Y_1: Retention time	Minimize		
Y_2: Peak area	In the range		
Y_3: Tailing factor	Minimize		
Y_4: Number of theoretical plates	In the range		

Method Optimization using Box-Behnken design (BBD) [49, 79]

Based on preliminary study, key factors significantly influencing the CAA were identified. A BBD experiment was then conducted to systematically examine the impact on these factors on CAAs. Experimental design consisted of 13 different runs, each with specific settings for the selected factors. For the analysis of observed responses statistical software was utilized, for the selection of the best fit model such as linear, polynomial, quadratic, 2FI, etc. and to develop model equations.

Here is an example of how to express a general polynomial equation:

$$\mathbf{Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2}$$

In this equation,

Y: Response or dependent variable

β_1 and β_2: Linear coefficients

β_{12}: Interaction coefficients between two factors;

X_1 and X_2: Coded levels of independent variables;

X_1X_2: Interaction term and

β_{11}, and β_{22}: Quadratic coefficients obtained from the observed response;

β_0: Intercept (average response)

X_1^2 and X_2^2: Quadratic terms.

To determine the impact of the related independent variable on the response, the sign and magnitude of the coefficients are crucial. Coefficient magnitude is inversely proportional to effect size; i.e. higher is the coefficient indicates a more pronounced effect, and vice versa. Whether it has a positive or negative influence on the reaction under study is indicated by the additional sign of the magnitude. The fitted model and its individual terms were tested for statistical significance using ANOVA. A p-value below 0.05 was considered to be statistically important. To explore association between two sets of independent and dependent variables, 2D-contour plots and 3D-response surface plots were generated. Further, overlay plot were used to investigate the interaction between variables.

Method Validation [80,81]

In accordance with ICH Q2 (R1) standards, the suggested chromatographic technique was tested for system compatibility, linearity, specificity, LOD, LOQ, accuracy, precision, and robustness.

System Suitability and Specificity

Six replicate injections of a standard CAV solution with a concentration of 15 µg/mL were used to determine system suitability. Various critical parameters were calculated: Rt, PA, NTPs, and Tf. For PA and Rt, % RSD values found below the permissible limit of 2%. Additionally, a specificity study was conducted to examine the impact of the mobile phase on Carvedilol phosphate detection.

Linearity

The linearity of the method was examined by analyzing six different concentrations of Carvedilol phosphate standard solutions, ranging from 5-30 µg/mL, in triplicate. The relationship among concentration and peak area was determined by plotting a calibration curve`. Moreover, the line equation was calculated, which included the intercept, slope, and coefficient of correlation (r^2).

LOD and LOQ

LOD and LOQ were calculated using equations

$$LOD = \frac{3.3XSD}{\alpha} \qquad \text{……..(1)}$$

$$LOQ = \frac{10XSD}{\alpha} \qquad \text{…….(2)}$$

Where, α: Slope of linear calibration curve.

SD: Standard deviation

Precision

Both intra-day and inter-day variances were examined to determine precision of established approach. On the same day, three different concentrations (5 µg/mL, 15 µg/mL, and 25 µg/mL) were examined for intraday precision. Alternatively, to determine the inter-day precision, same concentrations were examined on three

different days. As a measure of precision, % RSD values of the peak area were determined.

Accuracy

A recovery study was conducted at three distinct concentration levels to ascertain the accuracy. In a nutshell, tablet powder corresponding to 50%, 100%, and 150% of the medicine was added to 10 mg of bulk powder. The sample recovery percentage is calculated by diluting the powder combination with mobile phase and then analyzing the results.

Robustness

By intentionally adjusting the chromatographic parameters, including mobile phase composition (MeOH:0.1% OPA (64:36 and 66:34, v/v)), flow rate (±0.1 mL/min), and column temperature (±1°C), the method's robustness was assessed. The effect of these changes on CAAs were monitored.

Assay of Marketed tablets

Twenty Carvedilol phosphate tablets (Cardivas) were weighed individually, and powdered. Carefully measuring powder equivalent to 10 mg of Carvedilol phosphate was transferred to a 50 mL volumetric flask. Powder was thoroughly dissolved when 20 mL of methanol was added to the flask and stirred using a vortex mixer. After that, methanol was added to dilute the solution to volume, and a 0.45µm membrane filter was used for filtration. Using the established chromatographic method, the CAV content was determined after an adequate dilution of filtrate with mobile phase.

3.2 DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR NEBIVOLOL HYDROCHLORIDE IN BULK AND FORMULATION

Equipment and Chromatographic Parameters

The HPLC experiment was carried out using the latest version of Chemstation software, together with an autosampler and an Agilent 1100 system. In the stationary phase, there was an Agilent C18 column with dimensions 250 × 4.6 mm and a particle size of 5 μm. In the mobile phase, there was a 71:29 ratio of MeOH to 0.1% aqueous acetic acid. A flow rate of 0.8 mL/min, a column temperature of 25°C, sample injection volume of 20 μL, and the detection wavelength was adjusted to 282 nm during analysis. After passing each sample through a 0.45μm nylon filter, it was used to prepare the solvent system and then degassed. Sonication was done using a sonicator (MJL lab) to dissolve the drug and drug powder in the solvent.

Preparation Mobile Phase

Diluting 1 mL of acetic acid with Milli-Q water up to 1000 mL yielded a 0.1% v/v aqueous acetic acid solution, which was then used to prepare the mobile phase. The final mixture was a 71:29 v/v ratio of MeOH to 0.1% v/v aqueous acetic acid, which was achieved by further mixing an adequate amount of the resultant solution with methanol. Afterwards, a 0.45μm nylon membrane filter is used to filter the mobile phase. The resulting solution is set aside for future use.

Preparation of standard stock solution

10 mg of Nebivolol hydrochloride was dissolved in 10 mL of methanol by sonication to make a stock solution. To obtain a stock solution with a concentration of 100 μg/mL, 1 mL was transferred to a 10 mL volumetric flask and then diluted with

mobile phase to a final volume of 10 mL. Using the mobile phase as a diluent, further Nebivolol hydrochloride working solutions were prepared by a series of dilutions. A 0.45µ syringe filter was then used to filter the final solution.

AQbD enabled RP-HPLC method development

The term QbD refers to a methodical strategy for product and process development that starts with defined goals and places an emphasis on understanding the product and process via the application of sound scientific principles and effective risk management. Improved scientific knowledge of a product's key aspects and attributes is an important part of QbD. It involves making use of scientific understanding during creation to create controls and testing, and then applying that knowledge to improve the product over time. AQbD is an approach that taken an active role in developing and implementing in this study. First, ATP was identified. Then, CQAs and RA were examined using the Ishikawa (fishbone) diagram. Next, optimization and factor screening were done through the use of DOE.

Analytical Target Profile (ATP) and Critical Quality Attributes (CQAs)[76,77]

To begin executing QbD strategy, it is essential to develop an ATP. In order to guarantee the accuracy and precision of an analytical technique, the ATP entails outlining the precise quality criteria that should be present in the method. When determining the ATP, several factors are considered, including the method's applicability, the instrument's features, the drug's qualities, and the procedures used for sample preparation. The achievement of the target ATP depends on the selection of CQAs. CQAs are quantifiable characteristics of the analytical process that are associated with its quality. Table 6 shows several examples of CQAs, which include Tf, Rt, PA, resolution between peaks, NTPs, and more.

Table 6: QTPP, CQAs and Process Parameters of Proposed RP-HPLC Method

Sr. No.	AQbD Parameters	Description
1	Quality Target Product Profile (QTPP)	Flow rate, Mobile phase composition, Wavelength of detection, pH, Temperature, and Column attributes, Injection volume, Pressure, Buffer attributes
2	Critical Quality Attributes (CQAs)	Retention time, Peak area, Theoretical plates, Peak purity, Tailing factor.
3	Process Parameters	Specificity & Selectivity, Linearity, range, Precision, Robustness, Ruggedness, Accuracy, LOD, LOQ.

Risk Assessment studies and Factor screening studies [78,82]

The main focus of risk assessment study is to determine the risks and probabilities of failure related with method development. Figure 2 shows the results of the present study's usage of an Ishikawa Fish-Bone diagram for risk assessment, which included an analysis of the cause-and-effect relationship between CPP and CMAs with important CAAs. Design expert software was also used to optimize the chromatographic conditions. Optimization of CPPs, CMPs, and their interactions on CQA led to the selection of the BBD. During optimization, the three-level design with decreased trial runs was employed.

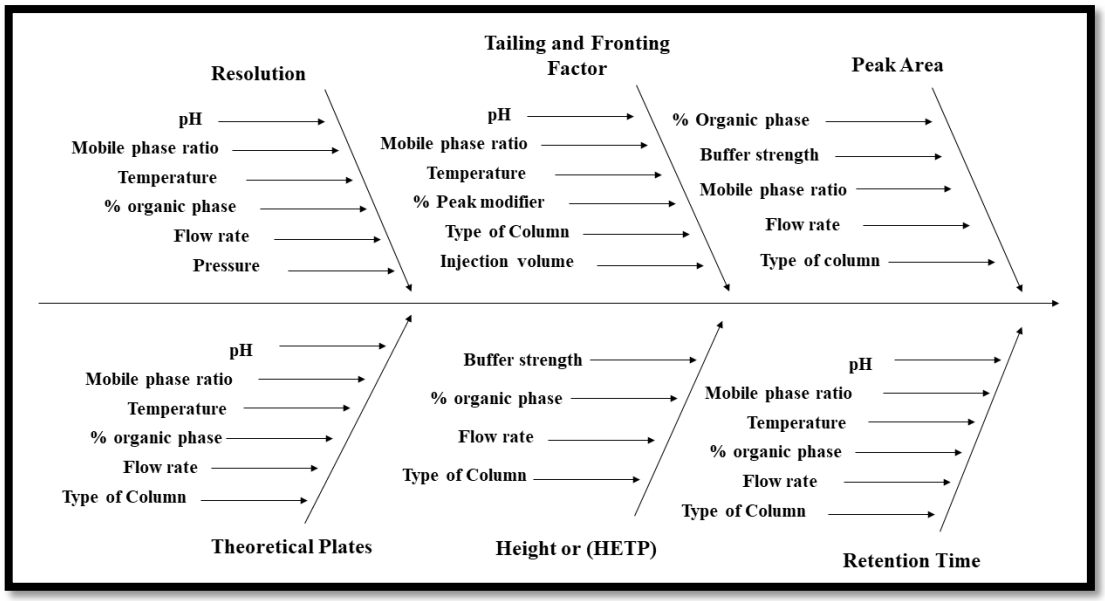


Figure 3: Ishikawa fish bone diagram

Optimization study [49]

To determine the effect of independent variables on various responses, Analytical Quality by Design was used. As BBD achieves excellent outcomes with a small number of experimental trials, BBD was chosen for the optimization. An analytical method was enhanced by applying the three-factor, three-level BBD. The components of the mobile phases, namely the methanol % (A), flow rate (B), and UV wavelength (C), were examined. Their combined and individual effects were evaluated in relation to the following responses: Y1-retention time (Rt), Y2-peak area, and Y3-tailing factor in minutes. In order to find the best combination, seventeen separate experimental runs were performed using different compositions using software (Table 18). The experimental data was fit using a variety of kinetic models, including linear, second-order, and quadratic models, in order to choose the best model. To evaluate the impact of the variables on the responses, polynomial equations, 3D plots, and 2D contour plots were employed. In order to determine the model's relevance and find the best fit, statistical analysis were performed, which included ANOVA and regression analysis.

For the purpose of selecting the most significant 2nd order quadratic polynomial equation (Eq. 1), the "Analysis of Variance" (ANOVA) F test was employed.

$$\mathbf{Y=\beta_0+ \beta_1A+ \beta_2B+ \beta_2C+ \beta_{12}AB+ \beta_{13}AC+ \beta_{23}BC+ \beta nA^2+ \beta_{22}B^2+ \beta_{33}C^2}$$

In the above equation, Y is response, intercept β_0, and "regression coefficients" β_1–β_{33} are in this context. A, B, and C are the variables that are being studied.

Several parameters were used to evaluate and compare the Model that was generated via the design process. The "correlation coefficient, adjusted correlation coefficient, predicted correlation, and adequate precision" were part of these criteria. Response surface analysis, which comprised creating "2D-contour and 3D-response surface plots," was necessary for extracting useful information from the model. The model forecasted the ideal analytical state by means of a numerical optimization technique. The "Analytical Design Space" was then used to find these optimal settings. A visual representation of this was created using a "overlay plot" that included a flag with all the pertinent parameters.

Table 7: BBD (variables and their levels used)

Factor	Levels used, actual (coded)	
	Low (-1)	High (+1)
Independent variables		
A: Solvent conc. in mobile phase (% v/v)	70 (-1)	72 (+1)
B: Flow rate (ml/min)	0.7 (-1)	0.9 (+1)
C: Wavelength (nm)	281	283
Dependent variables		
Y_1: Retention time	Minimize	
Y_2: Peak area	In the range	
Y_3: Tailing factor	Minimize	

Method validation[80,81]

The modified RP-HPLC procedure was validated according to the ICH Q2 (R1) standards. The study examined a number of parameters, including linearity range, system suitability, LOD and LOQ, precision (both intraday and inter-day), accuracy, robustness, and ruggedness. Additionally, the effectiveness of the optimized approach in evaluating the quality of NBV marked tablets was tested.

System Suitability and Specificity

Several responses, including Rt, PA, and Tf, were studied to determine the system's suitability. All of these measures were done three times. Additionally, a specificity study was conducted to examine the impact of the mobile phase on Nebivolol hydrochloride detection.

Linearity and Sensitivity analysis

The NBV solution with varying concentrations, ranging from 10 µg/mL to 50 µg/mL, was prepared by serially diluting portions of the stock solution. After injecting these solutions into the system, the peak area of the chromatograms that came out was calculated. In addition, the method's linearity was established by plotting the concentration versus peak area graph along the X axis and the Y axis, respectively. The linearity of the approach is indicated by the value of the regression coefficient (r^2). Formulas were used to determine additional LOD and LOQ.

$$LOD = \frac{3.3 \times Standard\ deviation}{Slope} \qquad LOQ = \frac{10 \times Standard\ deviation}{Slope}$$

Precision

By observing the closeness of results obtained by three separate injections of solutions of the same concentration, precision of the method was determined. The precision was assessed both inter-day (between different days) and intraday (within a single day). The results were used to calculate the percentage RSD value.

Accuracy study

The accuracy of the proposed method was determined using the recovery method. The following formula was used to determine the percentage recovery of the drug following spiking a 10 µg/mL solution at 80%, 100%, and 120% of its concentration level.

$$\%\ Recovery = \frac{Recovered\ Concentration}{Initial\ Concentration} \times\ 100$$

Robustness

Few deliberate changes were made in the optimized analytical parameters in order to assess the method's robustness. The changes were made in solvent system (MeOH: aq. acetic acid, 70:30 and 72:28 v/v), wavelength of scanning (281-283 nm) and flow rate (0.7-0.9 mL/min).

Ruggedness

Analysis of Nebivolol hydrochloride solution was carried out by two separate analysts using optimized method in order to determine the ruggedness of the method. Finding the % RSD of the results allowed to assess the method's robustness.

Assay of Marked tablets

Weighed and ground twenty Nebivolol hydrochloride tablets (Nebicia Hydrochloride-5 mg, manufactured by Daxia Healthcare) to obtain powder. The 10 mg of NBV powder sample was transferred to a 10 mL volumetric flask. In order to have the powder sample thoroughly dissolved, it was diluted with 2 mL of solvent solution and subjected to sonication for 15 minutes. Solvent was added till the volume reached the mark. The solution was filtered. The obtained solution was further serially diluted to get the final solution of 30 µg/mL. The sample was analysed and the tablets assay was calculated.

3.3 POLYMORPHISM STUDY

FORMATION OF MULTICOMPONENT POLYMORPHS OR COCRYSTALS

Selection of Cocrystal former [83,84]

Cocrystals are primarily formed through H-bonding interactions between drug and co-former. CAV having capacity to accept five H-atoms and donate three H-atoms. Similarly, NBV having capacity to donate and accept H-atom making it highly favourable for co-crystal formation. The chemical structures of the medicines and selected co-formers are as depicted in Figure 4.

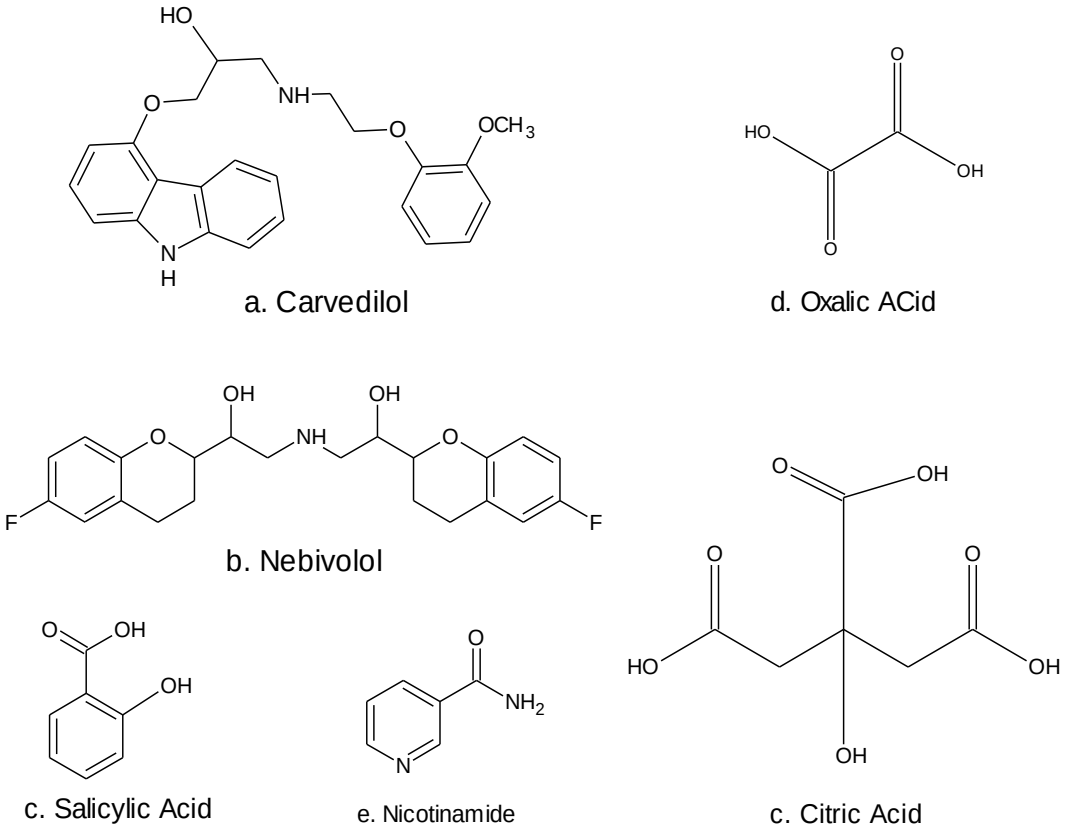


Figure 4: Chemical structure of drug and coformers

Ultrasound-Assisted Solution Co-crystallization (USSC)

Cocrystals were prepared using Ultrasound-assisted Co-crystallization technique (Sonics Vibro Cell, Model: Ultrasound Probe Sonicator VC750) at a controlled temperature of 15-20°C in a suitable solvent. The obtained cocrystals were subjected to DSC, FTIR, P-XRD, and SEM analysis.

Selection of co-former and its ratio

Four co-formers—nicotinamide, oxalic acid, salicylic acid, and citric acid—were chosen based on mechanochemistry; they are all good proton donors and acceptors and may create hydrogen bonds.

Preparation of Multicomponent Polymorphs (Carvedilol and Nebivolol)

Stoichiometric ratio (1:1, 1:2, 1:3) of carvedilol (CAV)/Nebivolol (NBV) and co-former were mixed in suitable solvent. In a glass sonoreactor vessel with a jacket, the resulting solution was pulsed with 6-12 ultrasonic waves using a high-intensity ultrasound probe. After ten seconds of each pulse, with 2 seconds of relaxation time. During sonication, a water bath was used to circulate ice-cold water through the glass sonoreactor jacket in order to maintain a temperature of 15-20°C. After filtering out the solids, the residue was rinsed with cold methanol and kept for drying overnight. The prepared co-crystals were collected, further dried, and stored in desiccators at room temperature for subsequent studies. [85]

Differential Scanning Calorimeter

To obtain DSC peak, Shimadzu DSC-60, Serial No-0004544, Shimadzu Corp. Japan-00642, thermal analyzer (TA-60) was used, with a flow controller (FC-60A). With a reference pan removed, the temperature was raised from 25 to 350 °C at a

continuous rate of 10 °C/min while a 2mg sample was placed in an aluminium pan and subjected to a flow of N2-gas at a rate of 20 mL/min. [86]

Various co-formers and their ratios are as shown in Table 8 and 9.

Table 8: Details of stoichiometric ratio of Carvedilol Phosphate (CAV) API and co-formers

API & Co-former	1:1	1:2	1:3	Solvent
CAV:Oxalic acid (W1,W2,W3)	1 gm:0.175 gm	1 gm:0.350 gm	1 gm:0.525 gm	25 mL
CAV:Salicylic acid (X1,X2,X3)	1 gm:0.268 gm	1 gm:0.536 gm	1 gm:0.804 gm	
CAV:Citric acid (Y1,Y2,Y3)	1 gm:0.370 gm	1 gm:0.740 gm	1 gm:1.110 gm	
CAV:Nicotinamide (Z1,Z2,Z3)	1 gm:0.230 gm	1 gm:0.460 gm	1 gm:0.690 gm	

Table 9: Details of stoichiometric ratio of Nebivolol hydrochloride (NBV) API and co-formers

API & Co-former	1:1	1:2	1:3	Solvent
NBV: Oxalic acid (A1,A2,A3)	0.5 gm:0.100 gm	0.5 gm:0.200 gm	0.5 gm:0.300 gm	25 mL
NBV:Salicylic acid (B1,B2,B3)	0.5 gm:0.156 gm	0.5 gm:0.312 gm	0.5 gm:0.468 gm	
NBV:Citric acid (C1,C2,C3)	0.5 gm:0.217 gm	0.5 gm:0.434 gm	0.5 gm:0.651 gm	
NBV:Nicotinamide (D1,D2,D3)	0.5 gm:0.135 gm	0.5 gm:0.270 gm	0.5 gm:0.405 gm	

Fourier Transform Infra-Red Spectrophotometer

The infrared spectrum of API and its Co-crystals was recorded on Nicolet impact 410 FT-IR using KBr pellets. (Shimadzu IR Affinity-1 using DRS 8000). Data were collected from software. [86-87]

Powder X-ray Diffraction

P-XRD diffractogram were obtained using- Rigaku SmartLab SE diffractometer (Rigaku, Tokyo, Japan), source- Cu_K_β_1D, Geometry-parallel beam geometries, Detector used- one-dimensional semiconductor array (Rigaku D/tex 250(H), Rigaku, Tokyo, Japan), Reflection mode- 2Θ= 5–90°at 25°C during measurements, by a step of 0.01°, scan speed of 10°min^{-1} (Cu-K_βsource, 40 kV, 30 mA). [88-89]

Scanning Electron Microscopy

SEM (JEOL JSM-6360LV, Japan) was used to examine the surface morphology of both the API and its co-crystal. Sputtered with gold before scanning, the samples were subjected to an acceleration voltage of 10 kilovolts.[90]

4. RESULTS AND DISCUSSION

4.1 DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR CARVEDILOL PHOSPHATE IN BULK AND FORMULATION

Preliminary Studies for method development

The goal of this research was to create an RP-HPLC method for the quantification of Carvedilol Phosphate that will be easy to use, highly accurate, and very stable. To get a strong, symmetrical peak free of tailing, the process was fine-tuned by adjusting parameters such mobile phase composition, organic modifier concentration, and column temperature.

Various mobile phase compositions, including Acetonitrile (ACN), Methanol (MeOH), Formic acid (FA), and Acetic acid, were used at various flow rates, injection volumes, concentrations, etc., according to the literature review.

Varying concentrations of methanol and water were used to conduct preliminary experiments. The results showed unacceptable resolution, with a tailing factor greater than 2 (Figure 5). To address this, methanol was replaced with acetonitrile for further investigation. However, using different ratios of acetonitrile and water produced broad, poorly resolved peaks with tailing (Figure 6). A well-defined, sharp peak of Carvedilol Phosphate was formed and the tailing factor decreased from 2.0 to 1.363 when methanol and a 0.1% aqueous solution of formic acid were used as the mobile phase (Figure 7). When ACN and 0.1% aqueous solution of formic acid was tried as mobile phase, the CDL peak eluted very near to the negative peak. Further decrease in ACN concentration to separate the CDL peak from negative peak led to increased retention time. Hence further trails were done with MeOH and formic acid. Further increase in the concentration of formic acid up

to 0.5% v/v reduced the tailing factor from 1.363 to 1.20 with improved peak shape. (Figure 9). It was noted that the tailing factor is significantly affected by column temperature. The tailing factor went down from 1.281 to 1.222 as the temperature decreased from 40°C to 25°C (Figure 10). A strong peak was achieved and the tailing factor was reduced to 1.20 when 0.1% OPA was used in place of formic acid (Figure 10C). In an experiment using a mobile phase consisting of ACN and 0.1% OPA, the tailing factor was more than 1.3 (Figure 10 D-E). Based on the observations the optimized conditions were as follows: Shim-pack GIST C18 column, a solution of 0.1% OPA in water and MeOH (35:65 v/v), a flow rate of 1.0 ml/min, a column temperature of 30°C, an injection volume of 10µl for each sample, and a detection wavelength of 240nm.

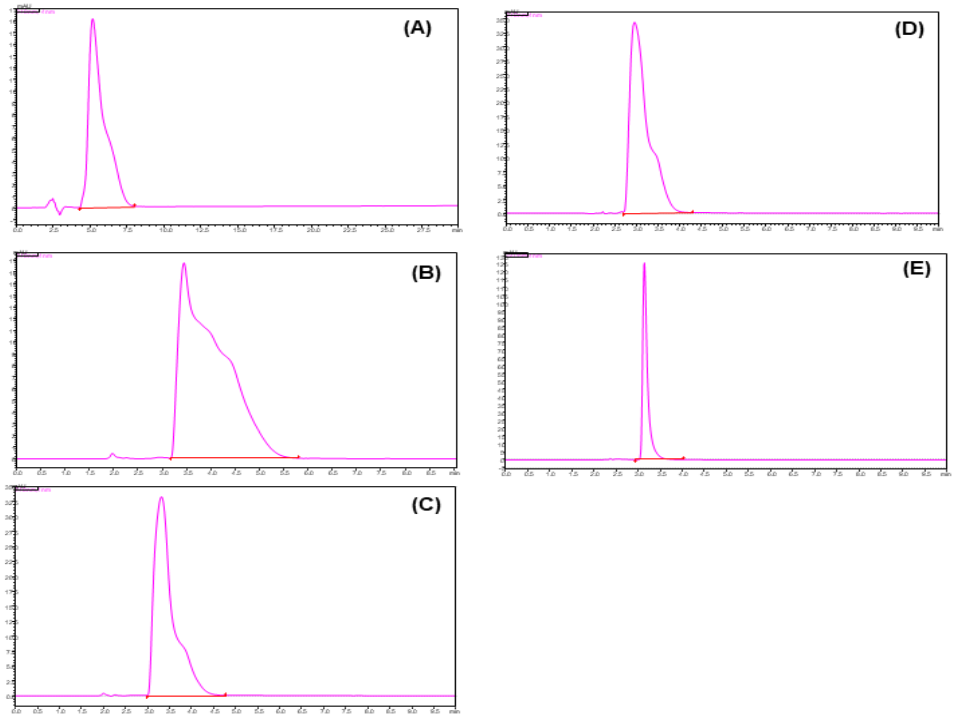


Figure 5: Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (A) MeOH:Water 50:50 v/v (B) MeOH:Water 60:40 v/v (C) MeOH:Water 70:30 v/v (D) MeOH:Water 80:20 v/v (E) MeOH:Water 90:10 v/v at 1mL/min flow rate, 40°C and 10µl sample injection volume

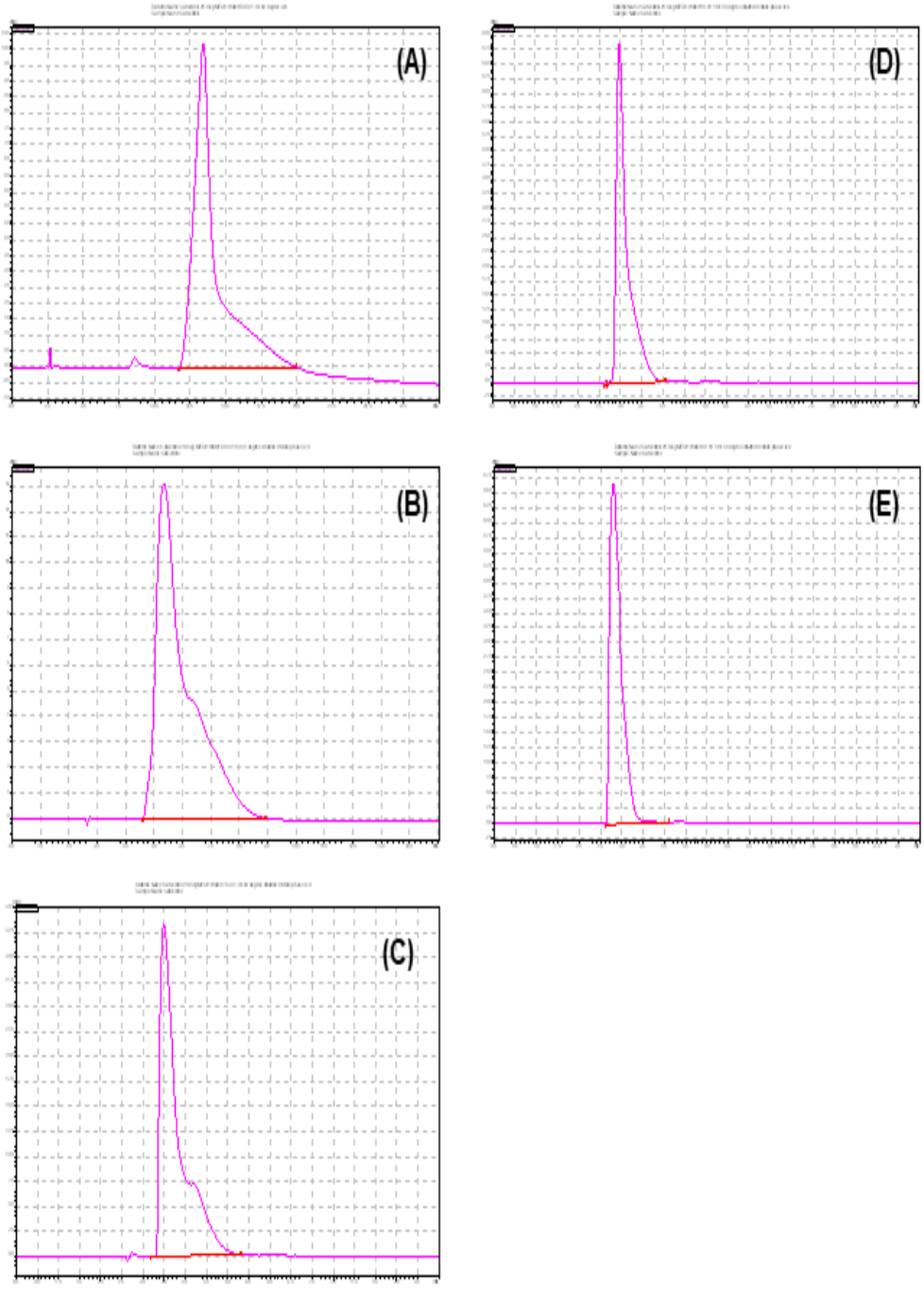


Figure 6: Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (A) ACN:Water 50:50 v/v (B) ACN:Water 60:40 v/v (C) ACN:Water 70:30 v/v (D) ACN:Water 80:20 v/v (E) ACN:Water 90:10 v/v at 1 mL/min flow rate, 40°C and 10 µL sample injection volume

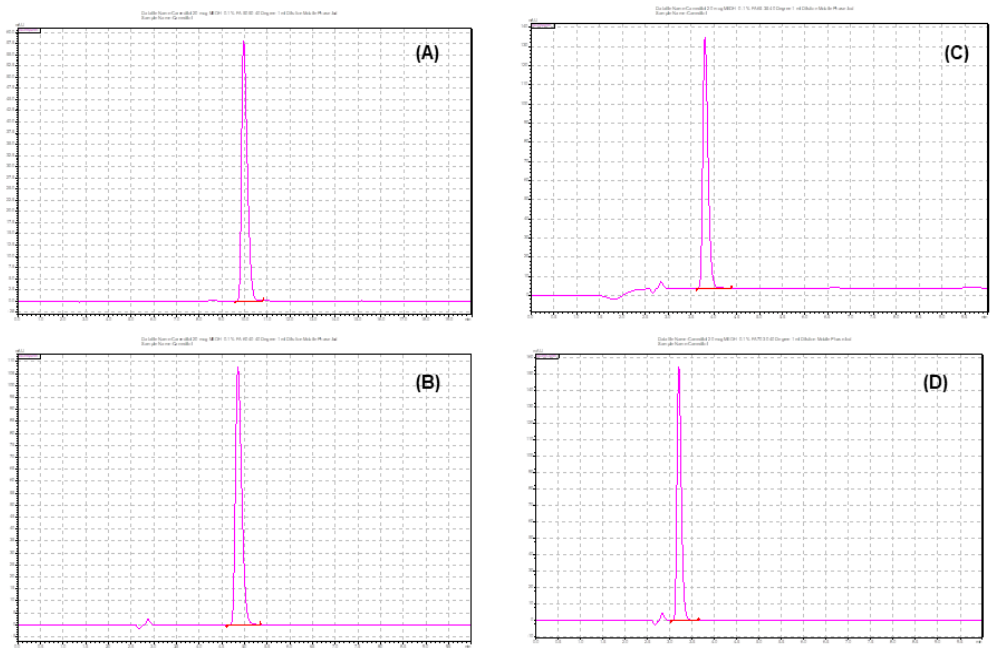


Figure 7: Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (A) MeOH:0.1%FA 50:50 v/v (B)MeOH:0.1%FA 60:40 v/v (C) MeOH:0.1%FA 65:35 v/v (D) MeOH:0.1%FA 70:30 v/v at 1 mL/min flow rate, 40°C and 10 µL sample injection volume

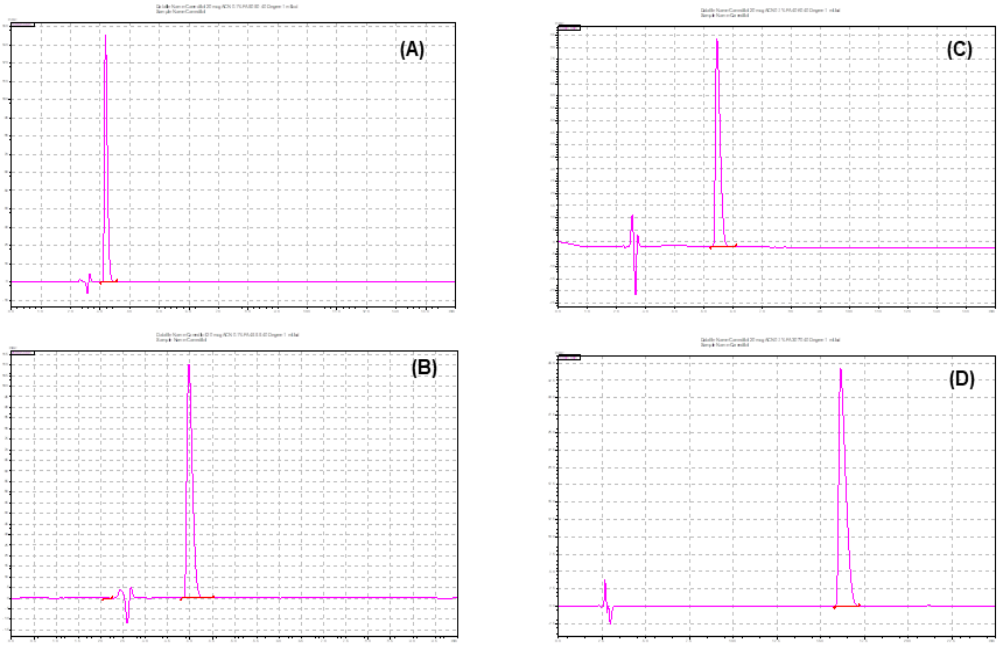


Figure 8: Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (A) ACN:0.1%FA 50:50 v/v (B) ACN:0.1%FA 45:65 v/v (C) ACN:0.1%FA 40:60 v/v (D) ACN:0.1%FA 30:70 v/v at 1 mL/min flow rate, 40°C and 10 µL sample injection volume

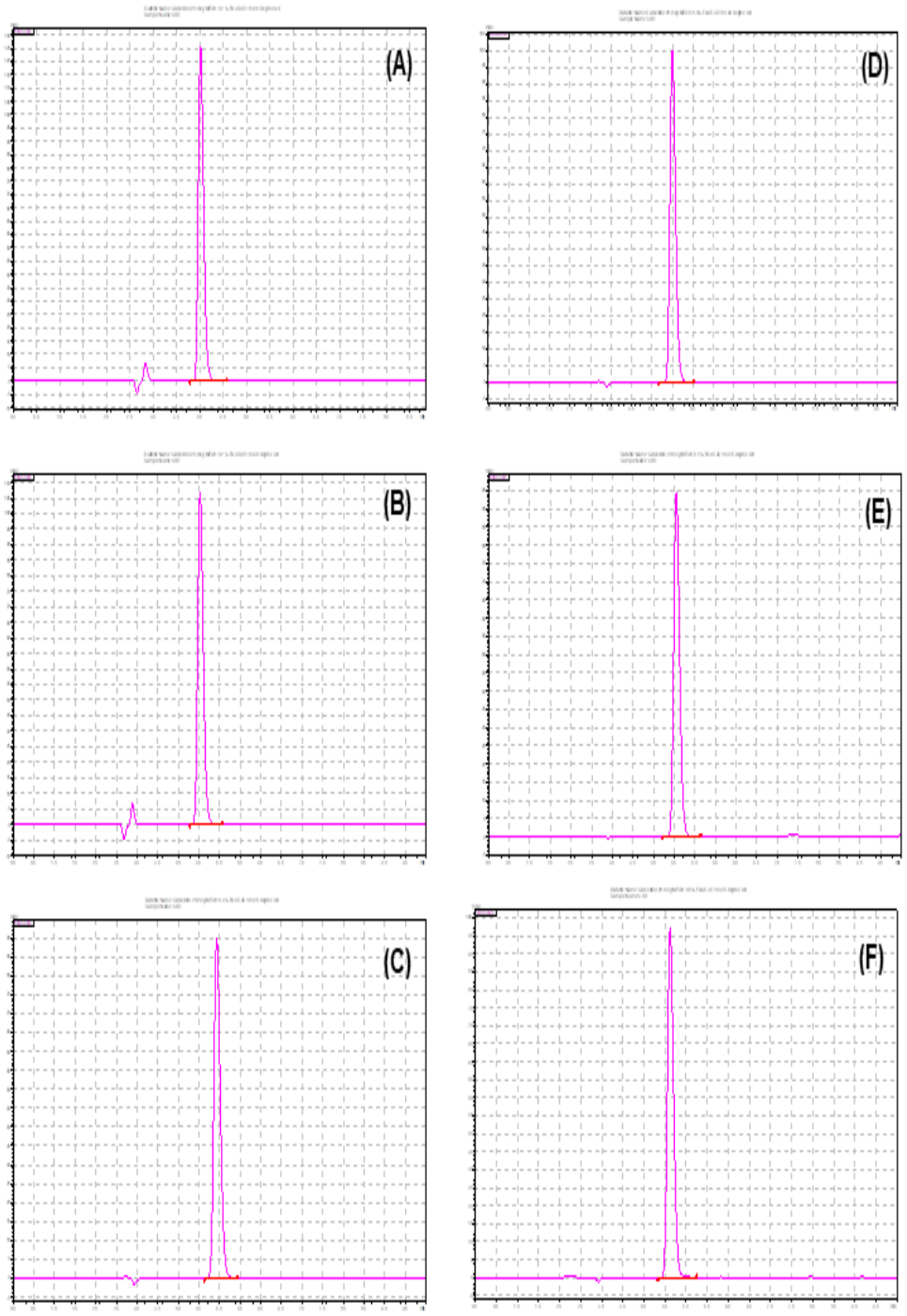


Figure 9: Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (A) MeOH:0.2%OPA 65:35 v/v at 40°C (B) MeOH:0.2%OPA 65:35 v/v at 30°C (C) MeOH:0.2%OPA 65:35 v/v at 20°C (D) MeOH:0.3%OPA 65:35 v/v at 30°C (E) MeOH:0.4%OPA 65:35 v/v at 25°C and (F) MeOH:0.5%OPA 65:35 v/v at 25°C at 1 mL/min flow rate, and 10 µL sample injection volume

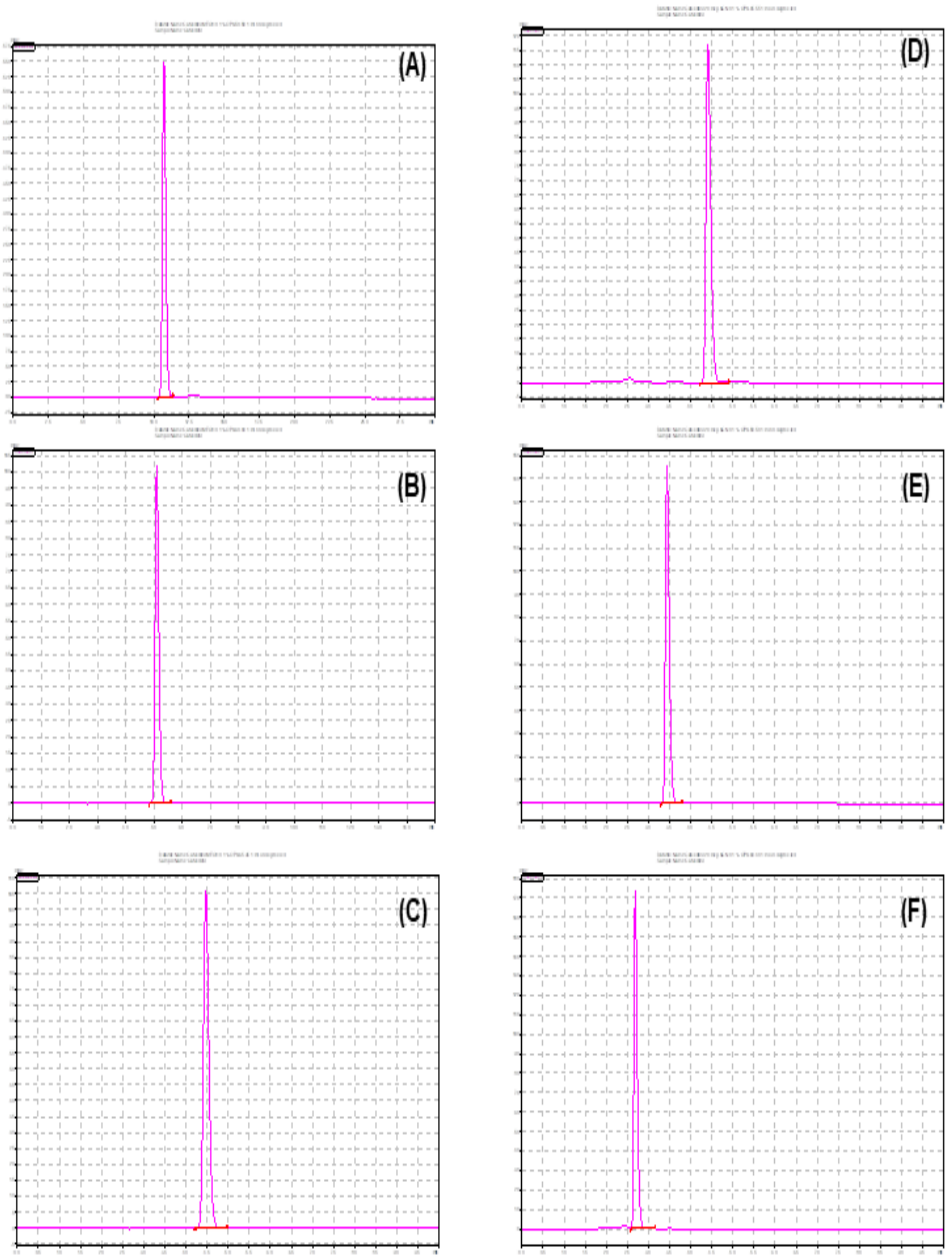


Figure 10: Preliminary trials to optimize chromatographic conditions for Carvedilol Phosphate method development (A) MeOH:0.1%OPA 50:50 v/v at 40°C (B) MeOH:0.1%OPA 60:40 v/v at 40°C (C) MeOH:0. 1%OPA 65:35 v/v at 30°C (D) ACN:0.1%OPA 45:55 v/v at 30°C (E) ACN:0.1%OPA 50:50 v/v at 40°C and (F) ACN:0.1%OPA 60:40 v/v at 30°C at 1 mL/min flow rate, and 10 µL sample injection volume

Risk assessment and Screening Studies [47]

The relationship between CMVs and CAAs can be better understood with the use of risk assessment. Research on potential dangers led to the selection of seven variables, which underwent additional testing using the Taguchi method. Using only a small number of trials, the screening study helped to identify the variables that have a major impact on the experimental outcomes. In the current study, half normal plots and pareto charts were utilized to ascertain how these factors affected CAA (Figure 11). The results were primarily influenced by two factors: the injection volume (X_2) and the methanol concentration (X_1).

Factor optimization using 3^2 BBD

Table 10 shows the results of thirteen experimental runs that were based on suggestions from the Design expert software. According to the results produced by the Design expert software, the best fit model for the dependent and independent variables was a quadratic model. Additionally, the model relevance was confirmed by an F-test result with a p-value less than 0.05. Table 8 shows the summary of the four responses. For each response, the R^2 value was ranged between 0.9972 and 1. Response data also fit well to the polynomials, according to the data. The predicted and adjusted R^2 values were quite near to each other, confirming the best fit even further (Difference <0.2). Equation 1 is the quadratic equation that was obtained, with the intercept being b0 and the coefficients of the interaction terms ranging from b1 to b22 (Table 11). Figure 12 depicts 2D contour plots and 3D response surface diagrams.

Table 10: 3^2 Box-Behnken design along with the observed values for optimization of RP-HPLC method for Carvedilol Phosphate

Run No	Independent Variables		Dependent variables			
	X_1	X_2	Y_1	Y_2	Y_3	Y_4
1	65.00	10.00	4.221	406576	1.191	4441
2	60.00	15.00	5.595	605931	1.172	5000
3	65.00	15.00	4.228	610665	1.206	4394
4	55.00	20.00	8.289	801457	1.18	5632
5	60.00	15.00	5.597	604773	1.173	4991
6	60.00	15.00	5.6	605588	1.174	4980
7	60.00	15.00	5.607	605613	1.175	4943
8	60.00	15.00	5.602	605547	1.174	4929
9	60.00	10.00	5.599	405319	1.115	5129
10	60.00	20.00	5.593	808590	1.192	4878
11	55.00	15.00	8.297	602148	1.16	5786
12	65.00	20.00	4.225	814187	1.217	4329
13	55.00	10.00	8.316	404127	1.138	5963

$X_{1:}$ Solvent concentration in mobile phase (% v/v); $X_{2:}$ Injection volume; $Y_{1:}$ Retention time; $Y_{2:}$ Peak area, Y_3 Tailing factor; $Y_{4:}$ Number of theoretical plates

Table 11: Regression analysis for the best fitting of different polynomial models and their responses (Y_1-Retention time, Y_2-Peak area, Y_3-Tailing factor, Y_4-Number of theoretical plates)

Models	SD	R^2	Adjusted R^2	Predicted R^2	PRESS	CV(%)	Remark
Response (Y_1)							
Linear	0.38	0.9460	0.9352	0.8938	2.80	6.39	
2FI	0.40	0.9460	0.9280	0.7816	5.75	6.73	
Quadratic	0.004	1.00	1.00	1.00	0.0006967	0.078	Suggested
Response (Y_2)							
Linear	1788.88	0.9999	0.9998	0.9997	84760000	0.30	
2FI	787.13	1.0000	1.0000	0.9999	13320000	0.13	
Quadratic	582.32	1.0000	1.0000	0.9999	13920000	0.09	Suggested
Response (Y_3)							
Linear	0.014	0.7750	0.7300	0.5447	0.003870	1.18	Suggested
2FI	0.014	0.7825	0.7100	0.2884	0.006048	1.22	
Quadratic	0.011	0.8988	0.8264	0.0690	0.007912	0.94	
Response (Y_4)							
Linear	76.63	0.9811	0.9773	0.9614	119900	1.52	
2FI	72.06	0.9849	0.9799	0.9446	172000	1.43	
Quadratic	26.87	0.9984	0.9972	0.9951	15310.77	0.53	Suggested

Table 12: Results of Analysis of Variance (ANOVA) and adequate precision for the various responses

Responses	Source	Mode	X_1	X_2	X_1X_2	X_1^2	X_2^2
Y_1 (Retention time)	F-value	2.50	1.184	6.66	11.42	57950.23	0.55
	p-value Prob > F	< 0.0001	< 0.0001	0.0364	0.0118	< 0.0001	0.4815
	Adequacy precision	1312.927					
Y2 (Peak area)	F-value	1.436	275.98	7.175	779.3	0.72	5.81
	p-value Prob > F	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.4257	0.0468
	Adequacy precision	1037.993					
Y3 (Tailing factor)	F-value	17.22	16.22	1814.10032			
	p-value Prob > F	0.0006	0.0025	0.0016			
	Adequacy precision	14.100					
Y4 (Number of theoretical plates)	F-value	858.41	4106.45	111.22	16.61	42.31	1.33
	p-value Prob > F	< 0.0001	< 0.0001	< 0.0001	0.0047	0.0003	0.2864
	Adequacy precision		89.691				

(X_1 and X_2 coded level-independent variables; X_1X_2-interaction terms; X_1^2 and X_2^2 and X_2^2-quadratic terms)

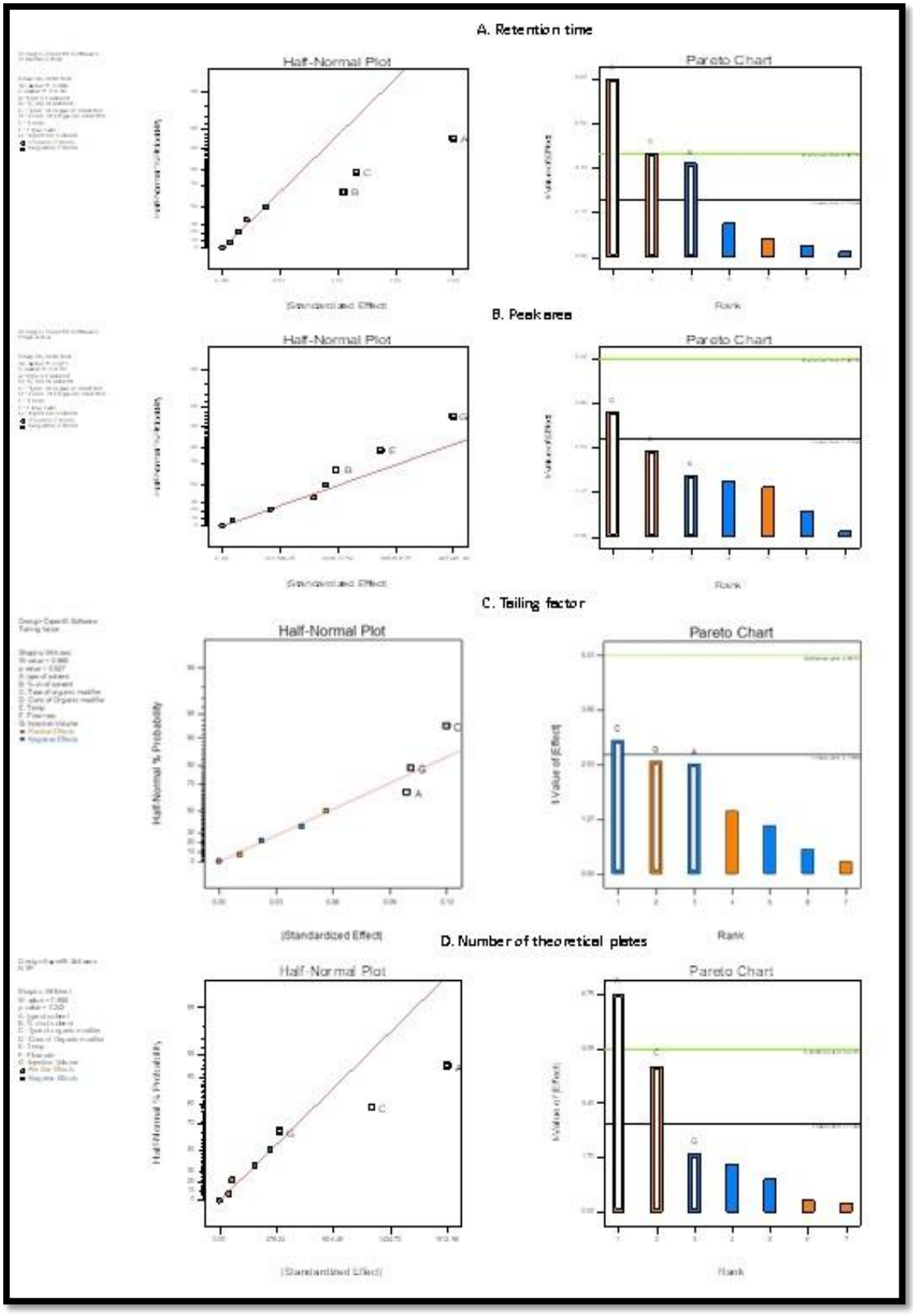


Figure 11: A-D Half-normal plots and Pareto charts indicating the factor effects of the responses, i.e., retention time, peak area, tailing factor and number of theoretical plates.

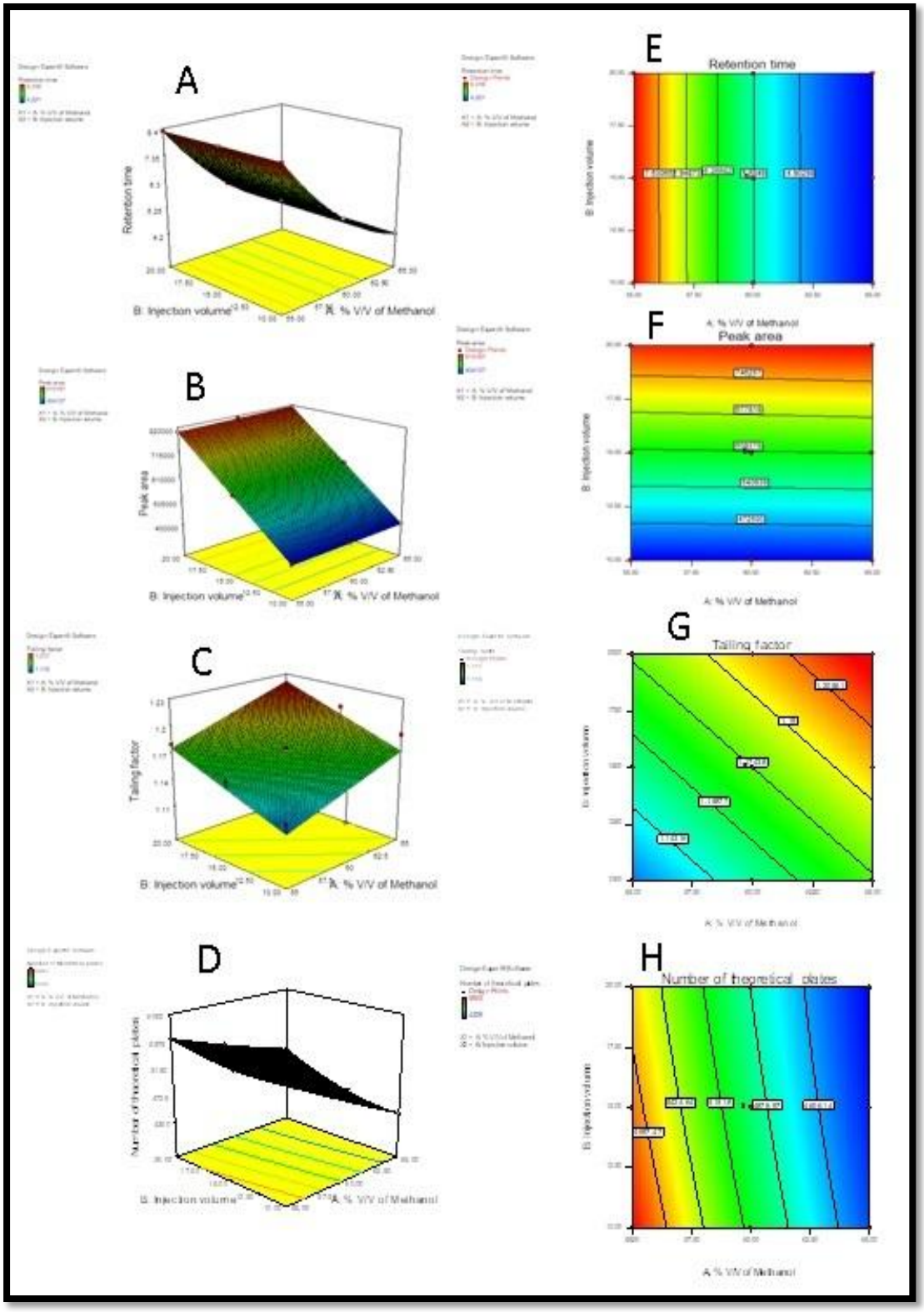


Figure 12: 3D-Response surface (A-D) and 2D-contour (E-H) plots showing the effect of %V/V of Methanol in mobile phase and injection volume on retention time, peak area, tailing factor and number of theoretical plates. X_1= A: Methanol in the mobile phase and X_2= B: Injection volume

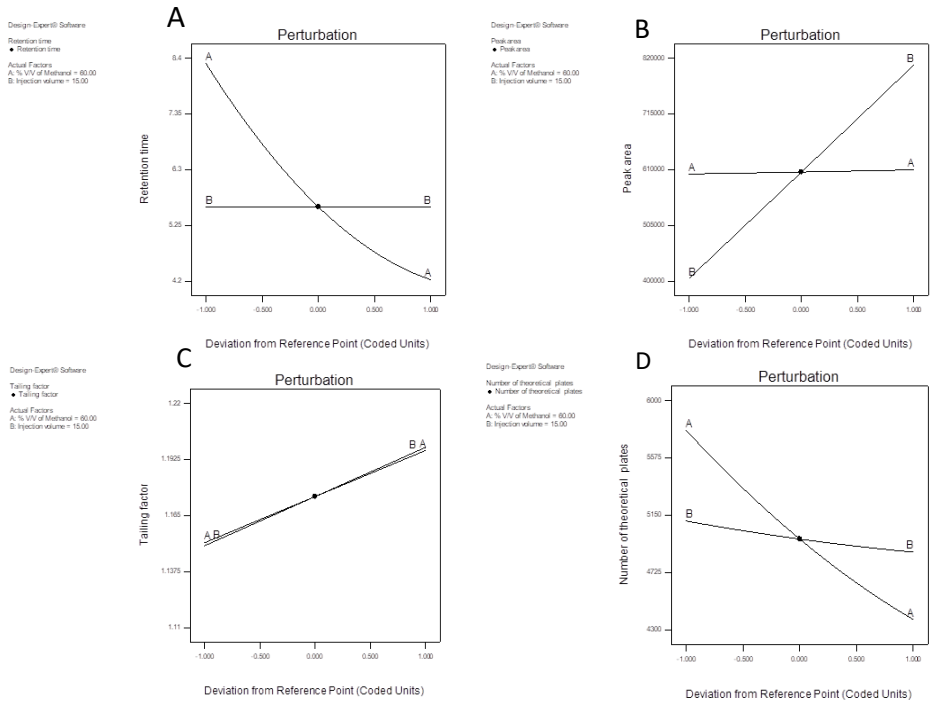


Figure 13: Perturbation plots (A-D) showing the effect of %V/V of Methanol in mobile phase and injection volume on retention time, peak area, tailing factor and number of theoretical plates. A: Methanol in the mobile phase and B: Injection volume

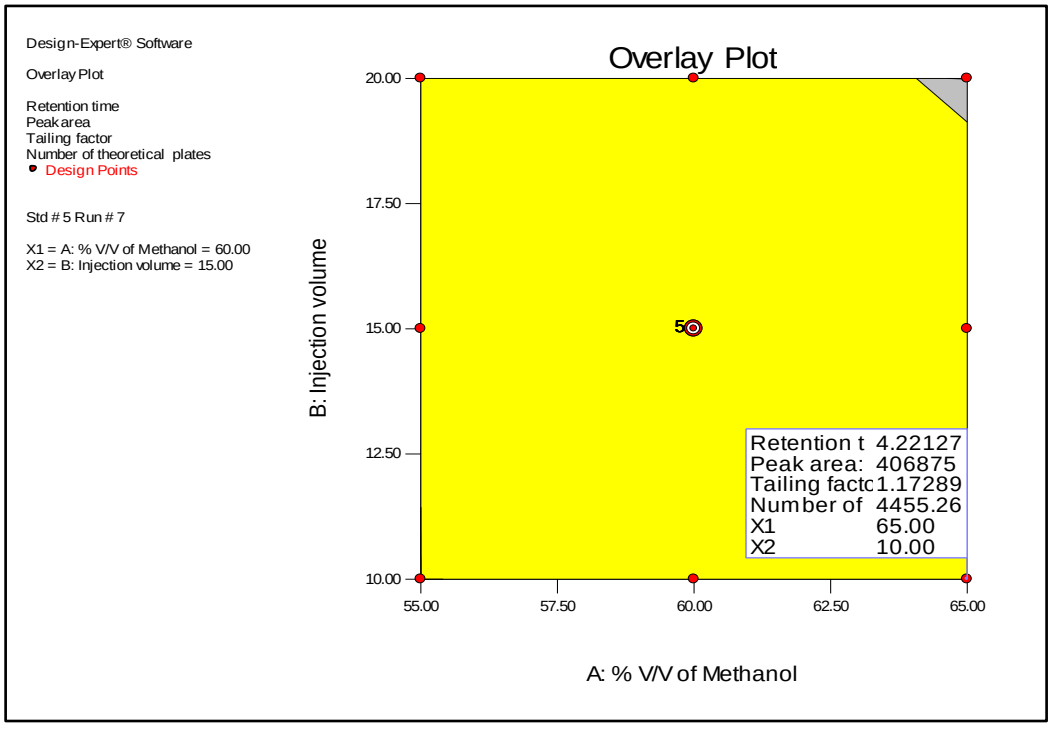


Figure 14: The overlay plot showing the optimized chromatographic conditions and their predicted responses.

Effect of independent variables on Retention time (Y_1):

The effect of the independent variables on retention time is shown below via the quadratic polynomial equation.

Retention time $(Y_1) = +5.60 - 2.04X_1 - 0.004833X_2 + 0.007750X_1X_2 + 0.66 X_1^2 - 0.002052X_2^2$

Both the amount of injection and the percentage of methanol in the mobile phase have a negative impact on retention time, as shown in the equation. When comparing the effects of injection volume and percentage v/v methanol on retention time (Rt), the larger value of the coefficient of X1 indicates a more significant relationship. The Rt is positively affected by the combined interaction factors of injection volume (X_1X_2) and percentage v/v of methanol. Analyzed retention time data using ANOVA; findings are shown in Table 12. With a significance level of 250401.42 for Model F, the possibility of noise is impacting the results is just 0.01%. The method accuracy is defined by the signal-to-noise ratio. Typically, a ratio greater than 4 is deemed suitable. A quadratic model may be employed to examine the outcomes in design space, as the ratio was determined to be 1312.927. Figure 12A, Figure 12E, and Figure 13A show the 3D-response surface plot, 2D-contour plot, and perturbation plot, respectively, that illustrate the relationship between retention time and the independent variables that were evaluated.

Effect of independent variables on peak area (Y_2):

The following quadratic polynomial equation shows how the independent factors affect peak area:

Peak area(Y_2) =+605700 + 3949.33 X_1+ 201400X_2+ 2570.25 X_1X_2+ 296.31 X_1^2+844.X_2^2

The data shows that the peak area is positively affected by both the injection volume and the percentage of v/v methanol in the mobile phase. The significant impact of injection volume on peak area is further demonstrated by the larger value of the coefficient of X2 when compared to the influence of % v/v methanol. The combined interaction terms % v/v of methanol, injection volume (X_1X_2), have positive effect on the peak area. Table 12 shows the results of ANOVA for peak area data. The value of 143571.34 for Model F represents its significance. Such a high value could have only 0.01% chance that noise is affecting the results. The signal to noise ratio was found to be 1037.993 which indicate that quadratic model can be suitable to represent the results in design space In Figure 12B, 12F, and 13B, respectively, 3D-response surface plots, 2D-contour plots, and perturbation plots were shown which illustrate the relationship between peak area and the independent variables that were explored.

Effect of independent variables on tailing factor (Y_3):

The linear equation that shows the effect of the independent variables on the tailing factor, is as shown below,

Tailing factor (Y_3) = +1.17 + 0.023 X_1 + 0.024 X_2

It can be seen from the equation that the tailing factor is positively affected by both the amount of injection and the percentage of methanol in the mobile phase. The coefficient values for X_1 and X_2 are similar, indicating that both factors have comparable influence on the tailing factor. The absence of interaction and squared terms in the model suggests that the independent variables did not significantly interact. An analysis of variance (ANOVA) with tailing factor data is presented in Table 12. Model F is statistically significant with a value of 17.22. With such a high value indicates only 0.06% chances of noise is impacting the results. A linear model may be appropriate for representing the data in design space, since the signal-to-noise ratio was determined to be 14.1. Figure 12C shows a 3D-response surface plot, Figure 13C shows a perturbation plot, and Figure 12G shows a 2D-contour plot, all of which illustrate the relationship between the tailing factor and the independent variables that were analyzed.

Effect of independent variables on number of theoretical plates (Y_4):

The quadratic equation that shows the effect of the independent variables on the number of theoretical plates is as shown below,

Number of theoretical plates (Y_4) = + 4973.24 - 702.83 X_1 -115.67 X_2 + 54.75 X_1 X_2+ 105.16X_1^2+ 18.66 X_2^2

The equation represents that both % v/v of methanol in mobile phase and injection volume have negative effect on the NTP. Further greater value of coefficient of X_1 witnesses the pronounced effect of % v/v of methanol in mobile phase on Y_4 in comparison to that of injection volume. The combined interaction terms % v/v of methanol, injection volume (X_1X_2), have positive effect on the Y_4. Table 12 shows the results of ANOVA for number of theoretical plates data. The value of 858.41 for Model F represents its significance. Such a high value could have only 0.01% chance that noise is affecting the results. The signal-to-noise ratio was found to be 89.691, indicating that the quadratic model is appropriate for representing the results in design space. The link between peak area and the analysed independent variables shown in 3D-response surface plots (Figure 12D), perturbation plots (Figure 13H), and 2D-contour plots (Figure 12H).

Analytical Method Validation

Validation of all analytical methods is a prerequisite for their application in routine qualitative or quantitative analysis.

System Suitability and specificity

When six replicates of Carvedilol phosphate (CAV) with a concentration of 15µg/mL were tested, the results of the system suitability test witnessed that the PA, Rt, NTP, and Tf values were not significantly different. In order to test the method's specificity, blank and spiked quality control samples were compared, wherein no interference was observed in either. The method's appropriateness and specificity for quantifying Carvedilol Phosphate in the formulation was confirmed by doing this exercise. The results shown in Table 13 and Figure 15.

Table 13: System suitability test parameters (n=6) at Concentration 15 µg/mL

Parameter	Retention time (Rt, min)	Peak area	Theoretical plates (N)	Tailing factor (T)
CAV	4.18	402732	4436	1.19
SD	0.007	380.81	35.51	0.0
%RSD	0.16	0.09	0.42	0.0

n = number of replicates; SD = Standard Deviation RSD = relative standard deviation

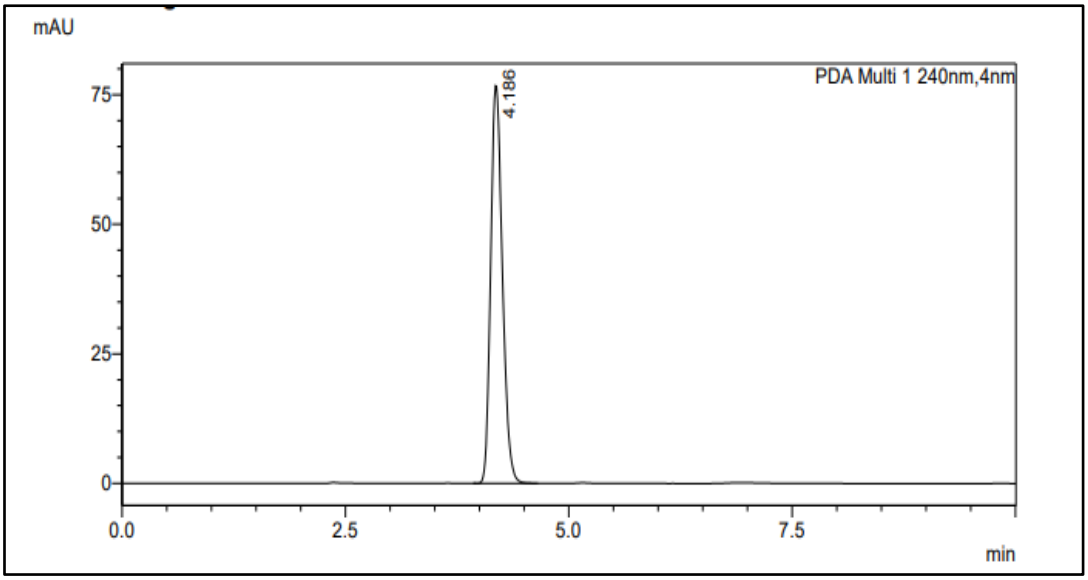


Figure 15. Chromatogram of Carvedilol phosphate in system suitability test

Linearity

The suggested analytical method's linearity was established using ICH recommendations. The concentration range of 5 to 30 µg/mL was used to check the linearity. The results witessed the linearity of the method with the regression coefficient value of 0.999. The resultant linear line has the equation y=53574x-57581. Figures 16 and 17a-f show the resulting calibration curve.

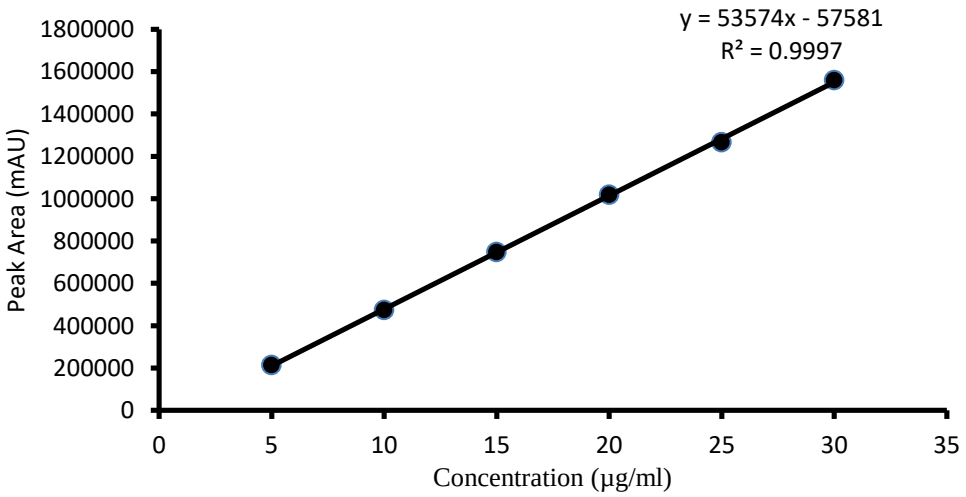


Figure 16. Calibration curve of Carvedilol Phosphate

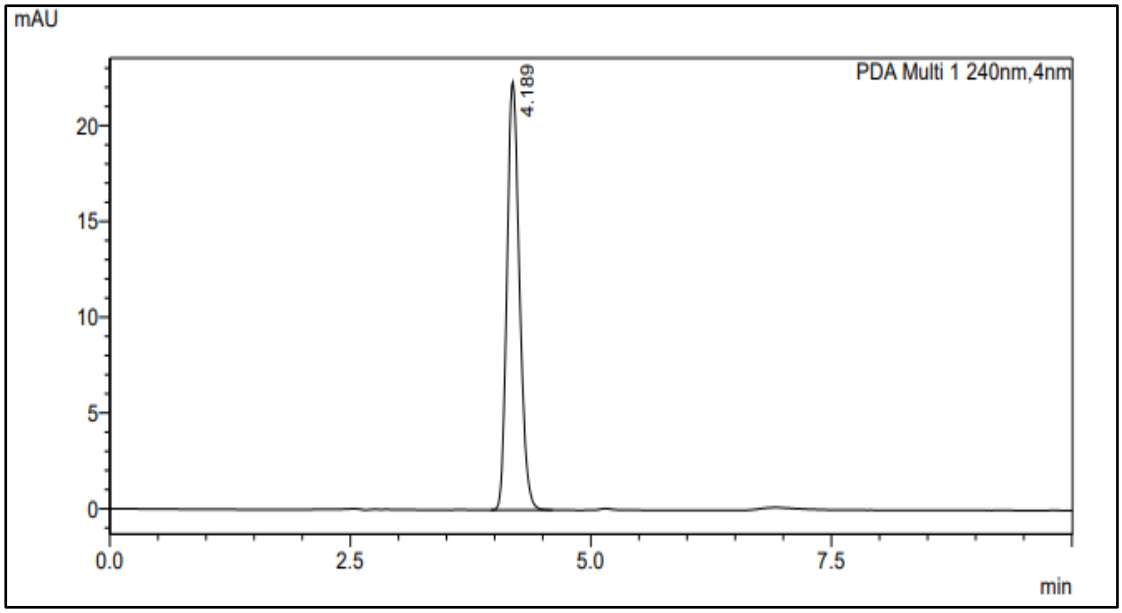


Figure 17a. Chromatogram of Carvedilol Phosphate (5 µg/mL)

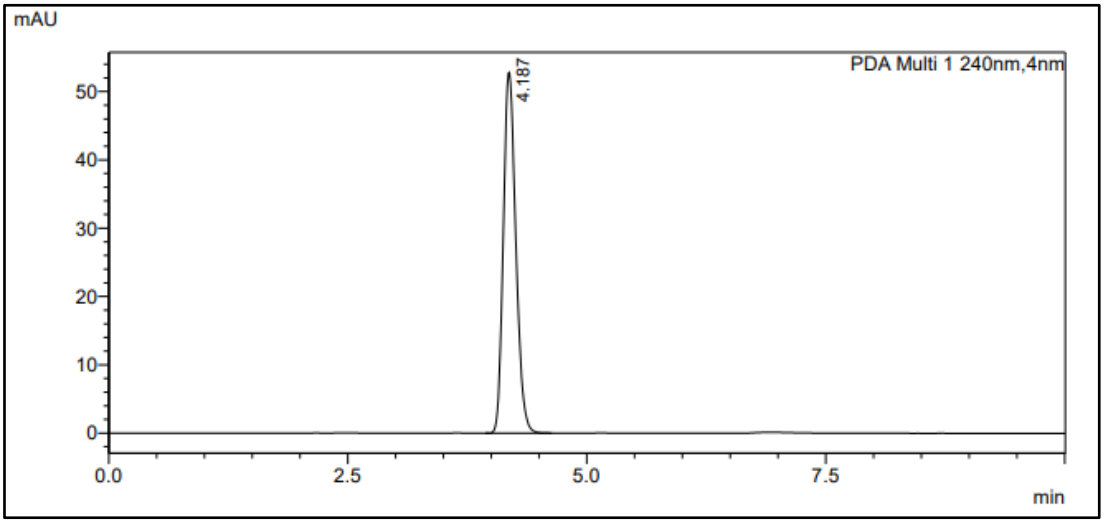


Figure 17b. Chromatogram of Carvedilol Phosphate (10 µg/mL)

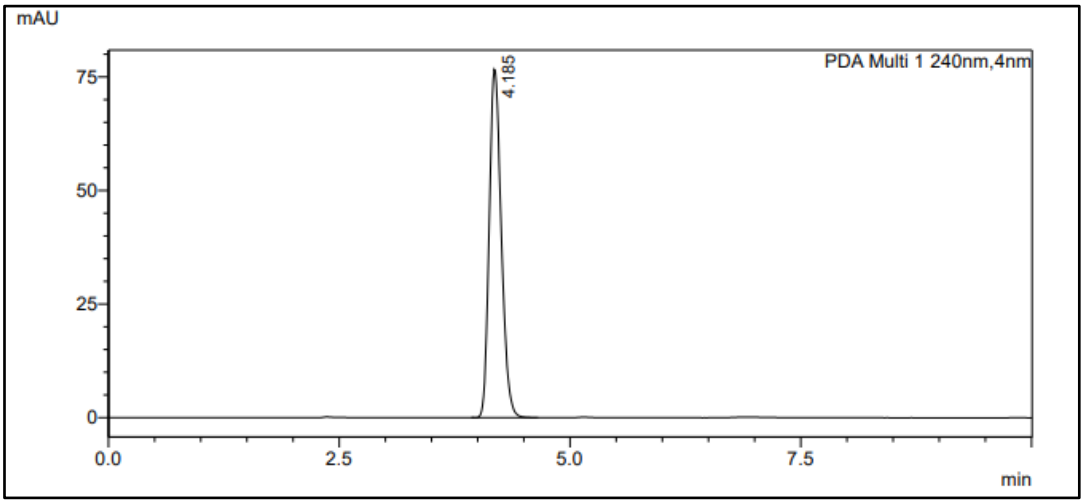


Figure 17c. Chromatogram of Carvedilol Phosphate (15 µg/mL)

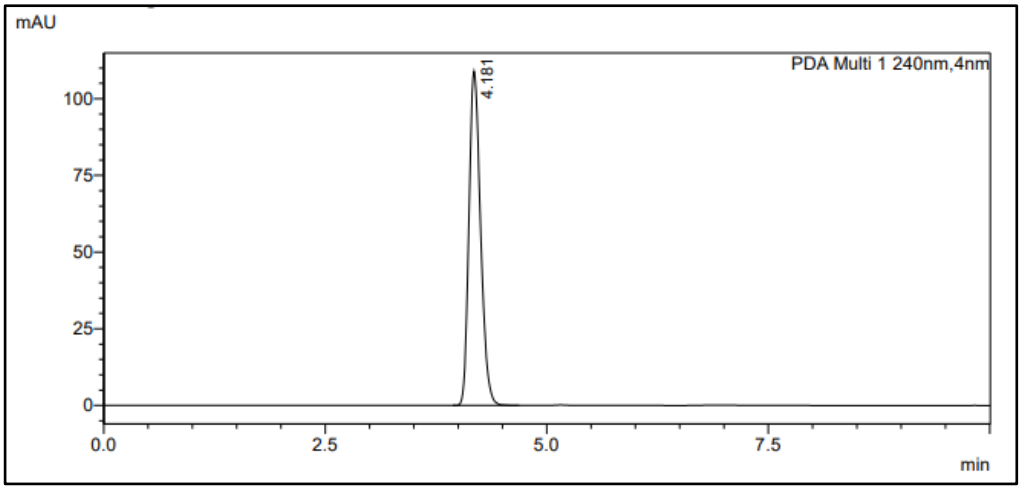


Figure 17d. Chromatogram of Carvedilol Phosphate (20 µg/mL)

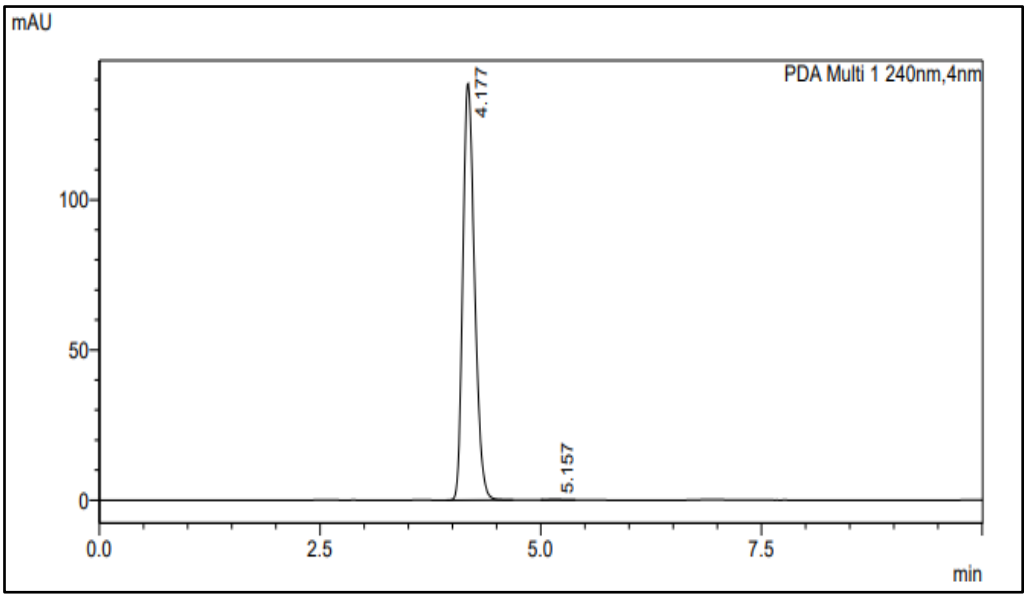


Figure17e. Chromatogram of Carvedilol Phosphate (25 µg/mL)

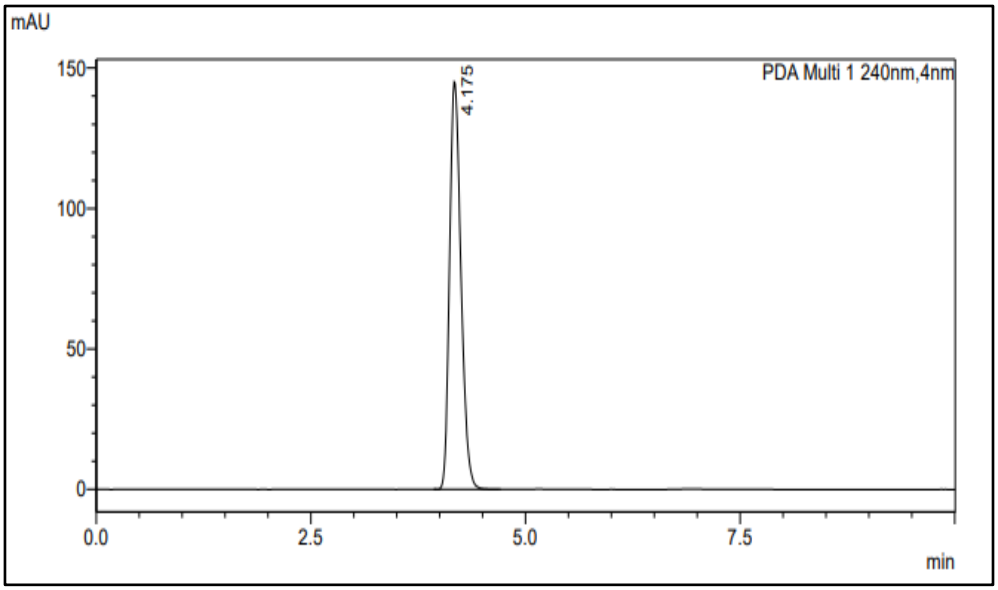


Figure 17f. Chromatogram of Carvedilol Phosphate (30 µg/mL)

LOD and LOQ

LOD and LOQ represents sensitivity of the method. LOD and LOQ values of developed method were found to be 0.61µg/ml and 1.86µg/ml, respectively. These findings show the method is highly sensitive.

Precision

The precision study findings showed that the peak area % RSD value lies within 1% during intra and inter days. The findings from the precision investigation are depicted in Table 14 and Figure 18.

Table 14: Intra-day and inter-day precision of Carvedilol Phosphate by RP-HPLC method

Precision Parameter	Concentration (µg/mL)	Amt. Found (µg/mL)	SD	%RSD
Intraday (n=6)	5.00	4.83	0.007	0.14
	15.00	14.09	0.009	0.07
	25.00	23.59	0.030	0.13
Interday-1 (n=3)	5.00	4.84	0.006	0.13
	15.00	14.10	0.008	0.06
	25.00	23.58	0.002	0.01
Interday-2 (n=3)	5.00	5.72	0.001	0.03
	15.00	15.93	0.005	0.03
	25.00	25.54	0.005	0.019
Interday-3 (n=3)	5.00	5.22	0.03	0.66
	15.00	14.78	0.01	0.09
	25.00	25.43	0.04	0.16

n= number of replicates; SD= Standard Deviation; RSD= relative standard deviation

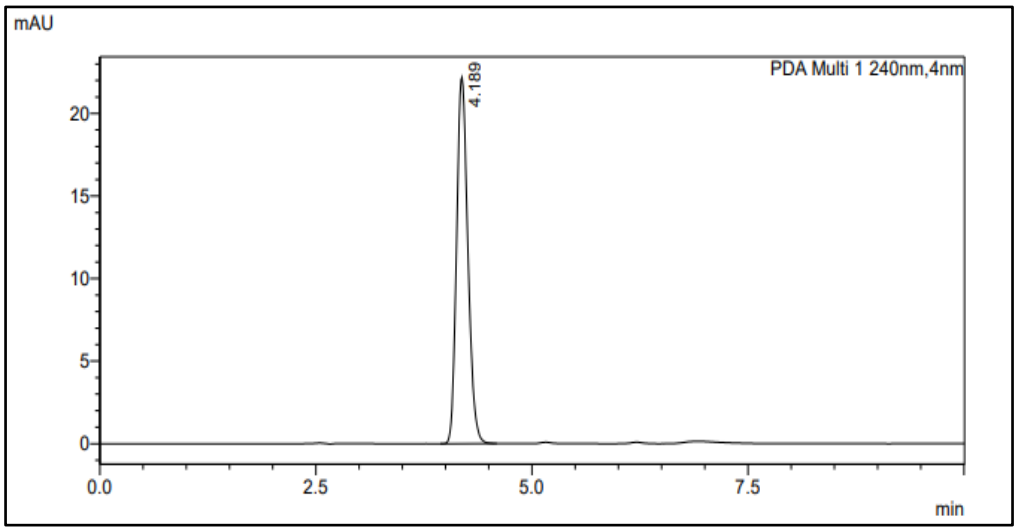


Figure 18: Precision Chromatogram of Carvedilol Phosphate (15 µg/mL)

Accuracy

The accuracy of the method is an indication of its ability to maintain conformity between experimental and theoretical responses. The results of accuracy study are shown in Table 15 and Figure 19a-c. The recovered concentration of CAV was found in the range between 99.41-99.82% with % RSD value of <2% with witnesses the accuracy of the method.

Table 15: Recovery studies of Carvedilol Phosphate by RP-HPLC (n = 3)

Level	Contents (mg)	Quantity added (mg)	Theoretical amount (mg)	Recovered amount (mg)	Recovery (%)	% RSD
50%	10	5	15	14.89	99.29	0.01
100%	10	10	20	19.80	99.02	0.07
150%	10	15	25	24.26	97.06	0.02

n = number of replicates; RSD = relative standard deviation

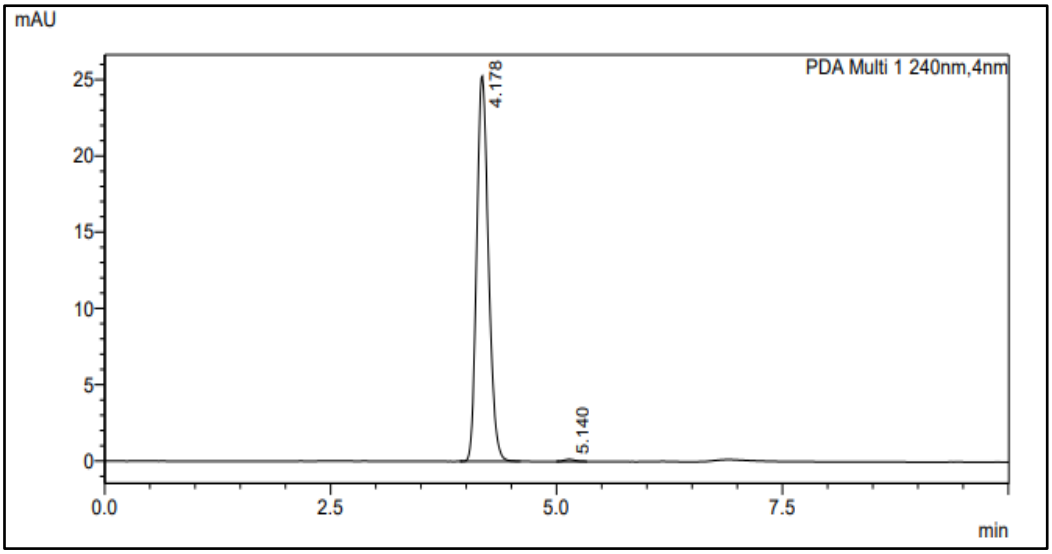


Figure 19a: Chromatogram of Carvedilol Phosphate (Accuracy 50%)

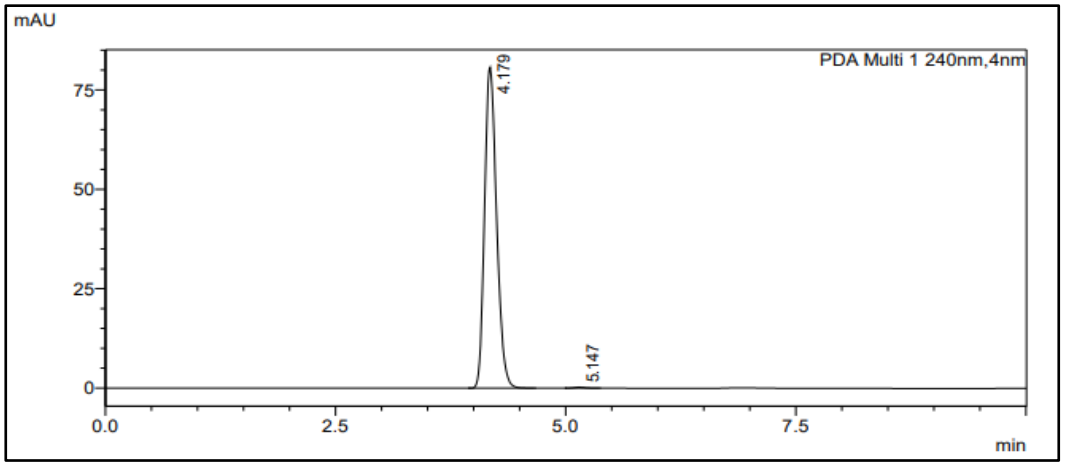


Figure 19b: Chromatogram of Carvedilol Phosphate (Accuracy 100%)

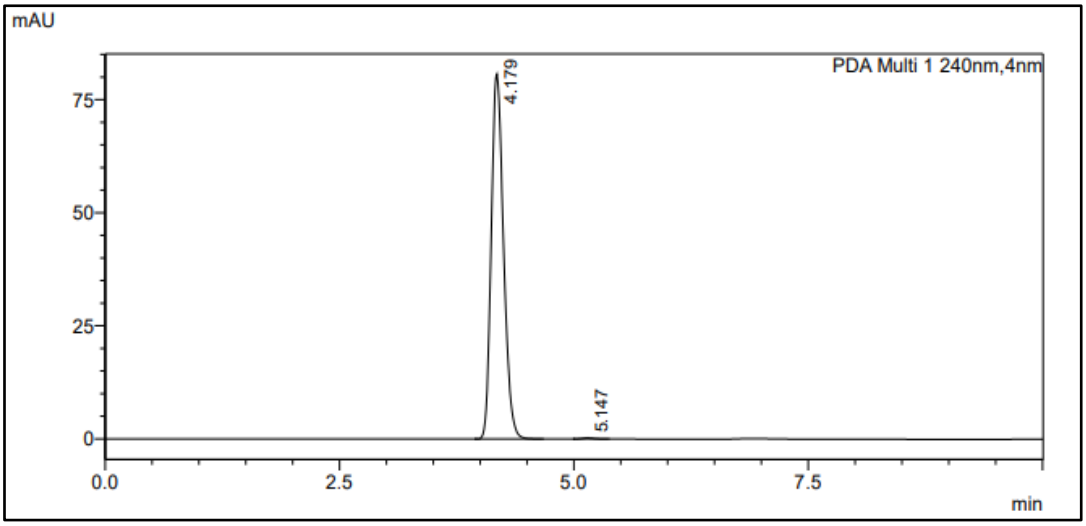


Figure 19c: Chromatogram of Carvedilol Phosphate (Accuracy 150%)

Robustness

In studied CAA, with minor changes in the method parameters, no any considerable changes have been observed and degree of % RSD was within <2%. Thus, the robustness of the developed technique was established for minor changes in the method parameters. Table 16 and Figure 20a-f shows the results of the robustness study.

Table 16: Results of robustness study (n=6) by RP-HPLC method

Parameter	Modification	% RSD of peak area	% RSD of retention time	Theoretical plates	Tailing factor	Percentage recovery
Flow rate	0.90 mL/min	0.10	0.04	4694	1.22	110.53
	Optimized	0.47	0.01	4325	1.21	100.00
	1.10 mL/min	0.05	0.03	3985	1.21	93.00
Mobile phase composition	64% MeOH	0.05	0.02	4216	1.22	101.27
	Optimized	0.47	0.01	4325	1.21	100.00
	66 % MeOH	0.02	0.04	4417	1.21	101.04
Column temperature	29 °C	0.05	0.11	4282	1.21	101.16
	Optimized	0.47	0.01	4325	1.21	100.00
	31 °C	0.01	0.01	4354	1.22	101.11

n = number of replicates; RSD = relative standard deviation

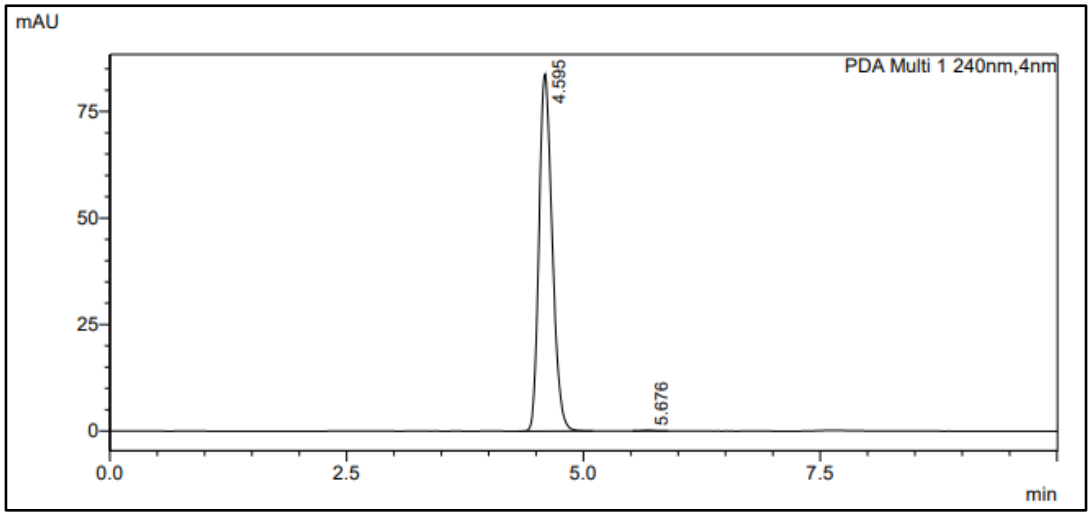


Figure 20a: Chromatogram of Carvedilol phosphate effect of change in flow rate of 0.9 mL/min

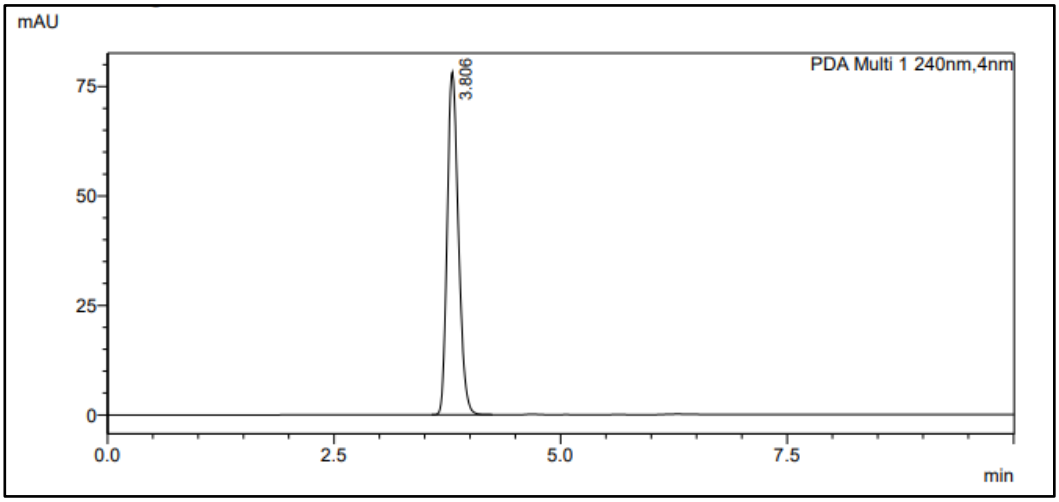


Figure 20b: Chromatogram of Carvedilol phosphate effect of change in flow rate of 1.1 mL/min

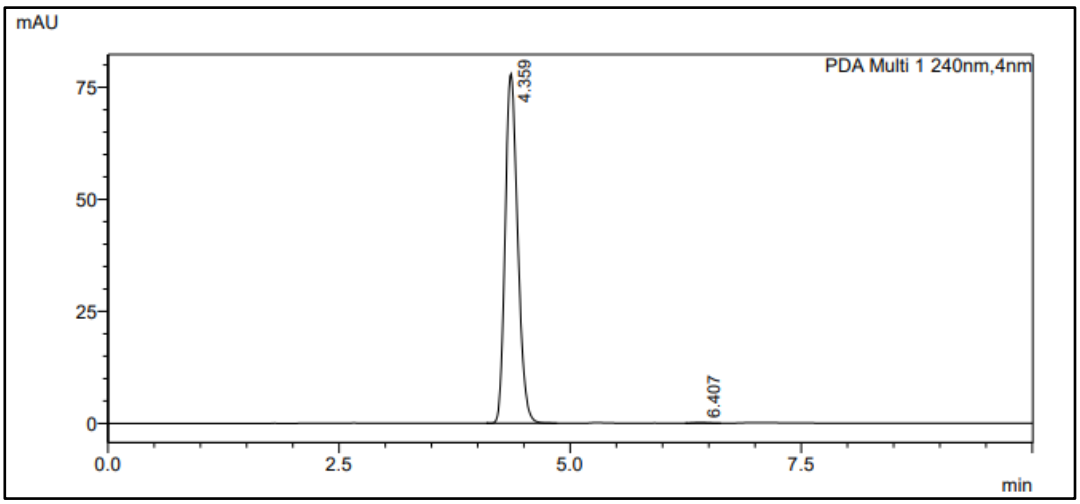


Figure 20c: Chromatogram of Carvedilol phosphate effect of change in concentration of mobile phase (MeOH:0.1%OPA,64:36)

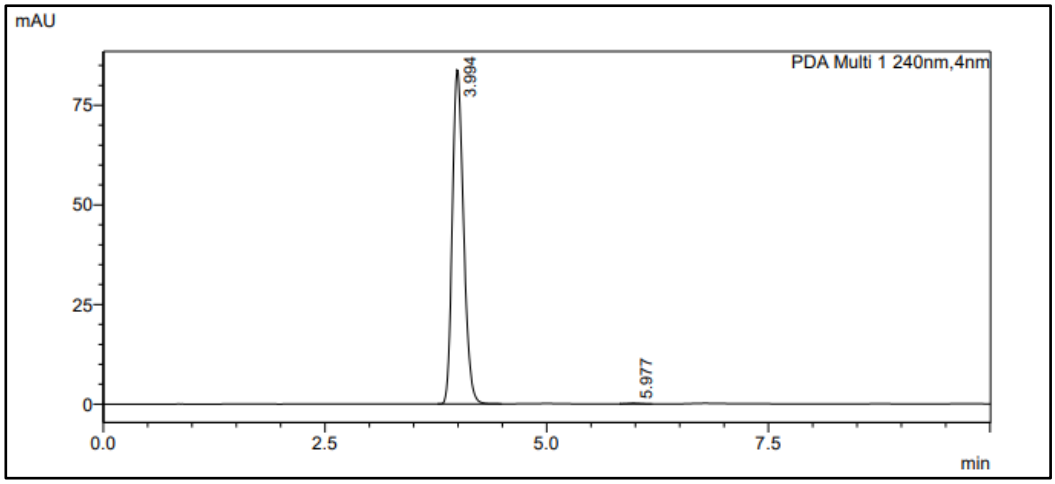


Figure 20d: Chromatogram of Carvedilol phosphate effect of change in concentration of mobile phase (MeOH:0.1%OPA,66:34)

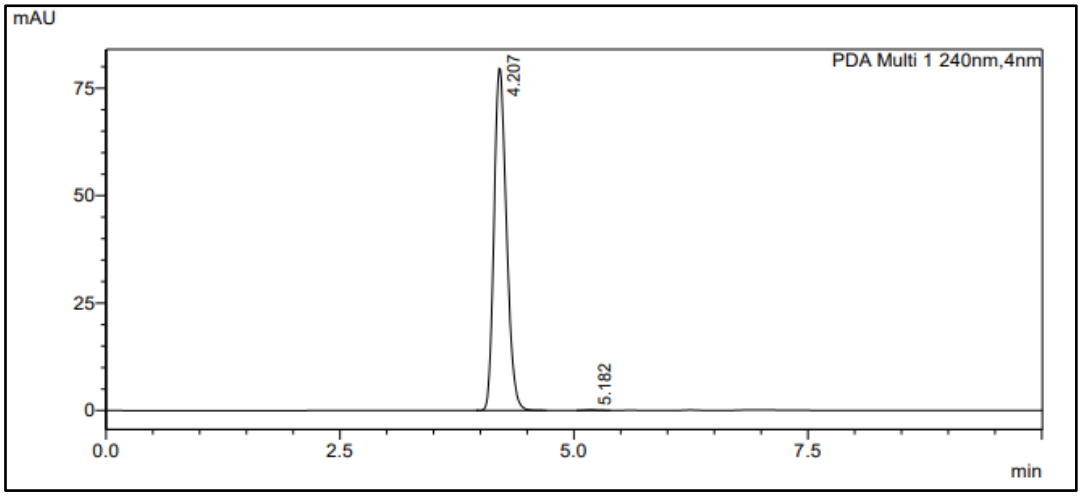


Figure 20e: Chromatogram of Carvedilol phosphate effect of change in column temperature (29°C)

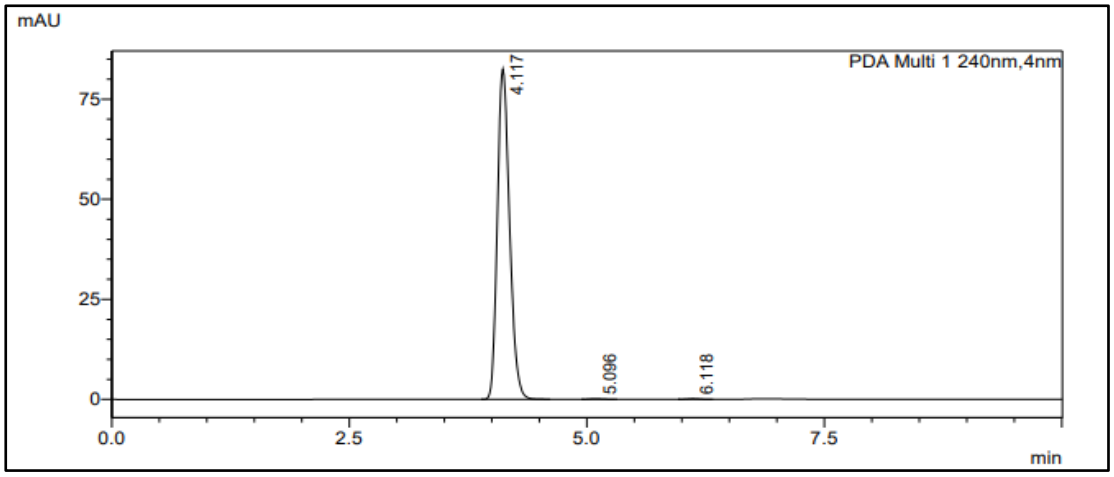


Figure 20f: Chromatogram of Carvedilol phosphate effect of change in column temperature (31°C)

Estimation of Carvedilol Phosphate by RP-HPLC method

The research utilizing BBD to optimize the RP-HPLC method for Carvedilol Phosphate resulted in excellent recovery, with a percent recovery of 105%, as shown in Table 17 and Figure 21. The retention time of Carvedilol Phosphate in the tablet formulation was similar to that of pure Carvedilol Phosphate API. The TF (tailing factor) and TP (theoretical plate) values were within acceptable ranges. Furthermore, no further peaks were observed in the chromatograms of any sample solutions, demonstrating the lack of unaccounted Carvedilol Phosphate in the dosage form. These findings support the established RP-HPLC technique as a dependable solution for repetitive QC of Carvedilol Phosphate in both API & tablet forms.

Tabel 17: Assay result of commercial tablets of Carvedilol Phosphate by RP-HPLC method

Sr. No.	Marketed formulation	Label Claim (mg/tablet)	Estimated amount (mg)	% Assay	SD	%RSD
1	Tablet	3.125	3.30	105.60	0.10	1.04

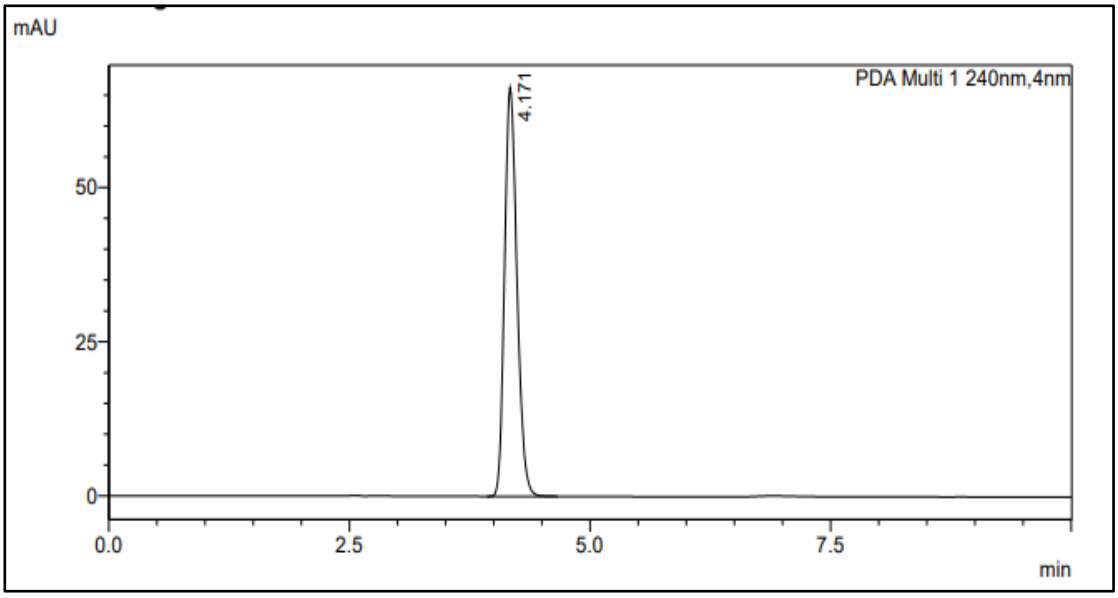


Figure 21: Assay chromatogram of Carvedilol Phosphate by RP-HPLC method

4.2 DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR NEBIVOLOL HYDROCHLORIDE IN BULK AND FORMULATION

Preliminary Studies for Method Development

Initial experimental runs were conducted to explore different combinations and identify the most suitable stationary phase based on the solvent system and column. Methanol was chosen as the solvent for the trial investigation, which was part of the effort to optimize the approach in terms of cost-effectiveness. For optimal separation under varying pH settings, aqueous solvents with varying amounts of acetic acid were also utilized. Further investigation was conducted using the Agilent C18 stationary phase after analyzing the outcomes of these early trials. (Table 18 and Figure 22 A-F)

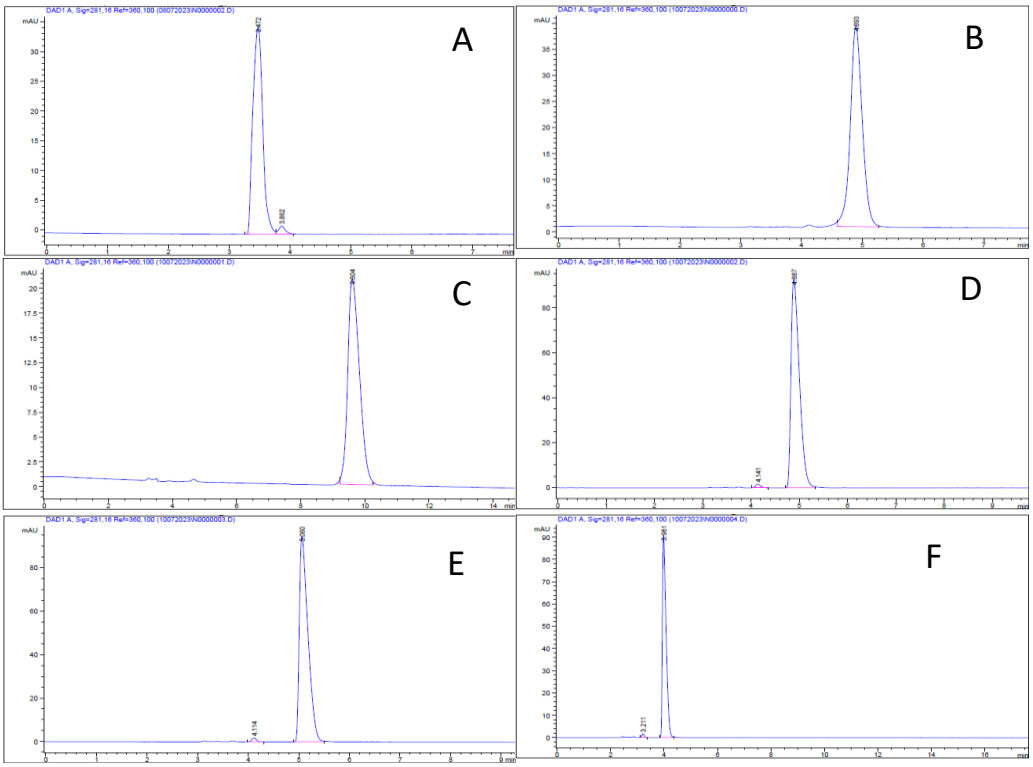


Figure 22 A-F: Preliminary trials to optimize chromatographic conditions for Nebivolol hydrochloride method development (A) MeOH:0.1%FA 71:29 v/v (B) MeOH:0.1%FA 71:29 v/v (C) MeOH:0.1%FA 71:29 v/v (D) MeOH:0.1%FA 71:29 v/v (E) MeOH:0.1%FA 71:29 v/v (F) MeOH:0.1%FA 71:29 v/v

Table 18: Results for Preliminary trial Runs

Figure	Mobile Phase Used	Observations
A	Methanol and 0.1% acetic acid in water (80:20)	Poorly resolved with tailing factor more than 2
B	Methanol and 0.1% acetic acid in water was changed (70:30 with pH 6).	poorly resolved with broad peaks and tailing
C	Concentration of Methanol was decreased to separate the Nebivolol Hydrochloride (NBV) (60:40)	NBV Peak from negative peak leads to increased retention time from 4.893 to 9.603min
D	Increase the concentration of Methanol from the mobile phase ratio 60:40 to 65:35	The retention time of NBV peak was reduced from 9.603 to 5.060 min, with tailing
E	Methanol and 0.1% acetic acid in the ratio 70:30 with pH 4.5	which results in sharp and well-defined peak of NBV, tailing factor was also reduced but height of peak more
F	Finally, Methanol and 0.1% acetic acid in water was changed (70:30) of NBV	Tailing factor was reduced (0.96) and good peak shape was obtained

Optimization of Chromatographic Condition

By utilizing AQbD, the RP-HPLC technique was optimized for the analysis of Nebivolol Hydrochloride. In the beginning, some basic experiments were conducted to observe the effects of different mobile phases (a mixture of solvents like methanol, water, acetic acid, and phosphate buffers) at varying flow rates to produce a clearly defined peak. There was a strong peak at 4.19 minutes at a flow rate of 0.8 ml/min and at 282 nm while using a mobile phase consisting of MeOH:0.1% aqueous acetic acid in a 71:29 ratio, according to the results. There was no sharp peak produced by any of the other compositions. The BBD technique was utilized for further optimization with regard to mobile phase, flow rate, wavelength. (Figure 23)

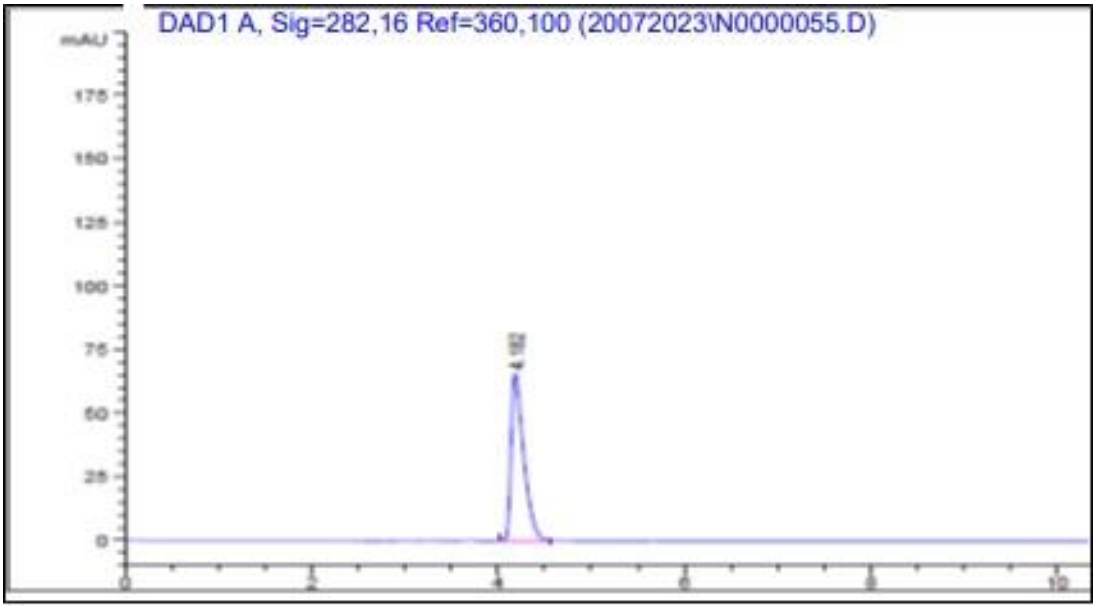


Figure 23: Preliminary trials to optimize chromatographic conditions for Nebivolol hydrochloride method development MeOH:0.1%FA 71:29 v/v

Risk assessment and Screening Studies [10]

Table 6 details the results of the examination of ATP, CQAs, and process parameters, as discussed in the methodology study. Analytical features and process parameters were investigated using an Ishikawa Fish-Bone diagram (Figure 3).

Chromatographic mode, flow rate, injection volume, and wavelength of detection were instrument-related characteristics, whereas solvent-system-related risk variables were sample extraction method, extraction duration, solvent choice, and mobile phase composition. The potential risk factors such as the flow rate, mobile phase makeup, and detecting wavelength were investigated.

Method Optimization

The analytical RP-HPLC parameters were optimized using the DoE program to acquire the best conditions for estimating Nebivolol Hydrochloride. Rt, PA, and Tf were considered dependent variables, and the effect of independent variables such as methanol (70 to 72%v/v), flow rate (0.7 to 0.9 ml/min), and wavelength (281 to 283 nm) was evaluated. Table 19 shows that the ideal chromatographic separation for Nebivolol Hydrochloride was determined with the use of seventeen experimental trial runs performed using the DoE program. For the purpose of selecting the design space, the following variables were used to establish the central composition point: flow rate (0.8 ml/min), wavelength (282 nm), and mobile phase ratio (MeOH:0.1% aqueous acetic acid 71:29% v/v). The design space was established by evaluating the effect of various variables on the responses. Several polynomial models, including linear, second-order, and quadratic ones, were used to fit the responses. With a high regression coefficient (R^2=0.9999) and a substantial advantage over other models ($p<0.0001$), the quadratic model was determined to be the best match. For every model, an analysis of variance (ANOVA) was carried out to find the "sum of squares, F value, mean square and p value" for each answer. Table 20 and 21 show the findings of the same. Figures 24-29 show the 3D-response surface plots and 2D-contour plots for each response, which indicate how many factors (the integration effect) influence a response at the same time.

Table 19: Box-Behnken design (BBD) based independent variable and their effect on dependent variables

Run No	Independent Variables			Dependent variables		
	A	B	C	RT	PA	TF
1	71	0.8	282	3.612	546.621	0.49
2	71	0.8	282	3.619	545.124	0.44
3	70	0.7	282	4.194	622.816	0.42
4	71	0.7	281	4.187	616.703	0.48
5	70	0.8	281	3.703	539.244	0.44
6	71	0.8	282	3.55	548.039	0.47
7	72	0.8	281	3.592	541.349	0.57
8	71	0.9	281	3.225	478.997	0.5
9	71	0.8	282	4.184	545.128	0.45
10	71	0.8	282	4.19	546.874	0.44
11	70	0.8	283	3.743	550.477	0.45
12	72	0.9	282	3.112	488.084	0.54
13	72	0.7	282	4.022	630.453	0.51
14	71	0.7	283	4.184	631.114	0.46
15	71	0.9	283	3.25	490.101	0.5
16	70	0.9	282	3.401	488.354	0.46
17	72	0.8	283	3.542	552.433	0.52

A: Solvent concentration in mobile phase (% v/v), B: Flow rate (ml/min), C: Wavelength (nm)

Table 20: Regression analysis for responses Retention time; Peak area, Tailing factor for fitting to different polynomial models

Models	SD	R^2	Adjusted R^2	Predicted R^2	PRESS	Adequate Precision	Remark
Retention time (RT)							
Linear	0.2016	0.7622	0.7074	0.7117	0.6407	11.1769	Suggested
2FI	0.2286	0.7648	0.6236	0.6618	0.7514		
Quadratic	0.2487	0.8052	0.5547	0.6465	0.7856		
Peak area (PA)							
Linear	5.86	0.9887	0.9860	0.9787	836.32	170.7498	
2FI	6.54	0.9891	0.9826	0.9544	1793.27		
Quadratic	1.15	0.9998	0.9995	0.9985	59.13		Suggested
Tailing factor (TF)							
Linear	0.0224	0.7516	0.6943	0.6292	0.0097	11.5231	Suggested
2FI	0.0234	0.7908	0.6653	0.5451	0.0119		
Quadratic	0.0171	0.9215	0.8206	0.7808	0.0057		

Table 21: Analysis of variance (ANOVA) for the responses (Y1: Retention time; Y2: Peak area, Y3: Tailing factor)

Results (ANOVA)	Retention time (Y1)	Peak area (Y2)	Tailing factor (Y3)
Regression			
Sum of squares	1.69	39318.28	0.0197
Degrees of freedom (df)	3	9	3
Mean Square	0.5646	4368.70	0.0066
F-value	13.89	3292.75	13.11
p-value	0.0002	< 0.0001	0.0003
Lack of fit tests			
Sum of squares	0.1030	3.09	0.0046
Degrees of freedom (df)	9	3	9
Mean Square	0.0114	1.03	0.0005
F-value	0.1076	0.6650	1.09
p-value	0.9971	0.6159	0.5046
Residual			
Sum of squares	0.5284	9.29	0.0065
Degrees of freedom (df)	13	7	13
Mean Square	0.0406	1.33	0.0005

Effect of independent factors on retention time (Y_1)

The model equation, 3D response, and 2D contour plot were used to show how the controlled variables affected retention time. In Figures 24 and 25, depicted the impact of the independent factors on retention time (A1-A3 and B1-B3). After applying software analytics to data collected from trial batches using a 3^3factorial design, it was discovered that interaction terms did not significantly impact retention time. Consequently, the following is the linear equation that was determined for Y1 (retention time):

Y1 (retention time) = +3.72 - 0.0966A - 0.4499B + 0.0015C.

The significance of the term B was determined by the fact that the related factor's P-value was less than 0.05. In addition, the model's F-value of 13.89 demonstrated its relevance. The probability of the F-value being due to signal (i.e., less than 0.02% noise) is 99.98%, indicating that the model is significant. R^2 was found to be quite near to both the anticipated value (0.7117) and the adjusted value (0.7074). Precession value of 11.1769, indicates that the signal is sufficient. Table 20 and 21 show the findings of the same. Wavelength (C) has a "positive effect" on retention time, in contrast to the "negative effect" of mobile phase composition (A) and flow rate (B). When it comes to retention time, the flow rate is the most important independent variable.

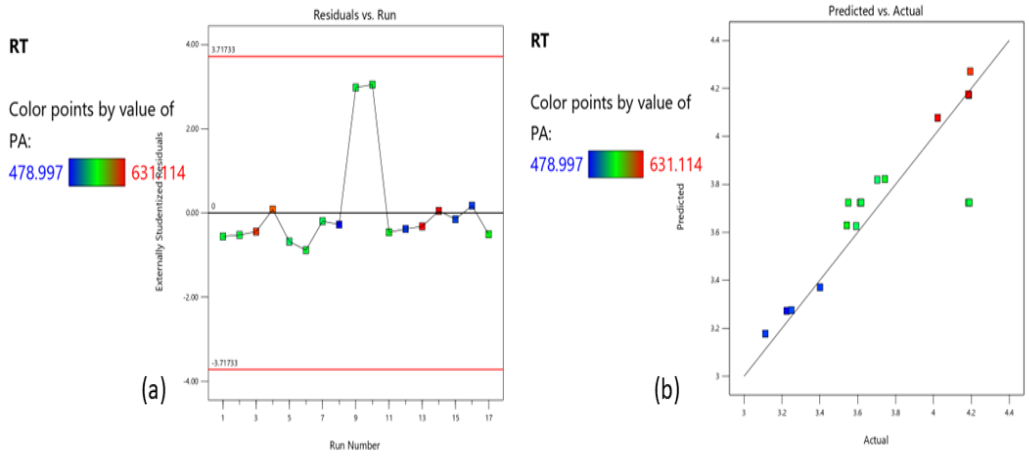


Figure 24: Plot (a) showing true versus predicted values, with residual plot (b) for retention time

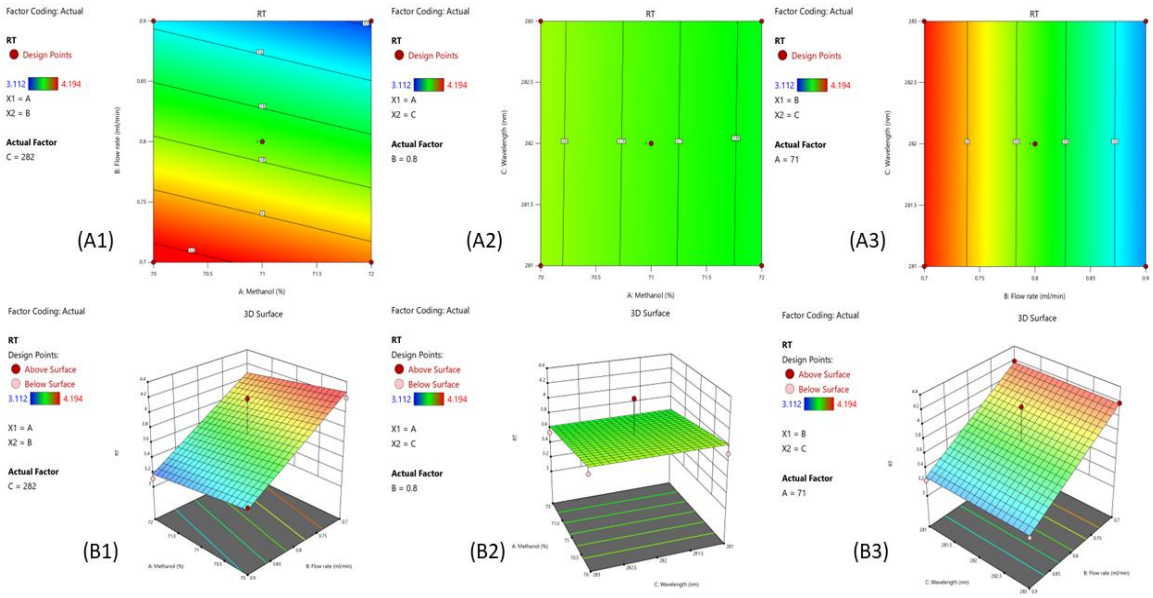


Figure 25: Relation plot (A1-A3 & B1-B3) 2-D contour plots and 3-D response surface plots showing the influence of CMP's i.e., (a) Solvent concentration in mobile phase (% v/v), (b) Flow rate (ml/min), (c) Wavelength (nm) on retention time (RT) as a CAA

Effect of independent factors on peak area (Y_2)

The model equation, 3D response, and contour plot were used to show how the controlled variables affected peak area. Both Figure 26 and Figure 27 (C1–C3 and D1–D3) display the impact of independent variables on peak area. Peak area is unaffected by either individual or interaction terms, consequently, the expression of the 2nd order quadratic equation that was found for Y_2 (peak area) is expressed below,

Y2 (**Peak area)** = +546.36 + 1.43A - 69.44B+ 5.98C - 1.98AB - 0.0371AC - 0.8266BC + $1.36A^2$ + 9.71 B^2 - $1.84C^2$

The significance of the terms A, B, C, AB, A^2, B^2, and C^2 was confirmed by determining the matching P-values which were less than 0.05. Further the significance of the model was determined by model F-value which was 3292.75. This witnessed that model is significant with 99.99% chance of F-value is due to signal (i.e. noise < 0.01%). The closeness between predicted (0.9985) and adjusted (0.9998) R^2 was observed. With a precession value of 170.7498, it is clear that the signal is sufficient. Table 20 and 21 show the findings of the same. Peak area is "positively affected" by mobile phase composition (A) and wavelength (C), but "negatively affected" by flow rate (B). One independent variable, flow rate, has a more noticeable effect on peak area than the other two.

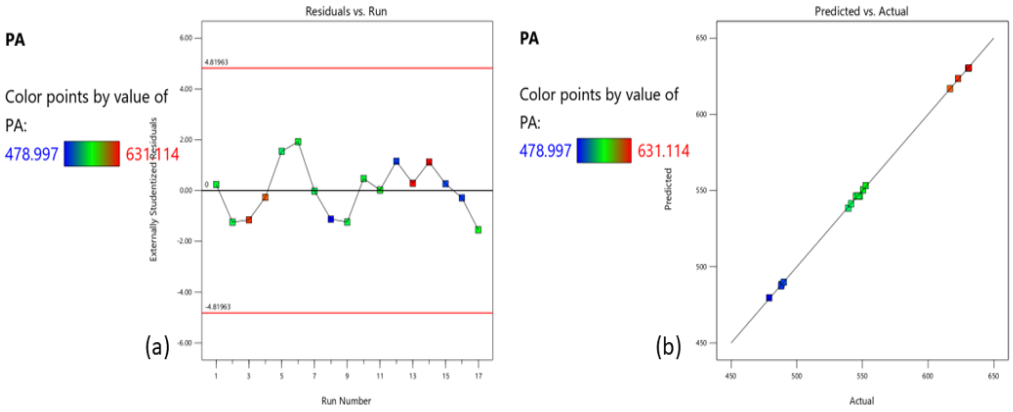


Figure 26: Plot (a) showing true versus predicted values, with residual plot (b) for peak area

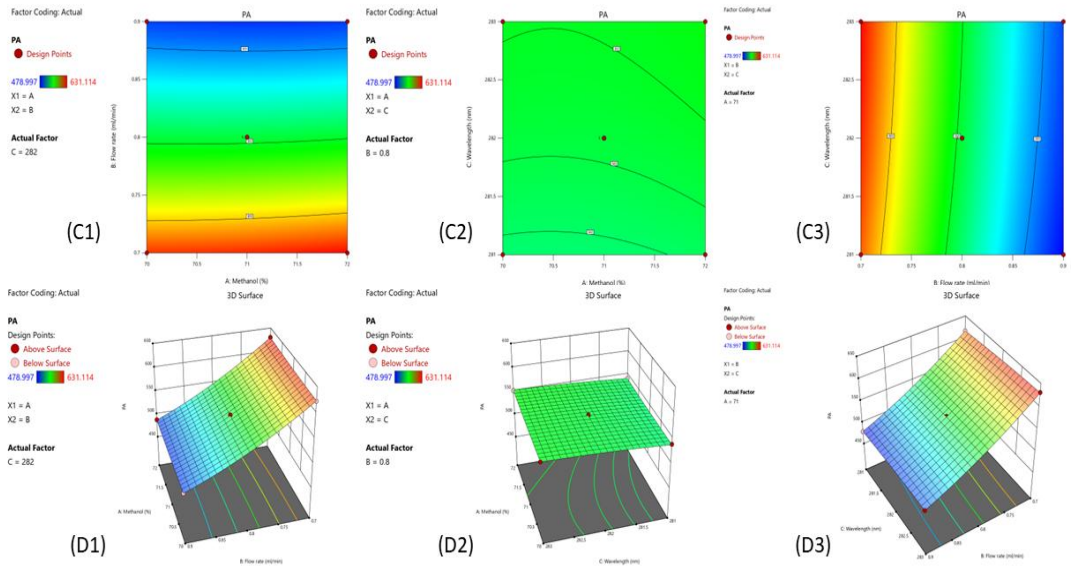


Figure 27: Relation plot (C1-C3 & D1-D3) 2-D contour plots and 3-D response surface plots showing the influence of CMP's i.e., (a) Solvent concentration in mobile phase (% v/v), (b) Flow rate (ml/min), (c)Wavelength (nm) on Peak area (PA) as a CAA

Effect of independent factors on tailing factor (Y_3)

Expressions such as 3D response, contour plot, and model equation were used to represent the effect of independent variables on the tailing factor. The effect of the independent variables on the tailing factor can be seen in Figures 28 and 29 (E1-E3 and F1-F3, respectively). Interaction terms were determined to have no meaningful impact on tailing factor when data handled using software analytics. The linear equation that was obtained for the tailing factor, Y_3 is expressed below,

Y_3 (Tailing factor) = +0.4788 + 0.0462A + 0.0162B - 0.0075C

Since the related factor P-values were less than 0.05, the A was considered to be statistically significant. A model F-value of 13.11 further established the model's importance. The likelihood of the F-value being due to signal (i.e., less than 0.03% noise) is 99.97%, indicating that the model is significant. There was a clear correspondence between the expected R^2 value of 0.6292 and the modified R^2 value of 0.6943. An acceptable precession value of 11.5231 demonstrates that the signal is sufficient. Table 20 and 21 show the findings of the same. While wavelength (C) has a "negative effect" on tailing factor, in contrast to the "positive effect" of mobile phase composition (A) and flow rate (B). When it comes to the tailing factor, the mobile phase is far more influential than the other two independent factors.

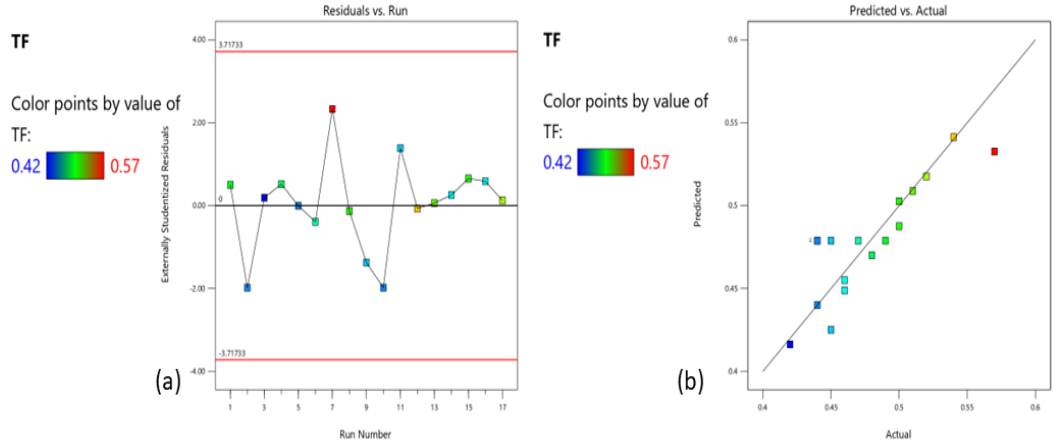


Figure 28: Plot (a) showing true versus predicted values, with residual plot (b) for tailing factor

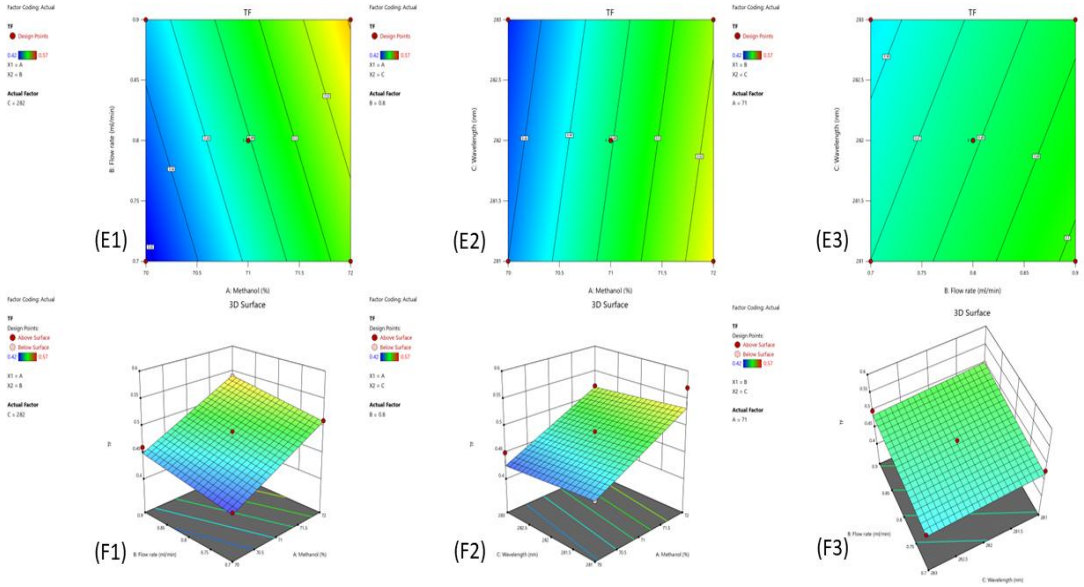


Figure 29: Relation plot (E1-E3 & F1-F3) 2-D contour plots and 3-D response surface plots showing the influence of CMP's i.e., (a) Solvent concentration in mobile phase (% v/v), (b) Flow rate (ml/min), (c)Wavelength (nm) on Tailing factor (TF) as a CAA

Optimization (Graphical and numerical)

The findings of the numerically optimized approach, with a highest desirability of 0.940, are shown in figure 30(b), while Figure 30(a) shows the overlay plot that illustrates the link between the independent variables and responses for the graphical optimization.

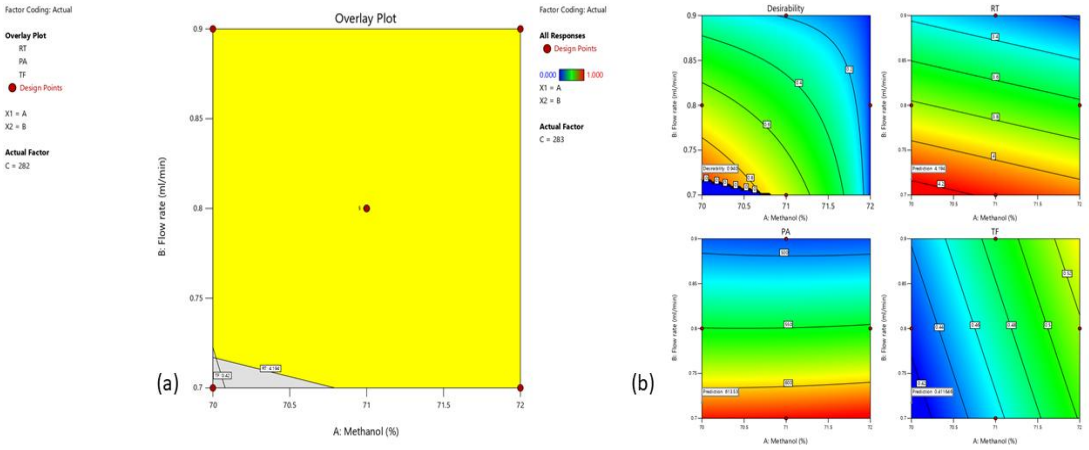


Figure 30: a. Overlay plot showing the optimized chromatographic conditions and their predicted responses, b. Graphs showing numerical optimization with highest desirability

Method validation

Method validation is a key aspect which is to be carried out before opting any analytical method for routine analysis.

System Suitability and Specificity

Critical variables such as chromatogram "retention time, peak area and tailing factor" were used to evaluate the developed analytical technique for system suitability. When the experiment was repeated three times with a concentration of 30 µg/mL of Nebivolol Hydrochloride, the findings demonstrated no statistically significant difference (%RSD <0.5). In order to test the method's specificity, blank and spiked quality control samples were analyzed, wherein no interference was observed in either. This confirmed the suitability and specificity of the method for quantifying Nebivolol Hydrochloride in the formulation (Table 22). The chromatogram of Nebivolol Hydrochloride is as shown in Figure 31.

Table 22: System suitability test parameters (n=6) at Concentration 30 µg/mL

Parameter	Retention time (Rt, min)	Peak area	Tailing factor
Mean	4.19	456.83	0.51
SD	0.007	17.35	0.08
%RSD	0.09	0.19	3.79

n = number of replicates; SD = Standard Deviation RSD = relative standard deviation

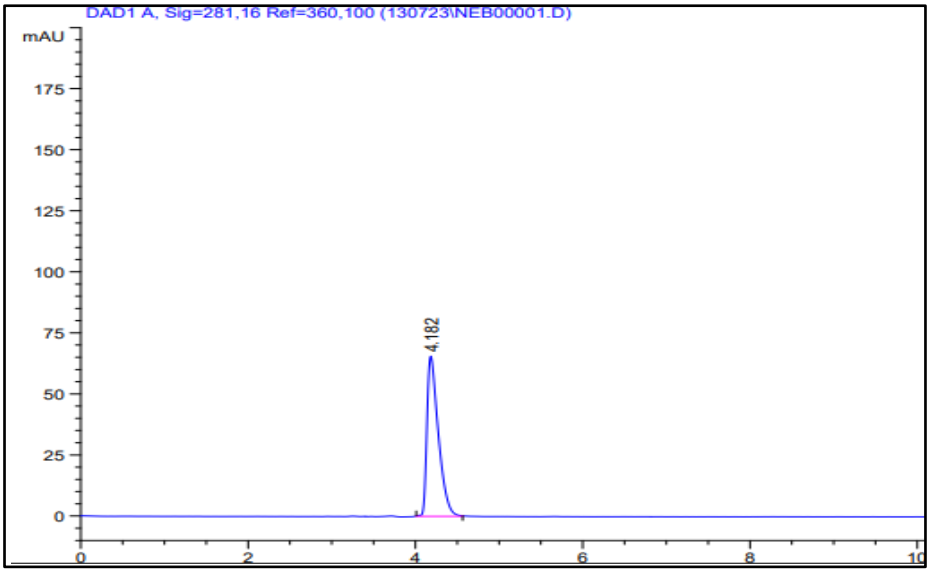


Figure 31: System suitability parameter of Nebivolol hydrochloride

Linearity and Sensitivity analysis

The suggested analytical method's linearity was established using ICH recommendations. Within the concentration range of 10 to 50 µg/mL, linearity results were obtained with a regression coefficient of (R^2) 0.999 and the equation of the resulting linear line being y=15.215x - 6.625 (Figure 32). The LOD and LOQ represents the sensitivity of the method. Results showed that the developed technique had a LOD of 0.54 µg/mL and a LOQ of 1.64 µg/mL These results demonstrate the method's high sensitivity.

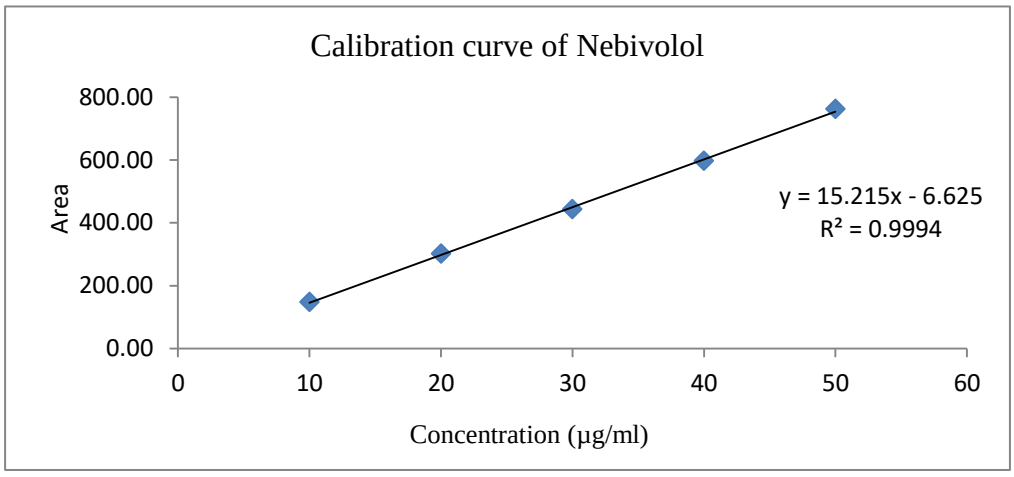


Figure 32. Calibration curve of Nebivolol hydrochloride by RP-HPLC method

Precision

Results of the precision study are presented in Table 23, the %RSD value for the peak area, measured in terms of both intraday and inter-day precision, was found to be less than 2%, suggesting that the method is precise for routine analysis.

Table 23: Intra-day and inter-day precision of nebivolol by RP-HPLC method

Precision Parameter	Concentration (µg/mL)	Mean Peak Area	SD	%RSD
Intraday	10	147.82	0.03	0.02
	30	441.76	0.44	0.10
	50	778.24	0.19	0.02
Interday-1	10	147.92	0.11	0.07
	30	442.70	0.43	0.10
	50	761.02	11.15	1.47
Interday-2	10	155.82	1.44	0.92
	30	452.35	0.69	0.15
	50	775.57	0.78	0.10
Interday-3	10	159.32	7.17	4.50
	30	447.87	0.75	0.17
	50	778.71	0.43	0.06

n = number of replicates; SD = Standard Deviation RSD = relative standard deviation

Accuracy study

The capacity of a method to match experimental findings with theoretical predictions is a measure of its accuracy. The concentration of Nebivolol Hydrochloride that was collected ranged between 102.49% and 103.44%, as shown in Table 24. The method's accuracy is further confirmed by the % RSD value, which is less than 2% Figure 33.

Table 24: Recovery studies of Nebivolol Hydrochloride (n = 3)

Level	Contents (µg/mL)	Quantity added (µg/mL)	Theoretical amount (µg/mL)	Recovered amount (µg/mL)	Recovery (%)	SD	% RSD
80%	10	8	18	18.28	103.44	0.02	0.02
100%		10	20	20.27	102.71	0.13	0.12
120%		12	22	22.30	102.49	0.21	0.20

n = number of replicates; SD= Standard deviation, RSD = relative standard deviation

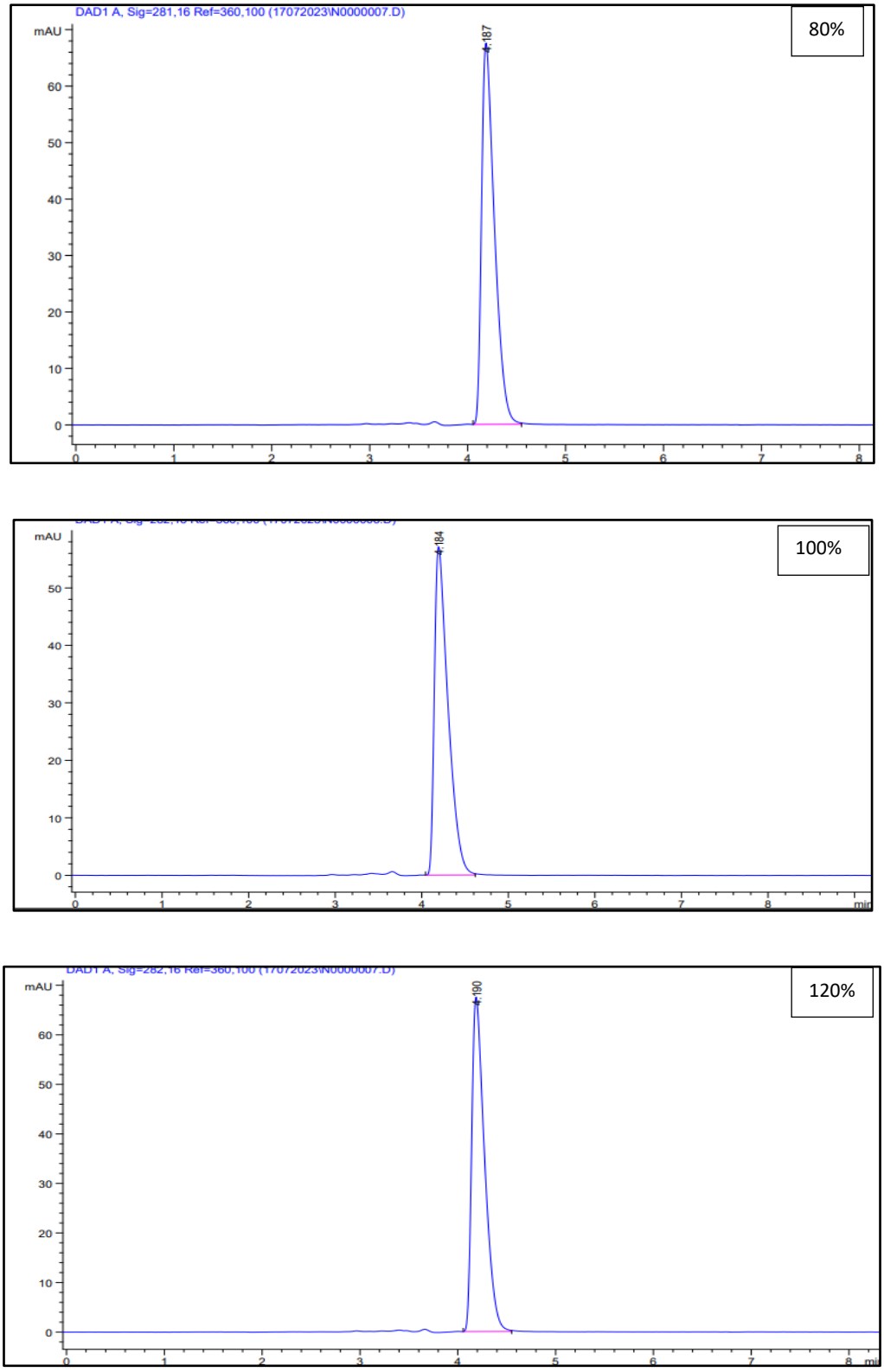


Figure 33: Chromatogram of Nebivolol hydrochloride

(Accuracy 80%, 100% & 120%)

Robustness

The methods robustness was assessed by changing the process parameters, and it was found that the percentage RSD value was below 2 %, well within the permitted limits. Since there was no noticeable change in the chromatogram, the devised RP-HPLC technique is in fact robust. (Table 25 and Figure 34a-e)

Table 25: Robustness study of the nebivolol by RP-HPLC method

Change of Flow Rate	**0.7 mL**			**0.9 mL**	
Sr No.	Conc µg/mL	Area	Sr No.	Conc µg/mL	Area
1	40	687.6149	1	40	535.0284
2	40	687.9306	2	40	534.8267
	Mean	**687.77**		**Mean**	**534.93**
	SD	**0.22**		**SD**	**0.14**
	%RSD	**0.03**		**%RSD**	**0.03**
Change of Mobile Phase ratio	**70 MeOH+30%**			**72 MeOH+28%**	
Sr No.	Conc µg/mL	Area	Sr No.	Conc µg/mL	Area
1	40	601.7283	1	40	602.4002
2	40	601.4721	2	40	602.5197
	Mean	**601.6**		**Mean**	**602.46**
	SD	**0.18**		**SD**	**0.08**
	%RSD	**0.03**		**%RSD**	**0.01**
Change of Wavelength Change	**281 nm**			**283 nm**	
Sr No.	Conc µg/mL	Area	Sr No.	Conc µg/mL	Area
1	40	594.9376	1	40	605.8355
2	40	594.5432	2	40	605.5332
	Mean	**594.7**		**Mean**	**605.68**

n = number of replicates; SD= Standard deviation, RSD = relative standard deviation

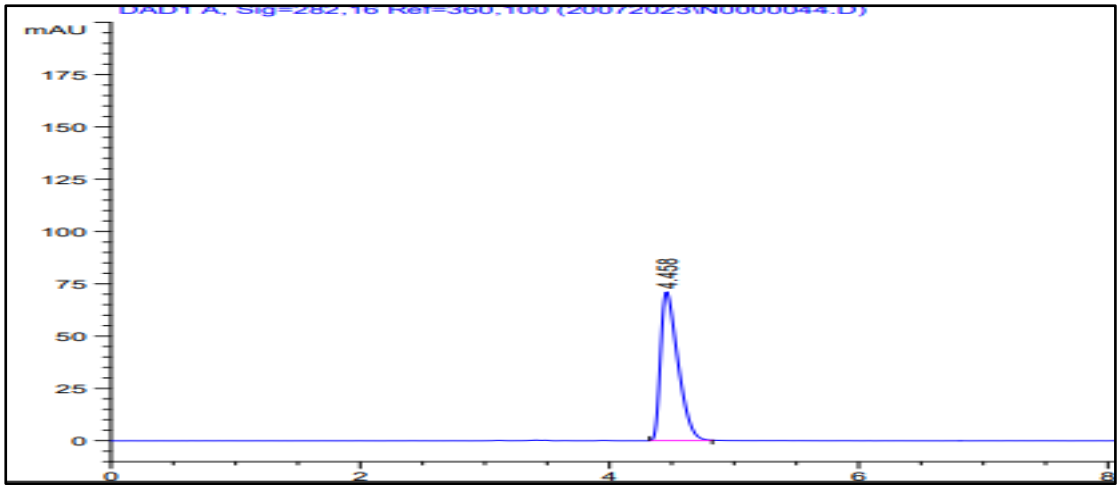


Figure 34a: Chromatogram of Nebivolol hydrochloride effect of change in flow rate of 0.7 ml/min

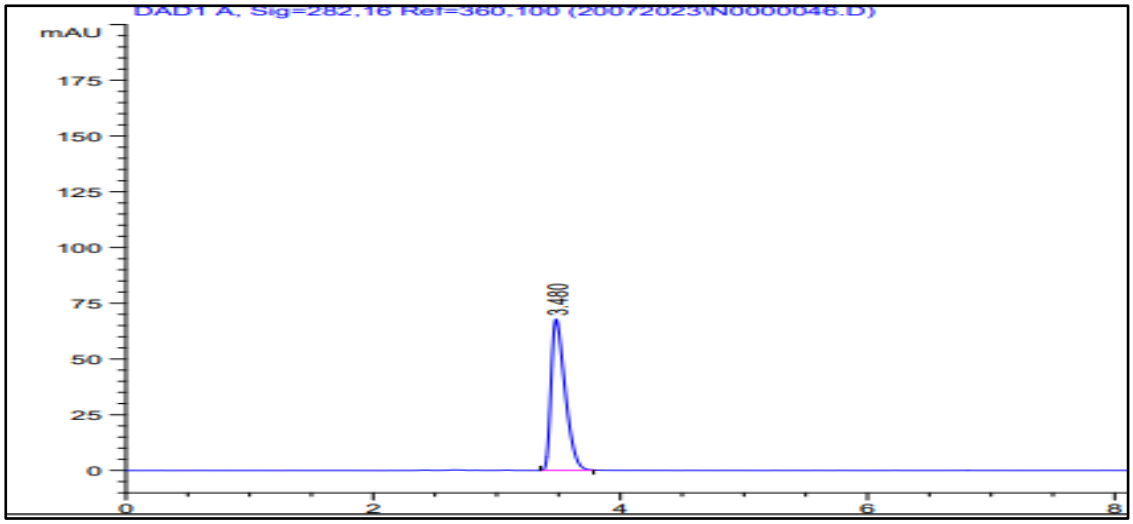


Figure 34b: Chromatogram of Nebivolol hydrochloride effect of change in flow rate of 0.9 ml/min

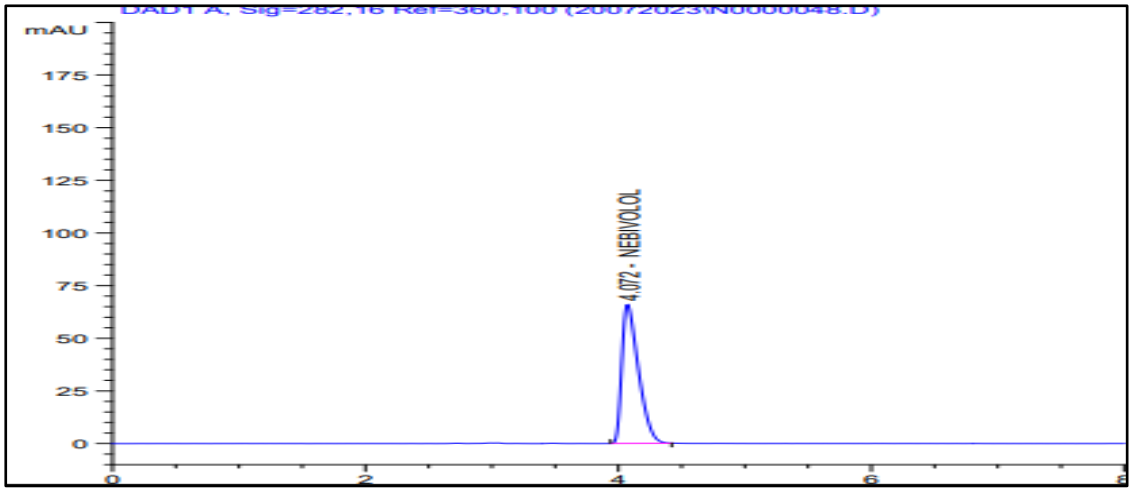


Figure 34c: Chromatogram of Nebivolol hydrochloride effect of change in concentration of mobile phase (70:30)

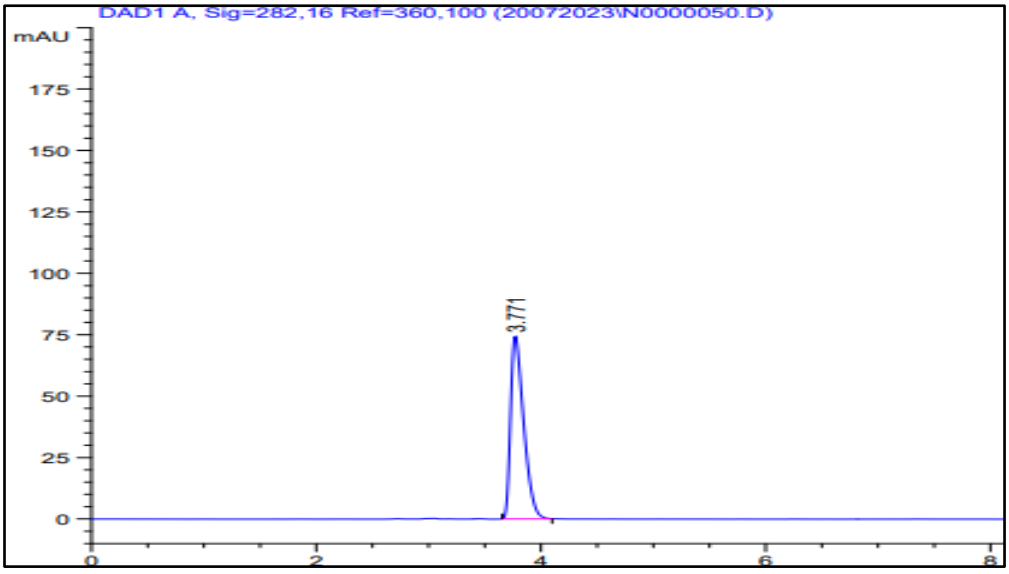


Figure 34d: Chromatogram of Nebivolol hydrochloride effect of change in concentration of mobile phase (72:28)

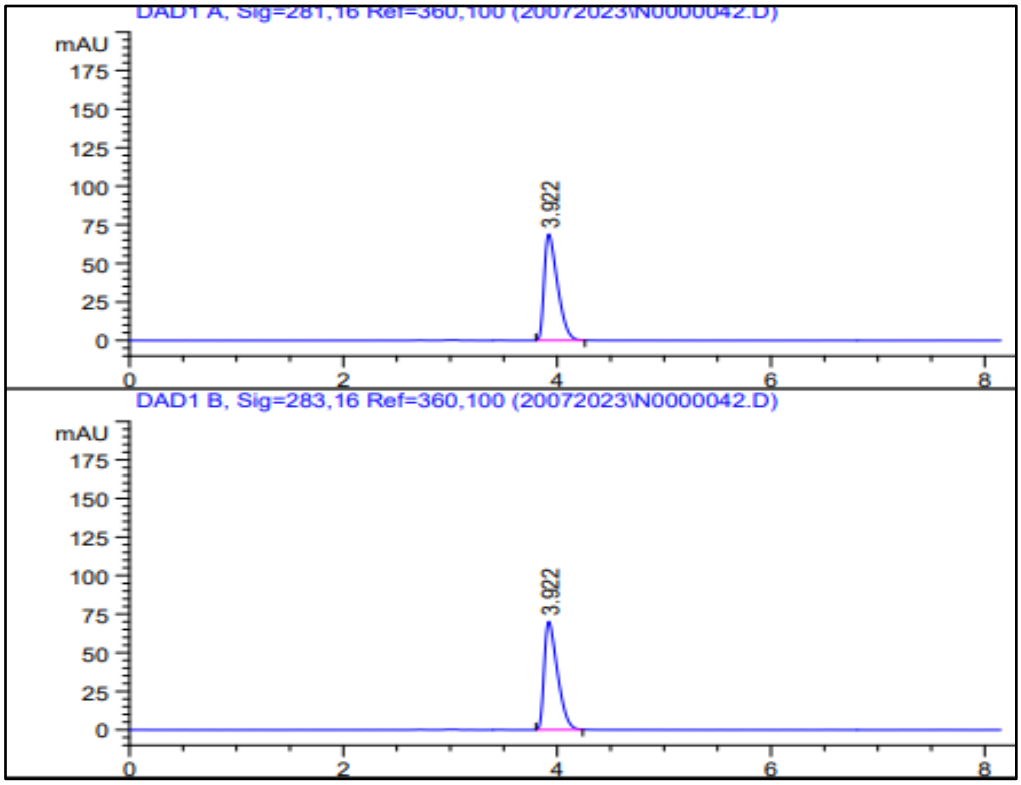


Figure 34e: Chromatogram of Nebivolol hydrochloride effect of change of wavelength (281nm, 283nm)

Ruggedness

Ruggedness of the method was ascertained by repetition of the analysis by different analysts. The observed responses showed no significant differences (%RSD <2%), indicating the ruggedness of the approach. (Table 26 and Figure 35)

Table 26: Ruggedness study of the Nebivolol hydrochloride by RP-HPLC method

Analyst	Conc µg/mL	Area	Amt Found
1	20.00	306.92	19.743261
2	20.00	306.627	19.723997
	Mean	**306.77**	**19.73**
	SD	**0.207**	**0.014**
	%RSD	**0.068**	**0.069**

n = number of replicates; SD= Standard deviation, RSD = relative standard deviation

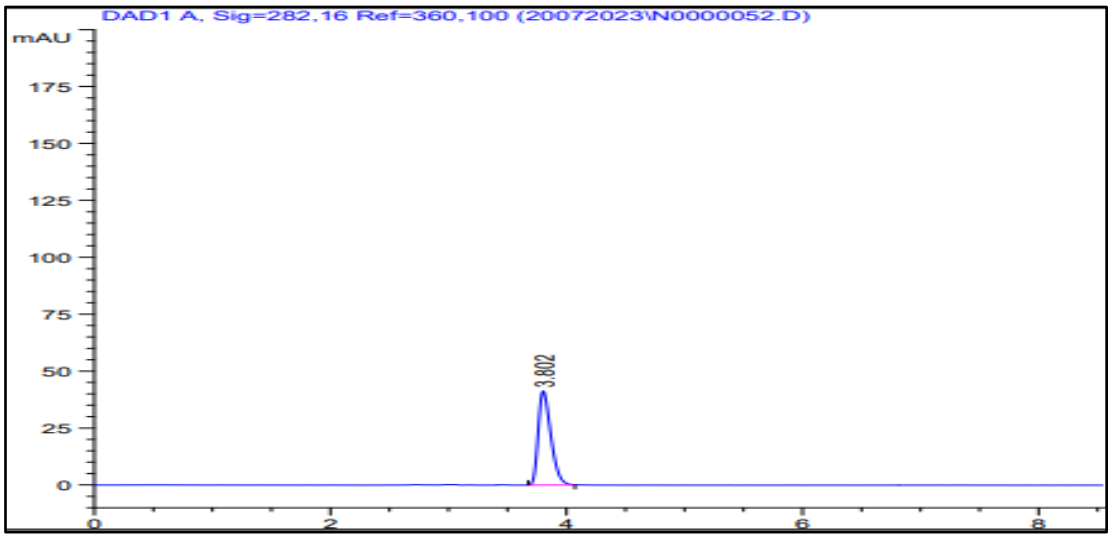


Figure 35: Ruggedness chromatogram of Nebivolol hydrochloride (20 µg/mL)

Estimation of Nebivolol hydrochloride in marketed formulation

The percent recovery was found to be 105.60% of the label claim of Nebivolol hydrochloride marketed formulation which meets the recommended standards (90.0% to 110.0%). Retention time of Nebivolol hydrochloride in the tablet formulation was similar to that of pure Nebivolol hydrochloride API. The TF (tailing factor) value were within acceptable ranges. Furthermore, no further peaks were observed in the chromatograms of any sample solutions, demonstrating no interference in the dosage form. Based on these results, the developed RP-HPLC approach has been proven to be a reliable method for routine quality control of Nebivolol hydrochloride in tablet and API form. In Figure 36, the chromatogram for the assay investigation is shown.

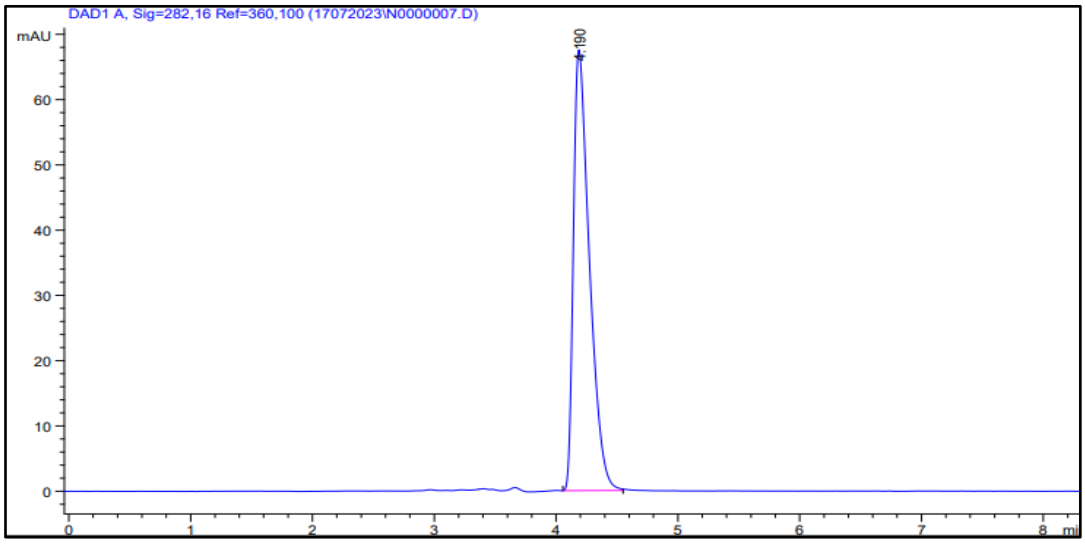


Figure 36: Assay chromatogram of Nebivolol hydrochloride

4.3 POLYMORPHISM STUDY

Characterization of Multicomponent Polymorphs by Analytical Techniques for Carvedilol Phosphate

Differential Scanning Calorimeter

The DSC thermogram for pure Carvedilol Phosphate exhibited a single peak at 123.11°C, consistent with its melting point (Figure 37). For Carvedilol-nicotinamide (Z1, 1:1) co-crystals, a sharp endothermic peak was detected at 112.55°C. This decrease in melting point may be attributable to the formation of new crystalline solid form (Figure 38). In case of Carvedilol-oxalic acid (W1, 1:1) cocrystal, a sharp endothermic peak was observed at 201.75°C. This increase in melting point may be signify the formation of different crystalline solid form, confirming successful formation of co-crystal (Figure 40)

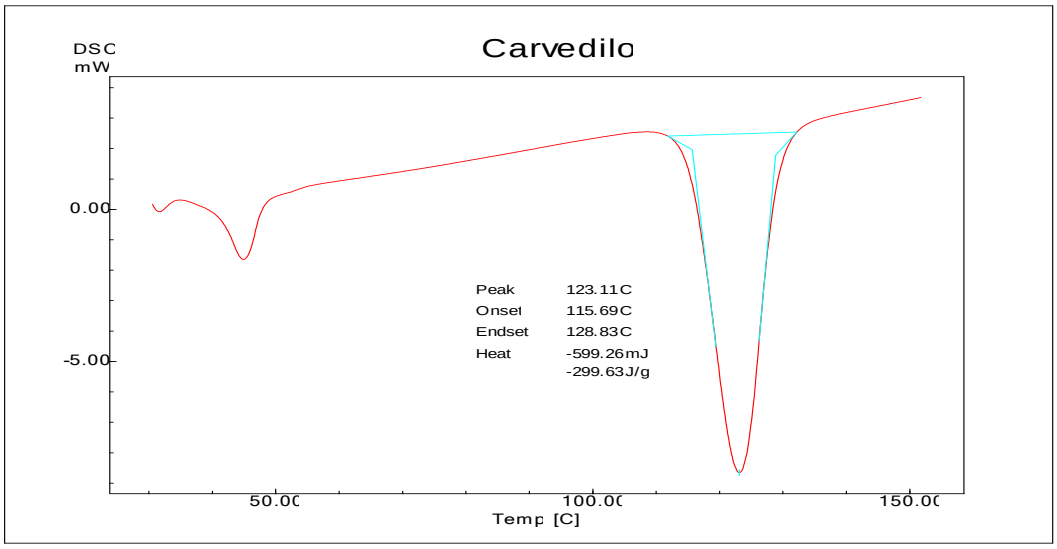


Figure 37: DSC thermogram of Carvedilol phosphate (API)

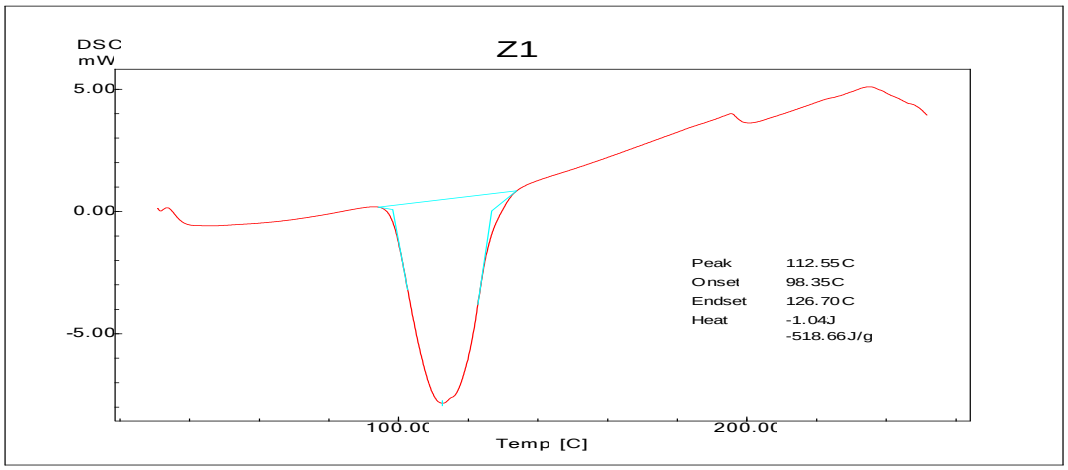


Figure 38: DSC thermogram of Carvedilol - nicotinamide (1:1)

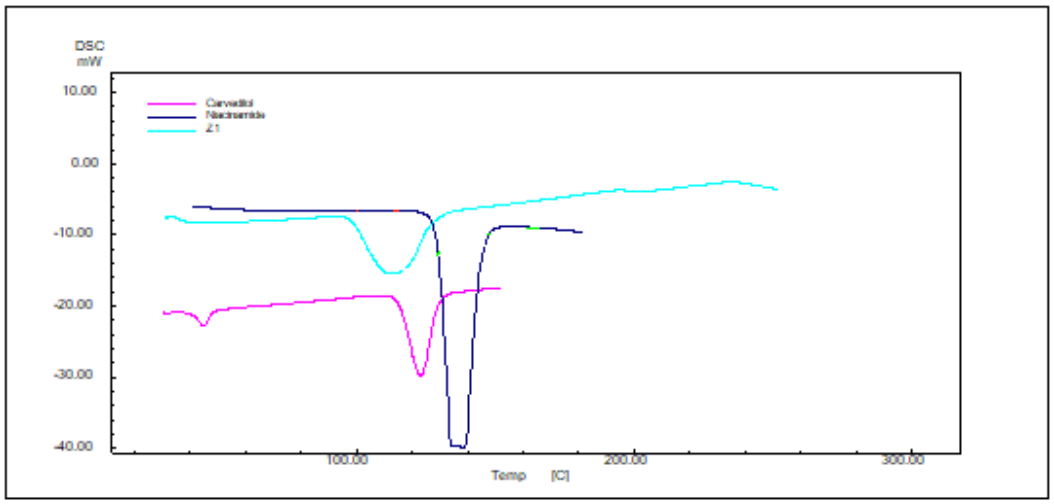


Figure 39: Overlay of DSC thermogram of Carvedilol Phosphate and Carvedilol-nicotinamide

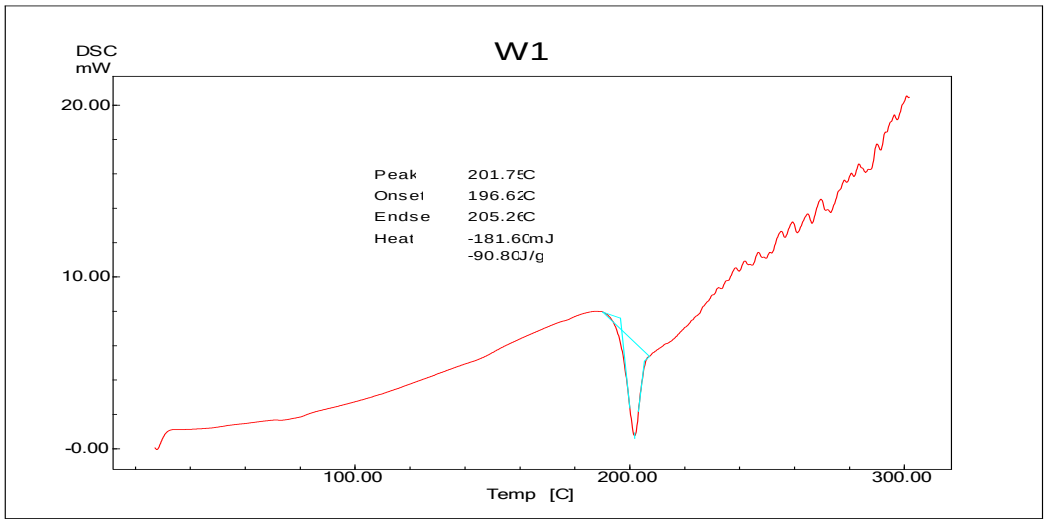


Figure 40: DSC thermogram of Carvedilol - oxalic acid (1:1)

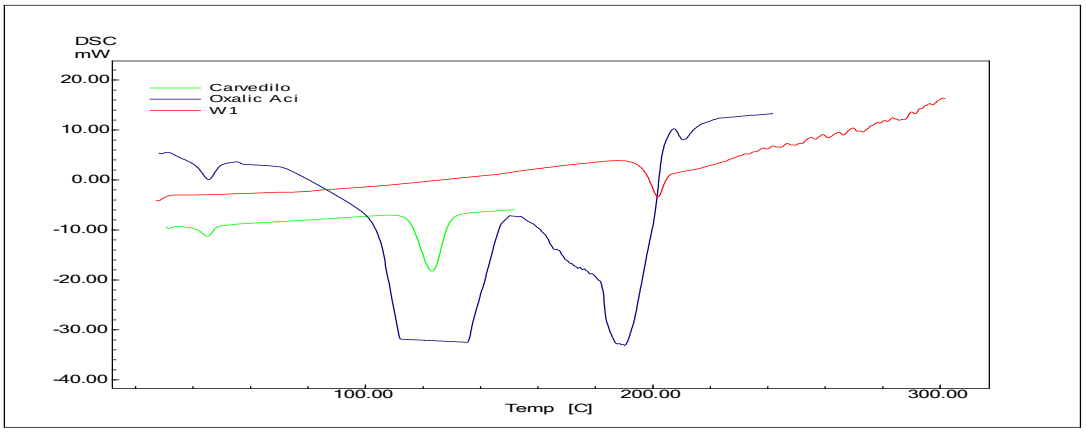


Figure 41: Overlay of DSC thermogram of Carvedilol and Carvedilol-oxalic acid

FTIR

Results of the FT-IR spectroscopy analysis of the drug and co-former interaction. The cocrystals of Carvedilol Phosphate showed certain extra peaks, which may be attributed to hydrogen bonds formed between drug and co-former.

Peaks at 3345.67 cm^{-1}, 2924.21 cm^{-1}, 1587.24 cm^{-1}, and 1257.64 cm^{-1} were noted in the infrared spectra of Carvedilol Phosphate (Figure 42). These peaks correspond to the combined stretching vibrations of N-H and O-H, C-H, N-H bending vibrations, and O-H bending and C-O stretching vibrations, respectively.

Typical IR peaks for vibrations of N-H stretching, O-H stretching, and C=O stretching was seen in the Nicotinamide spectra. Spectral analysis revealed a broadening and weakening of the characteristic band for N-H, which had previously been found at 3345.67 cm^{-1}, moving it to 3347.60 cm^{-1}. Unlike Carvedilol Phosphate, the cocrystal spectra did not include the bands at 1674.28 cm^{-1} and 2878.88 cm^{-1}. The weakening and disappearing of some peaks, further shifting of some peaks witness the possibilities of bond formation in cocrystals. (Figure 43-44)

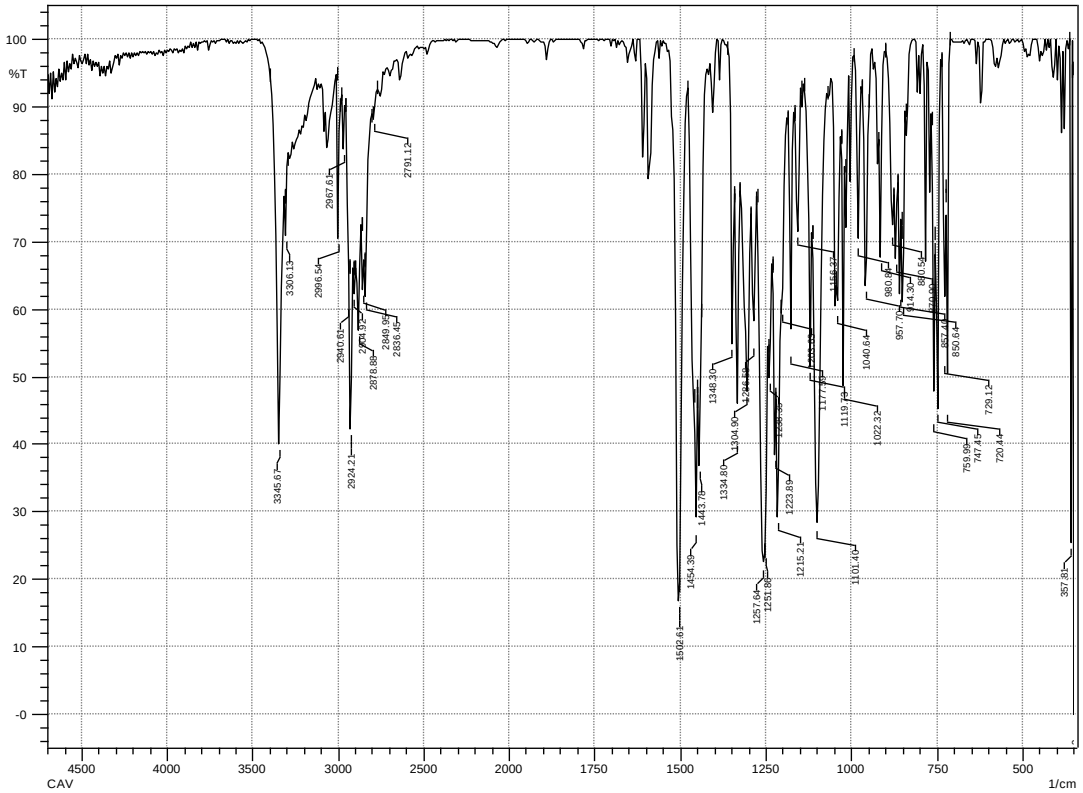


Figure 42: FT-IR spectra of Carvedilol Phosphate

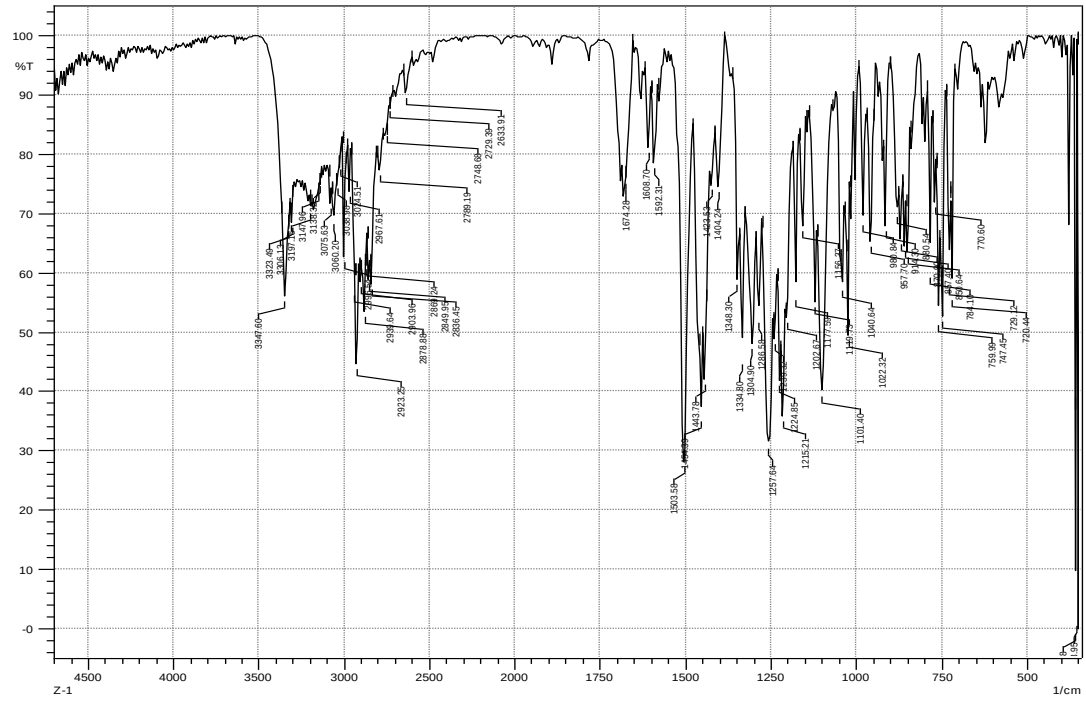


Figure 43: FTIR spectra of Carvedilol-nicotinamide cocrystals (Z1, 1:1)

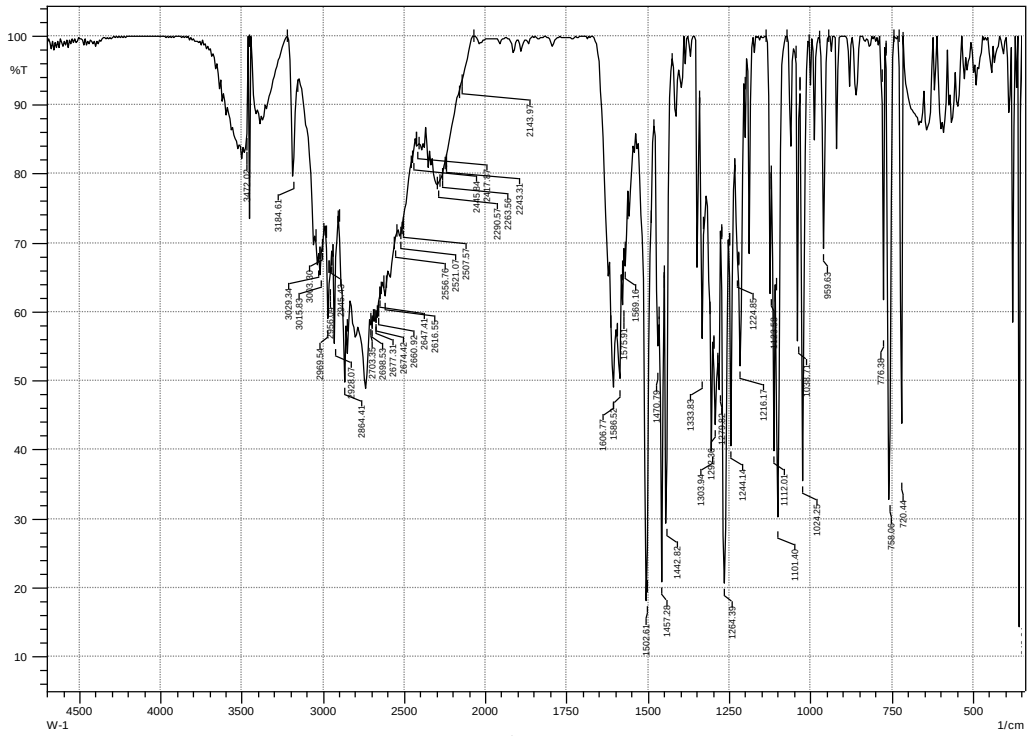


Figure 44: FTIR of Carvedilol-oxalic acid (W1, 1:1)

Powder X-Ray Diffraction

Powder X-Ray Diffraction (PXRD) pattern of pure Carvedilol Phosphate and its 1:1 cocrystals are shown in Figure 45. The sharp diffraction peak in PXRD pattern of both Carvedilol Phosphate, Carvedilol- nicotinamide (Z1) and Carvedilol-oxalic acid (W1) cocrystals prepared by USSC technique, confirms their crystalline nature. The difference in the 2Ɵ values between Carvedilol and cocrystals suggest the interaction between Carvedilol- nicotinamide (Z1) and Carvedilol-oxalic acid (W1). PXRD pattern of cocrystals exhibited distinct peak positions and intensity, indicating change in the internal structure due to interactions, which confirms the formation of cocrystals. The details of diffraction peak are shown in Table 27.

Table 27: Characteristic diffraction peaks of Carvedilol phosphate and its cocrystals

Sample ID	2Ɵ degree	d (angle)	Intensity
Carvedilol (CAV)	5.82, 11.64, 12.99, 14.83, 17.46, 18.43, 24.38, 26.18, 26.40	15.15, 7.59, 6.80, 5.96, 5.07, 4.80, 3.64, 3.40, 3.37	10917.1, 2674.91, 4906.17 7362.11, 9271.79 10472.45 4812.96, 3600.52, 3918.08
Carvedilol-nicotinamide (Z1)	5.99,11.86,13.17,14.98, 17.73,18.64, 24.49, 26.40, 27.51	14.73, 7.45, 6.71, 5.90, 4.99, 4.75, 3.63, 3.37, 3.23	5142.56, 2681.04, 4863.52 16769.77, 10520.81, 11435.1, 19547.37, 12749.87 6527.84
Carvedilol-oxalic acid (W1)	5.40, 11.62, 16.07, 16.69, 26.84, 26.94	16.33, 7.60, 5.50, 5.30, 3.31, 3.30	29690.78, 6807.66, 30867.35, 5721.69, 7494.02, 3892.35,

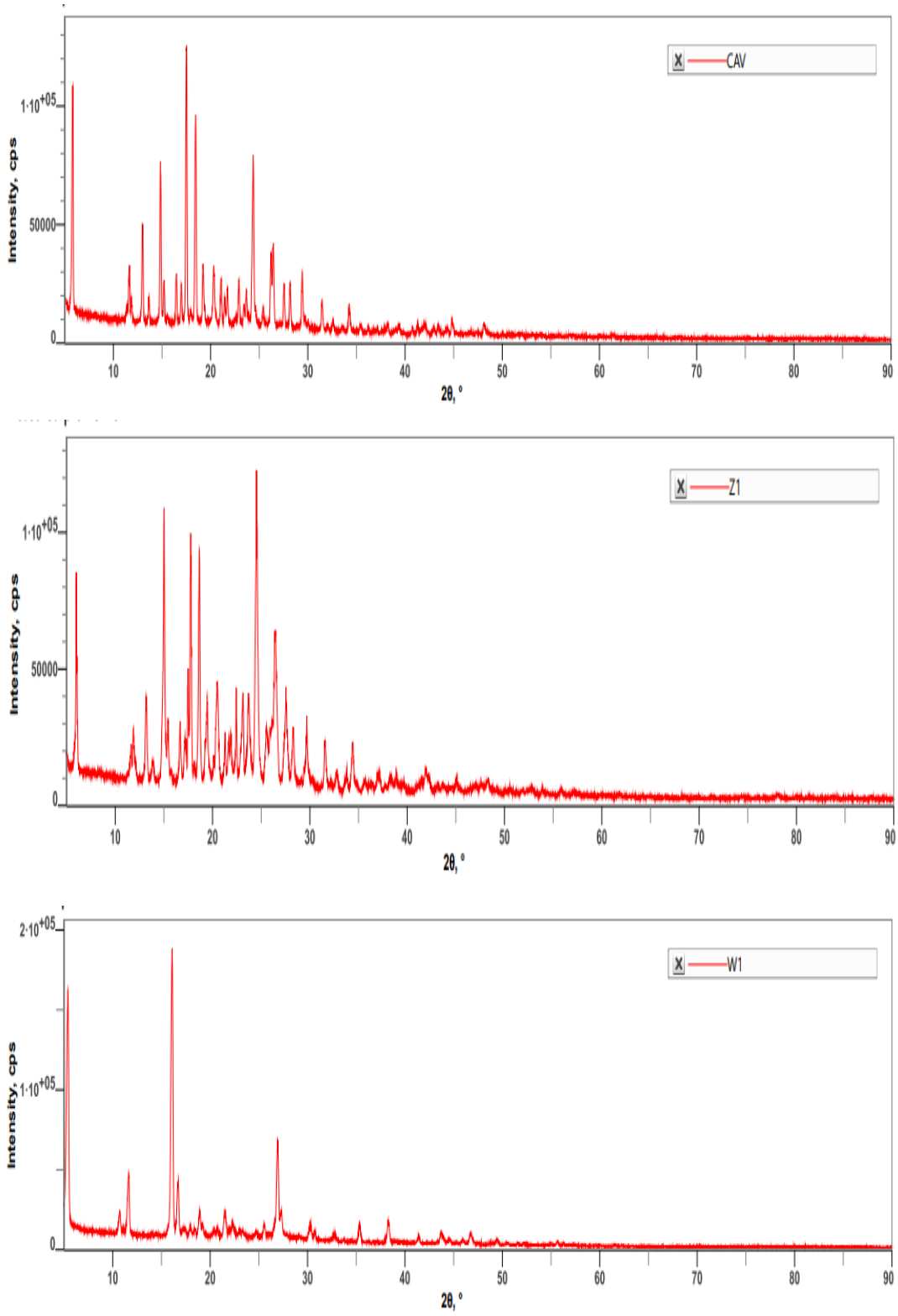


Figure 45: P-XRD diffraction patterns of Carvedilol phosphate, Carvedilol-nicotinamide (Z1, 1:1) and Carvedilol- oxalic acid (W1, 1:1) cocrystals

Scanning Electron Microscopy (SEM)

One crucial metric in the production of pharmaceuticals is the morphology of active medicinal ingredients. The physicomechanical properties of an API and its therapeutic activity are both affected by its crystal habits. Crystal habit differences between the drug and co-crystal were shown by SEM. SEM micrographs of Carvedilol (A), Carvedilol- oxalic acid (B, 1:1) and Carvedilol-nicotinamide (C, 1:1) cocrystals were utilized to analyze difference in their shape and surface morphology. Figure 46, shows that pure Carvedolol appears as irregular shaped single crystals. On the other hand, the SEM micrographs of cocrystals reveals short, rod-shape crystals with smooth surface.

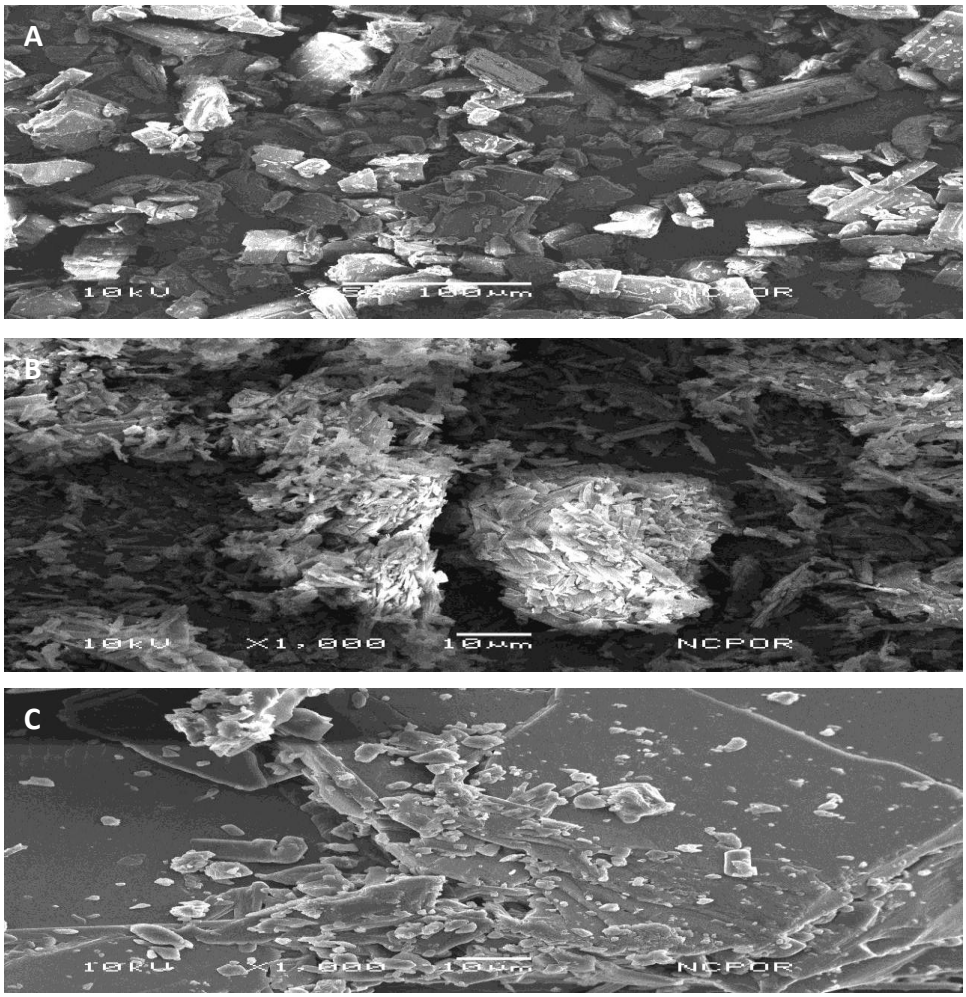


Figure 46: SEM micrographs of a A-Carvedilol pure drug, B- Carvedilol-oxalic acid (1:1) and C- Carvedilol-nicotinamide (1:1) co-crystal

Characterization of Multicomponent Polymorphs by Analytical Techniques for Nebivolol Hydrochloride

Differential scanning calorimetry (DSC)

DSC data acquired for both the plain nebivolol hydrochloride and its cocrystals. A sharp endothermic peak at 228.42°C was recorded for pure nebivolol hydrochloride (Figure 47). The co-crystals of nebivolol-salicylic acid (B1) displayed a sharp endothermic peak at 148.92 °C, which signifies a drop in the nebivolol hydrochloride melting point (Figure 48). This point is in the middle of the melting points of the two compounds, which confirms the formation of a new crystalline solid form. In case of nebivolol-nicotinamide co-crystals, the endothermic peak was observed at 125.62°C (D1, D2, & D3) which is lower than melting point of both nebivolol hydrochloride and nicotinamide (128–131 °C) which may confirm the formation of new crystalline solid form (Figure 50-52).

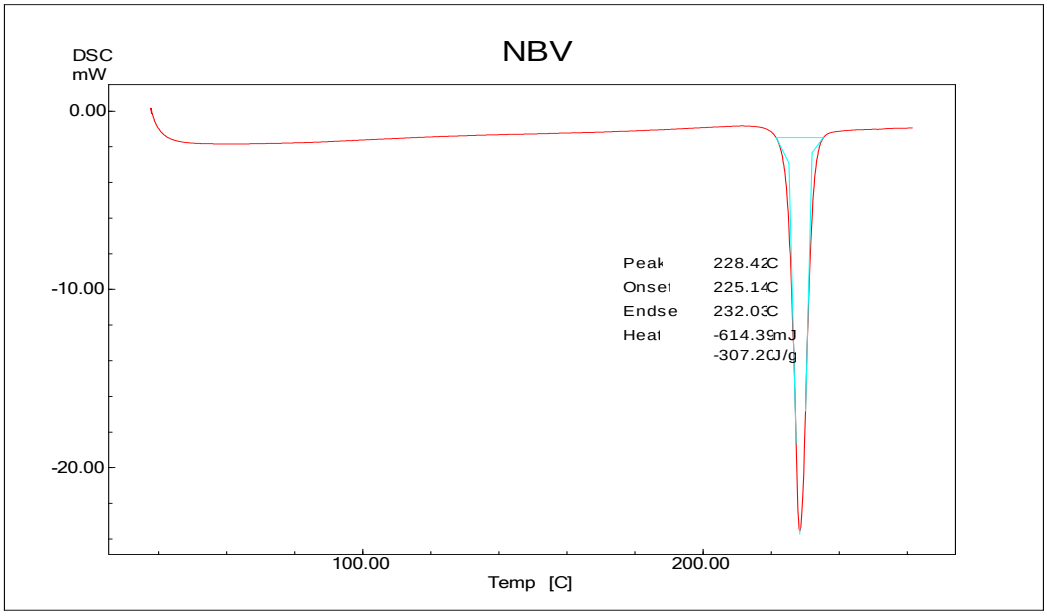


Figure 47: DSC thermogram of Nebivolol hydrochloride (API)

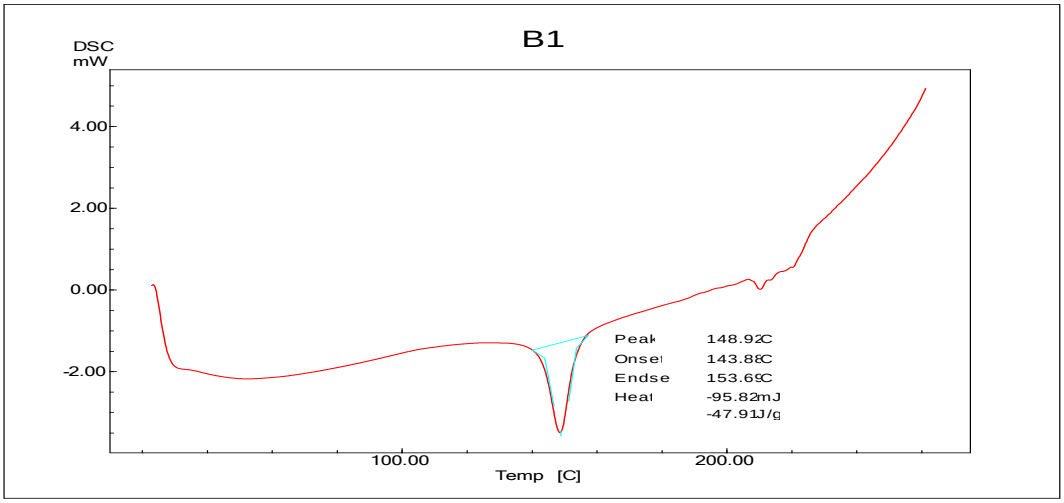


Figure 48: DSC thermogram of Nebivolol - salicylic acid (1:1)

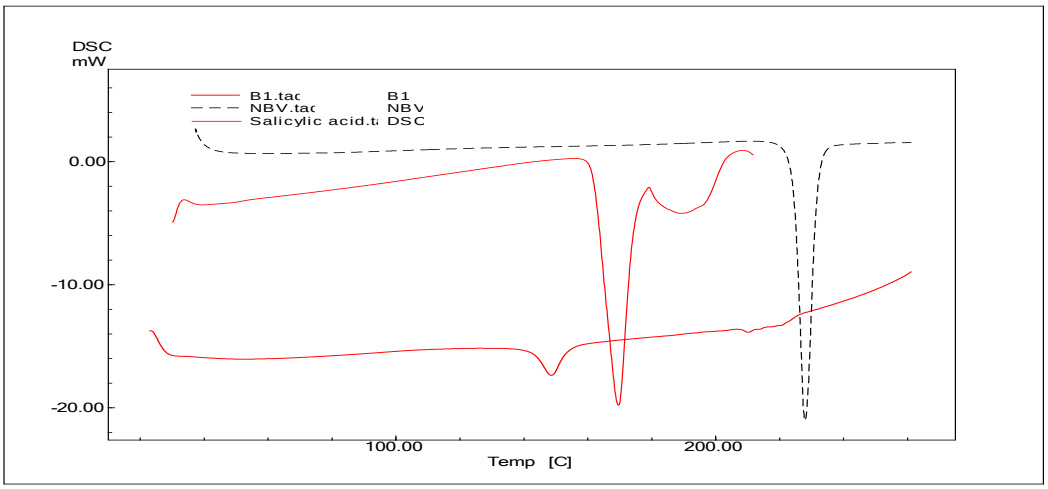


Figure 49: Overlay of DSC thermogram of Nebivolol - salicylic acid

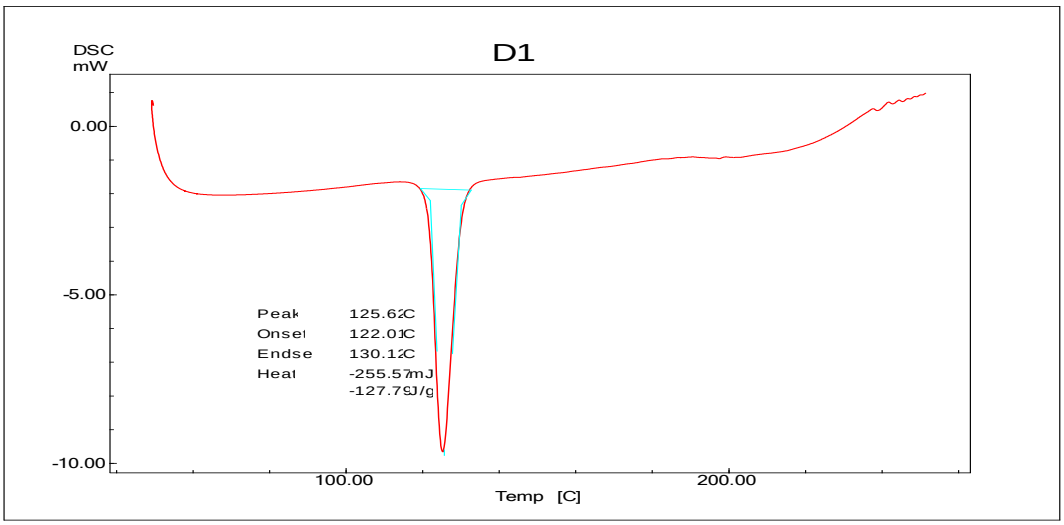


Figure 50: DSC thermogram of Nebivolol - nicotinamide (1:1)

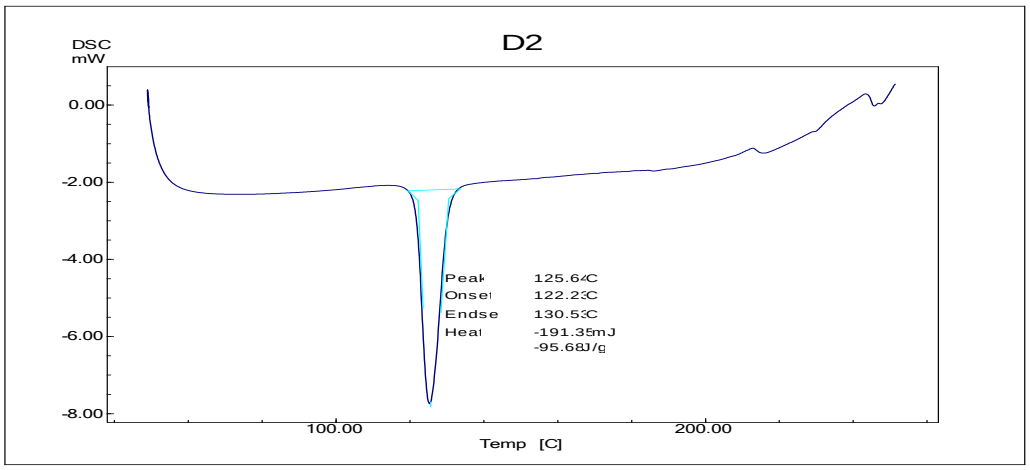


Figure 51: DSC thermogram of Nebivolol - nicotinamide (1:2)

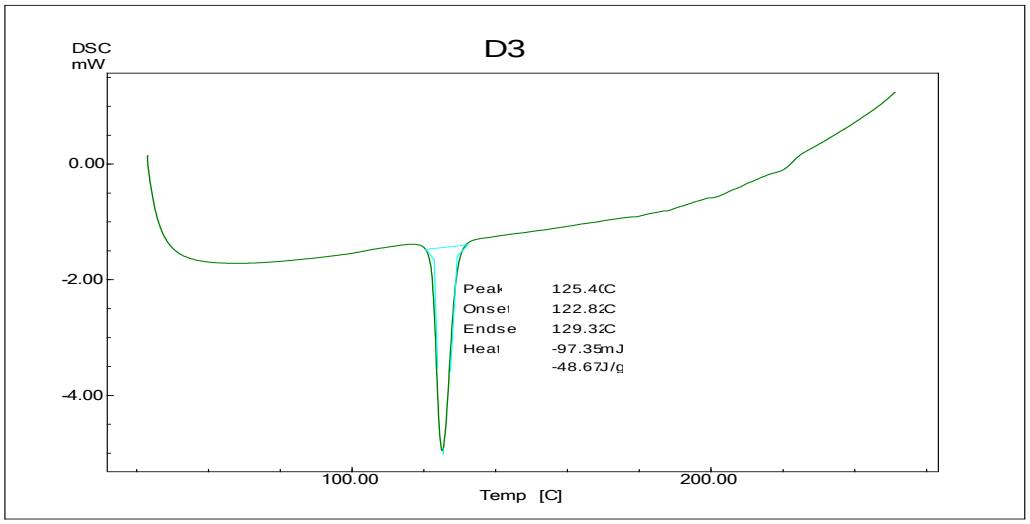


Figure 52: DSC thermogram of Nebivolol - nicotinamide (1:3)

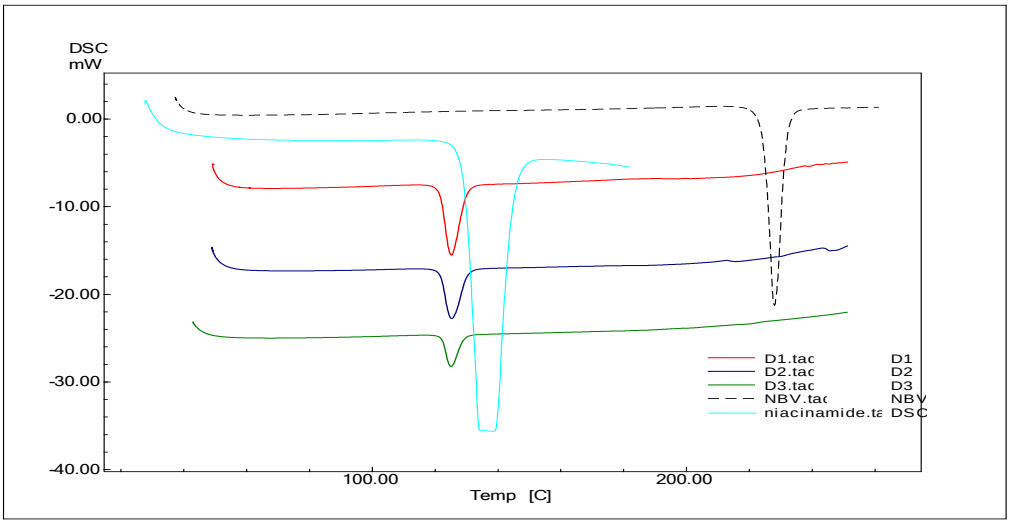


Figure 53: Overlay of DSC thermogram of Nebivolol - nicotinamide

Fourier-transform infrared spectroscopy (FTIR)

The use of Fourier transform infrared spectroscopy allowed for the recording and study of drug-cocrystal interactions. Various functional groups, such as C-F, C-X, N-H, O-H, C-C, and C-H stretching, are depicted as characteristic peaks in Figure 54 of the infrared spectra of nebivolol hydrochloride. Figure 55-58 displays the Fourier transform infrared spectra of pure nebivolol hydrochloride, nebivolol-salicylic acid cocrystals and nebivolol-nicotinamide. The presence of hydrogen bonding between the drug and co-former in nebivolol hydrochloride cocrystals is responsible for the observation of a few more peaks in addition to the drug's distinctive peaks. A peak in the range of 1700-1750 cm^{-1} was seen in the nebivolol - salicylic acid cocrystals (B1), which is attributed to the carboxylic acid moiety and signifies the existence of C=O stretching. The presence of the COOH functional group, which signifies the existence of C=O stretching which was found to cause an additional peak in the 1000-1300 cm^{-1} range. Figure 55 witnesses the hydrogen bonding which indicated by a peak in the 2400-3400 cm^{-1} stretch. The nebivolol-nicotinamide cocrystals (D1-D3) displayed N-H stretching at 3180 cm^{-1}, C-N stretching in the range of 1220-1050 cm^{-1}, and a peak at 1624 cm^{-1}, which indicate the C-N stretching of the amide. Figures 56-58 show that the C-X stretching of the halogen–hydrogen interaction is shown by peaks in the 400–800 cm^{-1} region in both cocrystals.

Nebivolol hydrochloride cocrystals FTIR study

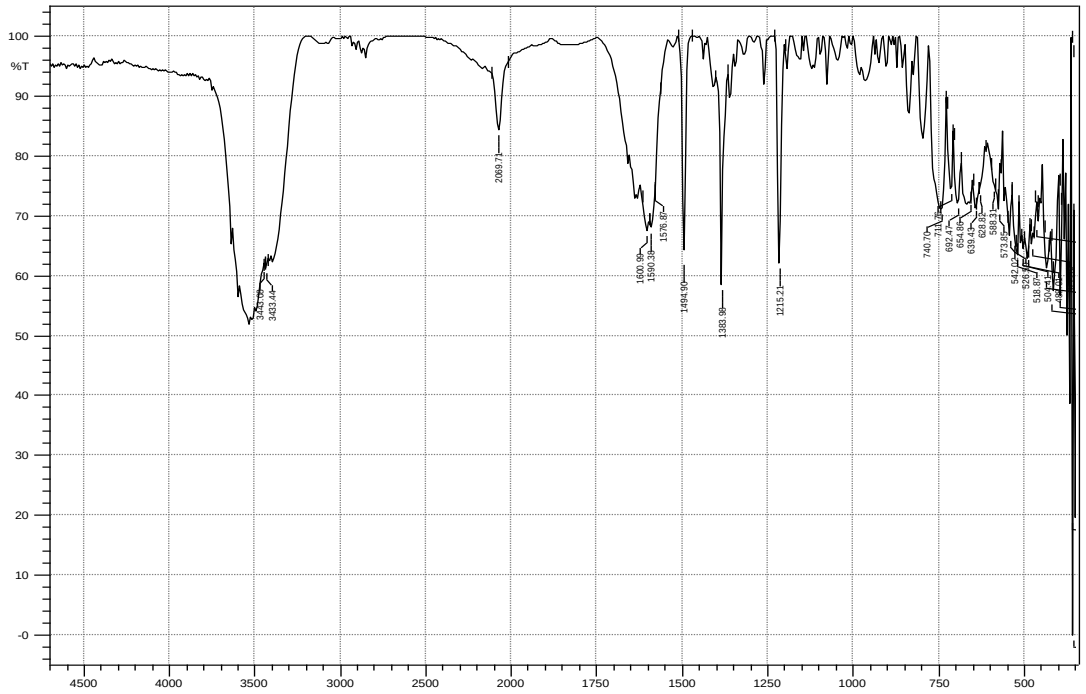


Figure 54: FTIR of Nebivolol hydrochloride

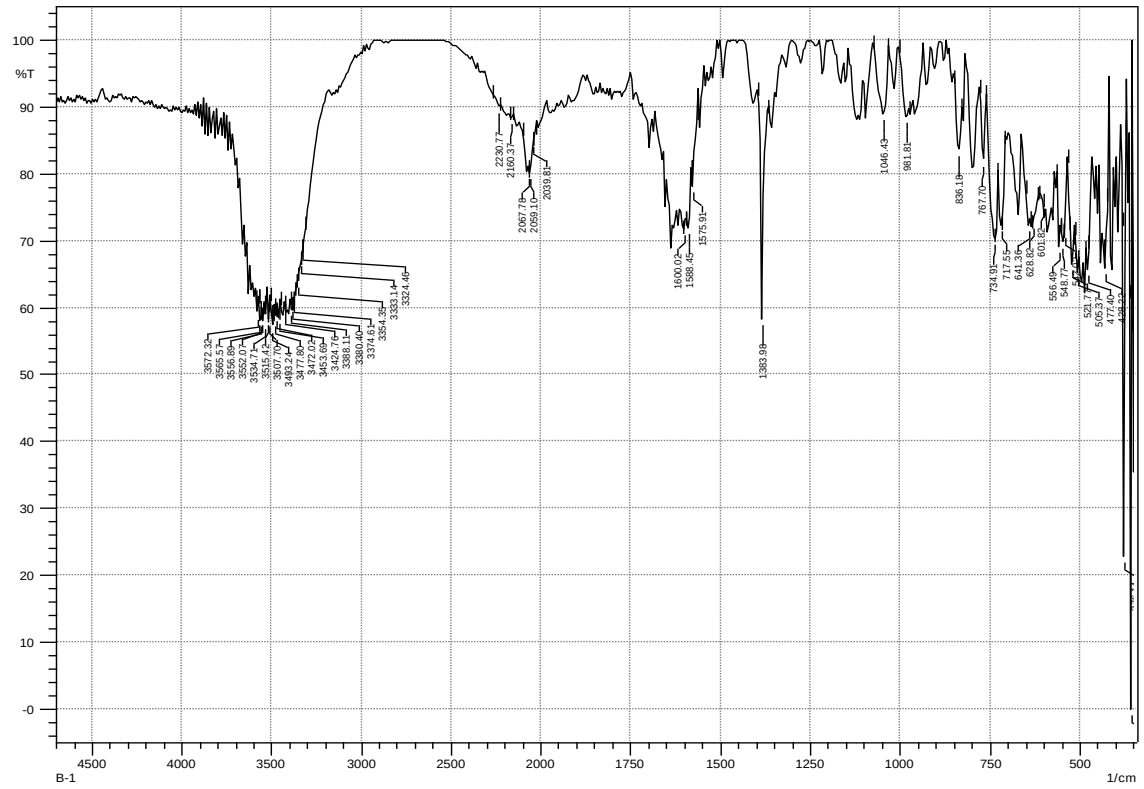


Figure 55: FTIR of Nebivolol - salicylic acid (1:1)

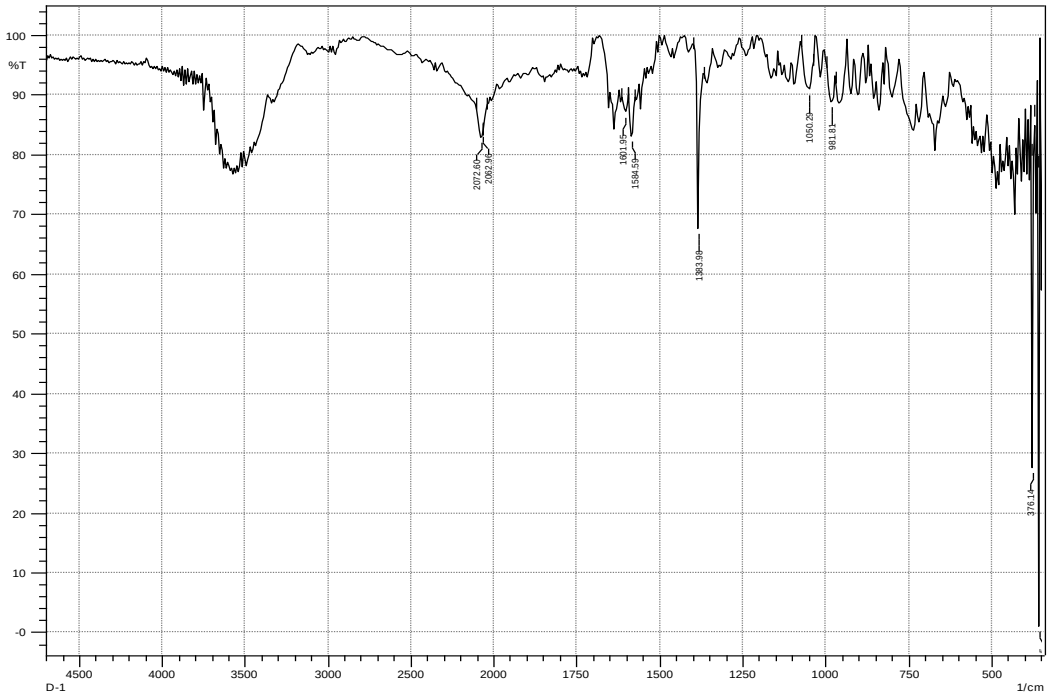


Figure 56: FTIR of Nebivolol - nicotinamide (1:1)

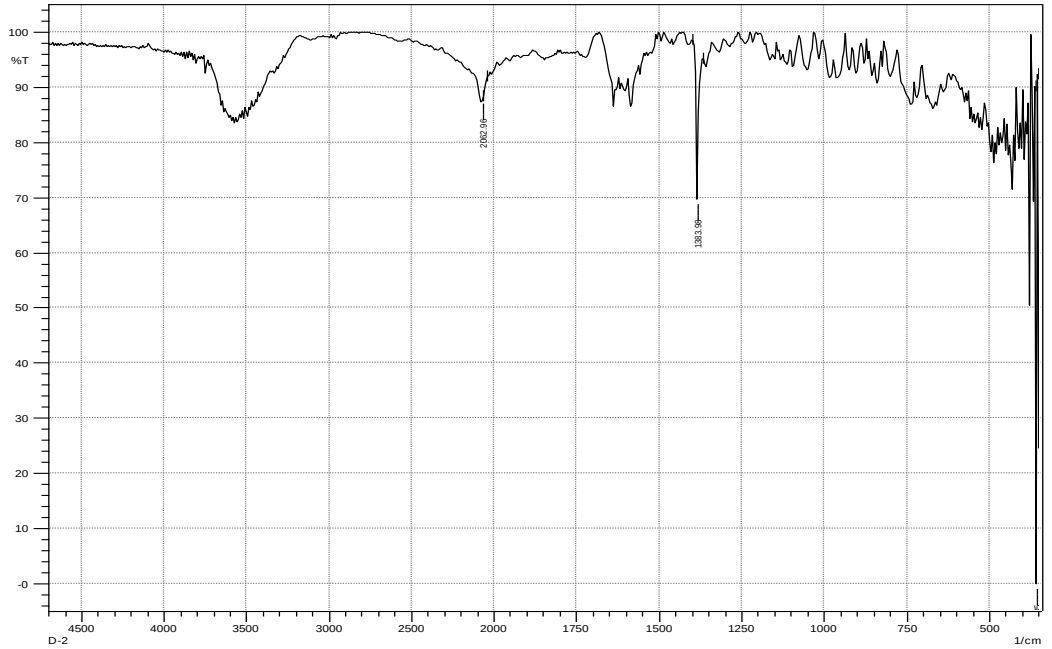


Figure 57: FTIR of Nebivolol - nicotinamide (1:2)

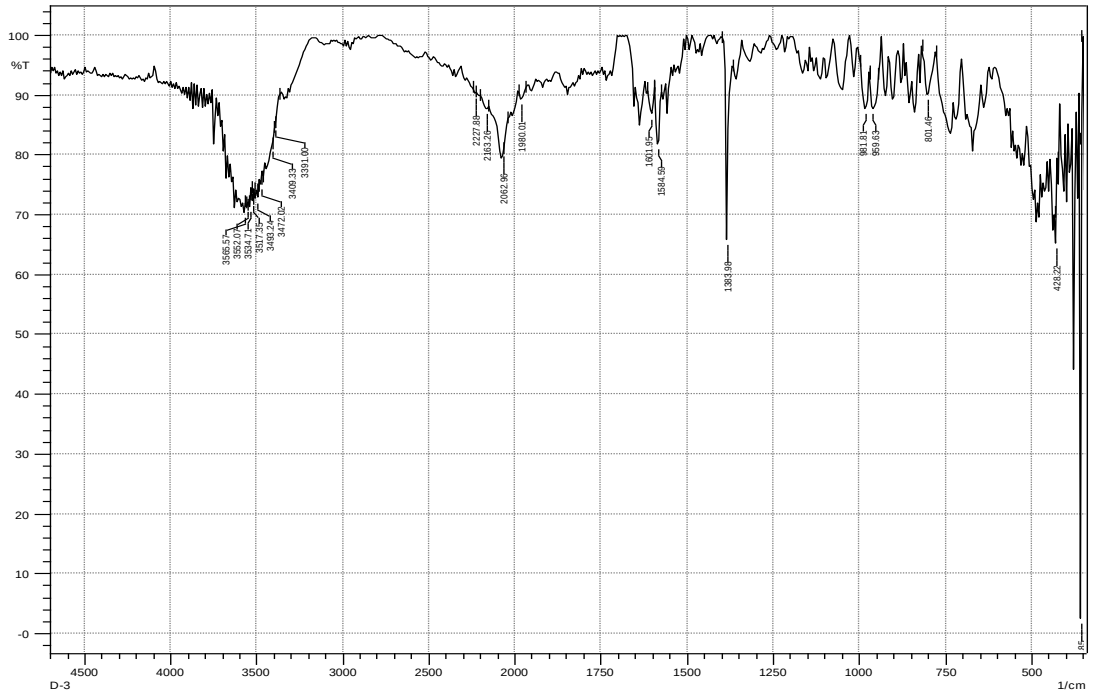


Figure 58: FTIR of Nebivolol - nicotinamide (1:3)

Powder X-Ray Diffraction

PXRD pattern of pure Nebivolol hydrochloride, Nebivolol-salicylic acid, and Nebivolol hydrochloride-nicotinamide, all prepared using the USSC method, have a prominent diffraction peak in the XRD pattern, proving that they are crystalline. The fact that the 2Ɵ values of Nebivolol hydrochloride and cocrystals are different indicates that Nebivolol hydrochloride, Nebivolol-salicylic acid (B1) and Nebivolol-nicotinamide (D1) interact with one other. The presence of clearly defined peaks and intensities in PXRD patterns of cocrystals provides further evidence that these solids have formed as a result of interactions. The specific information pertaining to diffraction peaks is as shown in Table 28 and Figure 59.

Table 28: Characteristic diffraction peaks of Nebivolol hydrochloride and its cocrystals

Sample ID	2Ɵ degree	d (angle)	Intensity
Nebivolol hydrochloride (NBV)	5.80, 11.54, 11.79, 13.24, 16.21, 17.27, 21.23, 22.31, 25.48, 31.64, 32.16	15.21,7.66, 7.49, 6.67, 5.46, 5.12, 4.18, 3.98, 3.49, 2.82, 2.78	1882.74,1992.54, 1689.87, 2532.62, 4354.51, 2830.56, 3542.97,4443.14, 6968.44, 1773.63, 1236.94
Nebivolol-salicylic acid (B1)	5.91,11.13,11.67, 16.36, 17.46, 22.36, 22.52, 25.41, 25.51	14.93, 7.94, 7.57, 5.41, 5.07, 3.97, 3.94, 3.50, 3.48,	1207.17, 1024.81, 1618.73, 2323.72, 6856.96,5165.3 1534.49, 820.17, 10329.64
Nebivolol - nicotinamide (D1)	5.84, 14.92, 16.29, 19.12, 21.29, 22.37, 25.41, 25.53	15.09, 5.92, 5.43, 4.63, 4.16, 3.97, 3.57, 3.50	469.56, 1384.32, 3091.77, 2235.31, 2573.45, 4225.56 2661.43, 1460.97

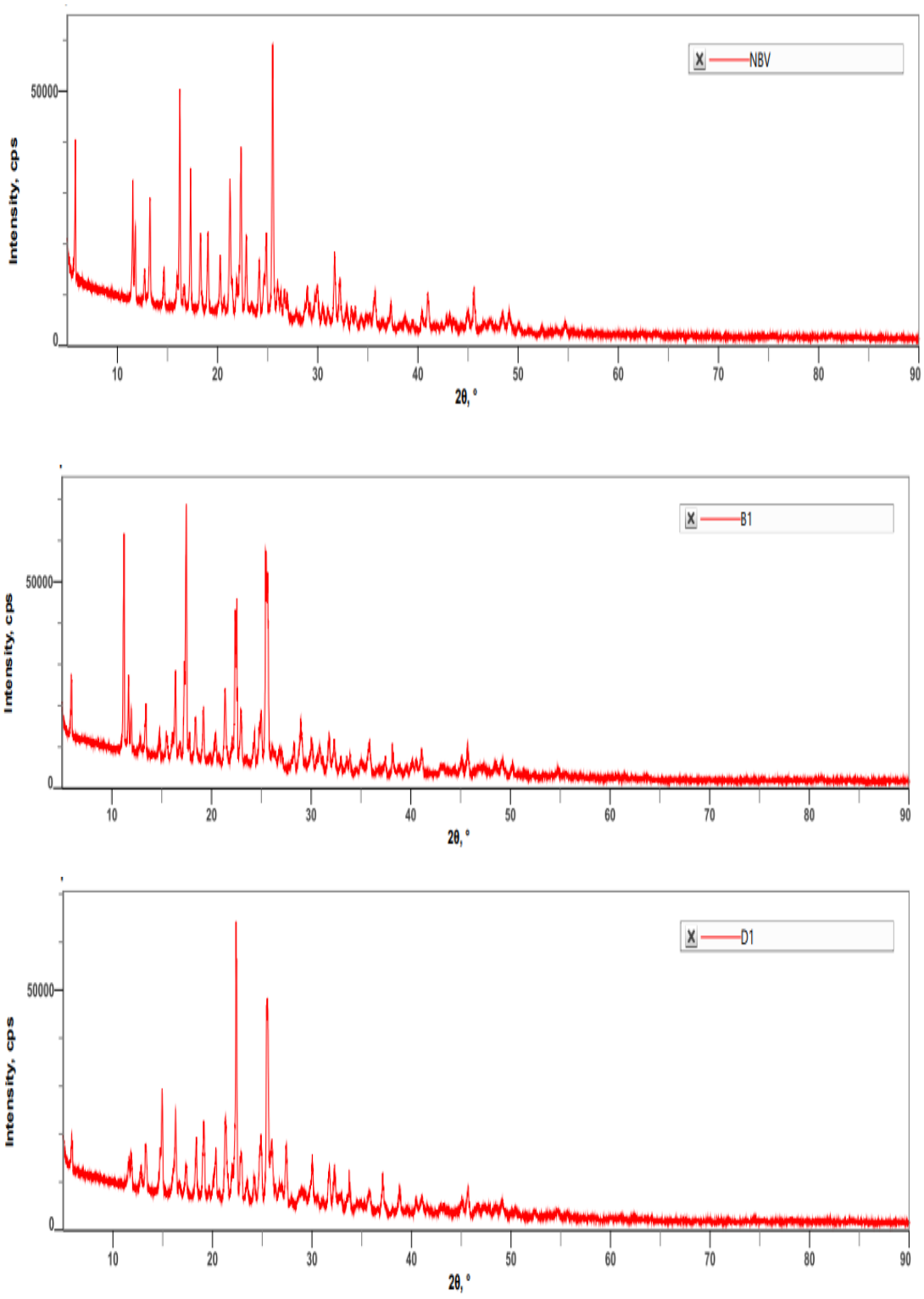


Figure 59: P-XRD diffraction patterns of Nebivolol hydrochloride (NBV), Nebivolol-salicylic acid (B1), Nebivolol-nicotinamide (D1)

Scanning Electron Microscope (SEM)

Crystal habit differences between the drug and co-crystal were shown by SEM (Figure 60). The Nebivolol-salicylic acid (B) and Nebivolol-nicotinamide (C) were characterized by short, irregularly shaped crystals and needle-shaped crystals, respectively, whereas the Nebivolol (A) had a spherical form and a smooth surface. A shift in crystal habits suggests the presence of co-crystals with new crystalline structures.

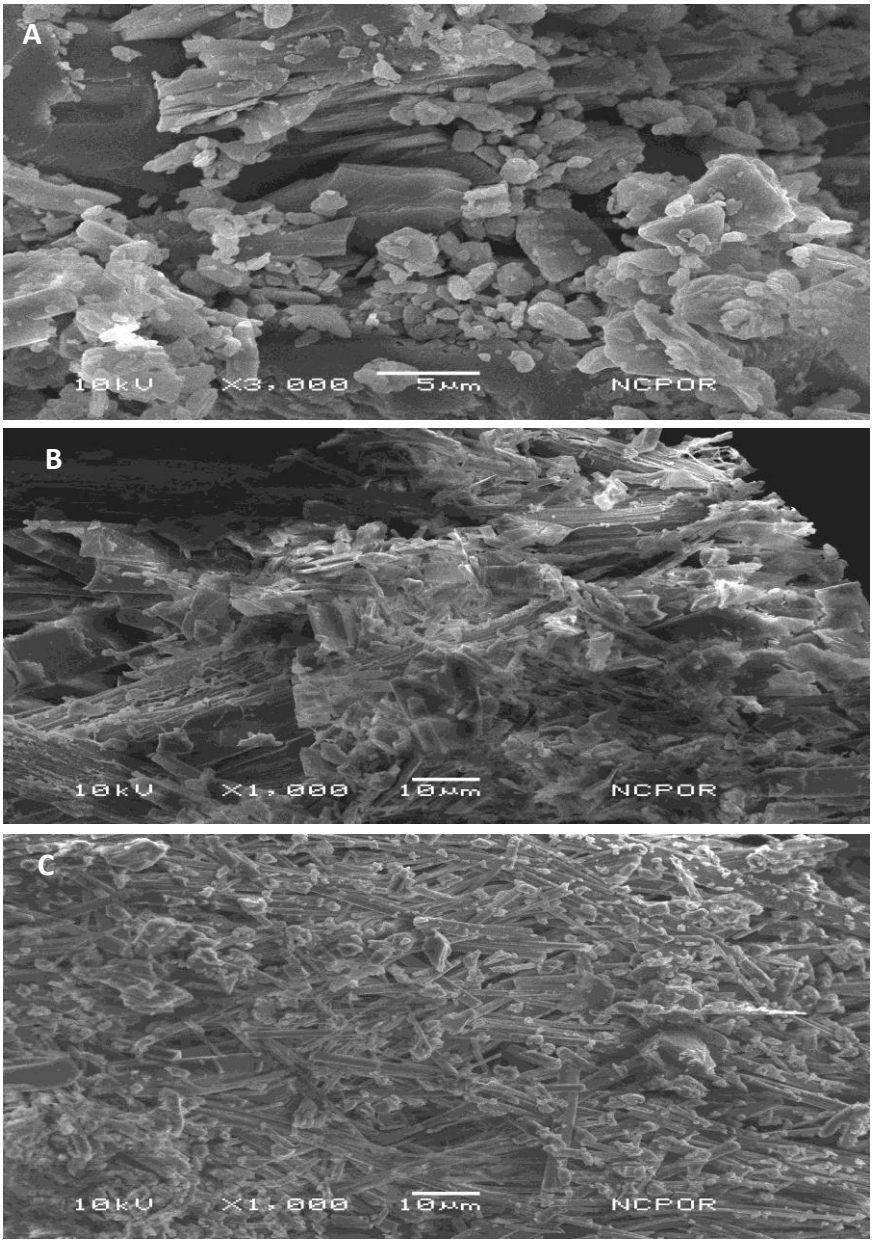


Figure 60: SEM micrographs of A-Nebivolol pure drug, B- Nebivolol-Salicylic acid and C- Nebivolol- Nicotinamide

5. SUMMARY

Pharmaceutical product development depends profoundly on comprehensive drug testing and analytical methodologies to ensure the safety, efficacy, and quality of medications. These processes, including techniques such as HPLC are integral across the drug development lifecycle, supporting task ranging from quality control to ensuring regulatory compliance.

Adoption of QbD principles alters traditional drug development by changing towards a proactive and organized approach. By identifying critical quality attributes and understanding the variability in manufacturing processes, QbD assures that quality is incorporated into products from the starts, improving their dependability and performance. The introduction of QbD to analytical process optimizes parameters and improves robustness, aligning with global regulatory standards. Additionally, insights into drug mechanisms, as illustrated in antihypertensive action, emphasize the importance of individualized therapeutic strategies to minimize side effects while maximizing efficacy. These approaches highlight the need of using innovative testing methodologies, quality frameworks, and regulatory rigor to advance pharmaceutical science and public health.

Summary of the RP-HPLC Method for Carvedilol Phosphate

In the present study, a robust RP-HPLC method for estimation of Carvedilol phosphate was developed and validated utilizing AQbD principles, which includes Taguchi design and BBD for optimization. The analysis was performed using a Shimadzu Prominence-i series LC-2030c 3D plus HPLC system which was equipped with a Shim-pack GIST C18 column (250 x 4.5 mm i.d., 5 µm) having guard column C18 (ODS;4x3.0mm i.d., Phenomenex, USA) and controlled using lab solutions

version 5.97 SP1 software. Chromatographic separation was carried out with a mobile phase comprising of methanol and 0.1% orthophosphoric acid (OPA) in a 65:35 (v/v) ratio, at a flow rate of 1 mL/min. Detection was performed at 240 nm, with an injection volume of 10 µL, maintaining thr column temperature at 30 °C. Method development involved evaluating various combinations of mobile and stationary phases to achieve optimal separation. Methanol was selected over acetonitrile due to its cost-effectiveness and efficiency. The final optimized conditions yielded a retention time of 4.18 minutes with excellent peak resolution. The validation of the developed method was conducted in accordance with regulatory guidelines, covering essential parameters including linearity, LOD, LOQ, system suitability, precision, accuracy and robustness studies. The method demonstrated linearity in the range of 5–30 µg/mL, with a correlation coefficient (R^2) of 0.999, confirming excellent linearity and reliability. The LOD and LOQ were calculated as 0.61 µg/mL and 1.86 µg/mL, respectively, using standard statistical approaches. System suitability was assessed by evaluating parameters such as peak area, retention time, theoretical plates, and tailing factor, all of which exhibited %RSD values < 2%, indicating high system performance. Precision studies revealed %RSD values of < 2% for intra- and inter-day analysis, confirming the reproducibility of the method. Accuracy was established through recovery studies using the standard addition method, with recoveries ranging between 99.41% and 99.82%. Robustness was evaluated by introducing minor variations in flow rate, mobile phase composition, and column temperature, with all %RSD values remaining below 2%, confirming method reliability under varied conditions. Additionally, the method was successfully applied to quantify Carvedilol in marketed tablets, demonstrating its suitability for routine quality control analysis. In conclusion, the developed and validated RP-HPLC method is reliable, precise, and

robust, making it a valuable tool for the estimation of carvedilol in pharmaceutical formulations.

Summary of the RP-HPLC Method for Nebivolol Hydrochloride

A reliable RP-HPLC method for the estimation of Nebivolol hydrochloride was developed and validated using an AQbD based approach and BBD optimization. The analysis was done on a Agilent 1100 (Autosampler) system which was equipped with a photodiode array (PDA) detector, using an Agilent C18 column (250 x 4.5 mm i.d., 5 µm particle size). Chromatographic separation was carried out with mobile phase comprising of methanol and 0.1% aqueous acetic acid in a 71: 29 (v/v) ratio, at a flow rate of 0.8 mL/min. The detection was performed at 282 nm, with an injection volume of 20 µL, column temperature maintained at 25 °C. Method development process involved combination of mobile and stationary phase to achieve optimal separation. Methanol was chosen over acetonitrile due to its cost-effectiveness and efficiency. The optimized conditions yielded a retention time of 4.19 minutes with excellent peak resolution. Validation of the developed method was conducted in accordance with regulatory guidelines, covering essential parameters including linearity, LOD, LOQ, system suitability, precision, accuracy and robustness studies. The method exhibited linearity in the range of 10-50 µg/mL, with a correlation coefficient (R^2) of 0.999, confirming excellent linearity and reliability. LOD and LOQ were determined to be 0.54 µg/mL and 1.64 µg/mL, respectively. System suitability was assessed by evaluating parameters such as peak area, retention time, theoretical plates, and tailing factor, all of which exhibited %RSD values <2%, indicating high system performance. Precision studies revealed %RSD values of <2% for intra- and inter-day analysis, confirming the reproducibility of the method. Accuracy was established through recovery studies using the standard addition method, with

recoveries ranging between 102.49% and 103.44%. Robustness was evaluated by introducing minor variations in flow rate, mobile phase composition, and column temperature, with all %RSD values remaining below 2%, ruggedness of the method was evaluated by analyzing the prepared samples with different analyst and % RSD values shows <2%, confirming method reliability under varied conditions. In addition, the method was successfully applied to quantify Nebivolol in marketed tablets, demonstrating its suitability for routine quality control analysis. In conclusion, the developed and validated RP-HPLC method proved to be reliable, precise, and robust, making it a valuable tool for the estimation of Nebivolol hydrochloride in pharmaceutical formulations.

Polymorphism study

The current research focuses on the crystalline product formation by the interaction of API (Carvedilol and Nebivolol) with different conformers. Specifically, crystals were prepared using Ultrasound-assisted Co-crystallization technique (Sonics Vibro Cell, Model: Ultrasound Probe Sonicator VC750) at a controlled temperature of 15-20°C in methanol. The work successfully identified and characterized (DSC, FTIR, SEM and P-XRD as analytical techniques) a variety of multicomponent polymorphic forms. While various crystal forms have been observed, Carvedilol-nicotinamide (CAV-NTA), Carvedilol-oxalic acid (CAV-OXA), nebivolol-salicylic acid (NBV-SA) and nebivolol-nicotinamide (NBV-NTA) were unique in their presentation as a two-component polymorphic system. Comparative assessment of selected polymorphic forms clearly witnesses their advantages and further scope for their formulation development in future.

6. CONCLUSION

In the proposed study, a method which can be employed for routine analysis of Carvedilol phosphate has been discovered using RP-HPLC method. The study was initiated with predefining the ATP. CAAs were selected, on the basis of ATP levels. The Taguchi design was exploited for screening of seven CMAs which were optimized using Box-Behnken Design. The interaction and major impacts of studied factors were further explored with 2D- contour plots and 3D-response surface plots. Numerical equations were generated using software to provide deeper insights into interaction effects. The newly developed techniques were further validated asper ICH principles, including linearity, system suitability, precision, LOD-LOQ, accuracy, and robustness. The designed and validated approach can detect and quantify carvedilol in pure and dosage form.

The developed and validated analytical method by QbD approach for the estimation of Nebivolol hydrochloride in pharmaceutical formulations exhibit excellent performance in terms of precision, reliability and robustness. The study utilized BBD for optimization. Different analytical parameters such as mobile phase, wavelength and flow rate were optimized The HPLC method for NBV, using methanol and aqueous acetic acid as solvent demonstrated remarkable linearity, LOD-LOQ, sensitivity, accuracy and robustness with minimal solvent interference.

The present study demonstrated that multicomponent polymorphic forms can be easily prepared by using Ultrasound-assisted Co-crystallization technique (Sonics Vibro Cell, Model: Ultrasound Probe Sonicator VC750) at a controlled temperature of 15-20°C in a suitable methanol. The formation of multicomponent polymorphs viz. Carvedilol-nicotinamide (CAV-NTA), Carvedilol-oxalic (CAV-OXA),

Nebivolol-salicylic acid (NBV-SA) and Nebivolol-nicotinamide (NBV-NTA) was confirmed through DSC, FTIR, P-XRD, and SEM. The obtained multicomponent polymorphs of Carvedilol and Nebivolol provides scope for development of new formulations with enhanced therapeutic performance.

7. BIBLIOGRAPHY

1. Jalalpure S.S, Kurangi B.K, Suryawanshi S.S. Quality Control and Standardization of Phytomedicines. 1st ed. Nirali Prakashan. 2021; ISBN 13: 9789354512704.
2. Ravisankar P, Gowthami S, Rao GD. A review on analytical method development. IJPBR. 2014;2(3):1183-95.
3. Kealey D and Hanies PJ. Analytical chemistry. Instant notes, ed. B.D. Haines. Leeds, UK: BIOS scientific publishers Ltd; 2002.
4. Ganorkar SB, Shirkhedkar AA. Design of experiments in liquid chromatography (HPLC) analysis of pharmaceuticals: Analytics, applications, implications and future prospects. Rev Anal Chem. 2017;36(3):27.
5. Siddiqui MR, Alothman ZA, Rahman N. Analytical techniques in pharmaceutical analysis: A review. Arab J Chem. 2017;10:S1409-21.
6. Valentina D, et al. Recent advances in chromatography for pharmaceutical analysis. Anal chem, 2018;91(1):210-39.
7. Kumar SD, Kumar DRH. Importance of RP-HPLC in analytical method development: a review. Int J Pharm Sci Res. 2012;3.12:4626
8. Snyder LR, Kirkland JJ, Dolan JW. Introduction to modern liquid chromatography. John Wiley & Sons; 2011 Sep 20.
9. Dos Santos Moreira C, Lourenco FR. Development and optimization of a stability-indicating chromatographic method for Verapamil hydrochloride and its impurities in tablets using an analytical quality by design (AQbD) approach. Microchem J. 2020;154(1):1-10.

10. Kalyane D, Raval N, Polaka S, Tekade RK. Quality by design as an emerging concept in the development of pharmaceuticals. The Future of Pharmaceutical Product Development and Research. Academic Press. 2020;(1):1-25.
11. Terzic J, Popovic I, Stajic A, Tumpa A, Jancic-Stojanovic B. Application of Analytical Quality by Design concept for bilastine and its degradation impurities determination by hydrophilic interaction liquid chromatographic method. J Pharm Biomed Anal. 2016;125(5):385-93.
12. Grangeia HB, Silva C, Simoes SP, Reis MS. Quality by design in pharmaceutical manufacturing: A systematic review of current status, challenges and future perspectives. Eur J Pharm Biopharm. 2020;147(1):19-37.
13. Reid GL, Morgado J, Barnett K, Harrington B, Wang J, Harwood J, Fortin D. Analytical quality by design (AQbD) in pharmaceutical development. Am Pharm Rev. 2013;144191.
14. Peraman R, Bhandraya K, Reddy YP. Analytical quality by design: A tool for regulatory flexibility and robust analytics. Int J Anal Chem. 2015;1-9.
15. Dewi MK, et al. Quality by Design: Approach to Analytical Method Validation. Pharm Sci. 2022;1(1):38-46.
16. Kumari N. et al. Quality by design: a systematic approach for the analytical method validation. J Drug Deliv Ther. 2019; 9(3-s):1006-12.
17. Raman NV, Mallu UR, Bapatu HR. Analytical quality by design approach to test method development and validation in drug substance manufacturing. J chem. 2015;2015(1):1-8.
18. Karpinski PH. Polymorphism of active pharmaceutical ingredients. Chem Eng Technol. 2006;29(2):233-7.
19. Bernstein J. Polymorphism in molecular crystals. Clarendon Press. Oxford 2002.

20. Brog JP, Chanez CL, Crochet A, Fromm KM. Polymorphism, what it is and how to identify it: a systematic review. RSC Adv. 2013;3(38):16905-31.
21. Surov AO, Vasilev NA, Churakov AV, Stroh J, Emmerling F, Perlovich GL. Solid forms of ciprofloxacin salicylate: polymorphism, formation pathways, and thermodynamic stability. Cryst Growth Des. 2019;19(5):2979-90.
22. Jendrzejewska I, Goryczka T, Pietrasik E, Klimontko J, Jampilek J. X-ray and thermal analysis of selected drugs containing acetaminophen. Molecules. 2020;25(24):1-24.
23. Panzade PS, Shendarkar GR, Kulkarni DA. Hot melt extrusion: an emerging green technique for the synthesis of high-quality pharmaceutical cocrystals. J Pharm Innov. 2022;17(2):283-93.
24. Viciosa MT, Ramos JJ, Diogo HP. Thermal behavior and molecular mobility studies in the supercooled liquid and glassy states of carvedilol and loratadine. Int J Pharm. 2020;584:119410.
25. Aldridge S. The shape shifter. Chemistry World 2007: 64-70. Available from www.chemistryworld.org
26. Purohit R, Venugopalan P. Polymorphism: an overview. Resonance. 2009;14: 882-93.
27. Bernstein J, Colman P, Helliwell JR, Kantardjieff KA, Mak T, et al., IUCr Monographs on Crystallography. Polymorphism in Molecular Crystals. 2002.
28. Brittain HG. Polymorphism in pharmaceutical solids. Drugs and the pharmaceutical sciences. 1999;95:183-226.
29. Bernstein J, Hagler AT. Conformational polymorphism. The influence of crystal structure on molecular conformation. J Am Chem Soc.1978; 100(3): 1973-80.
30. Harris KD, Xu M. Combined analysis of NMR & powder diffraction data. NMR Crystallography. Wiley. 2009:275-86.

31. Levy GC, Lichter RL, Nelson GL. Carbon-13 nuclear magnetic resonance spectroscopy. John Wiley and sons, New York; 1980.
32. Strohmeier M, Orendt AM, Alderman DW, Grant DM. Investigation of the polymorphs of dimethyl-3,6-dichloro-2,5-dihydroxyterephthalate by 13C solid state NMR. J Am Chem Soc. 2001;123:1713-22.
33. Jarari N, Rao N, Peela JR, Ellafi KA, Shakila S, Said AR, Nelapalli NK, Min Y, Tun KD, Jamallulail SI, Rawal AK. A review on prescribing patterns of antihypertensive drugs. Clin Hypertens. 2015;22:1-8.
34. Jobe M, Mactaggart I, Hydara A, Kim MJ, Bell S, Badjie O, Bittaye M, Perel P, Prentice AM, Burton MJ. Evaluating the hypertension care cascade in middle-aged and older adults in The Gambia: findings from a nationwide survey. E Clinical Medicine. 2023;64:1-8.
35. Drug profile of Carvedilol https://pubchem.ncbi.nlm.nih.gov/compound/9827459
36. Adib N, Shekarchi M. A New HPLC Method for Determination of Carvedilol in Human Plasma and its Application in Bioequivalence Studies. Biosci Biotech Res Asia. 2015;12(1):387-91.
37. Rao UV, Haritha G, Krishnaiah T, Saikiran TG, Reddy TA, Nikhil US, Veerabomma HS, Veeram SD. Method development and forced degradation studies of carvedilol by RP-HPLC. Int J Pharm Anal Res. 2017;6:457-68.
38. Drug profile of Nebivolol https://pubchem.ncbi.nlm.nih.gov/compound/24866733
39. Yilmaz B. Reverse phase HPLC method for determination of nebivolol in pharmaceutical preparations. Int. J Pharm Sci Rev Res. 2010;1(2):14-7.
40. Kokil SU, Bhatia MS. Simultaneous estimation of nebivolol hydrochloride and valsartan using RP HPLC. Indian J Pharm Sci. 2009;71(2):111.

41. Cem ON, Kadir AL, Çigdem AL, Armagan ON. High-performance Liquid Chromatography Analysis of Nebivolol and Amlodipine and Some Related Compounds in Synthetic Mixture. Bezmialem Science 2021;9(3):264-70
42. Deshmukh SA, Vanjari SS, Patil RB, Radheshyam KK. Application of design of experiment based innovative approach in method development and validation of RP-HPLC for estimation of azilsartan in bulk and pharmaceutical tablet dosage form. Indian J Pharm Educ Res. 2020;54(3):657-6.
43. Beg S, Sharma G, Katare OP, Lohan S, Singh B. Development and validation of a stability-indicating liquid chromatographic method for estimating olmesartan medoxomil using quality by design. J Chromatogr Sci. 2015;53(7):1048-59.
44. Patel KY, Dedania ZR, Dedania RR, Patel U. QbD approach to HPLC method development and validation of ceftriaxone sodium. Futur J Pharm Sci. 2021;7:1-0.
45. Prajapati PB, Thakor MA, Bodiwala KB, Shah SA. Quality risk management-based AQbD approach to development of VEER chromatography method for the estimation of multiple combined formulations of anti-hypertensive drugs. J AOAC Int. 2021;104(3):605-19.
46. Palakurthi AK, Dongala T, Katakam LN. QbD based development of HPLC method for simultaneous quantification of Telmisartan and Hydrochlorothiazide impurities in tablets dosage form. Pract Lab Med. 2020;21:1-11.
47. Suryawanshi SS, Palled MS. Box-Behnken design assisted optimization and standardization of chromatographic methodology for quality assessment of metformin: analytical quality by design avenue. Indian J Pharm Educ Res. 2022 ;56(2s):s152-62.
48. Haque MA, Bakshi V, Thippani M, Manda RM, Boggula N. Development and Validation of UV Spectrophotometric method for the determination of

Dolutegravir by using Quality by Design (QbD) Approach. J Adv Sci Res.2021;12(03 Suppl 1):113-9.

49. Alruwaili NK. Analytical Quality by Design Approach of Reverse-Phase High-Performance Liquid Chromatography of Atorvastatin: Method Development, Optimization, Validation, and the Stability-Indicated Method. Int J Anal Chem. 2021;2021(1):1-12.
50. Thakur D, Kaur A, Sharma S. Application of QbD based approach in method development of RP-HPLC for simultaneous estimation of antidiabetic drugs in pharmaceutical dosage form. J Pharm Investig. 2017;47(3):229-39.
51. Ganorkar SB, Dhumal DM, Shirkhedkar AA. Development and validation of simple RP-HPLC-PDA analytical protocol for zileuton assisted with design of experiments for robustness determination. Arab J Chem. 2017;10(2):273-82.
52. Patil TS, Deshpande AS. Development of an innovative quality by design (QbD) based stability-indicating HPLC method and its validation for clofazimine from its bulk and pharmaceutical dosage forms. Chromatographia. 2019;82(2):579-90.
53. Mandpe SR, Parate VR, Naik JB. Experimental design approach, screening and optimization of system variables, analytical method development of flurbiprofen in nanoparticles and stability-indicating methods for high-pressure liquid chromatography. Futur J Pharm Sci. 2022;8(1):38.
54. Patel BA, Parmar SJ, Patel JB, Chauhan GR, Captain AD. Development and validation of chromatographic determination of carvedilol phosphate in bulk and pharmaceutical dosage form using fluorescence detector. Int J Pharm Sci Res. 2012;3(11):4404.
55. Rahman A, Haque MR, Sultan MZ, Rahman MM, Rashid MA. Enantiomeric determination of carvedilol by a newly developed and validated chiral HPLC method. Dhaka Univ J Pharm Sci. 2019;18(1):61-8.

56. Yıldırım S, Erkmen C, Uslu B. Novel trends in analytical methods for β-blockers: An overview of applications in the last decade. Crit Rev Anal Chem. 2022;52(1):131-69.
57. Fadhil AK, Hassan MJ, Rasheed AS. A comparative review of methods for estimation of some antihypertensive drugs in pharmaceutical production. Egypt J Chem. 2021;64(11):6301-21.
58. Raju TR. Development and validation of stability-indicating impurity profiling method for the carvedilol in API and pharmaceutical formulation. Int J Pharmacol Pharm Sci. 2015;2(6):22-31.
59. Bechara V, Subrahmanyam EV, Shabaraya R. New analytical methods and their validation for the estimation of carvedilol in bulk and marketed formulation. Int J Pharm Sci Res. 2015;6(2):421-4.
60. Awotwe-Otoo D, Agarabi C, Faustino PJ, Habib MJ, Lee S, Khan MA, Shah RB. Application of quality by design elements for the development and optimization of an analytical method for protamine sulfate. J Pharm Biomed Anal. 2012;62: 61-7.
61. Shah DA, Bhatt KK, Mehta RS, Baldania SL, Gandhi TR. Stability indicating RP-HPLC estimation of nebivolol hydrochloride in pharmaceutical formulations. Indian J Pharm Sci. 2008;70(5):591.
62. Sahoo MK, Giri RK, Barik CS, Kanungo SK, Kumar BR. RP-HPLC Method for the Estimation of Nebivolol in Tablet Dosage Form. J Chem. 2009;6(3):915-9.
63. Patel LJ, Suhagia BN, Shah PB. RP-HPLC and HPTLC Methods for the Estimation of Nebivolol hydrochloride in Tablet Dosage form. Indian J Pharm Sci. 2007;69(4):594.

64. Szabo ZI, Szabo T, EMŐKE R, Sipos E. Validated HPLC method for determination of nebivolol in pharmaceutical dosage form and in vitro dissolution studies. Stud Univ Babes-Bolyai Chem. 2014;59(4):195-203.
65. Kachhadia PK, Doshi AS, Joshi HS. Development and validation of a stability-indicating column high-performance liquid chromatographic assay method for determination of nebivolol in tablet formulation. J AOAC Int. 2008;91(3):557-61.
66. Reddy CA, Zeeyauddin K, Rajendra Y. Method Development and Validation for Nebivolol and Valsartan by RP-HPLC Method. Eur J Med Sci. 2022;9(1): 138-43.
67. Nekkala K, Shanmukhkumar J, Ramchandran D, Ramanaiah G, Srinivas G. Method Development and Validation of Stability Indicating RP-HPLC Method for Simultaneous Estimation of Nebivolol HCL and Valsartan in Bulk and its Pharmaceutical Formulations. Am J Adv Drug Deliv. 2014;2(5):624-637.
68. Ambhore J, Bangale G, Chandak M. Development of Stability Indicating Assay Method for Estimation of Nebivolol and Hydrochlorothiazide in Tablet Dosage Form. Austin J Anal Pharm Chem. 2018;5(1):1-9.
69. Annadi A, Shoheib RE, Mohamed A. Assessments of Valsartan in the presence of Nebivolol or Amlodipine in solid formulations and its discriminative dissolution behavior via RP-HPLC and RP-UPLC methods. Egypt J Chem. 2020;63(8): 2837-51.
70. Surov AO, Vasilev NA, Churakov AV, Stroh J, Emmerling F, Perlovich GL. Solid forms of ciprofloxacin salicylate: polymorphism, formation pathways, and thermodynamic stability. Cryst Growth Des. 2019;19(5):2979-90.
71. Fernandes GJ, Kumar L, Sharma K, Tunge R, Rathnanand M. A review on solubility enhancement of carvedilol a BCS class II drug. J Pharm Innov. 2018;13:197-212.

72. Barikah KZ. Traditional and Novel Methods for Cocrystal Formation: A Mini Review. Syst Rev Pharm. 2018;9(1):79.

73. Seevakan K, Bharanidharan S. Different Types of Crystal Growth Methods. Int J Pure Appl Math. 2018;119(12):5743-58.

74. Hiendrawan S, Widjojokusumo E, Veriansyah B, Tjandrawinata RR. Pharmaceutical salts of carvedilol: polymorphism and physicochemical properties. Aaps Pharmscitech. 2017;18(4):1417-25.

75. Aher S, Dhumal R, Mahadik K, Paradkar A, York P. Ultrasound assisted co-crystallization from solution (USSC) containing a non-congruently soluble cocrystal component pair: Caffeine/maleic acid. Eur J Pharm Sci. 2010;41(5): 597-602.

76. Patil TS, Deshpande AS. Development of an innovative quality by design (QbD) based stability-indicating HPLC method and its validation for clofazimine from its bulk and pharmaceutical dosage forms. Chromatographia. 2019;82(2):579-90.

77. Saini S, Sharma T, Patel A, Kaur R, Tripathi SK, Katare OP, Singh B. QbD-steered development and validation of an RP-HPLC method for quantification of ferulic acid: Rational application of chemometric tools. J Chromatogr B. 2020;1155:122300.

78. Beg S, Sharma G, Katare OP, Lohan S, Singh B. Development and validation of a stability-indicating liquid chromatographic method for estimating Olmesartan medoxomil using quality by design. J Chromatogr Sci. 2015;53(7):1048-59.

79. Saha P, Pandey MM. Design of experiment (DoE)-approach based RP-HPLC analytical method development and validation for estimation of efavirenz in bulk and formulations. J Chromatogr Sci. 2022;60(1):35-44.

80. Guideline IH. Validation of analytical procedures: text and methodology. Q2 (R1). 2005 Nov;1(20):05.
81. Bandopadhyay S, Beg S, Katare OP, Sharma T, Singh B. Integrated analytical quality by design (AQbD) approach for the development and validation of bioanalytical liquid chromatography method for estimation of valsartan. J Chromatogr Sci. 2020;58(7):606-21.
82. Bandi S, Adikay S. AQbD-based stability indicating development and validation of azilsartan medoxomil and cilnidipine by UPLC method. JAPS. 2023;13(5): 095-106.
83. Nikam VJ, Patil SB. Pharmaceutical cocrystals of nebivolol hydrochloride with enhanced solubility. J Cryst Growth. 2020; 534:125488.
84. Shilpa C, Sarika N, Neetu K, Shubham W. Co-Crystals: A Review. J Drug Deliv Ther. 2018; 8(6-s):350-8
85. Fernandes GJ, Rathnanand M, Kulkarni V. Mechanochemical synthesis of carvedilol cocrystals utilizing hot melt extrusion technology. J Pharm Innov. 2019;14:373-81.
86. Aher S, Dhumal R, Mahadik K, Paradkar A, York P. Ultrasound assisted cocrystallization from solution (USSC) containing a non-congruently soluble cocrystal component pair: Caffeine/maleic acid. Eur J Pharm Sci. 2010;41(5):597-602.
87. Shayanfar A, Asadpour-Zeynali K, Jouyban A. Solubility and dissolution rate of a carbamazepine–cinnamic acid cocrystal. J Mol Liq. 2013;187:171-6.
88. Manin AN, Voronin AP, Drozd KV, Manin NG, Bauer-Brandl A, Perlovich GL. Cocrystal screening of hydroxy benzamides with benzoic acid derivatives: a comparative study of thermal and solution-based methods. Eur J Pharm Sci. 2014; 65:56-64.

89. Hata N, Furuishi T, Tamboli MI, Ishizaki M, Umeda D, Fukuzawa K, Yonemochi E. Crystal Structural Analysis of DL-Mandelate Salt of Carvedilol and Its Correlation with Physicochemical Properties. Crystals. 2020;10(1):53.

90. Sanphui P, Bolla G, Nangia A, Chernyshev V. Acemetacin cocrystals and salts: structure solution from powder X-ray data and form selection of the piperazine salt. IUCr J. 2014;1(2):136-50.

8: ANNEXURES

LIST OF RESEARCH PAPER PUBLICATIONS

1. Randive KH, Maste MM, Kempwade AA. Exploring Analytical Quality by Design (AQbD) Enabled RP-HPLC Method for Carvedilol. Indian J of Pharmaceutical Education and Research. 2024 Apr 1;58(2):651-60.
2. Randive KH, Maste MM, Kempwade AA. Exploration of AQbD Approach for Development of RP-HPLC Method of Nebivolol. International Journal of Pharmaceutical Investigation. 2024 Jul 1;14(3).

LIST OF POSTER AND ORAL PRESENTATIONS

Sl. No.	**Title of the poster/Oral Presentation**	**Name of the conference**	**Organized by**
1	Exploring Analytical Quality by Design (AQbD) Enabled RP-HPLC method for Antihypertensive Agents	Recent Development and Challenges in Pharmaceutical sciences	Krishna Institute of Pharmacy, Karad
2	Exploration of AQbD approach for Development of stability indicating RP-HPLC method of Nebivolol	Recent Advances in Drug Design	Annasaheb Dange College of Pharmacy, Ashata

LIST OF CONFERENCES/WORKSHOP ATTENDED

1. Two Days National Conference on "Recent Development and Challenges in Pharmaceutical sciences" Organized by, Krishna Institute of Pharmacy, Karad. On 4th and 5th March 2023.
2. Two Days National Conference on "Recent Advances in Drug Design" Organized by, Annasaheb Dange College of Pharmacy, Ashata. On 5th and 6th Feb. 2024.
3. National Level Workshop on "Design of Experiments" Organized by Department of Pharmaceutics in Collaboration with IIC KLE College of Pharmacy, Nipani. On 16th Sept. 2023.
4. Workshop and Hands on Training on "HPLC" Organized by KLE College of Pharmacy, Belagavi, On 4th April 2024.
5. Workshop on "Pursuits of Artificial Intelligence in Pharmacy Education" Organized by KLE College of Pharmacy, Belagavi, On 14th May 2024.

Ind. J. Pharm. Edu. Res., 2024; 58(2):651-660.
https://www.ijper.org

Original Article

Exploring Analytical Quality by Design (AQbD) Enabled RP-HPLC Method for Carvedilol

Krantisinha Hanumant Randive[1,2], Meenaxi Maruti Maste[1,*], Amolkumar Ashok Kempwade[3]

[1]Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research Belagavi, Karnataka, INDIA.
[2]Department of Pharmaceutical Chemistry, KLES's College of Pharmacy, Nipani, Karnataka, INDIA.
[3]Department of Pharmaceutics, KLES's College of Pharmacy, Nipani, Karnataka, INDIA.

ABSTRACT

Background: Carvedilol (CDL) is a beta-blocker that helps to lower the risk of heart attack related mortality and is used to treat congestive heart failure and excessive blood pressure. Objectives: The present study deals with the development and validation of a reverse-phase high performance liquid chromatography method for rapid and reliable analysis of CDL by utilizing the Analytical Quality by Design (AQbD) approach. **Materials and Methods:** Critical Analytical Attributes (CAAs) were selected based on predefined Analytical Target Attributes (ATPs). Further screening of Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs) having a significant impact on assigned CAAs was done by Taguchi Design. The optimized mobile phase consists of 0.1% Orthophosphoric Acid (OPA) and methanol in a ratio of 35:65% v/v. The flow rate was maintained at 1 mL/min, the column temperature was 30°C, and the analysis was carried out at 240 nm. Based on the results obtained, fine tuning of the CAAs was done by opting for the 3^2-Box-Behnken design. **Results:** The area under the curve was found to be linear within the studied range of 5-30 µg/mL (R^2=0.999). The retention time was found to be 4.18 min, with a sharp, well-defined peak. The percentage recovery of the drug during the accuracy study was found to be in the range between 97.06 and 99.29%. The detection and quantification limits were found to be 0.61 µg/mL and 1.86 µg/mL, respectively. **Conclusion:** The developed method was found to be precise with a RSD of <1%. The method can be effectively utilized for routine analysis of bulk drug and dosage forms in quality control labs.

Keywords: Analytical Quality by Design, Carvedilol, RP-HPLC, Box-Behnken design, Taguchi Design.

Correspondence:
Dr. Meenaxi M Maste
Professor, Department of Pharmaceutical Chemistry, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi-590010, Karnataka, INDIA.
Email: menaimm@gmail.com; meenaximaste@klepharm.edu
ORCiD ID: 0000-0002-1928-4483

Received: 29-04-2023;
Revised: 18-07-2023;
Accepted: 05-01-2024.

INTRODUCTION

Production of quality pharmaceutical product is a highest priority of any pharmaceutical industry. To ascertain the quality of any product, highly efficient, precise and accurate analytical method is required. The assessment of product with poor analytical method leads to generation of inaccurate and misleading data. It is highly risky if the analytical method used during product development stage is poorly developed as it affects on the quality attributes of product developed. This may also in turn result into life threatening health hazards to patients consuming these products and spoils the reputation of pharmaceutical industry.

Various pharmaceutical regulatory agencies like USFDA, ICH have taken these issues very seriously and to tackle such crucial issues they have designed some guidelines to the industry. In such effort, ICH has recently implemented ICH Q14 guidelines pertaining to development of analytical procedures and revision of Q2 (R1), Q2 (R2). Further these agencies are emphasizing the use of Analytical Quality by Design (AQbD) approach for development of analytical method.[1]

Initially Quality by Design (QbD) approach was utilized by formulation scientists to imbibe the quality in the product during its development stage itself. Further the need of accurate and precise analytical method faced by industry explored its use in analytical method development and flourished as AQbD approach.[2-7] As compared to traditional time and chemical consuming trial and error approach, AQbD has equipped analytical scientist with simple and rapid tool for development of highly efficient analytical methods. AQbD approach helps analytical scientists to understand various Critical Analytical Attributes (CAA) which affects on performance of analytical results. It accompanies the quality risk management principles with design of experiments to understand the risks and associated interactions among the method variables. It further defines

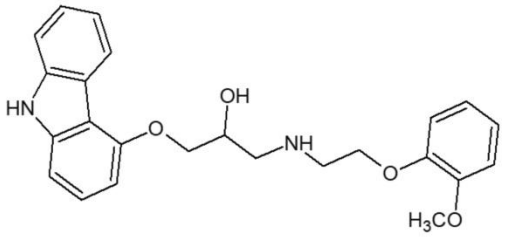
DOI: 10.5530/ijper.58.2.73

Copyright Information :
Copyright Author (s) 2024 Distributed under Creative Commons CC-BY 4.0

Publishing Partner : EManuscript Tech. [www.emanuscript.in]

quality target method profile and operable design space where the method will provide the consistent results without significant error. Thus, it will help the analytical scientist to develop a highly robust method for routine analysis of drug and drug products.[8]

Carvedilol is chemically, (2RS)-1-(9H-Carbazol4-yloxy)-3-[[2-(2-methoxy phenoxy) ethyl] amino] propan-2-ol having molecular formula of $C_{24}H_{26}N_2O_4$ and a molecular weight of 406.47 g/ mol.[9]



Structure of Carvedilol

Carvedilol, non-selective β-adrenergic blocking agent is indicated for mild to moderate heart failure and hypertension having cardiomyopathic or ischemic origin. In comparison to other β blocker, CDL has least inverse agonist activity which provide additional benefits with respect to morbidity and mortality in CHF.[10]

Reported methods suffer from certain lacuna. To mention few, complex procedures for extraction, time consuming methods, high retention time and use of costlier solvents etc. Further the developed methods have utilized traditional trial and error approach.[11] To the best of our knowledge, there is no study published which explores an AQbD approach to establish an analytical method for CDL analysis. Thus, the aim of the present research work was to develop sensitive, specific, precise, reliable and accurate RP-HPLC method using an AQbD enabled approach for the quick analysis of CDL in bulk and dosage form.

MATERIALS AND METHODS

Chemicals reagents and equipment used

Carvedilol (CDL) was obtained from Teva Pharmaceutical Pvt. Ltd., Gujarat, India as a gift sample. All chemicals required for the experiment (HPLC-grade) were procured from authentic sources.

Shimadzu Prominence-i series LC-2030c 3D plus was used to carry out the chromatographic (RP-HPLC) estimation. The system was equipped with Shim-pack GIST C18 column (250 x 4.5 mm i.d., 5 µm,) having guard column C18 (ODS; 4x3.0 mm i.d., Phenomenex, USA). A Lab solutions Version 5.97 SP1software was used. The mobile phase was consisting of 0.1% OPA and MeOH (35:65 v/v) which was forced through the column at a flow rate of 1.0 mL/min, keeping column temperature at 30ºC and detection wavelength of 240 nm. The 10 µL sample was injected at each sample time with 10 min of chromatographic run time.

Preparation of Sample solution

For preparation of CDL stock solution, 10 mg of CDL was dissolved in 10 mL of MeOH. A working sample solution was prepared by serial dilutions of stock solution with mobile phase to get final solution having concentration of 10 µg/mL.

AQbD enabled RP-HPLC method development

Analytical Target Profile (ATP) and Critical Analytical Attributes (CAA)

Defining Analytical Target Profile (ATP) is a primary step towards the QbD approach. ATP is nothing but predetermining the various quality attributes of analytical method which are desired by an analyst. This ATP will act as guiding principle for designing the quality and accuracy of the method. While deciding the ATP various factors considered are method application, instrument characteristics, drug characteristics, sample preparation etc. Selection of Critical Analytical Attributes (CAA) helps to meet the desired ATP. CAA's are the attributes of the chromatogram which can be measured and directly correlated with the quality of the developed analytical method. The Tailing factor (Tf), Retention time (Rt), Number of Theoretical Plates (NTPs), peak area, resolution between various peaks, etc. are few examples of CAA.[12,13]

Risk Assessment studies and Factor screening studies by Taguchi design

Determination of risks and probabilities of failure in the method development is important aspect of QbD which is done by risk assessment study. The risk assessment was established using a Taguchi orthogonal array design with seven components at two levels, where the cause effect relationship between CPP and CMAs was examined. For this study Design expert ® software (Version 7.0.0, Stat-Ease Inc., Minneapolis, MN, USA) was used.[14]

Various critical method variables selected were Injection volume, flow rate, temperature, concentration of organic modifier, and type of organic modifier, type of solvent and % v/v of solvent. Each factor was varied at 2 different levels. Table 1 shows various factors along with their levels.

Method development and Optimization by Box-Behnken Design (BBD)

On the basis of preliminary study, factors which significantly affected CAA were further screened by using Box-Behnken design. As per the design space 13 different runs were carried out and their effect on CAA was studied by software. Table 2 shows various runs with their experimental conditions. The observed responses were noted and further the data was fed to software to

Table 1: Taguchi design matrix for screening.

Standard trial no.	Type of solvent	% v/v of solvent	Type of organic modifier	Conc. of organic modifier	Temperature	Flow rate	Injection volume
1	MeOH	65.00	OPA	0.20	30.00	0.80	20.00
2	ACN	65.00	OPA	0.05	30.00	1.00	20.00
3	ACN	60.00	FA	0.20	40.00	1.00	20.00
4	ACN	60.00	FA	0.05	30.00	0.80	10.00
5	MeOH	65.00	FA	0.05	40.00	1.00	10.00
6	ACN	65.00	OPA	0.20	40.00	0.80	10.00
7	MeOH	60.00	OPA	0.20	30.00	1.00	10.00
8	MeOH	60.00	OPA	0.05	40.00	0.80	20.00
Factors selected for screening studies				Levels of factors			
				Low (-1)		High (+)	
Type of solvent				ACN		MeOH	
% v/v of solvent				60		65	
Type of organic modifier				FA		OPA	
Conc. organic modifier				0.05		0.2	
Temperature				30		40	
Flow rate				0.8		1.0	
Injection volume				10		20	

MeOH: Methanol; ACN: Acetonitrile; FA: Formic acid; OPA: Orthophosphoric acid.

get best fit model like linear, quadratic, polynomial, 2FI etc. and to generate the equations of the model.[15]

Polynomial equation can be represented in generalized form as

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2$$

Wherein response or dependant variable is denoted as Y. β_0 is the intercept which is an average arithmetic mean of all quantitative outcomes of 13 runs, β_1 and β_2 are linear coefficients; β_{12} interaction coefficients between two factors; β_{11} and β_{22} quadratic coefficients obtained from the observed response; X_1 and X_2 are coded levels of independent variables; X_1X_2 is interaction term and X_1^2 and X_2^2 are quadratic terms respectively. The magnitude of coefficients and their signs play an important role in identifying the effect of corresponding independent variable on responses. The effect is directly related to the magnitude of coefficient i.e. higher is the value of magnitude of coefficient more significant is the effect and vice versa. Further sign of the magnitude denotes its positive or negative effect on the studied response. Analysis of Variance (ANOVA) was used to determine the statistical significance of the model. The $p<0.05$ was considered as significant for model and model terms. Both 2D-contour plots and 3D-response surface plots were used to determine the correlation between the independent and dependent variables. Further interaction studies were carried out by overlay plots.

Method validation

In order to validate the proposed chromatographic method, it was assessed for linearity, specificity, accuracy, precision, robustness, and system applicability in accordance with the ICH Q2 (R1) guidelines.[16]

System Suitability

The system suitability was assessed by injecting six replicate injections of a standard solution of CDL at a concentration of 15 µg/mL. Various factors like NTP, Rt, PA, and Tf have been determined. RSD values for PA and Rt were less than 2%, which is within the acceptable range.

Linearity

The linearity of the method was established with six different concentrations of CDL standard solutions ranging from 5-30 µg/mL in triplicate. The plot between concentrations and peak area was obtained to get calibration curve. Further equation of the line was obtained to get slope, intercept and coefficient of correlation (R^2).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Equations 1 and 2 were used to determine the magnitude of the detection and quantification limits.

Table 2: 3^2 Box-Behnken designs along with the observed values for optimization of RP-HPLC method for CDL

Run No	Independent Variables		Dependent variables			
	X_1	X_2	Y_1	Y_2	Y_3	Y_4
1	65.00	10.00	4.221	406576	1.191	4441
2	60.00	15.00	5.595	605931	1.172	5000
3	65.00	15.00	4.228	610665	1.206	4394
4	55.00	20.00	8.289	801457	1.18	5632
5	60.00	15.00	5.597	604773	1.173	4991
6	60.00	15.00	5.6	605588	1.174	4980
7	60.00	15.00	5.607	605613	1.175	4943
8	60.00	15.00	5.602	605547	1.174	4929
9	60.00	10.00	5.599	405319	1.115	5129
10	60.00	20.00	5.593	808590	1.192	4878
11	55.00	15.00	8.297	602148	1.16	5786
12	65.00	20.00	4.225	814187	1.217	4329
13	55.00	10.00	8.316	404127	1.138	5963

X_1: Solvent concentration in mobile phase (% v/v); X_2: Injection volume; Y_1: Retention time; Y_2: Peak area; Y_3: Tailing factor; Y_4: Number of theoretical plates.

$$LOD = \frac{3.3 \times SD}{\alpha} \quad \text{.....(1)}$$

$$LOQ = \frac{10 \times SD}{\alpha} \quad \text{.....(2)}$$

Where SD represents the standard deviation of response of lowest concentration (intercept) and α is slope of linear calibration curve.

Precision

To determine the precision of the established method, intra-day and inter-day variations in responses were studied. Intraday precision was carried out by analysing the three different concentrations (5 µg/mL, 15 µg/mL and 25 µg/mL) three times in a day whereas inter-day precision was established with analysis of three different concentrations on three different days. Peak areas were determined and their % RSD values were calculated.

Accuracy

To determine the accuracy, % recovery studies were performed at three different concentration levels. Briefly, to the bulk powder of drug (10 mg) the tablet powder equivalent to 50%, 100% and 150% of drug was added. The powder mixture is diluted with mobile phase and analyzed to determine the % recovery of the samples.

Robustness

Robustness of the method was checked by intentionally varying the chromatographic conditions such as flow rate (±0.1 mL/min), mobile phase composition (MeOH: 0.1% OPA (64:36 and 66:34, v/v), and column temperature (±1°C) and studying the effect on each of the CAAs. The system suitability attributes were calculated, including NTP, Tf, % RSD of PA, Rt and % recovery.

Assay of Marketed tablets

Twenty marketed tablets of Carvedilol (Cardivas, Batch no-SIC 3318A, Mfg. 12/2021, Exp. 11/2024, Sun Pharma. Laboratories ltd.) were accurately weighed and triturated. A powder sample containing 10 mg of CDL was carefully weighed and placed in a 50 mL volumetric flask containing 20 mL of methanol. The mixture was vortex mixed to dissolve the drug completely. Further volume was made with MeOH, which was then filtered through a 0.45 µm membrane filter (Nylon). Suitable dilution of the filtrate with the mobile phase was carried out and CDL content was determined using the developed method.

RESULTS AND DISCUSSION

Preliminary method development

Review of available literature has witnessed the utilization of mobile phase compositions like acetonitrile, methanol, formic acid and acetic acid at different flow rate, injection volume, concentration etc.

Present study deals with development of a simple, precise and robust RP-HPLC method for the determination of Carvedilol (CDL). The method was optimized by changing method parameters such as mobile phase composition, organic modifier concentration and column temperature in order to obtain an intense and symmetrical peak with no tailing.

Preliminary studies were carried out by using methanol and water at various proportions. The results were not acceptable as poor resolution with tailing factor of more than 2 was observed. Further studies were conducted by replacing methanol with acetonitrile. The use of acetonitrile and water in different ratios has resulted into poorly resolved broad peaks with tailing. When MeOH

and 0.1% aqueous solution of formic acid was tried as mobile phase well defined sharp peak of CDL was obtained and tailing factor reduced from 2.0 to 1.363. When ACN and 0.1% aqueous solution of formic acid was tried as mobile phase, the CDL peak eluted very near to the negative peak. Further decrease in ACN concentration to separate the CDL peak from negative peak led to increased retention time. Hence further trails were done with MeOH and formic acid. Further increase in the concentration of formic acid up to 0.5% v/v reduced the tailing factor from 1.363 to 1.20 with improved peak shape. It was observed that column temperature plays an important role on tailing factor. When the temperature decreased from 40°C to 25°C, the tailing factor reduced from 1.281 to 1.222. When formic acid was replaced with 0.1% OPA, tailing factor was reduced (1.20) and sharp peak was obtained (Figure 1). When ACN and 0.1% OPA was tried as mobile phase, tailing factor was more than 1.3. Based on the results obtained RP-HPLC method was developed and optimized on Shim-pack GIST C18 column using 0.1% OPA in water and MeOH (35:65 v/v) by maintaining 1.0 mL/min flow rate, 30°C column temperature and 240 nm as detection wavelength.

Risk assessment and Screening Studies[12]

Risk assessment plays an important role in understanding the association between CMV's and CAA's. Based on risk assessment studies, seven factors were selected and their further screening was done based on Taguchi design. The screening study helps to find out the factors which significantly contribute the experimental results within few experimental trials. To represent the effect of these factors on CAA, half-normal plots and Pareto charts were used (Figure 2). Two factors i.e. concentration of methanol (X_1) and injection volume (X_2) were mainly contributing factors on the studied responses.

Factor optimization using 3^2 BBD

The results obtained through the Design expert software has suggested that quadratic model is a best fit model for correlating the independent and dependant variables. F test value with $p<0.05$ also witnessed the significance of the model. Summary of 4 responses is represented in Table 3. The R^2 value for all the responses was ranged between 0.9972-1. The data depicts that response data also fits good to the polynomials. Further confirmation of the best fit was confirmed by closeness of predicted and adjusted R^2 value (Difference <0.2). Quadratic equation obtained is represented as equation 1, wherein b_1 to b_{22} are the coefficients of interaction terms while b_0 is intercept. The 3D-response surface diagrams and 2D-contour plots are as shown in (Figure 3).

Effect of independent variables on Retention time (Y_1)

The quadratic polynomial equation depicting the independent variables effect on retention time is as represented below

$$\text{Retention time } (Y_1) = +5.60 - 2.04X_1 - 0.004833X_2 + 0.007750X_1X_2 + 0.66X_1^2 - 0.002052X_2^2$$

The equation represents that both % v/v of methanol in mobile phase and injection volume have negative effect on the retention time. Further greater value of coefficient of X_1

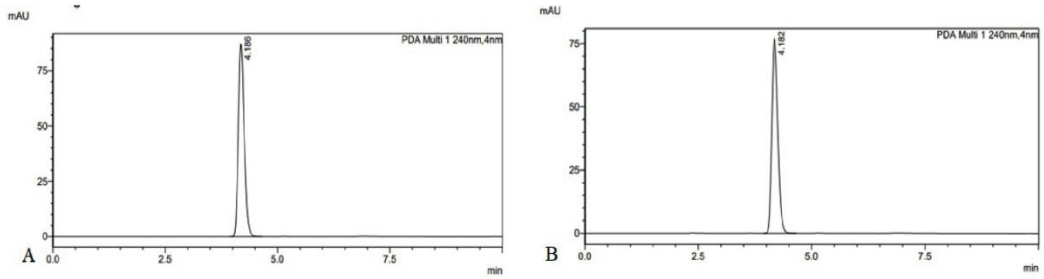


Figure 1: RP-HPLC Chromatogram of A) Carvedilol Standard B) Carvedilol Assay.

Table 3: Analysis of Variance (ANOVA) test results and adequate precision for various regression analysis responses.

	R^2	Adjusted R^2	Predicted R^2	SD	CV (%)	F Value	*p* Value	Adequacy precision
Retention time (min)	1.00	1.00	1.00	0.004	0.078	250401.1	<0.0001	1312.927
Peak area	1.0000	1.0000	0.9999	582.32	0.09	143571.34	<0.0001	1037.993
Tailing factor	0.8988	0.8264	0.0690	0.011	0.94	17.22	0.0006	14.100
Number of theoretical plates	0.9984	0.9972	0.9951	26.87	0.53	858.41	<0.0001	89.691

witnesses the pronounced effect of % v/v methanol on retention time in comparison to that of injection volume. The combined interaction terms % v/v of methanol, injection volume (X_1X_2), have positive effect on the retention time. Table 3 shows the results of ANOVA for retention time data. The value of 250401.42 for Model F represents its significance with such a high value, there is just a 0.01% possibility that noise is influencing the responses. The signal to noise ratio determines the precision of the method. Normally the value of the ratio more than 4 is considered as appropriate. The ratio was found to be 1312.927, indicating that a quadratic model may be used to analyze the results in design space. The 3D-response surface plots, 2D-contour plots and perturbation plots depicting the correlation between retention time and studied independent variables have been shown in Figure 3A, 3E and 4a-A respectively.

Effect of independent variables on peak area (Y_2)

The quadratic polynomial equation depicting the independent variables effect on peak area is as represented below

$$\text{Peak area}(Y_2) = +605700 + 3949.33\,X_1 + 201400X_2 + 2570.25\,X_1X_2 + 296.31\,X_1^2 + 844.X_2^2$$

The equation represents that both % v/v of methanol in mobile phase and injection volume have positive effect on the peak area. Further greater value of coefficient of X_2 witnesses the pronounced effect of injection volume on peak area in comparison to that of % v/v methanol. The combined interaction terms % v/v of methanol, injection volume (X_1X_2), have positive effect on the peak area. Table 3 shows the results of ANOVA for peak area data. The value of 143571.34for Model F represents its significance. Such a high value could have only 0.01% chance that noise is affecting the results. The signal to noise ratio was found to be 1037.993 which indicate that quadratic model can be suitable to represent the results in design space. The 3D-response surface plots, 2D-contour plots and perturbation plots depicting the correlation between peak area and studied independent variables have been shown in Figure 3B, 3F and 4a-B respectively.

Effect of independent variables on tailing factor (Y_3)

The linear equation depicting the independent variables effect on tailing factor is as represented below

$$\text{Tailing factor}\ (Y_3) = +1.17 + 0.023\,X_1 + 0.024\,X_2$$

The equation represents that both % v/v of methanol in mobile phase and injection volume have positive effect on the tailing factor. The values of coefficient of X_1 and X_2 have almost same which witnesses that both the factors have equal effect on the studied response. Absence of interaction terms and squared terms indicate that there was no significant interaction between the independent variables in case of tailing factor. Table 3 shows the results of ANOVA for tailing factor data. The value of 17.22 for Model F represents its significance. Such a high value could have only 0.06% chance that noise is affecting the results. The signal to noise ratio was found to be 14.1 which indicate that linear model can be suitable to represent the results in design space. 3D-response surface plots, perturbation plots and 2D-contour plots depicting the correlation between tailing factor and studied independent variables have been shown in Figure 3C, 3G and 4a-C respectively.

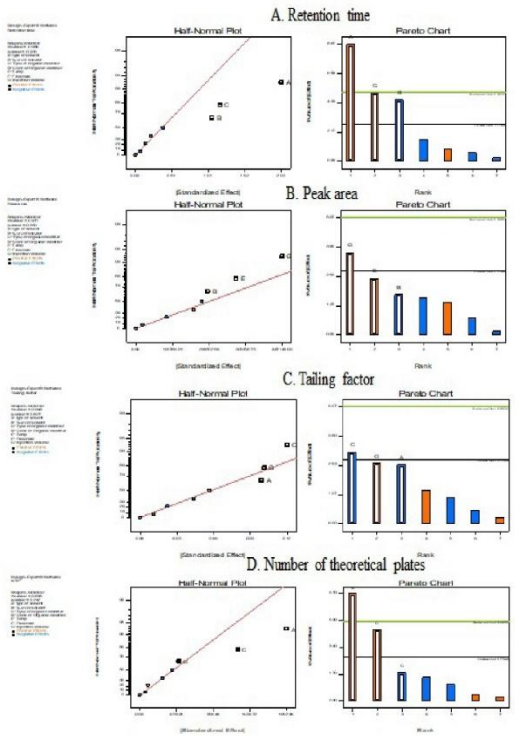


Figure 2: A-D Half-normal plots and Pareto charts indicating the factor effects of the responses, i.e., retention time, peak area, tailing factor and number of theoretical plates.

Table 4: System suitability test parameters (*n*=6) Concentration=15 µg/mL.

Compound	Retention time (Rt, min)	RSD % of retention time	Peak area	RSD % of peak area	Theoretical plates (N)	Tailing factor (T)
CDL	4.18	0.09	697961	0.19	4361	1.21

n=number of replicates;RSD=Relative standard deviation.

Effect of independent variables on number of theoretical plates (Y_4)

The quadratic equation depicting the independent variables effect on number of theoretical plates is as represented below

$$\text{Number of theoretical plates } (Y_4) = +4973.24 - 702.83 X_1 - 115.67 X_2 + 54.75 X_1 X_2 105.16 X_1^2 + 18.66 X_2^2$$

The equation represents that both % v/v of methanol in mobile phase and injection volume have negative effect on the NTP. Further greater value of coefficient of X_1 witnesses the pronounced effect of % v/v of methanol in mobile phase on Y_4 in comparison to that of injection volume. The combined interaction terms % v/v of methanol, injection volume (X_1X_2), have positive effect on the Y_4. Table 3 shows the results of ANOVA for number of theoretical plate's data. The value of 858.41 for Model F represents its significance. Such a high value could have only 0.01% chance that noise is affecting the results. The signal-to-noise ratio was found to be 89.691, indicating that the quadratic model is appropriate for representing the results in design space. Figure 3D, 3H, and 4a-D exhibit 3D-response surface plots, perturbation plots, and 2D-contour plots demonstrating the correlation between peak area and studied independent variables, respectively.

Analytical Method Validation

Validation of an analytical method is considered as a prerequisite for its application in day to day qualitative and quantitative analysis.

System Suitability

System suitability test results have witnessed that there is no significant difference in PA, Rt, NTP and Tf values when it is studied with six replicates of CDL having concentration of 15 µg/mL. The results are as shown in Table 4.

Table 5: Analytical method validation results.

Precision									
Analyte	Content (µg/mL)	Intraday (*n*=6)		Inter day (*n*=3)					
				Day 1		Day 2		Day 3	
		Found (µg/mL)	% RSD	Found (µg/mL)	% RSD	Found (µg/mL)	% RSD	Found (µg/mL)	% RSD
CDL	5.00	4.84	0.14	4.84	0.13	5.72	0.03	5.22	0.66
	15.00	14.10	0.07	14.10	0.06	15.93	0.03	14.78	0.09
	25.00	23.59	0.13	23.58	0.01	25.54	0.01	25.43	0.16
Recovery studies (*n*=3)									
Compound		Contents (mg)		Quantity added (mg)		Theoretical amount (mg)	Recovered amount (mg)	Recovery (%)	% RSD
CDL		10		5 (50%)		15	14.89	99.29	0.01
				10 (100%)		20	19.80	99.02	0.07
				15 (150%)		25	24.26	97.06	0.02
Robustness study (*n*=6)									
Parameter	Modification	CDL							
		% RSD of peak area		% RSD of retention time		Theoretical plates		Tailing factor	Percentage recovery
Flow rate	0.90 mL/min	0.10		0.04		4694		1.22	110.53
	Optimized	0.47		0.01		4325		1.21	100.00
	1.10 mL/min	0.05		0.03		3985		1.21	93.00
Mobile phase composition	64% MeOH	0.05		0.02		4216		1.22	101.27
	Optimized	0.47		0.01		4325		1.21	100.00
	66 % MeOH	0.02		0.04		4417		1.21	101.04
Column temperature	29°C	0.05		0.11		4282		1.21	101.16
	Optimized	0.47		0.01		4325		1.21	100.00
	31°C	0.01		0.01		4354		1.22	101.11

n=number of replicates;RSD=relative standard deviation.

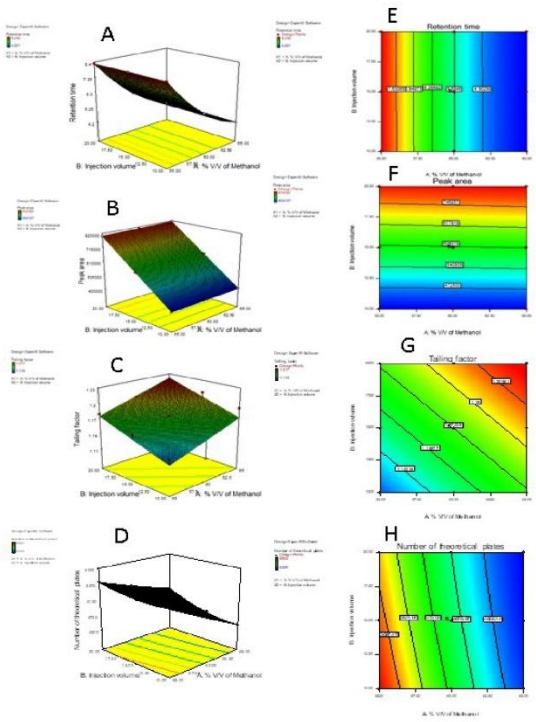


Figure 3: 3D-Response surface (A-D) and 2D-contour (E-H) plots showing the effect of %V/V of methanol in mobile phase and injection volume on retention time, peak area, tailing factor and number of theoretical plates. X_1=A: Methanol in the mobile phase and X_2=B: Injection volume.

Linearity

ICH guidelines were used to establish the linearity of the proposed analytical method. Linear results were obtained within studied concentration range of 5-30 µg/mL with regression coefficient value of 0.999. Equation of the linear line obtained is y=53574x-57581. The obtained calibration curve is as shown in Figure 5.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ represent sensitivity of the method. LOD and LOQ values of developed method were found to be 0.61 µg/mL and 1.86 µg/mL, respectively. These findings show the method is highly sensitive.

Precision

The results of precision study have depicted that the % RSD value of peak area lies within 1% during intra and inter day variation. The results of precision study have been as shown in Table 5.

Accuracy

The accuracy of the method is an indication about its ability to maintain conformity between experimental and theoretical responses. The results of accuracy study are shown in Table 5 The recovered concentration of CDL was found in the range between 99.41-99.82% with % RSD value of <2% which witnesses the accuracy of the method.

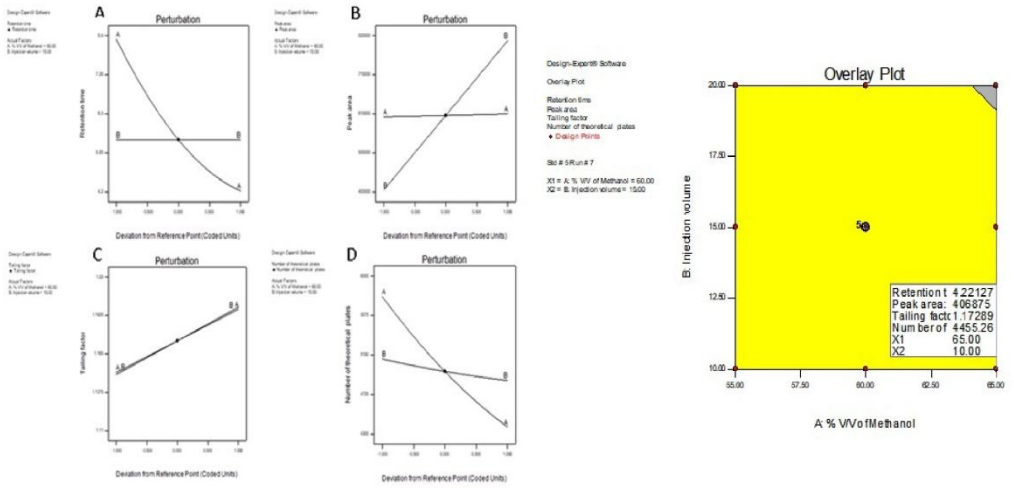


Figure 4: a) Perturbation plots (A-D) showing the effect of %V/V of methanol in mobile phase and injection volume on retention time, peak area, tailing factor and number of theoretical plates. A: Methanol in the mobile phase and B: Injection volume. b) Overlay plot showing the optimized chromatographic conditions and their predicted responses.

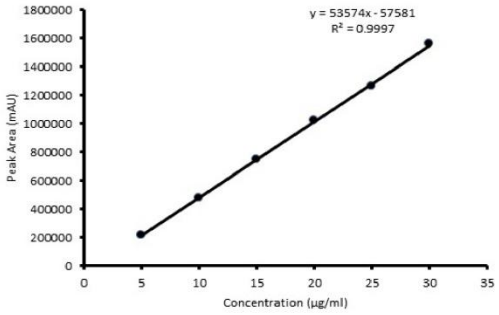


Figure 5: Calibration curve of Carvedilol.

Robustness

In studied CAA, with minor changes in the method parameters, no considerable changes have been observed and degree of % RSD was within <2%. Thus, the robustness of the developed technique was established for minor changes in the method parameters. Table 5 shows the results of the robustness study.

Assay of Marketed tablets

The assay of the marketed tablet was done by developed method and drug content was found to be 105.60% which complies with prescribed standards (90.0% to 110.0%). The chromatogram for assay study is as shown in Figure 1.

CONCLUSION

The present study is pertaining to development and validation of simple, reliable and economical RP-HPLC method for analysis of CDL in bulk and dosage form using AQbD approach. The study was started with predefining the ATP. On the basis of ATP, CAAs were selected. The Taguchi design was utilized to screen seven CMAs to further select two most prominent factors. Further optimization of the significant CMAs was carried out by using 3^2 Box-Behnken Design. The interaction and main effects between studied parameters were further explored with 2D-counter plots and 3D-response surface plots. Various numerical equations were derived from software to get further in-depth insights of interaction effects. The developed method was further validated as per ICH guidelines by utilizing parameters like linearity, system suitability, precision, LOD, LOQ accuracy and robustness. The developed and validated method is suitable to detect and quantify the CDL in bulk and dosage form.

ACKNOWLEDGEMENT

Authors would like to thanks Dr. Sunil S Jalalpure, Principal, KLE College of Pharmacy, KAHER, Belagavi, Karnataka, India for his constant support and providing required analytical infrastructure to carry out the research work. The authors are grateful to Dr. Mallesh for his inputs and valuable guidance about QbD and DOE. Authors are also thankful to the Principal KLE College of Pharmacy, Nipani, Karnataka, India for constant support to carry out my research work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AQbD: Analytical quality by Design; **ANOVA:** Analysis of Variance; **ATP:** Analytical Target Attributes; **BBD:** Box-Behnken design; **CAA:** Critical Analytical Attributes; **CMAs:** Critical Material Attributes; **CPPs:** Critical Process Parameters; **CDL:** Carvedilol; **DOE:** Design of Experiment; **ICH:** International Council for Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **OPA:** Ortho Phosphoric Acid; **PA:** Peak Area; **QbD:** Quality by Design; **RA:** Risk Assessment; **RSD:** Residual Standard Deviation; **RT:** Retention Time; **NTP:** Number of Theoretical Plates; **RP-HPLC:** Reverse Phase-High Performance Liquid Chromatography; **TF:** Tailing Factor.

SUMMARY

In the present study, Analytical Quality by Design (AQbD) approach is employed to develop and validate a RP-HPLC method for the rapid and reliable analysis of CDL. Based on the results, the CAAs were optimized using a 3^2 Box-Behnken design. The optimized mobile phase is composed of 0.1% ortho phosphoric acid and methanol in a 35:65%v/v ratio. The flow rate was kept constant at 1 mL/min, column temperature of 30°C, and the analysis was carried out at 240 nm. Analytical Quality by Design (AQbD) enabled optimized RP-HPLC method was standardized in terms of validating attributes such as system suitability, linearity, LOD and LOQ, precision, accuracy and robustness using ICH: Q2 (R1) guidelines. The area under the curve was determined to be linear within the tested range of 5-30 µg/mL (R^2=0.999). The retention time was found to be 4.18 min, with a sharp, well-defined peak. The % recovery of the drug during accuracy study was found to be in the range between 97.06 to 99.29%. The detection and quantification limit were found to be 0.61 µg/mL and 1.86 µg/mL respectively. The developed method was found to be precise with %RSD <1%. The values of precision and robustness parameters was found to be within the required acceptance limit with % RSD <2%. Hence it is concluded that the AQbD based developed RP-HPLC method can be effectively utilized for routine analysis of bulk drug as well as dosage from.

REFERENCES

1. Suryawanshi SS, Palled MS. Box-Behnken design assisted optimization and standardization of chromatographic methodology for quality assessment of metformin: analytical quality by design avenue. IJPER. 2022; 56(2s):s152-62. doi: 10.5530/ijper.56.2s.86.
2. Awotwe-Otoo D, Agarabi C, Faustino PJ, Habib MJ, Lee S, Khan MA, *et al.* Application of quality by design elements for the development and optimization of an analytical method for protamine sulfate. J Pharm Biomed Anal. 2012; 62: 61-7. doi: 10.1016/j.jpba.2012.01.002, PMID 22316620.

3. Bossunia MTI, Urmi KF, Shaha CK. Quality-by-design approach to stability indicating RP-HPLC analytical method development for estimation of canagliflozin API and its validation. Pharm Methods. 2017; 8(2): 92-101. doi: 10.5530/phm.2017.8.15.
4. Garg NK, Sharma G, Singh B, Nirbhavane P, Katare OP. Quality by design (QbD)-based development and optimization of a simple, robust RP-HPLC method for the estimation of methotrexate. J Liq Chromatogr Relat Technol. 2015; 38(17): 1629-37. doi: 10.1080/10826076.2015.1087409.
5. Ganorkar SB, Dhumal DM, Shirkhedkar AA. Development and validation of simple RP-HPLC-PDA analytical protocol for zileuton assisted with design of experiments for robustness determination. Arab J Chem. 2017; 10(2): 273-82. doi: 10.1016/j.arabjc.2014.03.009.
6. Panda SS, Ravi Kumar Bera VV, Beg S, Mandal O. Analytical Quality by Design (AQbD)-oriented RP-UFLC method for quantification of lansoprazole with superior method robustness. J Liq Chromatogr Relat Technol. 2017; 40(9): 479-85. doi: 10.1080/10826076.2017.1327442.
7. Thakur D, Kaur A, Sharma S. Application of QbD based approach in method development of RP-HPLC for simultaneous estimation of antidiabetic drugs in pharmaceutical dosage form. J Pharm Investig. 2017; 47(3): 229-39. doi: 10.1007/s40005-016-0256-x.
8. Maddala VL, Ray PC, Bulusu LS, Rao KM. Development and validation of an RP-HPLC method for fast, sensitive and simultaneous determination of few (eight) active pharmaceutical ingredient residues on stainless steel surface in manufacturing plants. Rasayan J Chem. 2015; 8: 316-20.
9. Beattie K, Phadke G, Novakovic J. Carvedilol. Profiles Drug Subst Excipients Relat Methodol. 2013; 38: 113-57.
10. Silva RA, Wang CC, Fernández LP, Masi AN. Flow injection spectrofluorimetric determination of carvedilol mediated by micelles. Talanta. 2008; 76(1): 166-71. doi: 10.1016/j.talanta.2008.02.029, PMID 18585259.
11. Al-Adl SM, Abdel-Aziz LM, Mohamed MAM. HPLC determination of carvedilol, candesartan cilexetil and hydrochlorothiazide in their bulk and tablet dosage forms. Anal Chem Lett. 2017; 7(2): 188-200. doi: 10.1080/22297928.2017.1326840.
12. Patil TS, Deshpande AS. Development of an innovative quality by design (QbD) based stability-indicating HPLC method and its validation for clofazimine from its bulk and pharmaceutical dosage forms. Chromatographia. 2019; 82(2): 579-90. doi: 10.1007/s10337-018-3660-8.
13. Saini S, Sharma T, Patel A, Kaur R, Tripathi SK, Katare OP, *et al.* QbD-steered development and validation of an RP-HPLC method for quantification of ferulic acid: rational application of chemometric tools. J Chromatogr B Analyt Technol Biomed Life Sci. 2020; 1155: 122300. doi: 10.1016/j.jchromb.2020.122300, PMID 32771967.
14. Beg S, Sharma G, Katare OP, Lohan S, Singh B. Development and validation of a stability-indicating liquid chromatographic method for estimating olmesartan medoxomil using quality by design. J Chromatogr Sci. 2015; 53(7): 1048-59. doi: 10.1093/chromsci/bmu165, PMID 25583970.
15. Alruwaili NK. Analytical quality by design approach of reverse-phase high-performance liquid chromatography of atorvastatin: method development, optimization, validation, and the stability-indicated method. J Environ Anal Chem. 2021; 2021: 1-12.
16. ICH. Validation-of-analytical-procedures-text-and-methodology, Guideline on validation of analytical procedures: text and methodology. International Conference on Harmonization (ICH: Q2); 2005.

Cite this article: Randive KH, Maste MM, Kempwade AA. Exploring Analytical Quality by Design (AQbD) Enabled RP-HPLC Method for Carvedilol. Indian J of Pharmaceutical Education and Research. 2024;58(2):651-60.

Int. J. Pharm. Investigation, 2024; 14(3):915-927.
https://www.jpionline.org

Original Article

Exploration of AQbD Approach for Development of RP-HPLC Method of Nebivolol

Krantisinha Hanumant Randive[1, 2], Meenaxi Maruti Maste[1,*], Amolkumar Ashok Kempwade[3]

[1]Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research Belagavi, Karnataka, INDIA.
[2]Department of Pharmaceutical Chemistry, KLES's College of Pharmacy, Nipani, Karnataka, INDIA.
[3]Department of Pharmaceutics, KLES's College of Pharmacy, Nipani, Karnataka, INDIA.

ABSTRACT

Background: Nebivolol hydrochloride (NBV) is a highly selective beta-adrenoceptor antagonist belonging to the third generation of beta-blockers. It is primarily utilized in the management of cardiovascular conditions. Since NBV is marketed in the tablet dosage form, ensuring its quality control and evaluation is important. **Objectives:** Analytical Quality by Design (AQbD) is a novel tool for the optimization of chromatographic methods. The present study explores the AQbD approach for optimization of RP-HPLC method for analysis of Nebivolol (NBV). **Materials and Methods:** In method development, Box-Behnken Design was used wherein mobile phase, wavelength and flow rate were considered as independent variables while retention time, peak area and tailing factor were responses. The optimized set of parameters was a mobile phase (MeOH:0.1% aqueous acetic acid in 71:29%v/v), flow rate of (0.8 mL/min) and wavelength (282 nm). **Results and Discussion:** The method was found to be linear in a concentration range of 10 to 50 µg/mL with regression value (r^2) of 0.999. The LOD and LOQ were obtained at concentration of 0.54 µg/mL and 1.64 µg/mL. The %RSD values for precision and robustness were found to be <2%. The % recovery was found to be 102.49% to 103.44% during accuracy study. Ruggedness of the method was confirmed by different analysts. **Conclusion:** The optimized and validated RP-HPLC method, for NBV analysis, is robust and precise, making it suitable for routine use in pharmaceutical analysis.

Keywords: AQbD, Nebivolol, RP-HPLC, Box-Behnken Design, Validation.

Correspondence:
Dr. Meenaxi M Maste
Professor and HoD, Department of Pharmaceutical Chemistry, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi-590010, Karnataka, INDIA.
Email: menaimm@gmail.com; meenaximaste@klepharm.edu
ORCID ID:0000-0002-1928-4483

Received: 21-03-2024;
Revised: 16-04-2024;
Accepted: 01-05-2024.

INTRODUCTION

Nebivolol hydrochloride (NBV) is a highly selective beta-adrenoceptor antagonist of the third generation of beta-blockers. It possesses a unique property, activating 3-adrenergic receptors to increase nitric oxide release, thereby improving endothelial function, inducing vasodilation, increasing arterial compliance and decreasing peripheral vascular resistance. Nebivolol possesses the molecular formula $C_{22}H_{25}F_2NO_4$ and has a molecular weight of 405.4 g/mol. It is denoted chemically as 1-(6-fluorochroman-2-yl)-[2-(6-fluorochroman-2-yl)-2-hydroxyethyl) amino] ethanol (Figure 1). The therapeutic efficacy of drug depends on its quality. Thus, use of sensitive analytical method for the analysis of bulk drugs during its development is a prerequisite to build a quality into a product.[1,2]

Analytical techniques, especially Liquid Chromatographic (LC) methods like High Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography (UPLC), are essential in drug product development and manufacturing. However, with the increasing complexity of drug products, method development becomes challenging. Robustness, the ability to consistently generate reliable results, is a crucial attribute. Conventional One-Factor-at-a-Time (OFAT) approaches often lead to non-optimized methods lacking insights into robustness, resulting in routine usage failures.[3]

In response to these challenges, the pharmaceutical industry is increasingly adopting the Quality by Design (QbD) approach in analytical methods. This approach emphasizes understanding the product and process from the outset and controlling specific Critical Quality Attributes (CQA) to ensure product quality. The implementation of Quality by Design is required by the European Medicines Agency (EMA) and the approval of new drug applications by the FDA is contingent upon the incorporation of analytical QbD principles.[4,5]

Box-Behnken Design (BBD) is indeed a valuable approach for optimizing chromatographic conditions and it typically employs

OPEN ACCESS
EPUBL
DOI: 10.5530/ijpi.14.3.101

Copyright Information :
Copyright Author (s) 2024 Distributed under Creative Commons CC-BY 4.0

Publishing Partner : EManuscript Tech. [www.emanuscript.in]

a three-factor analysis integrated into a staged process. One of the key advantages of BBD is its ability to significantly reduce the total number of experimental runs required for optimization when compared to other techniques. This makes BBD a highly practical and efficient method for optimizing chromatographic parameters in analytical methods. The implementation of Quality by Design (QbD) in experimental processes is considered a novel and valuable technique. When applied in the development and validation of method using the HPLC analytical technique, optimized chromatographic condition under QbD principles can results in substantial savings in both cost and time. This is especially advantageous for routine analysis in laboratory settings, as it enhances the efficiency and reliability of results.[6]

The development of AQbD and QBD, in general, is supported by international standards, such as those from the International Conference of Harmonisation (ICH), like Q8 and the newly developed Q14 guideline. These standards provide a systematic and scientifically sound approach to product development, emphasizing the control of CQAs and robustness in analytical methods.[4,6]

To further enhance method development, the concept of Analytical Quality by Design (AQbD) has emerged. AQbD involves predetermining the Analytical Target Profile (ATP), establishing Critical Method Attributes (CMA) and their specifications and identifying Critical Method Parameters (CMPs). Design of Experiments (DoE) is integral to AQbD, employing statistical tools and mathematical modeling to explore factor effects and their interactions on specific responses (CMAs).[7-9]

The thorough literature survey revealed that, even though various analytical methods have been developed and validated for analysis of nebivolol but these methods lack the utilization of AQbD approach. In this context, in the present study AQbD approach is applied to develop a highly efficient RP-HPLC method for screening of NBV, in both bulk drugs and pharmaceutical products. The study employed an experimental design approach to depict the connection between various factors and responses, ensuring method robustness through validation studies with the aim to create a simple, rapid, sensitive, robust and reliable method.

MATERIALS AND METHODS

Reagents and Chemicals used

Nebivolol (NBV) sample used in this study was kindly provided as a gift by 4care Life Science PVT. Ltd. located in Bagdol, Gujrat. HPLC grade methanol was supplied by Merck India Limited and to ensure its purity, the solvent system was filtered through a 0.45 µm membrane filter (Nylon) purchased from Pall India Pvt. Ltd., Mumbai. Further, solvent system was degassed and sonicated using sonicator. The commercial tablets containing NBV were purchased from local pharmacies in Belagavi, Karnataka, India (Neirite Hydrochloride-5 mg Mfg. by Jaslin Pharma). These tablets were subjected to a comprehensive quality analysis utilizing a BBD-assisted RP-HPLC methodology.

Equipment and Chromatographic Parameters

HPLC: Agilent 1100 (Autosampler).

Software: Chemstation-10.01.

Stationary phase: Agilent C_{18} (250×4.6 mm i.d. 5µm).

Mobile phase: Methanol (MeOH): 0.1% aqueous acetic acid (71:29).

Detection Wavelength: 282 nm.

Flow rate: 0.8 mL/min.

Column Temperature: 25°C.

Sample size: 20 µL.

All the samples were filtered through 0.45 µm nylon filters, then utilized for solvent system preparation and degassed. To solubilize the drug and powdered drug sample in the solvent, a sonicator (MJL lab) was employed. Before use, all glassware, including pipettes, measuring cylinder and volumetric flask, was calibrated.

Preparation Mobile Phase

To prepare the mobile phase, 0.1% v/v aqueous acetic acid solution was prepared by diluting 1 mL of acetic acid solution with Milli-Q water upto 1000 mL. Appropriate quantity of resulting solution is further mixed with methanol in such way that final composition was consisted of MeOH:0.1% v/v aqueous acetic acid solution (71:29) ratio on v/v basis. Further the prepared mobile phase is subjected to filtration through 0.45 µm nylon membrane filter. The obtained solution is kept aside till its further use.

Preparation of standard stock solution

To prepare a stock solution of NBV, 10mg of NBV was dissolved in 10 mL of methanol and sonicated to ensure its dissolution. From this, 1 mL was transferred to a 10 mL volumetric flask and diluted to 10 mL using mobile phase as a diluent to get stock solution of 100 µg/mL. Further NBV working solution were prepared through serial dilutions using mobile phase as the diluent and then the final solution was filtered using a 0.45 µ syringe filter.

Preliminary Method Development

To determine the most appropriate stationary phase, initial experimental runs were performed to explore potential combinations and selection of column and solvent system suitability. Preliminary trials were carried out to optimize methodology in terms of cost-effectiveness, methanol is selected as the solvent for trial study. Additionally, aqueous solvent with different concentrations of acetic acid were selected to achieve effective separation under different pH conditions. After

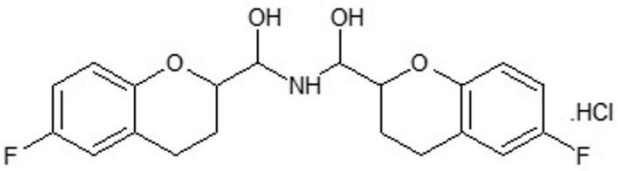


Figure 1: Chemical structure of Nebivolol hydrochloride.

analyzing the outcomes of these initial trials, the Agilent C_{18} stationary phase used for further investigation.

AQbD enabled RP-HPLC method development

Quality by Design (QbD), is described as a systematic approach to development that commences with predetermined objectives and emphasizes the comprehension of both product and process based on robust scientific principles and effective risk management. QbD involves gaining a better scientific understanding of the essential factors and qualities of a product. It includes designing tests and controls based on our scientific knowledge during development and using the information gathered throughout the product's life to continually enhance it. In this study, we have actively engaged with various aspects of AQbD (Analytical Quality by Design) and implemented them in practical methodologies. The process initiated with the identification of the Analytical Target Profile (ATP), followed by an examination of Critical Quality Attributes (CQAs) and Risk Assessment (RA) using the fishbone approach. Subsequently, our focus shifted towards the application of Design of Experiments (DOE), facilitating factor screening and the optimization of RP-HPLC method.

Analytical Target Profile (ATP) and Critical Quality Attributes (CQAs)

Formulating an Analytical Target Profile (ATP) is a crucial initial step in implementing the Quality by Design (QbD) approach. The ATP involves defining the specific quality attributes expected from an analytical method, providing a guiding framework for ensuring the method's precision and accuracy. Factors like method application, instrument characteristics, drug properties and sample preparation methods are taken into consideration when establishing the ATP. Selecting Critical Quality Attributes (CQAs) is essential for achieving the desired ATP. CQAs represent measurable chromatographic attributes directly linked to the quality of the analytical method developed. Examples of CQAs include parameters such as Tailing factor (Tf), Retention time (Rt), Number of Theoretical Plates (NTPs), peak area and resolution between peaks.

Risk Assessment studies and Factor screening studies

The primary focus of risk assessment study is to determine the risks and probabilities of failure associated with method development. In the present study, Ishikawa Fish-Bone (IFB) diagram was developed to establish risk assessment, wherein examination of cause effect relationship between CPP and CMAs with Critical Analytical Attributes (CAAs) was studied. Further, optimization of chromatographic conditions was carried out by design expert software. The BBD was opted to optimize the CPPs or CMPs and interactions thereof on CQA. The three factors, three level configuration with reduced experimental runs utilized during optimization.[10,11]

Optimization study

Analytical Quality by Design was employed to assess the effect of independent variables on various responses. For the optimization, a BBD (Design expert 13 version software) was selected because it gives optimal results with a relatively few numbers of experimental trials. The three-factor, three-level BBD was applied to enhance an analytical method. The factors under scrutiny included the composition of mobile phases, specifically the percentage of methanol (A), flow rate in mL/min (B) and UV wavelength in nanometre (C) their individual and combined impact were assessed with respect to selected responses: Y1-Retention time (Rt) measured in min, Y2-Peak Area (PA) and Y3-tailing factor. Using software, a total seventeen experimental runs were conducted with various composition to identify the optimal combination (Tables 2 and 3). To ascertain the most suitable model, various kinetic models- including linear, 2nd order and quadratic models- were utilized to fit the experimental data. Polynomial equations, 3D plots and 2D contour plots were utilized to assess the variables' effect on the responses. Statistical analyses, including "ANOVA and regression analysis", were conducted to ascertain the model's significance and identify the most suitable fit.[12]

The "Analysis of Variance" (ANOVA) F test was utilized to select best fit 2nd order quadratic polynomial equation (Eq. 1) based on its level of significance

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_2C+\beta_{12}AB+\beta_{13}AC+\beta_{23}BC+\beta nA^2+\beta_{22}B^2+\beta_{33}C^2$$

Where Y refers to response selected; while β_0 indicates intercept; β_1-β_{33} denote "regression coefficients". The factors under investigation are denoted by A, B and C.

The Model, developed through the design, underwent evaluation and comparison based on several parameters. These parameters encompassed the "correlation coefficient, adjusted correlation coefficient, predicted correlation and adequate precision". To

gain insights from the model involved conducting response surface analysis, which included generation of "2D-contour and 3D-response surface plots". Through the utilization of a numerical optimization method, the model predicted the optimal analytical condition. These optimized conditions were subsequently identified within the designated "Analytical Design Space". This was visualized as an "overlay plot" that incorporated a flag consisting of all relevant parameters.[12,13]

Method validation

ICH Q2 (R_1) guidelines were used to validate the optimized RP-HPLC method. Various parameters studied were linearity range, system suitability, LOD and LOQ limits, interday and intraday precision, accuracy, ruggedness and robustness. Further the optimized method was checked for its efficacy in assessment of quality of marketed NBV tablets.[14]

System Suitability and Specificity

The suitability of the system was assessed by studying various responses viz. Retention time (Rt), Peak Area (PA) and Tailing factor (Tf). These measurements were carried out in three replicates. Further, the interference of the mobile phase in the detection of NBV was studied by specificity study.

Linearity and Sensitivity analysis

Aliquots of stock solution were diluted serially in order to get the NBV solution of different concentration viz. 10-50 µg/mL. These solutions were then injected into system and peak area of resulting chromatograms were determined. Further, the linearity of the method was determined by plotting the graph of concentration vs peak area along X and Y axis respectively. The regression coefficient (R^2) value is indicative of linearity of the method. Further LOD and LOQ were determined by the following formulae

$$LOD = \frac{3.3 \times Standard\ deviation}{Slope} \qquad LOQ = \frac{10 \times Standard\ deviation}{Slope}$$

Precision

Precision of the method was established by observing the closeness of results obtained by injecting solutions of same concentration in triplicate. The precision was determined both interday (within a day) and intraday (different days). Based on the results obtained % RSD value were calculated.

Accuracy study

The recovery method was used to determine the accuracy of the proposed method. The % recovery of the drug after spiking 10 µg/mL solution at 80%, 100% and 120% of its concentration level was determined by using the following formula

$$\%\ Recovery = \frac{Recovered\ Concentration}{Initial\ Concentration} \times 100$$

Robustness

Few deliberate changes in the optimized analytical parameters were carried out to determine the robustness of the method. The parameters changed were solvent system composition, wavelength and flow rate.

Ruggedness

Analysis of the NBV solution was carried out by two different analysts using optimized method in order to determine the ruggedness of the method. The ruggedness of the method was determined by calculating the % RSD of the obtained results.

Assay of Marked tablets

Weighed and powdered 20 NBV tablets (Nebicia Hydrochloride-5 mg Mfg. by Daxia Healthcare) into a fine powder. The powder sample equivalent to 10 mg of NBV was transferred to volumetric flask of 10 mL capacity. The powder sample is diluted with 2 mL solvent system and further sonication was carried out 15 min in order to get the sample completely solubilize. The volume was made up to mark by solvent. Filtration of the resulting solution was carried out. The obtained solution was further serially diluted to get the final solution of 30 µg/mL. The analysis of this sample was carried out and the % assay of the tablet was determined.

RESULTS

Optimization of Chromatographic Condition

Optimization of RP-HPLC method for the analysis of NBV was achieved by implementing AQbD. Initially preliminary experiments were carried out wherein various mobile phases were tried in order to get well resolved, sharp peak (mobile phase was varied by using different combinations of the solvents like methanol, water, acetic acid and phosphate buffers with different flow rate). Based on the results obtained it was found that mobile phase consisting of MeOH:0.1% aqueous acetic acid in 71:29 ratio was giving optimal sharp peak at 4.19 min with a flow rate of 0.8 mL/min and at 282 nm. All other compositions failed to give sharp peak. Further optimization with respect to wavelength, mobile phase and flow rate was carried out by utilizing BBD approach.

Risk Assessment and Screening Studies[10]

As per the discussion in the methodology study, the findings of analysis of ATP, CQAs and process parameters are detailed in Table 1. Ishikawa Fish-Bone diagram (Figure 2) depicting the association of different inputs of process parameters and analytical attributes was explored. The probable risk factors related to the solvent system were method of extraction of sample, extraction time, choice of solvents and mobile phase composition while instrument-related parameters were chromatographic mode, flow rate, injection volume and wavelength of detection. The risk factors viz flow rate, mobile phase composition and wavelength

of detection are explored. Further for optimization by applying suitable experimental design.

Method Optimization

The DoE software was employed to optimize analytical RP-HPLC parameters in order to get the best condition for estimating NBV. The impact of independent variables such as methanol (70 to 72%v/v), flow rate (0.7 to 0.9 mL/min) and wavelength (281 to 283 nm), on dependent variables such as Rt, PA and Tf, was estimated. The DoE software, a total of 17 experimental trial runs were obtained, facilitating the identification of the optimum chromatographic separation for NBV (Tables 2 and 3). The central composition point was defined by the variables: mobile phase ratio (MeOH:0.1% aqueous acetic acid 71:29% v/v), flow rate (0.8 mL/min) and wavelength (282nm), were identified for the design space selection. The effect of these variables on the responses were evaluated to establish the design space. Response data were fitted into various polynomial models, such as linear, second order and quadratic. The quadratic model emerged as the best fit, as evidenced by its high regression coefficient (R^2=0.9999), significantly surpassing other models ($p<0.0001$). ANOVA tests were conducted for each model and parameters such as "sum of squares, F value, mean square and p value" for each response were determined. The results of the same are as depicted in Tables 4 and 5. The 3D-response surface plots and 2D-contour plots of each response, demonstrating the influence of many factors (integration effect) simultaneously on a response are shown in (Figures 3-8).

Table 1: QTPP, CQAs and Process Parameters of Proposed RP-HPLC Method.

Sl. No.	AQbD Parameters	Description
1	Quality Target Product Profile	Flow rate, Mobile phase composition, Wavelength of detection, pH, Temperature, and Column attributes, Injection volume, Pressure, Buffer attributes.
2	Critical Quality Attributes	Retention time, Peak area, Theoretical plates, Peak purity, Tailing factor.
3	Process Parameters	Specificity and Selectivity, Linearity, range, Precision, Robustness, Ruggedness, Accuracy, LOD, LOQ.

Effect of independent factors on retention time (Y1)

To depict the effect of variables under control on retention time, expressions like 3D response, counter plot and model equation were utilized. The effect of independent variables on retention time is as shown in Figures 3 and 4 (A1-A3 and B1-B3). When the data obtained after trial batches as per 3^3 factorial design is treated with software analytics, it was found that interaction terms have an insignificant effect on retention time. Thus, the linear equation obtained for Y1 (retention time) is given as follows

$$\text{Retention time (Y1)} = +3.72 - 0.0966A - 0.4499B + 0.0015C$$

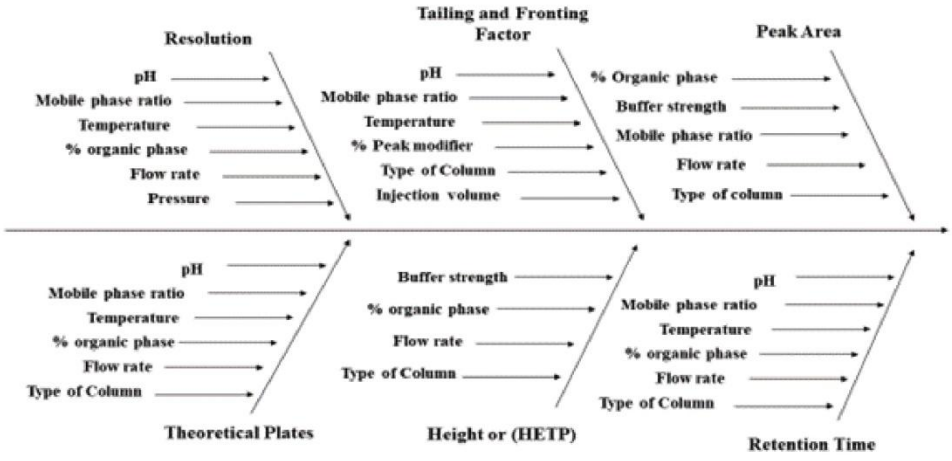


Figure 2: Ishikawa fishbone diagram.

The term, B were found to be significant as the *p*-value for corresponding factors was found to be less than 0.05. Further, the significance of the model was determined by the model F-value which was 13.89. This witnessed that model is significant with a 99.98% chance of F-value being due to signal (i.e. noise<0.02%). The closeness between predicted (0.7117) and adjusted (0.7074) R^2 was observed. The sufficiency of the signal is highlighted by an adequate precession value of 11.1769. The results of the same are depicted in Tables 4 and 5. The composition of mobile phase (A) and flow rate (B) have a "negative effect" on retention time, while wavelength (C) has a "positive effect" on retention time. The flow rate has more dominant effect on retention time as compared to the other two independent variables.

Table 2: Variables and their levels used in Box-Behnken design.

Factor	Levels used, actual (coded)	
	Low (-1)	High (+1)
Independent variables		
A: Solvent conc. in mobile phase (% v/v)	70 (-1)	72 (+1)
B: Flow rate (mL/min)	0.7 (-1)	0.9 (+1)
C: Wavelength (nm)	281	283
Dependent variables		
Y_1: Retention time	Minimize	
Y_2: Peak area	In the range	
Y_3: Tailing factor	Minimize	

Effect of independent factors on peak area (Y2)

To depict the effect of variables under control on retention time, expressions like 3D response, counter plot and model equation were utilized. The effect of independent variables on peak area is as shown in Figures 5 and 6 (C1-C3 and D1-D3). When the data obtained after trial batches as per 3^3 factorial design is treated with software analytics, it was found that, both individuals as well as interaction terms have insignificant effect on peak area. Thus, the 2nd order quadratic equation obtained for Y1 (peak area) is expressed as given below

$$\text{Peak area (Y2)} = +546.36+1.43A-69.44B+5.98C-1.98AB-0.0371AC-0.8266BC+1.36A^2+9.71B^2-1.84C^2$$

The term, A, B, C, AB, A^2, B^2 and C^2 were found to be significant as the P-values for corresponding factors were found to be less than 0.05. Further, the significance of the model was determined by the model F-value which was 3292.75. This witnessed that the model is significant with a 99.99% chance of F-value being due to signal (i.e. noise<0.01%). The closeness between predicted (0.9985) and adjusted (0.9998) R^2 was observed. The sufficiency of signal is highlighted by adequate precession value of 170.7498. The results of the same are as depicted in Tables 4 and 5. The composition of mobile phase (A) and wavelength (C) have "positive effect" on peak area, while flow rate (B) has "negative effect" on peak area.

Table 3: Box-Behnken Design (BBD) based independent variable and their effect on dependent variables.

Run No	Independent Variables			Dependent variables		
	A	B	C	RT	PA	TF
1	71	0.8	282	3.612	546.621	0.49
2	71	0.8	282	3.619	545.124	0.44
3	70	0.7	282	4.194	622.816	0.42
4	71	0.7	281	4.187	616.703	0.48
5	70	0.8	281	3.703	539.244	0.44
6	71	0.8	282	3.55	548.039	0.47
7	72	0.8	281	3.592	541.349	0.57
8	71	0.9	281	3.225	478.997	0.5
9	71	0.8	282	4.184	545.128	0.45
10	71	0.8	282	4.19	546.874	0.44
11	70	0.8	283	3.743	550.477	0.45
12	72	0.9	282	3.112	488.084	0.54
13	72	0.7	282	4.022	630.453	0.51
14	71	0.7	283	4.184	631.114	0.46
15	71	0.9	283	3.25	490.101	0.5
16	70	0.9	282	3.401	488.354	0.46
17	72	0.8	283	3.542	552.433	0.52

A: Solvent concentration in mobile phase (% v/v), B: Flow rate (mL/min), C: Wavelength (nm).

Table 4: Regression analysis for responses Retention time; Peak area, Tailing factor for fitting to different polynomial models.

Models	SD	R^2	Adjusted R^2	Predicted R^2	PRESS	Adequate Precision	Remark
Retention Time (RT)							
Linear	0.2016	0.7622	0.7074	0.7117	0.6407	11.1769	Suggested
2FI	0.2286	0.7648	0.6236	0.6618	0.7514		
Quadratic	0.2487	0.8052	0.5547	0.6465	0.7856		
Peak Area (PA)							
Linear	5.86	0.9887	0.9860	0.9787	836.32	170.7498	
2FI	6.54	0.9891	0.9826	0.9544	1793.27		
Quadratic	1.15	0.9998	0.9995	0.9985	59.13		Suggested
Tailing Factor (TF)							
Linear	0.0224	0.7516	0.6943	0.6292	0.0097	11.5231	Suggested
2FI	0.0234	0.7908	0.6653	0.5451	0.0119		
Quadratic	0.0171	0.9215	0.8206	0.7808	0.0057		

Table 5: Analysis of Variance (ANOVA) for the responses (Y1: Retention time; Y2: Peak area, Y3: Tailing factor)

Results (ANOVA)	Rt	PA	Tf
Regression			
Sum of squares	1.69	39318.28	0.0197
Degrees of freedom (df)	3	9	3
Mean Square	0.5646	4368.70	0.0066
F-value	13.89	3292.75	13.11
p-value	0.0002	<0.0001	0.0003
Lack of fit tests			
Sum of squares	0.1030	3.09	0.0046
Degrees of freedom (df)	9	3	9
Mean Square	0.0114	1.03	0.0005
F-value	0.1076	0.6650	1.09
p-value	0.9971	0.6159	0.5046
Residual			
Sum of squares	0.5284	9.29	0.0065
Degrees of freedom (df)	13	7	13
Mean Square	0.0406	1.33	0.0005

The impact of the flow rate on peak area has more pronounced compared to the other two independent variables.

Effect of independent factors on tailing factor (Y3)

To depict the effect of variables under control on tailing factor, expressions like 3D response, counter plot and model equation were utilized. The effect of independent variables on the tailing factor is as shown in Figures 7 and 8 (E1-E3 and F1-F3). When the data obtained after trial batches as per 3^3 factorial design is treated with software analytics, it was found that interaction terms have an insignificant effect on retention time. Thus, the linear equation obtained for Y3 (tailing factor) is given as follows

Tailing factor (Y3)=+0.4788+0.0462A+0.0162B-0.0075C

The term, A was found to be significant as the P-values for corresponding factors were found to be less than 0.05. Further, the significance of the model was determined by the model F-value which was 13.11. This witnessed that the model is significant with a 99.97% chance of F-value being due to signal (i.e. noise<0.03%). The closeness between predicted (0.0.6292) and adjusted (0.6943) R^2 was observed. The sufficiency of the signal is highlighted by an adequate precession value of 11.5231. The results of the same are depicted in Tables 4 and 5. The composition of mobile phase (A) and flow rate (B) have "positive effect" on tailing factor, while

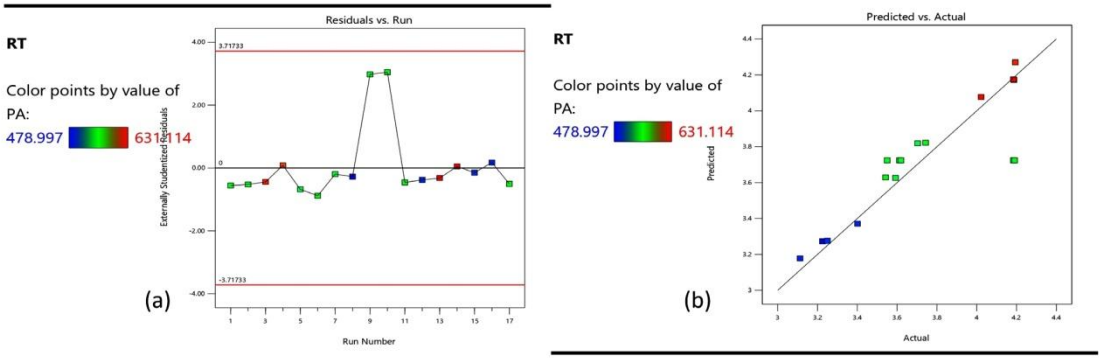


Figure 3: Plot (a) showing true versus predicted values, with residual plot (b) for retention time.

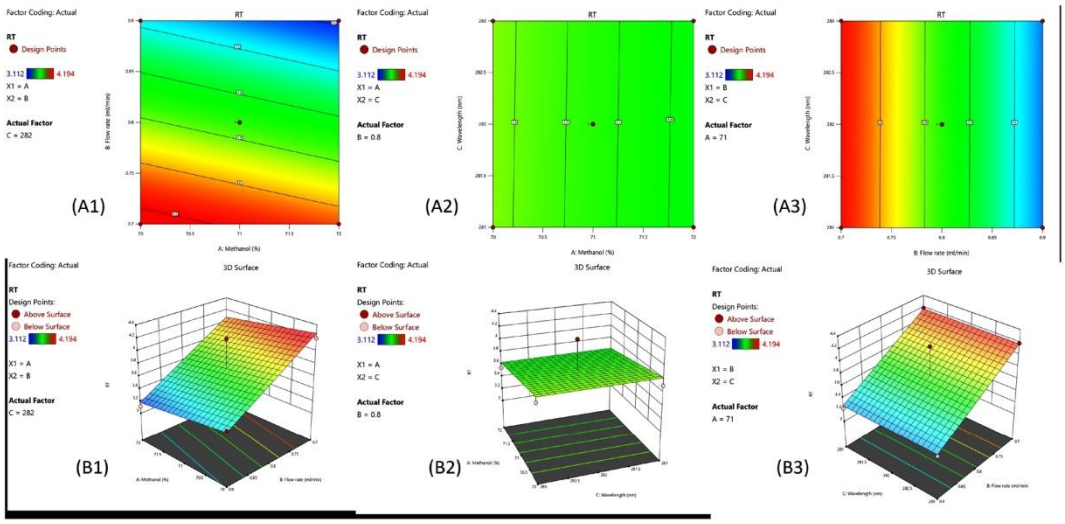


Figure 4: Relation plot (A1-A3 and B1-B3) 2-D counter plots and 3-D response surface plots showing the influence of CMP's i.e., (a) Solvent concentration in mobile phase (% v/v), (b) Flow rate (mL/min), (c)Wavelength (nm) on retention time (RT) as a CAA

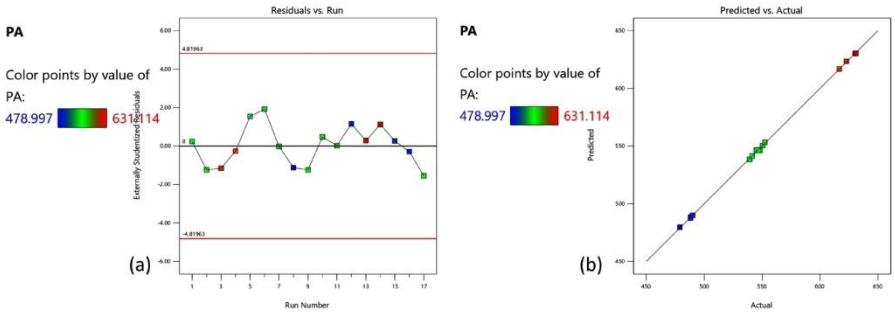


Figure 5: Plot (a) showing true versus predicted values, with residual plot (b) for peak area.

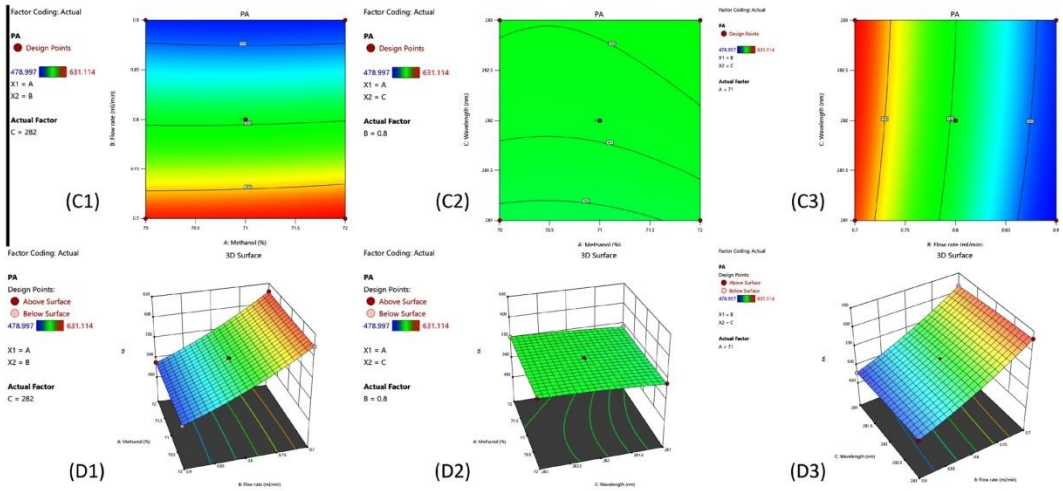


Figure 6: Relation plot (C1-C3 and D1-D3) 2-D counterplots and 3-D response surface plots showing the influence of CMP's i.e., (a) Solvent concentration in the mobile phase (% v/v), (b) Flow rate (mL/min), (c) Wavelength (nm) on Peak Area (PA) as a CAA.

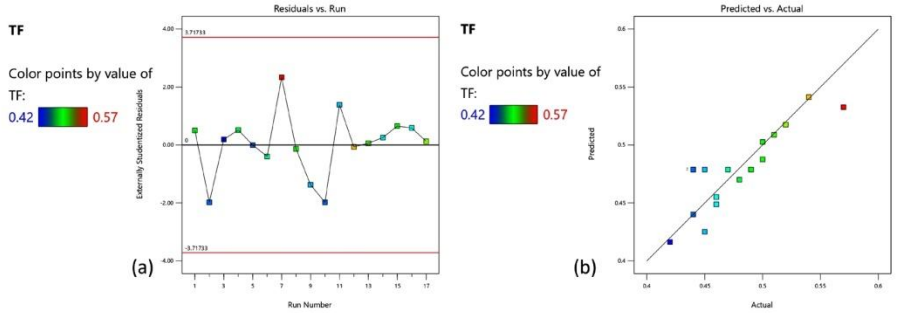


Figure 7: Plot (a) showing true versus predicted values, with residual plot (b) for tailing factor.

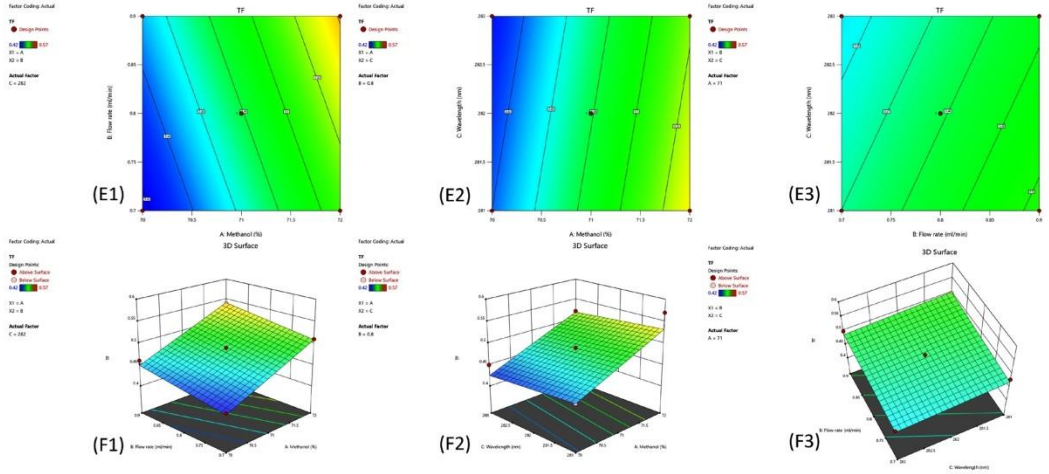


Figure 8: Relation plot (E1-E3 and F1-F3) 2-D counterplots and 3-D response surface plots showing the influence of CMP's i.e., (a) Solvent concentration in the mobile phase (% v/v), (b) Flow rate (mL/min), (c) Wavelength (nm) on the Tailing Factor (TF) as a CAA.

wavelength (C) has "negative effect" on tailing factor. The mobile phase has more dominant effect on tailing factor as compared to other two independent variables.

Optimization (Graphical and numerical)

Figure 9(a) shows the overlay plot depicting the relationship between independent variables and responses for graphical optimization, whereas Figure 9(b) depict the results of the numerically optimized method, having the highest desirability of 0.940.

Method validation

Validation of an analytical method is an important step before its implementation in routine qualitative and quantitative analyses.

System Suitability and Specificity

The developed analytical method was assessed for system suitability, focussing on critical attributes such as chromatogram "retention time, peak area and tailing factor". The results showed no significant difference, (%RSD<0.5) when it is studied with three replicates of NBV having a concentration of 30 µg/mL. The method specificity was assessed by analyzing both blank and spiked quality control samples which revealed that there is no interference in either case. This confirmed the suitability and specificity of the method for quantifying NBV in the formulation. Figure 10(a) presented an HPLC chromatogram of standard NBV.

Assay of Marketed tablets

The assay of the marketed tablet was carried out by the developed method and drug content was found to be 105.60% of the label claim which complies with prescribed standards (90.0% to 110.0%). The chromatogram for the assay study is as shown in Figure 10 (b).

Linearity and Sensitivity analysis

ICH guidelines were used to establish the linearity of the proposed analytical method. Linearity results obtained within the studied concentration range of 10 to 50 µg/mL, with a regression coefficient value of 0.999 and equation of the linear line obtained is y=15.215x-6.625. The LOD and LOQ represents the sensitivity of the method. The LOD and LOQ values for the method developed

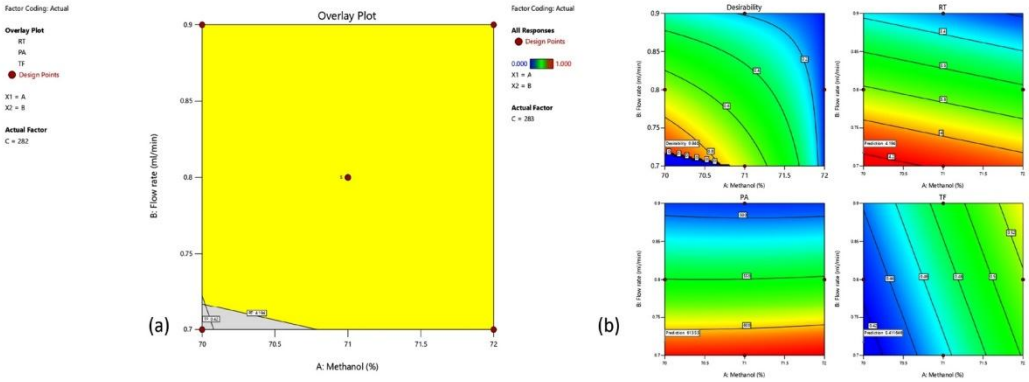


Figure 9: a. Overlay plot showing the optimized chromatographic conditions and their predicted responses, b. Graphs showing numerical optimization with the highest desirability.

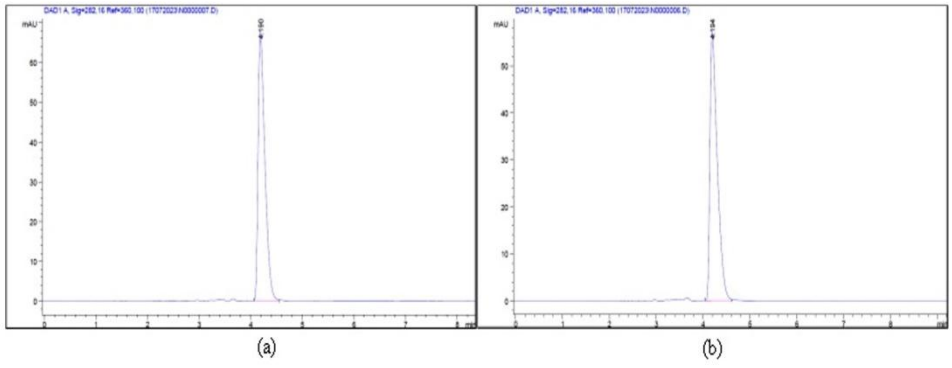


Figure 10: Chromatogram depicting (a) NBV Standard (b) NBV assay.

were found to be 0.54 µg/mL and 1.64 µg/mL respectively. These finding shows the high sensitivity of the method.

Precision

The results of precision study are presented in Table 6 the %RSD value for peak area in terms of interday and intraday precision study was obtained below 2% which indicates that method is precise for routine analysis.

Accuracy study

Method accuracy signifies its capability to align experimental results with theoretical expectations. The accuracy study results, as presented in Table 7, reveal that the recovered concentration of NBV falls within the range of 102.49 to 103.44%. Additionally, the % RSD value is less than 2%, demonstrating the high accuracy of the method.

Robustness

The robustness of the study evaluated the process parameters and it was observed that the % RSD value remained consistently below 2 percent, well within the specified limits. The chromatogram showed no noticeable change, indicating that the developed RP-HPLC procedure is indeed robust (Table 8).

Ruggedness

The ruggedness of the developed method was assessed by analyzing the prepared samples by different analysts. There were no any significant differences in observed responses (%RSD <2%) which indicates that the method is rugged (Table 8).

Table 6: Intraday and inter-day precision of NBV.

Analyte	Conc. (µg/mL)	Intraday (*n*=3)		Inter day (*n*=3)					
				Day 1		Day 2		Day 3	
		Peak area	% RSD	Peak area	% RSD	Peak area	% RSD	Peak area	% RSD
NBV	10.00	147.82	0.02	147.92	0.07	155.82	1.43	147.47	0.04
	30.00	441.76	0.10	442.70	0.10	451.85	1.57	442.85	0.05
	50.00	771.62	0.02	761.02	1.47	778.71	0.06	764.96	0.73

n=Number of replicates; RSD=Relative standard deviation.

Table 7: Recovery studies of NBV (*n*=3).

Compound	Contents (µg/mL)	Quantity added (µg/mL)	Theoretical amount (µg/mL)	Recovered amount (µg/mL)	Recovery (%)	% RSD
NBV	10	8 (80%)	18	18.28	103.44	0.02
		10 (100%)	20	20.27	102.71	0.12
		12 (120%)	22	22.30	102.49	0.20

n=Number of replicates; RSD=Relative standard deviation.

Table 8: Result of robustness and ruggedness study.

Robustness					
Change of Flow Rate	0.7 mL			0.9 mL	
Sl. No.	Conc µg/mL	Area	Sl. No.	Conc µg/mL	Area
1	40	687.6149	1	40	535.0284
2	40	687.9306	2	40	534.8267
	Mean	687.77		Mean	534.93
	SD	0.22		SD	0.14
	%RSD	0.03		%RSD	0.03
Mobile Phase	70 MeOH+30%			72 MeOH+28%	
Sl. No.	Conc µg/mL	Area	Sl. No.	Conc µg/mL	Area
1	40	601.7283	1	40	602.4002
2	40	601.4721	2	40	602.5197

Robustness					
Change of Flow Rate	**0.7 mL**			**0.9 mL**	
Sl. No.	**Conc µg/mL**	**Area**	**Sl. No.**	**Conc µg/mL**	**Area**
	Mean	601.6		Mean	602.46
	SD	0.18		SD	0.08
	%RSD	0.03		%RSD	0.01
Wavelength Change	281 nm			283 nm	
Sl. No.	Conc µg/mL	Area	Sl. No.	Conc µg/mL	Area
1	40	594.9376	1	40	605.8355
2	40	594.5432	2	40	605.5332
	Mean	594.7		Mean	605.68
	SD	0.28		SD	0.21
	%RSD	0.05		%RSD	0.04
Ruggedness					
Analyst	Conc µg/mL	Area		Amt Found	
1	20.00	306.92		19.743261	
2	20.00	306.627		19.723997	
	Mean	306.77		19.73	
	SD	0.207		0.014	
	%RSD	0.068		0.069	

RSD=Relative standard deviation.

DISCUSSION

The RP-HPLC method for analyzing NBV was fine-tuned using AQbD principles and BBD. Optimal conditions were found with a mobile phase of MeOH:0.1% aqueous acetic acid (71:29), a flow rate of 0.8 mL/min and a wavelength of 282 nm, resulting in well-defined peaks at 4.19 min. A risk assessment focused on key factors: Peak Area (PA), Retention time (Rt) and Tailing factor (Tf). Using DoE software, a quadratic model with a high R^2 value of 0.9999 was selected. The impact of variables on responses was clear: methanol and flow rate affected retention time negatively, while wavelength had a positive effect. Peak area was influenced positively by the mobile phase and wavelength but negatively by the flow rate. Tailing factor was positively affected by the mobile phase and flow rate but negatively by wavelength. Graphical and numerical optimization highlighted the relationship between variables and responses, with the numerically optimized method achieving a high desirability of 0.940. Method validation ensured system suitability, specificity, linearity (R^2=0.999), sensitivity (LOD=0.54 µg/mL, LOQ=1.64 µg/mL), precision (%RSD<2%), accuracy (recovery 102.49% to 103.44%, %RSD<2%) and robustness (%RSD<2%). Ruggedness was confirmed by different analysts. Analyzing marketed tablets using the method resulted in drug content within standards (105.60% of label claims). In conclusion, the optimized RP-HPLC method, validated for NBV analysis, is robust and precise, making it suitable for routine use in pharmaceutical analysis.

CONCLUSION

In the present study a simple, precise, robust RP-HPLC method was successfully developed for analysis of NBV with the aid of Analytical Quality by Design approach. The study utilizes Box-Behnken Design as a tool of optimization. Various analytical parameters optimized were mobile phase, wavelength and flow rate. The optimized conditions obtained were mobile phase (MeOH:0.1% aqueous acetic acid 71:29% v/v), wavelength (282 nm) and flow rate (0.8 mL/min). The effect of these analytical conditions on retention time, peak area and tailing factor was assessed. The developed method was validated as per ICH guidelines. The study concludes that AQbD can be effectively utilized for development of the RP-HPLC method for the analysis of NBV.

ACKNOWLEDGEMENT

The Authors would like to express their sincere gratitude to Dr. Sunil S Jalalpure, Principal, KLE College of Pharmacy, KAHER, Belagavi, Karnataka, India for his constant support and providing the necessary analytical infrastructure to carry out the research work. Additionally, authors are also thankful to the Principal KLE

College of Pharmacy, Nipani, Karnataka, India for consistent encouragement and support throughout the research work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AQbD: Analytical quality by Design; **ANOVA:** Analysis of Variance; **ATP:** Analytical Target Attributes; **BBD:** Box-Behnken design; **CAA:** Critical Analytical Attributes; **CMAs:** Critical Material Attributes; **CPPs:** Critical Process Parameters; **CQA:** Critical Quality Attributes **NBV:** Nebivolol; **DOE:** Design of Experiment; **ICH:** International Council for Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **OPA:** Ortho Phosphoric Acid; **PA:** Peak Area; **QbD:** Quality by Design; **RA:** Risk Assessment; **RSD:** Residual Standard Deviation; **RT:** Retention Time; **NTP:** Number of Theoretical Plates; **RP-HPLC:** Reverse Phase-High Performance Liquid Chromatography; **TF:** Tailing Factor.

REFERENCES

1. Ingle U, Lali A. Preparative separation of nebivolol isomers by improved throughput reverse phase tandem two-column chromatography. Chirality. 2020;32(11):1324-35. doi: 10.1002/chir.23278, PMID 32920878.
2. Nandania J, Rajput SJ, Contractor P, Vasava P, Solanki B, Vohra M. Quantitative determination of nebivolol from human plasma using liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2013;923-924:110-9. doi: 10.1016/j.jchromb.2013.01.034, PMID 23500355.
3. Nandania J, Rajput SJ, Contractor P, Vasava P, Solanki B, Vohra M. Quantitative determination of nebivolol from human plasma using liquid chromatography-tandem mass spectrometry. Journal of Chromatography B. 2013;923-924:110-9. doi: 10.1016/j.jchromb.2013.01.034.
4. Peraman R, Bhadraya K, Padmanabha Reddy Y. Analytical quality by design: a tool for regulatory flexibility and robust analytics. Int J Anal Chem. 2015;2015:868727. doi: 10.1155/2015/868727, PMID 25722723.
5. Hakiem AF, Hamdy AK, Ali HR, Gomaa M, Aboraia AS. In depth investigation of the retention behavior of structurally related β-blockers on RP-HPLC column: quality by Design and quantitative structure-property relationship complementary approaches for optimization and validation. J Chromatogr B Analyt Technol Biomed Life Sci. 2021;1166:122549. doi: 10.1016/j.jchromb.2021.122549, PMID 33545562.
6. Vogt FG, Kord AS. Development of Quality-by-Design analytical methods. J Pharm Sci. 2011;100(3):797-812. doi: 10.1002/jps.22325, PMID 21280050.
7. Alam P, Shakeel F, Taleuzzaman M, Foudah AI, Alqarni MH, Aljarba TM et al. Box-Behnken design (BBD) application for optimization of chromatographic conditions in RP-HPLC method development for the estimation of thymoquinone in Nigella sativa Seed Powder. Processes. 2022;10(6):1082. doi: 10.3390/pr10061082.
8. Suryawanshi SS, Palled MS. Box-Behnken design assisted optimization and standardization of chromatographic methodology for quality assessment of metformin: analytical quality by design avenue. IJPER. 2022;56(2s):s152-62. doi: 10.5530/ijper.56.2s.86.
9. Tome T, Žigart N, Časar Z, Obreza A. Development and optimization of liquid chromatography analytical methods by using AQbD principles: overview and recent advances. Org Process Res Dev. 2019;23(9):1784-802. doi: 10.1021/acs.oprd.9b00238.
10. Mandpe SR, Parate VR, Naik JB. Experimental design approach, screening and optimization of system variables, analytical method development of flurbiprofen in nanoparticles and stability-indicating methods for high-pressure liquid chromatography. Future J Pharm Sci. 2022;8(1):1-21. doi: 10.1186/s43094-022-00426-4.
11. Patil TS, Deshpande AS. Development of an innovative quality by design (QbD) based stability-indicating HPLC method and its validation for clofazimine from its bulk and pharmaceutical dosage forms. Chromatographia. 2019;82(2):579-90. doi: 10.1007/s10337-018-3660-8.
12. Saini S, Sharma T, Patel A, Kaur R, Tripathi SK, Katare OP et al. QbD-steered development and validation of an RP-HPLC method for quantification of ferulic acid: rational application of chemometric tools. J Chromatogr B Analyt Technol Biomed Life Sci. 2020;1155:122300. doi: 10.1016/j.jchromb.2020.122300, PMID 32771967.
13. Saini S, Sharma T, Patel A, Kaur R, Tripathi SK, Katare OP ET AL. QbD-steered development and validation of an RP-HPLC method for quantification of ferulic acid: rational application of chemometric tools. Journal of Chromatography B. 2020;1155. doi: 10.1016/j.jchromb.2020.122300.
14. Alruwaili NK. Analytical Quality by Design approach of reverse-phase high-performance liquid chromatography of atorvastatin: method development, optimization, validation and the stability-indicated method. Int J Anal Chem. 2021;2021:8833900. doi: 10.1155/2021/8833900, PMID 33628253.
15. Patil TS, Deshpande AS. Development of an innovative quality by design (QbD) based stability-indicating HPLC method and its validation for clofazimine from its bulk and pharmaceutical dosage forms. Chromatographia. 2019;82(2):579-90. doi: 10.1007/s10337-018-3660-8.
16. ICH. Validation-of-analytical-procedures-text-and-methodology, Guideline on validation of analytical procedures: text and methodology. International Conference on Harmonization (ICH: Q2); 2005.

Cite this article: Randive KH, Maste MM, Kempwade AA. Exploration of AQbD Approach for Development of RP-HPLC Method of Nebivolol. Int. J. Pharm. Investigation. 2024;14(3):915-27.



KRISHNA VISHWA VIDYAPEETH
(Deemed to be University)
Knowledge • Innovation • Excellence

Accredited by NAAC with A+ Grade (CGPA : 3.39 on 4 point scale), An ISO 9001:2015 Certified University

KRISHNA INSTITUTE OF PHARMACY, KARAD

CERTIFICATE

This is to certify that

Mr/~~Ms~~/~~Mrs~~/~~Dr~~ Krantisinha Hanumant Randive.

of KLE College of Pharmacy, Nipani has participated as a delegate & presented his/her worthy oral presentation titled Exploring Analytical Quality By Design (AQbD) Enabled Rp-Hplc Method for Antihypertensive Agent. at National Conference on **"Recent Developments & Challenges in Pharmaceutical Sciences"** on **4th & 5th March 2023.**

Dr. Akshada A. Koparde	Dr. Amol S. Shete	Dr. M. V. Ghorpade
Dean Academics, KIP, KVV, Karad	Dean I/C, KIP, KVV, Karad	Registrar, KVV, Karad
(Co-convener)	(Convener)	



Sant Dnyaneshwar Shikshan Sanstha's
ANNASAHEB DANGE COLLEGE OF B PHARMACY, ASHTA

Department of Pharmaceutical Chemistry under the aegis of IQAC

In association with

Shri Vasantrao Bandoji Patil Trust's
APPASAHEB BIRNALE COLLEGE OF PHARMACY, SANGLI

Two Days National Level Conference

On

"Recent Advances In Drug Design"

Certificate

This certificate is awarded to Prof./Dr./Mr./Mrs./Ms. Krantisinha H. Randive

from KLE College of Pharmacy, Belagavi

has actively presented poster entitled as Exploration of AQbD approach for development of Stability Indicating RP-HPLC method of Nebivolol.

under Faculty / Research Scholar / PG / UG / Diploma category in Two Days, National Level Conference on **"Recent Advances In Drug Design"** organized by **Annasaheb Dange College of B Pharmacy, Ashta** on 5th to 6th February 2024. His / Her poster presentation in conference is deeply appreciated.

Place : Ashta
Date : 6th Feb. 2024

Dr. R. R. Shah	Dr. Y. H. Momin	Dr. S. A. Tamboli	Prof. Dr. M. G. Saralaya	Prof. R. A. Kanal
Co-ordinator, ABCP, Sangli	Co-ordinator, ADCBP, Ashta	Convener & Principal, ABCP, Sangli	Convener & Principal, ADCBP, Ashta	Executive Director, SDSS



Sant Dnyaneshwar Shikshan Sanstha's
ANNASAHEB DANGE COLLEGE OF B PHARMACY, ASHTA

Department of Pharmaceutical Chemistry under the aegis of IQAC
In association with

Shri Vasantrao Bandujji Patil Trust's
APPASAHEB BIRNALE COLLEGE OF PHARMACY, SANGLI

Two Days National Level Conference
On
"Recent Advances In Drug Design"

Certificate

This certificate is awarded to Prof./Dr./Mr./Mrs./Ms. Krantisinha H. Randive
from KLE college of pharmacy, Belagavi
has actively participated as Delegate / Resource Person / Chairperson / Evaluator in Two Days, National Level Conference on "Recent Advances In Drug Design" organized by Annasaheb Dange College of B Pharmacy, Ashta on 5th to 6th February 2024. His / Her participation in conference is deeply appreciated.

Place : Ashta
Date : 6th Feb. 2024

Dr. R. R. Shah, Co-ordinator, ABCP, Sangli
Dr. Y. H. Momin, Co-ordinator, ADCBP, Ashta
Dr. S. A. Tamboli, Convener & Principal, ABCP, Sangli
Prof. Dr. M. G. Saralaya, Convener & Principal, ADCBP, Ashta
Prof. R. A. Kanai, Executive Director, SDSS



KLE COLLEGE OF PHARMACY, BELAGAVI
A Constituent Unit of
KLE Academy of Higher Education and Research
(Deemed-to-be University established u/s 3 of the UGC Act, 1956), Re-accredited A+ Grade by NAAC (3rd Cycle) Placed in Category 'A' by MoE (GoI)

BELAGAVI
NAAC 3rd Cycle 'A+' Grade
B.Pharm Program Accredited for 6 years (year 2019-25)
nirf39th Rank 2023

Certificate of Participation

Dr./Mr./Ms. Krantisinha . H. Randive
of K. L. E. College of Pharmacy, Nipani.
32/34
Has participated as a delegate in the Workshop & Hands on Training on

"High Performance Liquid Chromatography (HPLC)"

on 04th April 2024, organized by
Department of Pharmaceutical Chemistry, KLE College of Pharmacy, Belagavi.

PROGRAM CO-ORDINATOR
Prof. (Dr.) M. S. Palled
KLE College of Pharmacy, Belagavi.

CHIEF CO-ORDINATOR
Prof. (Dr.) Sunil S. Jalalpure
Principal
KLE College of Pharmacy, Belagavi.

PROGRAM CO-ORDINATOR
Mr. Shailendra Suryawanshi
KLE College of Pharmacy, Belagavi.



KLE COLLEGE OF PHARMACY, BELAGAVI

A Constituent Unit of

KLE Academy of Higher Education and Research

(Deemed-to-be University established u/s 3 of the UGC Act, 1956), Re-accredited A+ Grade by NAAC (3rd Cycle) Placed in Category 'A' by MoE (GoI)

BELAGAVI

B.Pharm Program Accredited for 6 years (year 2019-25)

NAAC 3rd Cycle 'A+' Grade

nirf 39th Rank 2023

CERTIFICATE

Dr./Mr./Ms. Randive . Krantisinha . H.

of K. L. E. College of Pharmacy, Nipani

Has actively participated as a Resource Person/Delegate in the workshop

"PURSUIT OF ARTIFICIAL INTELLIGENCE IN PHARMACY EDUCATION"

on 14th May 2024, organized by Department of Pharmaceutical Chemistry,

KLE College of Pharmacy, Belagavi

Mr. Kiran Gaikwad
PROGRAM CO-ORDINATOR

Mr. Nikhil Gawas
PROGRAM CO-ORDINATOR

Prof. (Dr.) M. M. Maste
PROGRAM CONVENER

Prof. (Dr.) Kalpana Patil
CHIEF CONVENER (DOPE)

Prof. (Dr.) Sunil S. Jalalpure
Principal
CHIEF CO-ORDINATOR

Errata

Sl. NO	Particulars	Appropriate (Yes/No)	Changes Suggested	Correction made/clarification
1	**Title of the thesis**	**Yes**	NIL	Not Applicable
2	**Abstract**	**Yes**	NIL	Not Applicable
3	**Introduction**	**Yes**	**Numerous grammatical errors noticed** 1. Spectral methods examine, the light absorbed or emitted by pharmaceuticals or medications. (Page 2) **2.** This approach has various limitations like numbers of experiments to be carried out are more which is time consuming as well as economically not affordable as large amount of chemicals are wasted. 3."methodical consider to development that starts with established goals and emphasises both product under-standing and process control,	**Changes suggested were rectified and corrected in revised thesis** 1. Spectral methods examine light absorbed or emitted by pharmaceuticals **substances.** (Page 2) 2. This approach have few limitations, **to exemplify requirement of more experimentation** which is time consuming as well as economically not affordable as large amount of chemicals are wasted. 3. "**systematic approach** to development that starts with established goals and **emphasizes** both product **understanding** and process control, based on sound science and **quality**

			based on sound science and quality threat management. (Page no 4) 4. That way, we can look at how different factors affect the method's output all at once. So, without wasting chemicals on a huge number of experiments, a simple, robust, and accurate analytical method can be devised in a short amount of time. Additionally, the regulatory bodies are increasingly leaning towards and relying on QbD methods. That is why AQbD is so important right now for getting the analytical results as per requirement (Page no 4) 5. (Form I is needle type, Form II is prism type), (page no 7)	**risk management.** (Page no 4) 4. **This approach helps us to simultaneously investigate the effect of various variables on outcome of the method. Thus simple, robust and precise analytical method can be developed in small span of time without wastage of chemicals on large number of trials. The regulatory authorities are also showing more orientation and reliance towards QbD approaches. Thus AQbD is currently playing a key role in achieving the desired performance in analytical field.** (Page no 4) 5. (Form I is needle **shaped,** Form II is prism **shaped),** (page no 7)
4	**Objectives of the study**	**Yes**	NIL	Not applicable

5	**Review of Literature**	**Yes**	**Grammatical errors noticed** 6. The list of antihypertensive drugs selected in proposed research work is given in Table 1. (page no 16) 7. Carvedilol phosphate is shown in Table 2 (page no 17) 8. Nebivolol hydrochloride is shown in Table 3. (page no 17) 9. An Ishikawa fish-bone (IFB) diagram was assessed the risks and identified essential quality attributes. (page no 23) 10. The stability of the approach under stress conditions was demonstrated by its successful separation of carvedilol from its impurities and degradation products. (page no 26)	**Grammatical errors were rectified and corrected in revised thesis** **6.** The list of antihypertensive drugs selected **for the** proposed research work is given in Table 1. (page no 16) 7. **The drug profile of** Carvedilol phosphate is shown in Table 2. (page no 17) 8. **The drug profile of** Nebivolol hydrochloride is shown in Table 3. (page no 17) 9. An Ishikawa fish-bone (IFB) diagram was **used to assess** the risks and identified essential quality attributes. (page no 23) 10. **The approach successfully separated carvedilol from its impurities and degradation products under stress conditions.** (page no 26)

6	**Materials and methods**	**Yes**	**Grammatical errors noticed** 11. fishbone technique (page no 44) 12. Powder X-Ray Diffraction (page no 54)	**Grammatical errors were rectified and incorporated in the revised thesis** 11. **Ishikawa (fishbone) diagram** 12. **Powder X-ray Diffraction** (page no 54)
7	**Results and Discussion**	**Yes**	**Grammatical errors noticed** 13. Various mobile phase compositions, including Acetonitrile (ACN), Methanol (MeOH), Formic acid (FA), and Acetic acid, have been used at various flow rates, injection volumes, concentrations, etc., according to the literature review. (page no 55) 14. Varying concentrations of methanol and water were used to conduct preliminary experiments. With a tailing factor of higher than 2 (Figure 5), the findings showed unacceptable resolution. (page no 55) 15. The Carvedilol Phosphate peak eluted very close to the negative peak when ACN and a 0.1% aqueous solution of formic acid were used	**Grammatical errors were rectified and corrected** 13. Various mobile phase compositions, including Acetonitrile (ACN), Methanol (MeOH), Formic acid (FA), and Acetic acid, **were** used at various flow rates, injection volumes, concentrations, etc., according to the literature review. (page no 55) 14. Varying concentrations of methanol and water were used to conduct preliminary experiments. **The results showed unacceptable resolution, with a tailing factor greater than 2 (Figure 5)** (page no 55) 15. **When ACN and 0.1% aqueous solution of formic acid was tried as mobile phase, the**

			as the mobile phase (Figure 8). An increase in retention duration was seen after a further reduction in ACN concentration which was used to distinguish the Carvedilol Phosphate peak from the negative peak. Therefore, MeOH and formic acid were used in additional trials. As the formic acid content was increased to 0.5% v/v, the tailing factor was lowered from 1.363 to 1.20, and the peak shape was improved (Figure 9). (Page no 55)	**Carvedilol Phosphate peak eluted very near to the negative peak. Further decrease in ACN concentration to separate the CDL peak from negative peak led to increased retention time. Hence further trails were done with MeOH and formic acid. Further increase in the concentration of formic acid up to 0.5% v/v reduced the tailing factor from 1.363 to 1.20 with improved peak shape.** (Page no 55)
			16. With only a small number of trials, the screening research can help identify the variables that have a major impact on the experimental outcomes. (Page no 61)	16. **Using** only a small number of trials, the screening **study helped to** identify the variables that have a major impact on the experimental outcomes. (Page no 61)
			17. The effect of the independent variables on retention time is shown below via the quadratic polynomial equation. (page no 68)	17. The effect of the independent variables on retention time **is represented by quadratic polynomial equation below.** (page no 68)
			18. An increase in the peak area is observed when the injection volume (X1X2) and the percentage of methanol by volume (X2/V) interact positively. Analyzed data on peak areas are shown in Table 12. Model F significance is indicated by its value of 143571.34. With such a	18. **The combined interaction terms % v/v of methanol, injection volume (X_1X_2), have positive effect on the peak area. Table 12 shows the results of ANOVA for peak area data. The value of 143571.34 for Model F represents its significance. Such a high value**

			high figure, the possibility of noise influencing the results is only 0.01%. The results can be better represented in design space using a quadratic model, as the signal-to-noise ratio was determined to be 1037.993. (page no 69)	**could have only 0.01% chance that noise is affecting the results. The signal to noise ratio was found to be 1037.993 which indicate that quadratic model can be suitable to represent the results in design space.** (page no 69)
			19. Coefficient values for X1 and X2 are quite close to one another, indicating that the two factors are equally influential on the response under study. In the event of a tailing factor, the lack of interaction terms and squared terms suggests that the independent variables did not interact significantly with one another. (page no 70)	19. **The coefficient values for X_1 and X_2 are similar, indicating that both factors have comparable influence on the tailing factor. The absence of interaction and squared terms in the model suggests that the independent variables did not significantly interact.** . (page no 70)
			20. Both the volume of injection and the percentage of methanol in the mobile phase have a negative impact on the NTP, as shown in the equation. The significant impact of the percentage v/v of methanol in the mobile phase on Y_4 is more noticeable when the coefficient of X_1 is bigger than the effect of injection volume. X_1 and X_2, the combined interaction terms of injection volume and percentage v/v of methanol, positively affect Y_4. The ANOVA	20**. The equation represents that both % v/v of methanol in mobile phase and injection volume have negative effect on the NTP. Further greater value of coefficient of X1 witnesses the pronounced effect of % v/v of methanol in mobile phase on Y4 in comparison to that of injection volume. The combined interaction terms % v/v of methanol, injection volume (X1X2), have positive effect on the Y4. Table 12 shows the**

			findings for the number of theoretical plates is shown in Table 12. A significance level of 858.41 for Model F indicates its importance. With such a high figure, the possibility of noise influencing the results is only 0.01%. With a signal-to-noise ratio of 89.691, the data is best represented in design space by the quadratic model. (page no 71)	**results of ANOVA for number of theoretical plates data. The value of 858.41 for Model F represents its significance. Such a high value could have only 0.01% chance that noise is affecting the results. The signal-to-noise ratio was found to be 89.691, indicating that the quadratic model is appropriate for representing the results in design space.** (page no 71)
			21. The method's sensitivity is represented by LOD and LOQ. The devised approach yielded LOD values of 0.61 µg/mL and LOQ values of 1.86 µg/mL, respectively. These results demonstrate the sensitivity of the method. (page no 74)	21. **LOD and LOQ represents sensitivity of the method. LOD and LOQ values of developed method were found to be 0.61µg/ml and 1.86µg/ml, respectively. These findings show the method is highly sensitive.** (page no 74)
			22. The model equation, 3D response, and conter plot were used to show how the controlled variables affected retention time.	**22.**The model equation, 3D response, and **contour** plot were used to show how the controlled variables affected **peak area.**
			23. A model F-value of 3292.75 further established the model's importance. The probability of the F-value being due to signal (i.e., noise < 0.01%) is 99.99%, indicating that the model is significant. The adjusted R^2 value	**23. Further the significance of the model was determined by model F-value which was 3292.75. This witnessed that model is significant with 99.99% chance of F-value is due to signal (i.e. noise < 0.01%). The**

			of 0.999 and the predicted R^2 value of 0.9985 were quite near to one another. (Page no 92) 24. The method's appropriateness and specificity for quantifying Nebivolol Hydrochloride in the formulation was confirmed by doing this exercise. (page no 97)	**closeness between predicted (0.9985) and adjusted (0.9998) R^2 was observed.** 24. **This confirmed the suitability and specificity of the method for quantifying Nebivolol Hydrochloride in the formulation.**(page no 97)
8	**Summary**	**Yes**	NIL	Not applicable
9	**Conclusion**	**Yes**	NIL	Not applicable
10	**Bibliography / List of References / Annexures**	**Yes**	**Bibliography they were not properly mentioned** 1. Ravisankar P, Gowthami S, Rao GD. A review on analytical method development. Indian J Res Pharm Biotechnol. 2014;2(3):1183. (Page no 131) 2. Kalyane D, Raval N, Polaka S, Tekade RK. Quality by design as an emerging concept in the development of pharmaceuticals. The Future of Pharmaceutical Product Development and Research. 2020;(1):1-25. (Page no 132)	**Changes suggested were rectified and corrected in thesis under the bibliography section.** 1. Ravisankar P, Gowthami S, Rao GD. A review on analytical method development. IJPBR. 2014;2(3):1183-95. (Page no 131) 2. Kalyane D, Raval N, Polaka S, Tekade RK. Quality by design as an emerging concept in the development of pharmaceuticals. The Future of Pharmaceutical Product Development and Research. **Academic Press.** 2020;(1):1-25. (Page no 132)

			3. Bernstein J. Polymorphism in molecular crystal. IUCr Monographs on Crystallography-14. Oxford: Clarendon press; 2002. (Page no 133) 4. Raju TR. Development and validation of stability-indicating impurity profiling method for the carvedilol in API and pharmaceutical formulation. International Journal of Pharmacology. 2015;2(6):22-31. (Page no 137) 5. Nikam VJ, Patil SB. Pharmaceutical cocrystals of nebivolol hydrochloride with enhanced solubility. Journal of Crystal Growth. 2020; 534:125488. (page no 140)	3. **Bernstein J, Colman P, Helliwell JR, Kantardjieff KA, Mak T, et al., IUCr Monographs on Crystallography. Polymorphism in Molecular Crystals. 2002.** (Page no 133) 4. Raju TR. Development and validation of stability-indicating impurity profiling method for the carvedilol in API and pharmaceutical formulation. **Int J Pharmacol Pharm Sci.** 2015;2(6):22-31. (Page no 137) 5. Nikam VJ, Patil SB. Pharmaceutical cocrystals of nebivolol hydrochloride with enhanced solubility. **J Cryst Growth.** 2020; 534:125488. (page no 140)
11	**Annexures**	**Yes**	NIL	Not Applicable
12	**Grammar**	**Yes**	**Numerous grammatical errors noticed**	**Grammatical errors were rectified and same has been incorporated in thesis**

13	**Spelling mistakes**	**Yes**	**Spelling mistakes are noticed** dose forms (Page No 1) the rmoanalytical (Page No 2) cocrytals fluroquinolone (Page No 7) organised, objects (Page no 12) approximation (Page no 13) attraction (page no 19) characterise (page no 32) circumstances (page no 32) mixes (page no 33) analyses (page no 37) auto sampler (page no 43) conformer (page no 51,128)	**All spelling mistakes were rectified and same has been incorporated in revised thesis are as follows** **dosage forms (page no 1)** **thermoanalytical** (Page No 2) **cocrystals fluoroquinolone** (Page No 7) **Organized, Objective** (Page no 12) **Estimation** (Page no 13) **Attention** (page no 19) **Characterize** (page no 32) **Conditions** (page no 32) **Mixtures** (page no 33) **Analysis** (page no 37) **Autosampler** (page no 43) **co-former** (page no 51,128)

			analyser (page no 52) lineary,withessed (page no 73) 2D conter (page no 90) Replies	**analyser** (page no 52) **linearity,** witnessed (page no 73) **2D contour** (page no 90) Replaced as responses
14	**Any other observations**	**---**	NIL	Not Applicable