"QUANTITATIVE ESTIMATION OF AMLODIPINE BESYLATE AND NEBIVOLOL HYDROCHLORIDE BY CHEMOMETRICS USING UV-VIS SPECTROPHOTOMETRY IN THEIR COMBINED DOSAGE FORM"

A Thesis Submitted to

NIRMA UNIVERSITY

in Partial Fulfillment for the Award of the Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

BY

DIVYA H.PANCHAL (11MPH303), B. PHARM.

Under the guidance of

Dr. PRITI J MEHTA – GUIDE
Professor, Department of Pharmaceutical Analysis

Department of Pharmaceutical Analysis
Institute of Pharmacy
Nirma University
Ahmedabad-382481
Gujarat, India.

MAY 2013

CERTIFICATE

This is to certify that the dissertation work entitled ***"QUANTITATIVE ESTIMATION OF AMLODIPINE BESYLATE AND NEBIVOLOL HYDROCHLORIDE BY CHEMOMETRICS USING UV-VIS SPECTROPHOTOMETRY IN THEIR COMBINED DOSAGE FORM"*** *submitted by Ms. DIVYA H.PANCHAL with Regn. No.(11MPH303) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

Guide:

Dr. Priti J. Mehta
M. Pharm., Ph.D.
Sr. Associate Professor & Head,
Department of Pharmaceutical Analysis,
Institute of Pharmacy,Nirma University

Forwarded Through:

Dr. Manjunath Ghate
M. Pharm.,Ph.D.
Director
Institute of Pharmacy,
Nirma University

Date :17th May,2013.

DECLARATION

I hereby declare that the dissertation entitled ***"QUANTITATIVE ESTIMATION OF AMLODIPINE BESYLATE AND NEBIVOLOL HYDROCHLORIDE BY CHEMOMETRICS USING UV-VIS SPECTROPHOTOMETRY IN THEIR COMBINED DOSAGE FORM"****, is based on the original work carried out by me under the guidance of Dr.Priti J Mehta, Sr. Associate Professor and Head, Department of Pharmaceutical Analysis,Institute of Pharmacy,Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

Ms. DIVYA H.PANCHAL (11MPH303)
Department of Pharmaceutical Analysis
Institute of Pharmacy
Nirma University
Sarkhej - Gandhinagar Highway
Ahmedabad-382481
Gujarat, India

Date: 17 ***May,2013.***

Acknowledgements

*First of all, I am thankful to **almighty** for always being with me and blessing me .*

*I am highly thankful to **Dr. Manjunath Ghate** (I/C Director, Institute of Pharmacy, Nirma University, Ahmedabad) for providing all necessary help and facility for my work and also for his constant support and encouragement.*

*I am deeply grateful to Professor **Dr. Priti Mehta**, Ph.D., Head of the Department of Pharmacy, Institute of Pharmacy, Nirma University, for her detailed and constructive comments, and for her important support throughout this work. Her enthusiasm and perfection towards work was motivational for me.*

***Mr. Nrupesh R. Patel,** Assistant Professor, Institute of Pharmacy, Nirma University was a constant source of support and guidance with his endless patience, knowledge and depth on the subject matter.*

*I am extremely grateful to **Dr. Charmy S. Kothari, Omkar Sir, TejasSir** Dept of Pharmaceutical analysis, Institute of Pharmacy, Nirma University for their continuous encouragement and support throughout the course of this dissertation work.*

*I express my special thanks to **Mr.Ghanshyam C.Patel** for his support in stastical knowledge regarding my project. **Dr.Tejal A Mehta**, Associate Professor, Head, Dept. of Pharmaceutics and I/C Head,Dept of Pharmacology, Institute of Pharmacy, Nirma University **Dr. Vimal Kumar,**Professor, Dept. of Pharmacogonosy and Phytopharmaceuticals, Institute of Pharmacy, Nirma University, for their constant moral support and kind cooperation.*

*I would like to thank specially to Ph.D students **Omkar sir, Jignesh sir,Prerak sir, Som sir** for their selfless support, co-operation and valuable suggestion. I would like to thank my colleagues B**onny, Reenal, Richa, Upasna, Chintan, Harshal, Mitul,Raghav,Ravi and Samarth..** I would like to thank my seniors **Ajay, Margi, Kartik, Shikha, Keval, Shweta, Jay,Barkat, Ankit,** and juniors.*

*For the Infrared spectroscopic studies, **Raghav** provided us with the necessary FTIR studies of the final formulationswhich helped us in giving shape to our project. I would also like to acknowledge the Nirma University for providing us with such facilities.*

*My time at **Nirma University** was made enjoyable in large part due to the many friends and groups that became a part of my life. I am grateful for **Satejbhai's,Shreyas bhai's and Bipin bhai's** hospitality as I finished up my degree, and for many other people and memories.*

*I would like to thank **my family** for all their love and encouragement. For my parents who raised me with a love of science, supported me in all my pursuits and provided me with a vision and a goal to achieve in future. And most of all for my loving, supportive, encouraging, and patient family including **my darling mom, my caring dad, my brother and sister** whose thorough support during the final stages of this M.Pharm. is very much appreciated.*

Last, but not the least, I express my gratitude and apologize to anybody whose contributions, I could not mention in this page.

Date:

Divya H.Panchal

Institute of Pharmacy,

Nirma University,

Ahmedabad.

LIST OF ABBREEVIATIONS

I.P	: Indian Pharmacopoeia
B.P.	: British Pharmacopoeia
U.S.P.	: United States Pharmacopoeia
AMLO	: Amlodipine Besyalte
NEBI	: Nebivolol Hydrochloride
Conc.	: Concentration
Fig.	: Figure
U.V.	: Ultra Violet Spectroscopy
H.P.L.C.	: High Pressure Liquid Chromatography
H.P.T.L.C.	: High Pressure Thin Layer Chromatography
S.D.	: Standard Deviation
R.S.D.	: Relative Standard Deviation
%	: Percentage
°C	: Degree Centigrade
cm	: centimetre
µm	: micrometer
nm	: nanometer
mg	: milligram
µg	: microgram
ml	: mililiter
µl	: microliter
no.	: number
Rt	: Retention Time

Rf	: Retention Factor
Ref	: Reference
Sec	: Seconds
NaOH	: Sodium Hydroxide
HCl	: Hydrochloric Acid
SPSS	:Stastical Package For Social Science

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
1.1	Wavelength selection by simultaneous equation method	2
1.2	Wavelength selection by absorption ratio method	3
1.3	Example of an on-line process analysis for UV-VIS	5
1.4	Illustration of model dimensionality affects the fit and predictive ability.	17
1.5	Schematic description of the calibration set and test set used in multivariate calibration.	18
1.6	Geometrical interpretation of a PCA model	20
1.7	Schematic description of a decomposition of a matrix X with PCA using two PCs.	21
1.8	Geometrical interpretation of a PLS model consisting of one PLS component.	23
1.9	Schematic description of the matrices in PLS regression	24
1.10	Illustration of the conjunction between spectroscopy and chemometrics	25
1.11	Multivariate analysis flow	26
4.1	UV spectra of 20µg/ml AMLO Solution in methanol	47
4.2	UV spectra of 20µg/ml NEBI Solution in methanol	47
4.3 (a) and (b)	Recorded and Reported FTIR Spectra of AMLO	47-48
4.4	Recorded FTIR spectra of NEBI	48
6.1	Actual and Predicted Conc. of AMLO and NEBI	55
6.2	Representation of Component Number for AMLO and NEBI	56
6.3	Outlier Analysis	60
6.4 (a) and (b)	Peak Purity Spectra AMLO and NEBI	61-62
6.5	HPLC Chromatogram of Linearity of AMLO and NEBI (20-120µg/ml) standard.	63
6.6	Linearity curve of AMLO(a) and NEBI(b)	64
6.7	RP-HPLC overlay Chromatogram of Standard AMLO and NEBI with Marketed Formulation (Nebi AM*)	65

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
1.1	Properties of AMLO	10-11
1.2	Properties of NEBI	12-13
4.1	Melting Point of AMLO and NEBI.	46
4.2	Absorption Maxima of AMLO and NEBI, 20µg/ml, methanol	46
4.3	Reported and Recorded IR peaks of NEBI	48
5.1	UV-VIS spectrophotometer parameter	51
5.2	Training set using PLS and PCR method for AMLO and NEBI	51-52
6.1	Correlation matrix	54
6.2	Stastical Parameter of AMLO and NEBI by PLS and PCR method	57
6.3	Statistical parameters of chemometric methods in the calibration step	57
6.4	Recovery Results of AMLO and NEBI by PLS and PCR method	58-59
6.5	Outlier Analysis of training set	59-60
6.6	System Suitability Results of RP-HPLC method	62
6.7	Linearity of AMLO and NEBI standard	63
6.8	Assay results of RP- HPLC Method	65
6.9	Comparision of assay results of developed Chemometric UV-Spectroscopic and RP-HPLC Method	66
6.10	Comparison of Developed and Reported method	66

INDEX

Chapter No.	Title		Page No.
1	INTRODUCTION		
	1.1	Introduction to Multicomponent Formulation	1-7
	1.2.	Rationale of Drug Combination	8-9
	1.3	Introduction to Drug Profile	10-13
	1.4	Introduction to UV-VIS Spectrophotometry	14
	1.5	Introduction to Chemometrics	15-27
2	LITERATURE REVIEW		
	2.1	Amlodipine Besylate	28-38
	2.2	Nebivolol Hydrochloride	39-44
3	AIM OF PRESENT WORK		45
4	IDENTIFICATION OF DRUG		
	4.1	Melting Point	46
	4.2	UV-VIS Spectrophotometry	47
	4.3	FT-IR Spectrometer	48
5	Estimation of AMLO and NEBI by Chemometrics-Assisted UV-VIS Spectrophotometry		49-53
6	Comparison to RP-HPLC method by student t-test		54-66
7	Summary and Future Scope		67-68
8	References		69-83

Application of statistical and mathematical methods to solve chemical problems for maximal collection of useful information was known as “chemometrics”. Chemomatrics models, the information in the data can be extracted in an optimal way which allows us to make the best interpretation, decisions and optimization.Quantification was based on direct proportionality of concentration of analyte and absorbance in the mixture.The combination of UV-VIS spectrophotometer with chemometrics was used for the quantification of Amlodipine besylate(AMLO) and Nebivolol Hydrochloride (NEBI) used as antihypertensive and beta blocker respectively.A Partial least square (PLS) model and multivariate calibration was developed by using different concentration of both the drugs and absorbance was measured by selecting the wavelength in the range of 240-380nm. The overall % Recovery were found 99.54±1.3(PLS) and 99.31±1.39(PCR)for AMLO , 98.027±1.37(PLS) and 98.38±1.26(PCR)for NEBI respectively.The chromatographic separation was achieved on reverse-phase C_8 analytical column with mobile phase consisiting of mixture of 20mM ammonium phosphate buffer(pH=2.5),acetonitrile in the ratio of (50:50) and UV detection at 291nm. A comparison of the obtained results from PLS and PCR were also performed with those obtained from reported method. The obtained t-values indicating no significant differences between the results of the proposed and reported methods. Results showed that UV-VIS spectrophotometry combined with multivariate calibration has significant potential in quantitative analysis of Amlodipine besylate and Nebivlol.

Chapter 1
Introduction

1.1 INTRODUCTION TO MULTICOMPONENT FORMULATION [1]

The multicomponent formulations have gained lot of importance now a day due to greater patient acceptability, increased potency and decreased side effects. The quantitative analysis of such multicomponent formulations is very important. One of the quantitative procedures for multicomponent formulations is the simultaneous spectrophotometric method which utilizes the measurement of intensity of electromagnetic radiation emitted or absorbed by the analyte. The spectrophotometers have an inbuilt microprocessor for spectral data processing. The instrument computes accurate results within minimal time. Chemometric application for multicomponent formulation has many advantage because spectroscopy can generate overlapping spectral band and it is difficult to segregate spectra of one component. So, chemometric can help it out to identify exact spectral identity of each component.

Spectrophotometric simultaneous methods for multicomponent formulation:-

The spectrophotometric assay of drugs involves the measurement of absorbance of sample containing only one absorbing component. The pharmaceutical analyst frequently encounters the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances, which potentially interfere in the assay. The basis of all the spectrophotometric techniques for multi-component samples is:

(a) The absorbance of a solution is the sum of absorbance of the individual components;

(b)The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

In multicomponent formulations, the concentration of the absorbing substance is calculated from the measured absorbance using one of the following procedures:

1.1.1.Assay as a single-component sample:-The concentration of a component in a sample which contains other absorbing substances may be determined by a simple spectrophotometric measurement of absorbance, provided that the other components have a sufficient small absorbance at the wavelength of measurement.

1.1.2.Assay using absorbance corrected for interference:- If the identity, concentration and absorbtivity of the absorbing interferents are known, it is possible to calculate their contribution to the total absorbance of a mixture.

1.1.3.Simultaneous equation method[2]:- If a sample contains two absorbing drugs (X and Y) each of which absorbs at the λ max of the other, it may be possible to determine both drugs by the technique of simultaneous equations (Vierodt's method).

$Cx=A_2a_{y1}-A_2a_{y2}/A_{x2}a_{y1}-a_{x1}a_{y2}$……(1.1)

$Cy=A_1a_{x2}-A_2a_{x1}/A_{x2}a_{y1}-a_{x1}a_{y2}$……(1.2)

Where:

a)The absorptivity of X at λ_1 and λ_2, a_{x1} and a_{x2} respectively.

b)The absorptivity of Y at λ_1 and λ_2, a_{y1} and a_{y2} respectively.

c)The absorbances of the diluted sample at λ_1 and λ_2, A_1 and A_2 respectively.

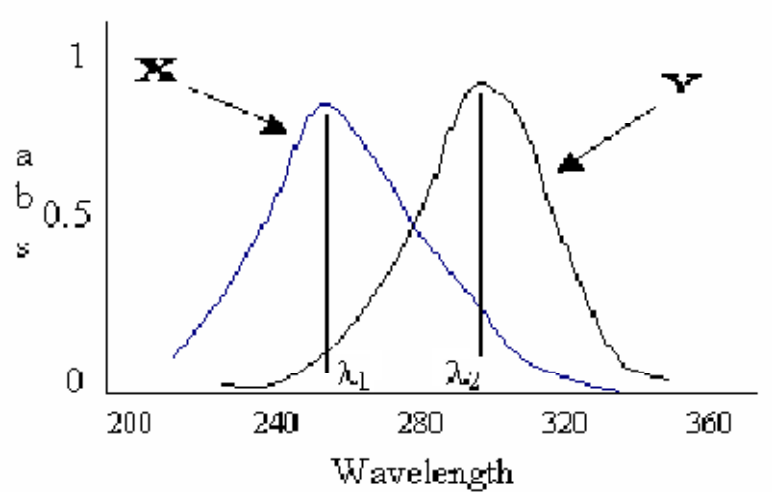


Figure-1.1: Wavelength selection by simultaneous equation method

1.1.4.Absorbance ratio method[2] :- The absorbance ratio method is a modification of the simultaneous equations procedure. Q-analysis is based on the relationship between absorbance ratio value of a binary mixture and relative concentrations of such a mixture. The ratio of two absorbance determined on the same solution at two different wavelengths is constant. This constant was termed as "Hufner's Quotient' or Q-value which is independent of concentration and solution thickness.

$Cx = Qm – Qy / Qx – Qy . A_1/ a_{x1}$……(1.3)

equation gives the concentration of X in terms of absorbance ratios, the absorbance of the mixture and the absorptivity of the compounds at the iso-absorptive wavelengths. Accurate dilutions of the sample solution and of the standard solutions of X and Y are necessary for the accurate measurement of A_1 and A_2 respectively.

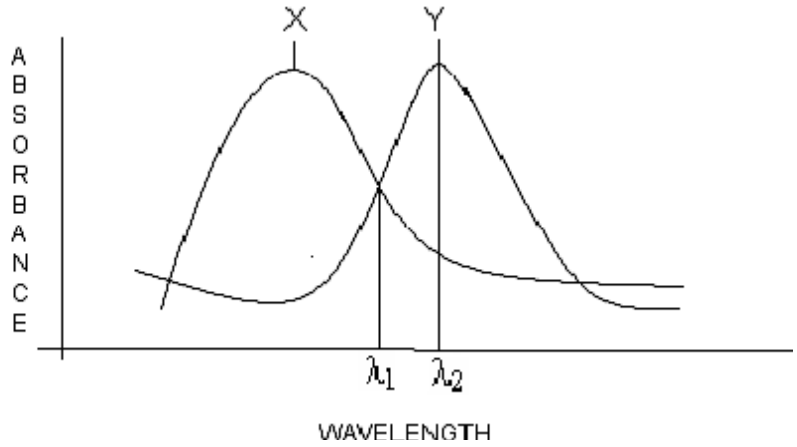


Figure-1.2: Wavelength selection by absorption ratio method

1.1.5.Geometric correction method:- A number of mathematical correction procedures have been developed which reduce or eliminate the background irrelevant absorption that may be present in samples of biological origin. If the wavelengths λ_1, λ_2 and λ_3 are selected to that the background absorbance B_1, B_2 and B_3 are linear, then the corrected absorbance D of the drug may be calculated from the three absorbance A_1, A_2 and A_3 of the sample solution at λ_1, λ_2 and λ_3 respectively as follows:

Let vD and wD be the absorbance of the drug alone in the sample solution at λ_1 and λ_3 respectively, i.e. v and w are the absorbance ratios vD/D and wD/D respectively.

$B_1 = A_1 – vD$, $B_2 = A_2 –D$ and $B_3 = A_3 –wD$…….(1.4)

Let y and z be the wavelengths intervals ($\lambda_2 – \lambda_1$) and ($\lambda_3 - \lambda_2$) respectively

$D= y(A_2 -A_3) + z(A_2 – A_1) / y (1-w) + z(1-v)$…….(1.5)

1.1.6.Orthogonal polynomial method:- The technique of orthogonal polynomials is another mathematical correction procedure, which involves more complex calculations than the three-point correction procedure.

$A(\lambda) = p P(\lambda) + p_1 P_1(\lambda) + p_2 P_2(\lambda) ….. p_n Pn(\lambda)$……(1.6)

Where A denotes the absorbance at wavelength λ belonging to a set of n+1 equally spaced wavelengths at which the orthogonal polynomials, P (λ) , P_1 (λ), P_2 (λ) ….. Pn (λ) are each defined.

1.1.7.Difference spectrophotometry: -Difference spectroscopy provides a sensitive method for detecting small changes in the environment of a chromophore or it can be used to demonstrate ionization of a chromophore leading to identification and quantitation of various components in a mixture. If the individual absorbances, A_{alk} and A_{acid} are proportional to the concentration of the analyte and path length, the Δ A also obeys the Beer-Lambert law and a modified equation may be derived:

$$\Delta A = \Delta abc \quad \text{.....(1.7)}$$

Where Δ a is the difference absorptivity of the substance at the wavelength of measurement.

If one or more other absorbing substances is present in the sample which at the analytical absorbance Ax in the alkaline and acidic solutions, its interference in the spectrophotometric measurement is eliminated:

$$\Delta A = (A_{alk} + Ax) - (A_{acid} + Ax) \quad \text{.....(1.8)}$$

The use of 0.1M sodium hydroxide and 0.1M hydrochloric acid to induce the Δ A of the analyte is convenient and satisfactory when the irrelevant absorption arises from pH-insenstive substances. Unwanted absorption from pH-sensitive components of the sample may also be eliminated if the pKa values of the analyte and interferents differ by more than 4.

1.1.8.Derivative spectrophotometry : - Derivative spectrophotometry is a useful means of resolving two overlapping spectra and eliminating matrix interferences or interferences due to an indistinct shoulder on side of an absorption band.

$$[A] = f(\lambda): \text{ zero order} \quad \text{.....(1.9)}$$

$$[dA/d\lambda] = f(\lambda): \text{ first order} \quad \text{.....(1.10)}$$

$$[d^2A/d\lambda^2] = f(\lambda): \text{ second order} \quad \text{.....(1.11)}$$

1.1.9.Process Analytical Technology(PAT) for spectrophotometry:

1.1.9.1 UV-Vis for On-Line Analysis [3-5] :-

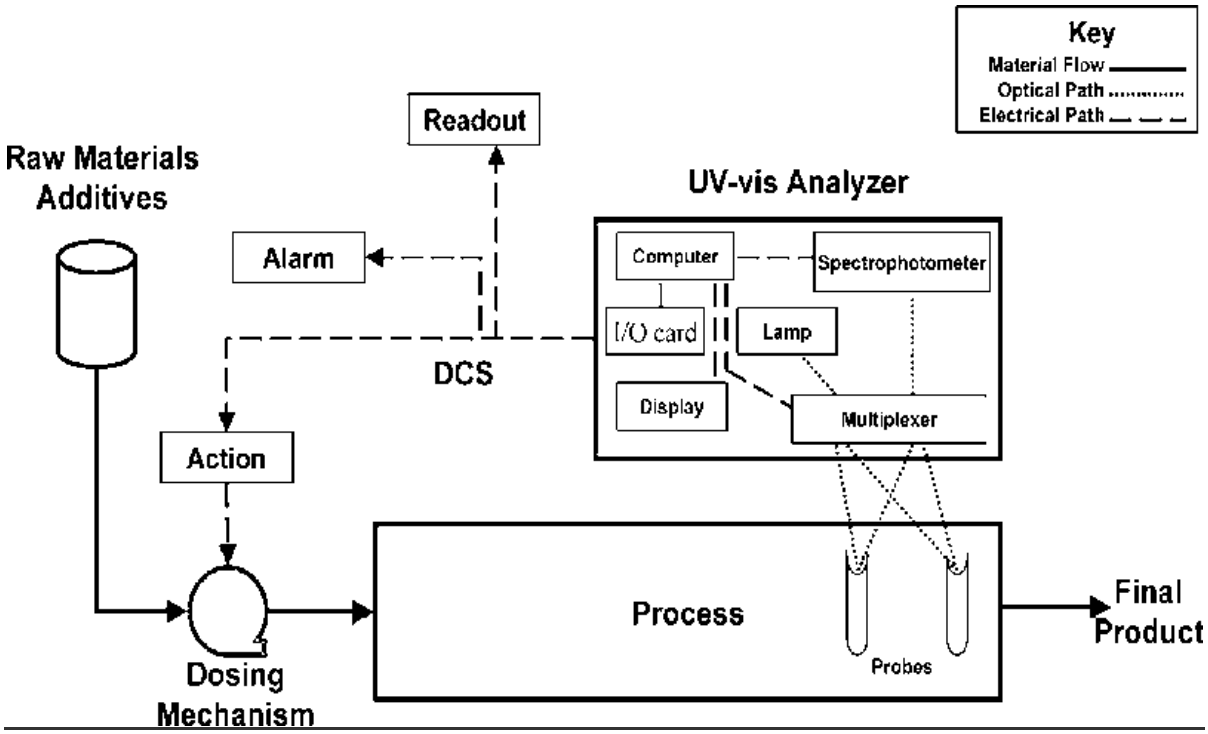


Figure 1.3.: Example of an on-line process analysis for UV-VIS

- The UV-vis analyzer contains a spectrophotometer and a light source. A fiber multiplexer may be added if multiple sampling points are required. Modern analyzers will also contain a control computer, display and some kind of data I/O interface to the process. These interfaces can include analog current outputs, digital alarm signals, or numerical streams sent to the plant's distributed control system (DCS). One or more probes are placed into the process. If the measurements made by the analyzer indicate that the process is drifting towards the control limits, the DCS may now take some action to change the operation of the process and bring it back to the optimal range. For example, the amount of reactant being fed may be adjusted, the temperature may be changed, or mixing speeds may be altered.

1.1.9.2.Near-Infrared Chemical Imaging as a Process Analytical Tool[6-9]:-

Conventional near-infrared (NIR) spectroscopy is a very rugged and flexible technique

that can be used in a wide range of chemical analysis.The additional application is the spatial perspective of NIR spectroscopic imaging offers to greater understanding and therefore control of the manufacturing process of complex composite materials and products.

1.1.9.2.1.The process analytical technology (PAT) initiative:-The PAT initiative of the US Food and Drug Administration (US FDA) strongly advocates the use and development of new measurement technologies for pharmaceutical manufacturing. The ultimate goal of the initiative is to maintain a stable process throughout manufacturing using real-time monitoring of critical process parameters. This goal cannot be reached immediately, as first the manufacturing process must be well characterized, and parameters that impact its stability must be identified. For example, do small changes in the blending, drying, pressing, coating or other manufacturing steps have on the final dosage form and once the critical parameters are identified, analytical techniques that can monitor those parameters must be identified, optimized for process applications, validated and deployed.

As the pharmaceutical manufacturing process becomes better understood, it is apparent that the metrics of potency and purity are no longer sufficient. The underlying tenant of the PAT initiative is to develop a better product through understanding of the manufacturing process. NIR-CI can be used to identify the elusive critical control parameters that will impact the performance of the finished product. The technique is fast and non-destructive and can be used independently, or in concert with other techniques such as dissolution analysis, to rapidly diagnose potential production problems. Incorporating NIR-CI analysis into the pre-formulation and formulation development phases of manufacturing can improve testing, limit scale-up difficulties and reduce the time to market. This presents a more robust process, imparting significant economic benefits to the pharmaceutical manufacturer. Near-infrared chemical imaging instrumentation is rugged and flexible, suitable for both the laboratory and the manufacturing environment.

1.1.9.3.Infrared Spectroscopy for Process Analytical Applications[10-14]:- The first industrial applications of IR spectroscopy were for quality and production control in the petrochemical industries, primarily for the analysis of fuels, lubricants, and IR

Spectroscopy. Early instruments were designed only for mid-IR absorption measurements and were limited to simple transmission cells.

The ideal is for process analysis to provide feedback to the process control system, there by ensuring optimum production efficiency. For some industries product quality is the most important controlling parameter, and this requires analytical controls throughout critical stages of production. This is a view shared by the current process analytical technologies (PATs) initiative that is being endorsed by the pharmaceutical industry. Other examples include high-tech manufacturing, such as in the semiconductor industry and value-added chemical production. In the petrochemical industries, which are dominated by continuous processes, sources of information that help to control the process itself and/or maintain production efficiency are the important attributes of a process analytical system. Considering all these factors and requirements, it is important to review the physical implementation, giving rise to terms such as ‘on-line,’ ‘off-line,’ ‘near-line,’ ‘at-line,’ and even remote and portable methods of analysis.

1.2 RATIONALE OF DRUG COBINATION [15-17]:-

Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product.

ANTIHYPERTENSIVES:

Antihypertensive drugs act through various pathways like angiotensive converting enzyme, angiotensin antagonist, calcium channel blockers, diruetics, adrenergic blockers and central sympatholytics.

Amlodipine is a dihydropyridine, long acting calcium channel blocker (L-type).They lower blood pressure by decreasing peripheral resistance without compromising cardiac output. While Nebivolol is third generation selective β1 blocker.

Combination therapy of anti-hypertensives is widely chosen so as to prevent hypertension through various mechanisms, longer therapeutic effect, synergism i.e. decreases the dose of both drugs. Fixed-dose combination of antihypertensive drugs can simplify dosing regimens, improve compliance, improve hypertension control, decrease dose-dependent side effects and reduce cost as the first-line treatment of hypertension [15].These potential advantages make it recommendable for the combination antihypertensive therapy to be used for severe initial hypertension [16].

Calcium antagonists are vasodilatory and tend to increase plasma renin, therefore combination with a β-blocker is theoretically used. Amlodipine, with its intrinsically long half-life alone or together with β- blocker, is likely to produce superior ischaemia reduction. The reduction of side-effects, obtained by adding a dihydropyridine derivate to a β-blocker, confirms the effectiveness of this combination [17].

Thus,the combination of Amlodipine Besylate (AMLO) and Nebivolol Hydrochloride (NEBI) is used in treatment of acute hypertension and also to prevent the occurrence of postural hypotension. Combination was approved in 14th Sept, 2004. [S-Amlodipine Besylate (2.5mg) and Nebivolol Hydrochloride (5mg)].

Marketed Pharmaceutical Dosage Form (avalaible in india):

Brand 1:-AMLOPRES-NB tab, Cipla (Nebivolol 10mg,Amlodipine 10mg)

Brand 2:-NEBIPRIL SA tab, Alkem (Nebivolol 10mg,Amlodipine 10mg)

Brand 3:-NEBILONG-AM tab, Micro Labs (Nebivolol 5mg,Amlodipine 5mg)

Brand 4:-NODON AM tab, Cadila (Nebivolol 5mg,Amlodipine 5mg)

Brand 5:-NEBISTAR-SA tab, Lupin (Nebivolol 5mg,Amlodipine 5mg)

Brand 6:-NEBI AM*tab, Otsira Genetica (Nebivolol 5mg,Amlodipine 5mg)

Brand 7:-NEBICARD-SM, Torrent (Nebivolol 2.5mg,Amlodipine 5mg)

Brand 8:-NEBINEX AM, Glenmark (Nebivolol 5mg,Amlodipine 5mg)

Brand 9:-AMLOVAS-SN, Macleods (Nebivolol 5mg,Amlodipine 2.5mg)

Brand 10:-ASIVOL-A, AS Pharma (Nebivolol 5mg,Amlodipine 2.5mg)

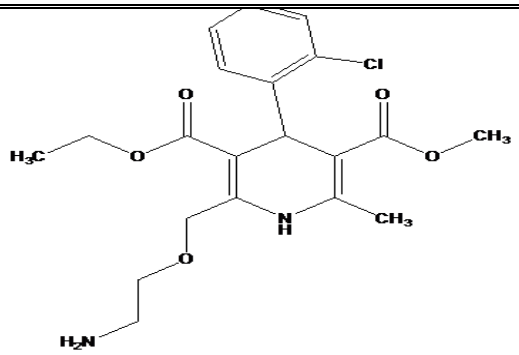
Brand 11:-NEBIVAS-SA, Intra Labs, (Nebivolol 5mg,Amlodipine 2.5mg)

Brand 12:-NUBETA-SM, AHPL, (Nebivolol 5mg,Amlodipine 5mg)

Brand 13:-NYFE-SA, Aamorb, (Nebivolol 2.5mg,Amlodipine 2.5mg)

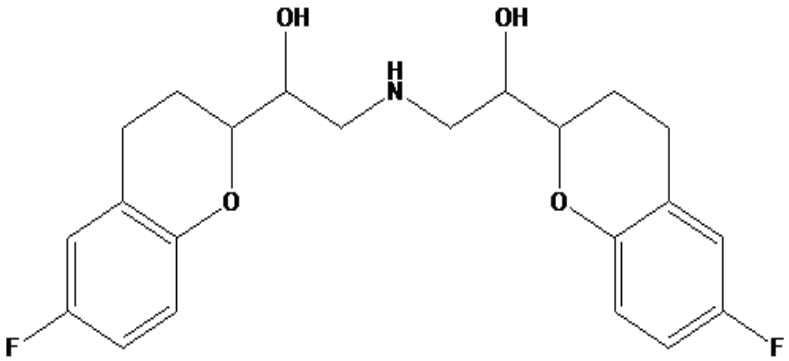
1.3 INTRODUCTION TO DRUG PROFILE [18-24]:-

Table 1.1.: Properties of AMLO

Physicochemical properties	
CAS no	88150-42-9(AMLO),111470-99-6 (AMLO Besylate)
Chemical Structure	 .$C_6H_6O_3S$
Chemical Name	2-[(2-Aminoethoxy)methyl]-4-(2–chlorophenyl)-1,4–dihydro–6–methyl–3,5–pyridinedicarboxylic acid 3–ethyl 5–methyl ester
Molecular Formula	$C_{20}H_{25}ClN_2O_5$ (AMLO) $C_6H_5SO_3H$ (Besylate)
Molecular Weight	408.9 (AMLO),567.1 (AMLO Besylate)
Official Status(AMLO Besylate)	I.P. 2010, B. P. 2010, U.S.P. 2012
FDA approval	14th May,1999
pKa	8.6
Log P	3.0.
Appearance	A white crystalline powder
Melting Point	178° to 179°C
Solubility	Slightly soluble in water and in isopropyl alcohol, sparingly soluble in dehydrated alcohol, freely soluble in methyl alcohol.
Dosage	Up to 10 mg (of the base) daily.
Pharmacological Properties	
Therapeutic Category	Calcium–channel Blocker, antihypertensive
Mechanism of action	Amlodipine is a dihydropyridine calcium-channel blocker with actions similar to those of Nifedipine.

Adverse Drug Effect	The commonest adverse effects were ankle oedema, flushing, headache, skin rash, fatigue, tachycardia and palpitations; associated with its vasodilator action and often diminish on continued therapy. Over dosage may be associated with bradycardia and hypotension, hyperglycaemia, metabolic acidosis and coma may also occur.
Pharmacokinetic Properties	
	Amlodipine is well absorbed after oral doses with peak blood concentrations occurring after 6 to 12 hours. The bioavailability varies but is usually about 60 to 65%.Amlodipine is reported to be about 97.5%bound to plasma proteins. It has a prolonged terminal elimination half-life of 35 to 50 hours and steady-state plasma concentrations are not achieved until after 7 to 8 days of use. Amlodipine is extensively metabolised in the liver; metabolites are mostly excreted in urine together with less than 10% of a dose as unchanged drug. Amlodipine is not removed by dialysis.

Table 1.2.: Properties of NEBI

Physicochemical Properties	
CAS No	*99200–09–6*
Chemical structure	 .HCl
Chemical name	2*α*,*α*′-[Iminobis(methylene)]bis[6–fluoro–3,4–dihydro–2*H*-1–benzopyran–2–methanol]
Molecular formula	$C_{22}H_{25}F_2NO_4$ (NEBI), $C_{22}H_{26}ClF_2NO_4$ (NEBI HCl)
Molecular weight	405.4 (NEBI), 441.9 (NEBI HCl)
Official Status(NEBI HCl)	I.P. 2010
FDA approval	12th July,2002
pKa	8.22
Log P	3.23
Appearance	White crystals
Melting point	140.7°C
Solubility	Soluble in methanol, dimethyl formamide, sparingly soluble in ethanol, PEG, PG and very slightly soluble in hexane, dichloromethane
Dosage	A daily oral dose of 5 mg
Pharmacological Properties	
Therapeutic category	Beta Blocker
Mechanism Of action	Nebivolol is a cardioselective beta1 blocker. It has vasodilating activity, which appears to be due to a direct action on the endothelium, possibly involving nitric oxide release . Beta blockers are competitive antagonists of catecholamines at beta-adrenergic receptors in a wide range of tissues.beta1 blockade mainly affects the heart, reducing heart rate, myocardial contractility, and rate of conduction of impulses through the

	conducting system (class II antiarrhythmic effect). It also leads to suppression of adrenergic-induced renin release and lipolysis.
Adverse Drug Effect	The most serious adverse effects are heart failure, heart block, and bronchospasm. Troublesome subjective effects include fatigue and coldness of the extremities; when beta blockers are used for long-term treatment of asymptomatic diseases such as hypertension, such effects may be an important determinant of patient compliance. Cardiovascular effects include bradycardia and hypotension; heart failure or heart block may be precipitated or worsened in patients with underlying cardiac disorders. Abrupt withdrawal of beta blockers may exacerbate angina and may lead to sudden death.
Pharmacokinetic Properties	
	Nebivolol is rapidly absorbed after oral doses. It is extensively metabolised in the liver by alicyclic and aromatic hydroxylation,*N*-dealkylation and glucuronidation; the hydroxy metabolites are reported to be active. The rate of aromatic hydroxylation by cytochrome P450 isoenzyme CYP2D6 is subject to genetic polymorphism ,and bioavailability and half-life vary widely. In fast metabolisers the elimination half-life of nebivolol is about 10 hours and that of the hydroxyl metabolites is about 24 hours. Peak plasma concentrations of unchanged drug plus active metabolites are 1.3 to 1.4 times higher in slow metabolisers and the half-life of nebivolol and its hydroxyl metabolites are prolonged. Nebivolol is about 98% bound to plasma proteins.It has high lipid solubility. It is excreted in the urine and faeces, almost entirely as metabolites.

1.4 INTRODUCTION TO UV-VIS SPECTROPHOTOMETRY [25] :-

Ultraviolet-visible Spectrophotometry (UV/Vis) refers to absorption spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared) ranges. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state. The Determination is usually carried out in solutions. UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules.

1.4.1 Principle of UV-VIS spectroscopy:

The absorption of ultraviolet or visible radiation by some species M can be considered to be a two-step process, the first of which involves excitation as shown by the equation:

M+hv=M*……(1.12)

where,M* represents the atomic or molecular particle in the electronically excited state resulting from absorption of the photon hv. The most common type of relaxation involves conversion of the excitation energy to heat,

M*=M+heat…….(1.13).

The absorbance of a solution is directly proportional to the path length, *b*, through the solution and the concentration, *c*, of the absorbing species. This relationship is called the Lambert-Beer law:

A= €**b***c*.……..(1.14)

where €*is the molar absorptivity. A limitation of this law is that it is only linear for solutions having an absorbance of about <1.5 AU. Hence with too high a concentration there is no longer a linear relationship between the absorption and the concentration and absorptivity of the absorbing interferents are known, it is possible to calculate their contribution to the total absorbance of a mixture**.**

1.5 INTRODUCTION TO CHEMOMETRICS:-

Chemometrics is the application of statistical and mathematical methods to chemical problems to permit maximal collection and extraction of useful information[26]

As defined by Massart "Chemometrics is a chemical discipline that uses mathematics, statistics, and formal logic (a) to design or select optimal experimental procedures; (b)to provide maximum relevant chemical information by analyzing chemical data; and (c) to obtain knowledge about chemical systems." [27]

There are techniques for collecting good data (optimization of experimental parameters, design of experiments, calibration, signal processing) and for getting information from these data (statistics, pattern recognition, modeling, structure property-relationship estimations.Collection of data for known cases generation of a mathematical model which is usually based on multivariate statistics or neural networks, interpretation of the model parameters in terms of the underlying chemistry, application of the model to new cases, and realizing how meaningful calculations have to be performed. [28]

1.5.1 VALIDATION:

Validation is a crucial step of the model concept in chemometrics. The validation is needed in order to be able to decide whether or not the conclusions drawn from it are reliable, i.e. to make sure that the results can be extrapolated to new data. An unvalidated model is useless since it is possible that the conclusions drawn result only from random noise.

Validation can be divided into two main types: a) internal validation and b) external validation. Internal validation means utilising the data that has been used to construct the model to validate it, while external validation means that new data that not has been previously used in the model is used for the validation. However, it is generally recommended that one should first use an internal validation of the model and then apply new data sets and perform a thorough external validation of the model before it is further used. The variation in a data set can be described with variance which is given by the square of the standard deviation.

$$s = \sqrt{\frac{\sum_{i=1}^{n} \left(x_i - \bar{x}\right)^2}{n-1}}$$

……(1.15)

where,x is the the mean of the number of measurements , n and xi are the individual values.

When performing a multivariate data analysis on a set of data, the sum of squares of the total variance of the data set (SStotal) can be divided into two parts:

SStotal = SSmodel + SS residual……..(1.16)

where,SSmodel is the sum of squares of the variance explained by the model and SSresidual is the residual or unexplained variance.

The aim of chemometric modelling is generally to capture the variance in a given data matrix in a simple model of low dimensionality. This means that the explained variance (SSmodel) should be as high as possible, leaving only a very small amount of unexplained variance (SSresidual). An important term in the internal validation of a model is therefore the explained variance:

Explained variance = (SStotal – SSresidual)/ SStotal…….(1.17)

This measure is often called the goodness of fit or the model validation term and thus describes the amount of variance explained by a model. It is sometimes expressed as a percentage.In order to obtain an estimate of the predictive ability of a regression model,a often used method is cross validation (CV) [29]

The basic principle of CV is as follows. In the used data set one sample is kept out of the calibration and a regression model is calculated from the remaining observations. The y value of the object taken away is then predicted with the calibration model constructed. The prediction error of this observation can then be calculated as the difference between the predicted and true value. This procedure is then repeated leaving out every sample in the calibration set once, the summed prediction error then indicating the predictive ability. This ‘leave one out’ cross validation is, however, generally valid only when the number of samples is small, for larger calibration sets, the procedure should be carried out by dividing the calibration set into smaller subgroups instead of single samples. By summing the prediction error sum of squares (PRESS) [30]

$PRESS = \Sigma (y_{observed} - y_{predicted})^2$……..(1.18)

Where, $y_{observed}$ is the true value and $y_{predicted}$ is the predicted y value in the cross validation, a measure of the predictive ability of the model is given. By comparing PRESS with the total sum of squares of the variance in the data set, the goodness of prediction measure can be calculated:

Goodness of prediction = (SStotal –PRESS) / SStotal …….(1.19)

Generally the objective with the model is to explain as much of the variance in the data with as few PC (principal component) as possible in order to minimize the influence of noise and to obtain a simple model. Furthermore, a model consisting of too few components will result in under-fit, i.e. the model has not deal with all the variance present in the data set. Correspondingly, a model with too many components will result in over-fit, since noise then is modelled to a higher extent. Over-fit also impairs the predictive ability of the model. This is illustrated in Fig.1.5.1 below. It is important, therefore, to find the optimum number of components to use in a model and CV is a method often used for finding the optimal model dimensionality.

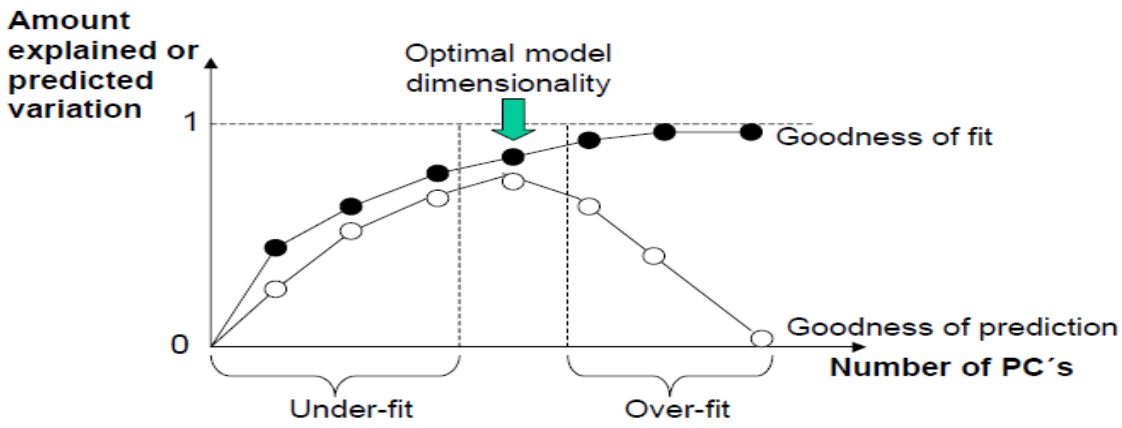


Figure 1.4: Illustration of how the model dimensionality affects the fit and predictive ability.

1.5.2 Multivariate calibration:

The term calibration can be defined as the use of empirical data and prior knowledge for determining how to predict unknown quantitative information Y from available measurements X, via a mathematical transfer function of some kind. Calibration can hence be described as the process of establishing this mathematical function (f) between the measured variable x and a dependent variable y:

$f(x) = y$…….(1.20)

One of the simplest forms of calibration is linear regression.

$y = kx + l$…….(1.21)

where, k is the regression coefficient and l is the intercept of the linear approximation.

In multivariate Calibration [31-33] numerous variables are used and the term multivariate calibration refers to the process of constructing a mathematical model that relates a property such as content or identity to the absorbance of a set of known reference samples at more than one wavelength. Multivariate calibration thus means using many variables simultaneously to quantify one or many target variables Y.

The best way to perform this validation is by using new samples not previously used, an external test set consisting of new samples (p) from which the same variables have been measured. By predicting the Y values of the samples in the external test set and then comparing the results with the true values, an estimate of the predictive ability of the model is obtained.

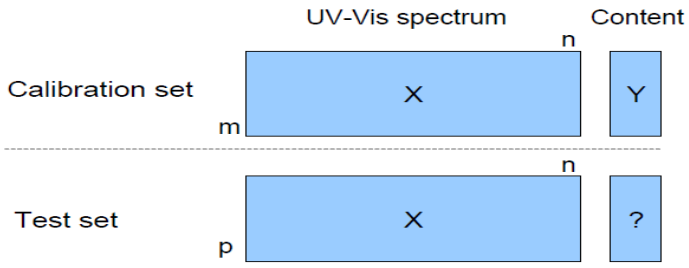


Figure 1.5. : Schematic description of the calibration set and test set used in multivariate calibration.

The calibration samples should in the calibration set span the calibration experimental domain as far as possible and experimental design can be used to find suitable samples to include in the calibration set. Also the best calibration is generally obtained when the

calibration model contains all the sources of variation that can occur in the actual measurement. The order in which the samples are analysed is one source of variation and randomisation of the order of analysis minimise the error caused by day-to-day variations. The evaluation of the predictive ability of a quantitative multivariate calibration model can be made, by means of the root mean square error of prediction (RMSEP) and relative standard error of prediction (RSEP) [34-35]

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{p} (y_{pred} - y_{obs})^2}{(p)}}$$

$$RSEP(\%) = 100 * \sqrt{\frac{\sum_{i=1}^{p} (y_{pred} - y_{obs})^2}{\sum (y_{obs})^2}}$$

……..(1.22)

where,y_{pred} is the predicted concentration in the sample and y_{obs} is the observed or reference value of the concentration in the sample, p is the number of samples in the test set.

RMSEP gives an estimate of the prediction error in the same unit as the initial data, while RSEP gives a relative measure of the prediction error in terms of percentage. RSEP has also been defined as the relative standard deviation of residuals of the concentrations. In recent years the use of multivariate calibration has become well established and standardised guidelines have been published. [36]

Multivariate calibration methods has been widely applied because many common analytical methods provide analysis of multiple species. In these methods,it is assumed that there are a series of mixtures for which the amounts of each component are known and for which a series of properties has been measured.The methods include principal components regression(PCR), partial least-squares (PLS), simulated annealing (SA), the genetic algorithm (GA), and artificial neural networks (ANN).

1.5.2.1 Principal components regression(PCR):- The measurement of the spectral characteristics of a series of calibration samples for which the concentrations are known provides a set of data in which there are significant correlations and collinearities. To

reduce these problems, a principal components analysis (PCA) is performed on the matrix of spectral characteristics.

The PCA produces a set of orthogonal vectors that are fully uncorrelated and by choosing a subset of the vectors can help to enhance the signal to noise ratio. This approach is called principal components regression. A principal components analysis maximizes the included variance in the analysis, but does not necessarily maximize the quality of the resulting predictions of concentrations from measurements on an unknown sample. PCA is a well-known chemometric method for the decomposition of two-way matrices. [37-38]

The variance in the data matrix X, with m observations and n variables, is decomposed by successively estimating principal components (PCs) that capture the variance in the data in scores and loadings. In Figure the geometrical interpretation of PCA on a small data set consisting of twelve observations and three variables is shown. The steps in the geometrical interpretation of PCA are as follows. Firstly the X-space is given a coordinate system where each variable gets an axis whose length corresponds to its scaling. In this example three variables mean three coordinate axes. Each observation in this space is represented by a point. The average of each variable is then calculated and subtracted (mean centring). This is equivalent to moving the swarm of points to the centre of the coordinate system. Thereafter a function is fitted to the data that describes as closely as possible the variance of the observations in the X-space. This is the first PC and is represented by the line in fig. :

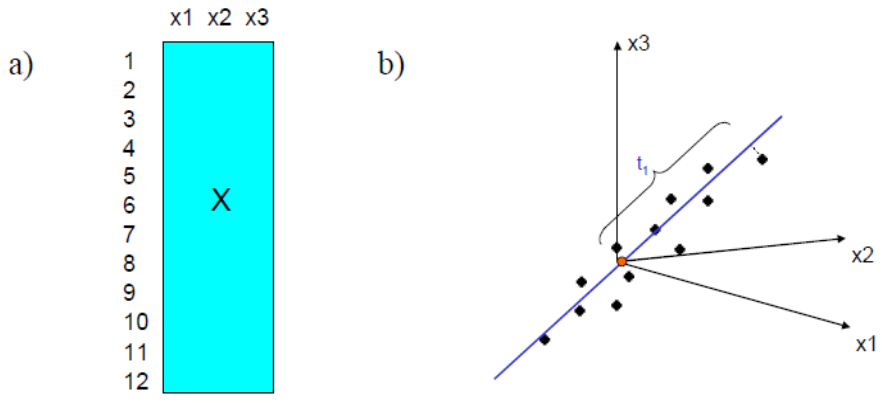


Figure 1.6.: Geometrical interpretation of a PCA model consisting of one PC: a) data set consisting of twelve objects and three variables, b) a geometric interpretation of the first PC calculated from the data set.

By projecting each point down to the line (Euclidian distance) and measuring the distance between the centre point and the projection point, the score value (t) of each observation is obtained. Since the data set consists of twelve objects, the same number of score values (t_1) are obtained for the first PC. The angle between the line and each variable axis determines the influence of each variable, the loading value (p). One loading value is given for each variable in the data set (p_1). In this example twelve score values and three loading values are thus given for the first PC. When the first PC has been calculated, the remaining unexplained variance is left in the residual matrix,E:

$X = TP' + E$ …….(1.23)

The decomposition of a matrix with PCA is schematically described in figure where the initial data matrix X is decomposed with PCA using two PCs.

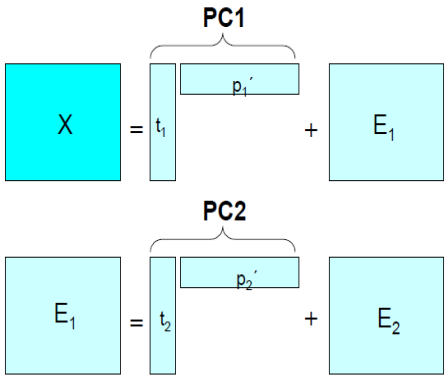


Figure 1.7.: Schematic description of a decomposition of a matrix X with PCA using two PCs.

After the first PC has been calculated, the next is calculated on the residual matrix E_1, which contains the variance not explained by the first PC.

$X = t_1p_1' + t_2p_2' + \ldots + t_ap_a' + E$ ……..(1.24)

The second PC is orthogonal to the first. More PCs (a) can be calculated as long as unexplained information is left. The significant number of principal components can be estimated by different methods, of which cross validation is an often-used method.

The variance of a principal component is described by the eigenvalue, which is proportional to the variance explained by a PC. The eigenvalue (λ) can be described as the length of the PC and estimated as the sum of squares of the scores:

$$\lambda = \sum_{m=1}^{M} t_{ma}{}^{2}$$

……..(1.25)

Where, t_{ma} = the score of object m for component a.

- **Advantages of PCR method** : It doesn't need the selection of wavelength; most of the time the whole spectrum is used. As one uses great number of wavelengths; the averaging effect attained and decrease in the chance for spectral noise can be utilized for mixtures with large constituents (highly complex). PCR also enables, sometimes to figure out samples with constituents, which are not present basically (originally) in the calibration mixture.
- **Application of PCR:-** PCR has widely been applied for the specrophtometric resolution of mixtures comprising two or more overlapping spectra [39-40]

1.5.2.2.Partial least squares regression:-PLS[41-42] has been established as a standard data analysis tool for multivariate data in the last ten to fifteen years and there are numerous applications in different fields of analytical chemistry. In PLS the variance in a data matrix X and a dependent matrix Y is decomposed by successively estimating PLS components that capture the variance and correlation between X and Y. In Figure the geometrical interpretation of PLS of two small data sets consisting of twelve objects, three x-variables (x1–x3) and three y-variables (y1–y3) is shown. The steps in the geometrical interpretation of PLS are as follows. Firstly, as in PCA, the X space gets a coordinate system where each variable gets an axis with the length corresponding to its scaling and in PLS this is also given for the Y space. Since the data sets in this example consisted of three x-variables and three y-variables, the X and Y spaces both get three axes.

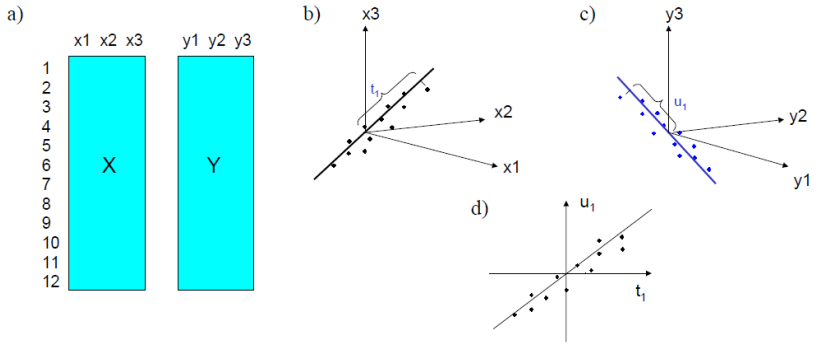


Figure 1.8.: Geometrical interpretation of a PLS model consisting of one PLS component. a) a data set consisting of twelve objects and three x and y-variables, b) geometric interpretation of the first PLS component in the X space, c) geometric interpretation of the first PLS component in the Y space, d) PLS inner relation.

Mean centring of both the x and y-variables then moves the swarm of points to the middle of the coordinate system. A function is then fitted to the data in a way that best describes the variance in the X and Y spaces as well as maximising the correlation between X and Y. This function is a line in both the X and the Y space and it is the first PLS component. PLS can be seen as the regression extension of PCA since it simultaneously fit two ´PCA-like´ models, one for the X space and one for the Y space, in such a way that the correlations between X and Y are maximised. In contrast to PCA, PLS is a maximum covariance method since the main aim of PLS is to predict the y-variables from the x-variables. As in PCA, the PLS decomposition summarises the variance in the data sets in new latent variables, scores and loadings. By projecting each point down to the line and measuring the distance between the centre point and the projection point, the scores for the X space ($\mathbf{t_1}$) and Y space ($\mathbf{u_1}$) are given. The PLS model can be regarded as consisting of an outer relation and an inner relation,

where,the outer relation describes the X and Y block individually, while the inner relation links the two blocks together. The outer relations is given by:

$X = TP' + E$ …….(1.26)

$Y = UC' + F$ …….(1.27)

Where, T is the score matrix and P´ the loading matrix of the X space, U is the score matrix and C´ the loading matrix of the Y space. E and F are the residual matrices of

the X and Y spaces respectively. By plotting the t_1 values of the twelve objects against the corresponding u1 values, the PLS inner relation showing the correlation structure between X and Y is obtained :

U=BT+H ……..(1.28)

where B is an identity matrix and H is a residual matrix. In PLS a type of additional loadings is calculated that expresses the correlation between X and Y, the weights, W.

The weights are related to the PLS regression coefficients, BPLS:

BPLS=W(P´W)-1C´ ……..(1.29)

The regression coefficients show the direction and magnitude of the influence of an x variable on a specific y-variable. The prediction of y-variables of new samples is given by:

Y=XBPLS + F……(1.30)

After the first PLS component has been calculated the next one can be calculated on residual matrices E and F. The number of significant PLS components (the model dimensionality) in a calibration model can be decided by means of cross validation.

The matrices in PLS regression are shown schematically in Figure and, for each new PLS component, t, u, w, p and c are calculated. The details of PLS regression have been thoroughly described elsewhere[43-44]

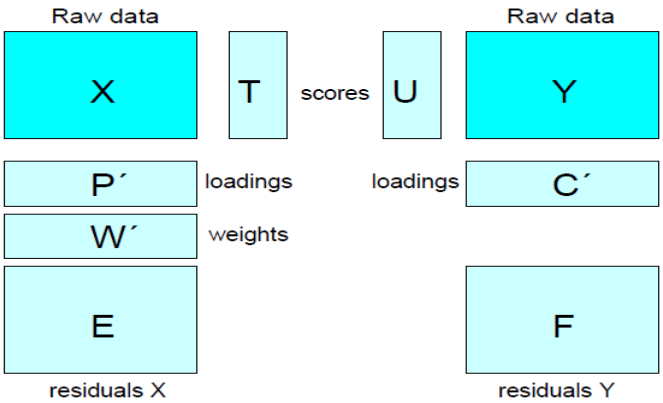


Figure 1.9.: Schematic description of the matrices in PLS regression.

1.5.3 Multivariate analysis and spectroscopy:-

There is a close relationship between multivariate analysis method and spectroscopy. This can be explained in detail as follows:

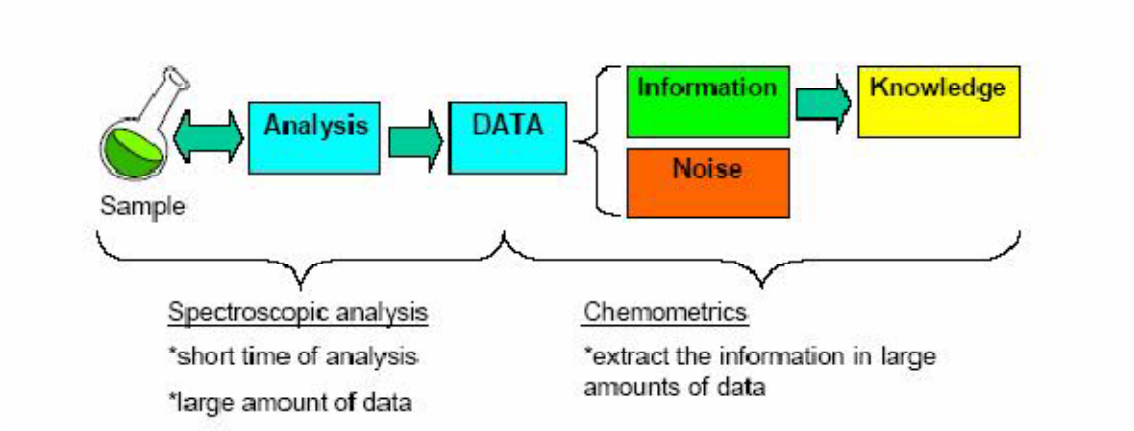


Figure-1.10.: Illustration of the conjunction between spectroscopy and chemometrics

Spectroscopic techniques are generally fast, with the analysis time from a few seconds to a few minutes and also produce large amounts of data for each sample analyzed. Roughly speaking, this data can be said to consist of two parts: information and noise. The information portion of the data is what eventually leads to knowledge generation about the sample, while the noise is a non informative section. A matter of concern is always to minimize and, if possible, to get rid of disturbing noise in the data since it impairs the information gained. This is where chemometrics comes in, since multivariate methods are constructed to extract the information from large sets of data.

Using multivariate data with many variables instead of univariate data offers many advantages in qualitative and quantitative spectroscopic analysis. The methods generally become more robust, precise and less sensitive to background interferences.One could therefore say that multivariate methods are the optimal choice for the evaluation of spectroscopic data and that the conjunction of spectroscopic analysis techniques with multivariate data analysis offers further possibilities in analytical chemistry.

Multivariate calibration thus means using many variables simultaneously to quantify one or many target variables Y. A calibration model is determined from a set of samples of known content of the calibration set. This can be done by means of partial least square (PLS) or principle component regression (PCR) and the resulting model is used to predict the content of new unknown samples from their digitized spectra.

The calibration model is then constructed and subsequently validated from the values of the independent and dependent variables. The best way to perform this validation is by using new samples not previously used, an external test set consisting of new samples (p) from which the same variables have been measured. By predicting the Y values of the

samples in the external test set and then comparing the results with the true values, an estimate of the predictive ability of the model is obtained .The multivariate analysis flow is shown in fig. :

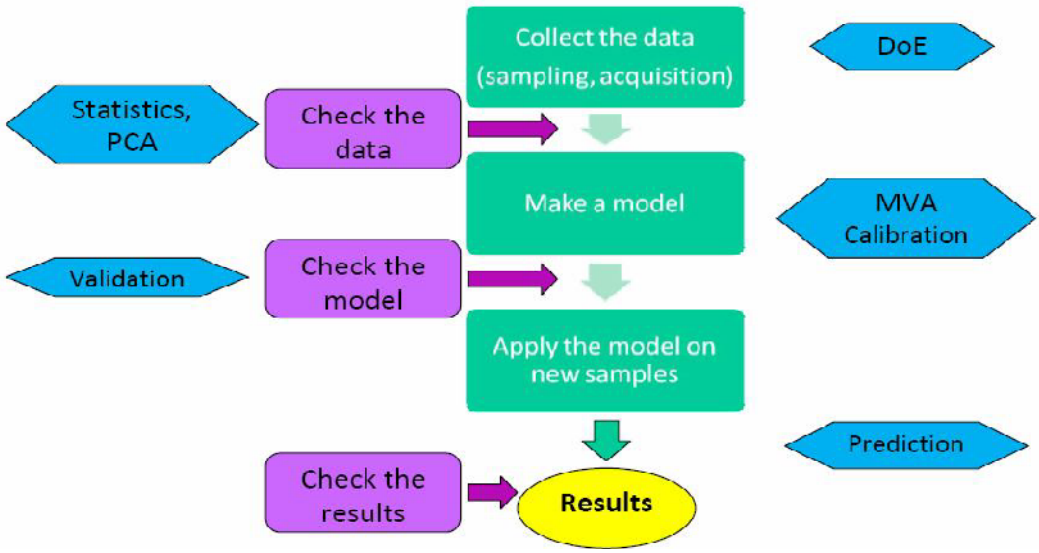


Figure-1.11.: Multivariate analysis flow

1.5.4. Importance of chemmometrics in Process Analytical Technology [45-46] :-

PAT is defined as: "systems for continuous analysis and control of manufacturing processes based upon real-time measurements or rapid measurements during processing of quality and performance attributes of raw and in-process materials and processes to assure acceptable end product quality at the completion of the process.PAT involves the use of process analytical chemistry tools, information management tools, feedback process control strategies and product and process design and optimization strategies.PAT used in pharmaceutical applications can improve the capability and the efficiency of pharmaceutical processes while maintaining or improving product quality .

The application of chemometrics in PAT requires the application of proven mathematical and statistical methods and a basic understanding of chemical phenomena under study. The process modeling will be provide for the derivation of the state of the process — thus the focus is chemical understanding, not statistical tool making. Once a model is implemented it must be monitored during the process using actual real-time

measurements of the process. With the monitoring system in place, the process is manually or automatically controlled with real-time feedback provided to individuals having a detailed understanding of the chemical and physical phenomena involved in the manufacturing process as well as an understanding of the limitations of the statistics to determine ultimate causal relationships.

The technical advantages of using chemometrics include:

- Speed in obtaining real-time information from data.
- The capability of extracting high quality information from less resolved data
- The capability of clear information resolution and discrimination power even when applied to second, third, and possibly higher-order data.
- The potential for providing methodology for cloning sensors — for making one sensor take data "precisely" as another sensor.
- Diagnostics for the integrity and probability that the information derived from sensor data is accurate; improved measurement quality; and, eventually, improved knowledge of existing processes.

Economic benefits of chemometrics include:

- Relatively low capital requirements,safer plant and process operations through real-time monitoring and prevention of potentially dangerous process upsets.
- Assurance that processes and plant environments are in compliance with environmental regulations.
- Increase in process plant operability through timely adjustments in processes possible using real-time data.
- Improved product quality through maintenance of tighter control limits, minimization of waste products through process optimization; product production cost minimization through tighter target limits and more accurate production scheduling; optimization of production capacity resulting from increased process operability and continuous product quality verification.
- Chemometric-based real-time measurements eliminate the greatest challenges to 100% compliance and analytical accuracy for measuring a process — namely, sampling error.

Chapter 2
Literature Review

2.1 LITERATURE REVIEW

2.1.1 AMLO

For determination of AMLO various techniques are avalaible alone and in combination with other drugs. HPTLC is official in I.P.2010 and HPLC is official in B.P 2010 and U.S.P 2012.

2.1.1.1. UV-Visible Spectrometric Techniques:

Table 2.1.1.: UV-Visible Spectroscopic Techniques of AMLO

Sr no	matrix	Other analyte	Method	Description	Ref
1.	Tablet		Colorimetric	**Solvent/Reagent:**-ion-pair complex due to the action of Orange-II **λ max:**- 487nm **Linearity:**- 2-30 µg/ml-	[47]
2.	Tablet		Colorimetric	**Solvent/Reagent:**-acid-dye bromophenol blue at pH 3.2 in chloroform **λ max:**- 414nm **Linearity:**- 6-30 µg/mL	[48]
3.	Tablet		Titrimetry and complex formation	**Solvent/Reagent:**- bleach the dyes Methyl Orange or Indigo Carmine **Linearity:**- 0.5-3.0 µg/mL	[49]
4.	Tablet		Colorimetric	**Solvent/Reagent:**- bromo phenol blue 0.05%. 2.4 buffer solution **λ max:**- 413 nm **Linearity:**- 4-20 µg/mL	[50]
5.	Tablet		Charge-Transfer Complex Formation	**Solvent/Reagent:**-p-Chloranilic Acid	[51]
6.	Tablet		Charge-Transfer Complex Formation	**Solvent/Reagent:**-2,3-dichloro 5,6-dicyano 1,4-benzoquinone (DDQ)	[52]

				and ascorbic acid in N,N dimethylform amide medium (DMF) **λ max:-** 580 nm and 530nm **Linearity:-** 1-25µg/mL and 10-140 µg/mL	
7.	Tablet &bulk dosage form		Ion pair Complex formation	**Solvent/Reagent:-** methyl orange in Acidic medium **λ max:-** 520 nm **Linearity:-** 1 to 10µg/ml	[53]
8.	Tablet &bulk dosage form		Ion pair Complex formation	**Solvent/Reagent:-** Bromothymol blue and coupling product with 3-methyl-2-enzothiazolinone Hydrazonehydrochloride (MBTH) in the presence of ceric ammonium sulphate **λ max:-** 405 nm And 603nm **Linearity:-** 5 to 40µg/ml	[54]
9.	Tablet		Colorimetric	**Solvent/Reagent:-** Erichrome Black- T at pH 3.0 and indigo caramine at pH 2.8	[55]
10.	Tablet		Colorimetric	**Solvent/Reagent:-** bromocresol green (BCG),bromophenol blue (BPB) and methylene blue (MB) in acidic medium. **λ max:-** 409.0nm(BCG),409.0 nm (BPB) and 668.2 nm (MB). **Linearity:-** 0-80µg/ml	[56]
11.	Tablet	Nebivolol Hydrochloride	Simultaneous Equation method and Q-analysis Method	**Solvent/Reagent:-** Methanol **λ max:-** 238nm **Linearity:-** 10-5µg/mL	[57]
12.	Tablet	Atenolol	Derivative Spectroscopy	**Solvent/Reagent:-** Aqueous Methanol **λ max:-** 361 nm	[58]

13.	Tablet	Atorvastatin calcium	Simultaneous Equation ethod And Q- analysis Method	**Solvent/Reagent:-** Methanol **λmax:-** 361 nm And 238.8 nm (as an isoabsorptive point) and 246nm. **Linearity:-** 0.5-30 µg/mL	[59]
14.	Tablet	Atorvastatin calcium	Simultaneous Equation method And Q- analysis Method	**Solvent/Reagent:-** Methanol **λ max:-** 361 nm And 238.8 nm (as an isoabsorptive point) and 246 nm. **Linearity:**-0.5-30 µg/mL	[60]
15.	Tablet	Atorvastatin calcium	Simultaneous Equation method	**Solvent/Reagent:-** Methanol **λ max:-** 246.6 nm **Linearity:-** 5-30 µg/mL	[61]
16.	Tablet	Atorvastatin calcium	Simultaneous Equation method And Dual Wavelength method	**Solvent/Reagent:-** Methanol **λ max:-** 245 nm,259.9 nm **Linearity:-** 0-20 µg/mL	[62]
17.	Tablet	Atorvastatin calcium	Multicomponent mode	**Solvent/Reagent:-** 50%v/v aqueous Methanol **λ max:-** 245 and 363 nm. **Linearity:-** 7 to 13mg/ml	[63]
18.	Tablet	Atorvastatin calcium	Simultaneous Equation method	**Solvent/Reagent:-** Methanol **λ max:-** 364 nm **Linearity:-** 1-50µg/mL	[64]
19.	Tablet	Atorvastatin calcium	Simultaneous Equation method	**Solvent/Reagent:-** methanol **λmax:-** 264 nm	[65]
20.	Tablet	Benidipine Hydrochloride	Coloured complex of drug with rhodizonic acid	**Solvent/Reagent:-** chloroform **λ max:-** 450 nm **Linearity:-** 0.1-.5mg/ml	[66]

21.	Tablet	Enalapril Maleate and Lisinopril	First derivative spectroscopy	**Solvent/Reagent:-** 0–1 M HCl	[67]
22.	Tablet	Hydrochlorothiazide	Simultaneous Equation method, Q-absorbance Equation,First order Derivative Spectroscopy	**Solvent/Reagent:-** Methanol **max:-** 238.5 nm,257.5 nm,271 nm **Linearity:-** 1-10µg/mL,1-20µg/mL 1-20µg/mL	[68]
23.	Tablet	Losartan Potssium	Simultaneous Equation method, Q-absorbance Equation	**Solvent/Reagent:-** Methanol **λ max:-** 237.5 nm and 242.5 nm (as an isoabsorptive point) and 237.5 nm **Linearity:-** 2-20µg/mL	[69]
24.	Tablet	Losartan Potassium and hydrochlorothiazide	Simultaneous Equation method And Area under curve method	**Solvent/Reagent:-** Methanol **λ max:-** 236.5 nm **Linearity:-** 5-25µg/ml	[70]
25.	Tablet	Losartan Potassium and Hydrochlorothiazide	Multivariate Calibration Models	**λ max:-** 230.5-350.4 nm **Linearity:-** 1-5µg/mL	[71]
26.	Tablet	Nebivolol hydrochloride	Q-analysis method	**Solvent/Reagent:-** methanol **λ max:-** isoabsorptive point 268nm **Linearity:-** 2-40µg/mL	[72]
27.	Tablet	Nebivolol hydrochloride	Absorbance Correction Method And Absorbance ratio method	**λ max:-** 365 nm and isoabsorptive point and 280 nm **Linearity:-** 5-25µg/mL	[73]
28.	Tablet	Olmesartan Medoxomil	Simultaneous Equation and area under curve	**Solvent/Reagent:-** Methanol **λ max:-** 237.5 nm And 242.5-232.5nm **Linearity:-** 10-50µg/ml	[74]
29.	Tablet	Olmesartan Medoxomil	Simultaneous Equation method	**λ max:-** 360 nm **Linearity:-** 2 to 20ìg/mL	[75]
30.	Tablet	Ramipril	Simultaneous Equation method	**Solvent/Reagent:-** Methanol **λ max:-** 238 nm **Linearity:-** 5- 25µg/mL	[76]
31.	Tablet	Valsartan	Fractional	**Linearity:-** 1.08–17.27	[77]

			Wavelet transform (FWT) calibration method	µg/mL	
32.	Tablet	Valsartan	Multivariate Calibration method	**Linearity:-** 1.08–17.27 µg/mL	[78]
33.	Tablet	Valsartan	simultaneous equation method and Absorption Correction method	**Solvent/Reagent:-** Methanol **ƛ max:-** 238 nm And 236.0 nm isobestic point **Linearity:-** 5-30µg/mL	[79]

2.1.1.2. HPLC Techniques:

The column used in most of the techniques is C-18.

Table 2.1.2: HPLC Techniques of AMLO

Sr no	matrix	Other analyte	Description	Ref
34.			**Mobile phase:-**Ammonium acetate:methanol (30:70) **ƛ max:-** 237nm	[24]
35.			**Mobile phase:-** Methanol:acetonitrile:buffer(35 :15:50) **ƛmax:-** 237nm	[25]
36.	Tablet		**Mobile phase:-** 0.05 M ortho-phosphoric acid buffer, methanol and acetonitrile in the ratio of 50:35:15 **ƛ max:-** 361nm	[80]
37.	Human plasma		**Mobile phase:-** derivatization with 4-chloro-7-nitrobenzofurazan (NBDCl), **Linearity:-** 0.25–18.00ng/mL	[81]
38.	pharmacokinetics in rabbits		**Mobile phase:-** 0.04 M ammonium acetate:acetonitrile (38:38:24,v/v/v) containing 0.02%triethylamine (final pH 7.1). **Rt:-** 10.6min **Linearity:-** 2.5 to 100ng/mL	[82]

39.	pharmacokinetics in rabbits		**Mobile phase:-** water and acetonitrile under gradient conditions **Linearity:-** 0.5-10ng/mL	[83]
40.	Human plasma		**Mobile phase:-** water and acetonitrile under gradient conditions (both containing 0.3%formic acid) and **Linearity:-** 0.15–16.0ng/mL	[84]
41.	Manufacturing equipment surfaces		**Mobile phase:-** sodium phosphate buffer (pH 2.5) **Linearity:-** 0.25–16ng/mL	[85]
42.	Human serum		**Mobile phase:-** sodium phosphate buffer (pH 2.5) **Linearity:-** 0.25–16ng/mL	[86]
43.	Human plasma	Metoprolol succinate	**Mobile phase:-** methanol–water containing 0.5% formic acid (8:2, v/v). **Linearity:-** 1-15ng/mL	[87]
44.	Tablet	Atenolol	**Mobile phase:-** ammonium acetate buffer (pH 4.5 ± 0.05 with glacial acetic acid), acetonitrile and methanol (35:30:35 v/v). **Rt:-** 2.4 min **ƛ max:-** 237nm	[88]
45.	Tablet	Atorvastatin calcium	**Mobile phase:-** 0.02 M potassium Dihydrogen phosphate:acetonitrile:methanol (30:10:60,v/v/v)adjusted to pH 4 using ortho phosphoric acid **Rt:-** 4.5 min **ƛ max:-** 240nm **Linearity:-** 0.1-20µg/mL	[89]
46.	Tablet	Atorvastatin calcium	**Mobile phase:-** methanol: acetonitrile: 50mM KH 2 PO 4 (20:50:30;pH 3.5) **Rt:-** 3.2 min **ƛ max:-** 240nm **Linearity:-** 5-100µg/mL	[90]
47.	Tablet	Atorvastatin calcium	**Mobile phase:-** phosphate buffer (1ml ortho phosphoric acid in 1000 ml 0f water) acetonitrile and methanol (53:43:4, v/v) **Rt:-** 3.37min	[91]

			ƛ max:- 246nm **Linearity:-** 40-60µg/mL	
48.	Tablet	Benazepril Hydrochloride	**Mobile phase:-** acetonitrile–0.025 M NaH2PO4 buffer (pH 4.5)(55:45, v/v) **ƛ max:-** 237nm **Linearity:-** 2–30µg/mL	[92]
49.	Tablet	Benazepril Hydrochloride	**Mobile phase:-** phosphate buffer pH 3.0(0.01 M aqueous potassium dihydrogen phosphate, pH 3.0adjusted with orthophosphoric acid)solvent mixture (equal mixture of acetonitrile and methanol) in the ratio of 45:55 (v/v) **Linearity:-** 5.21 −15.63ìg/mL	[93]
50.	Tablet	Benazepril Hydrochloride	**Mobile phase:-** phosphate buffer and acetonitrile in the proportion of 65:35 (v/v)with apparent pH adjusted to 7.0 **ƛ max:-** 240nm **Linearity:-** 6–14µg/mL	[94]
51.	Tablet	Antihypertensive formulations	**Mobile phase:-** methanol-water (60:40v/v) **ƛmax:-** 215nm	[95]
52.	Tablet	Hydrochlorothiazide, and Valsartan	**Mobile phase:-** sol-A (pH 3.00±0.05 of 0.01M Potassium dihydrogen Phosphate)and sol-B (Acetonitrile).gradient **Rt:-** 6.0 min **ƛ max:-** 237nm **Linearity:-** 5-75µg/mL	[96]
53.	Tablet	Losartan Potassium, Hydrochlorothiazide	**Mobile phase:-** 0.025 M phosphate buffer (pH3.7):acetonitrile (57:43v/v) **ƛ max:-** 232nm **Linearity:-** 2-14µg/mL	[97]
54.	Tablet	Genotoxic alkyl benzenesulfonates	**Mobile phase:-** 65:35 (v/v) mixture of 1% triethyl amine, pH adjusted to 3.0 with orthophosphoric acid and acetonitrile **Linearity:-** 75–180µg/mL	[98]
55.	Tablet	Metoprolol	**Mobile phase:-** 0.02 M phosphate buffer solution: acetonitrile (70:30v/v, pH 3.0). **Rt:-** 2.57min	[99]

			ƛ max:- 221nm **Linearity:-** 10-110µg/mL	
56.	Human plasma	Metoprolol succinate	**Mobile phase:-** methanol–water containing 0.5% formic acid (8:2, v/v). **Linearity:-** 1 –15ng/mL	[100]
57.	Tablet	Nebivolol Hydrochloride	**Mobile phase:-** Ammonium acetate buffer (pH 4.5):acetonitrile 50:50,v/v). **Rt:-** 9.59Min **ƛmax:-** 274nm **Linearity:-** 240-640µg/mL	[101]
58.	Tablet	Nebivolol Hydrochloride	**Mobile phase:-** 0.005M ammonium acetate solution,acetonitrile and triethylamine in the ratio 60:40:0.1 (v/v) and pH 3.0 was adjusted with orthophosphoric acid **Rt:-** 3.911min **ƛmax:-** 269nm **Linearity:-** 10-30µg/mL	[102]
59.	Tablet	1,4-dihydropyridines	**Mobile phase:-** acetonitrile–10 mM acetate buffer (72:28, v/v) **Rt:-** 4.96min **Linearity:-** 4.5–15µg/mL	[103]
60.	Tablet	Olmesartan Medoxomil	**Mobile phase:-** buffer–Acetonitrile(60:40, v/v). **Linearity:-** 5–100µg/mL	[104]
61.	Tablet	Olmesartan Medoxomil	**Mobile phase:-** 0.05M potassium dihydrogen phosphate buffer:acetonitrile (50:50v/v) **Rt:-** 3.69min **ƛmax:-** 238nm **Linearity:-** 4-20µg/mL	[105]
62.	Tablet	Telmisartan	**Mobile phase:-** 0.05 M potassium dihydrogen ortho phosphate: acetonitrile (60:40% v/v) **Rt:-** 5.47min **ƛ max:-** 237nm **Linearity:-** 1-50µg/mL	[106]
63.	Tablet	Valsartan	**Mobile phase:-** acetonitrile: KH2PO4 (50:50v/v) adjusted to pH 3.5 by phosphoric acid. **ƛ max:-** 238nm	[107]

			Linearity:- 5- 40µg/mL	
64.	Tablet	Valsartan	**Mobile phase:-** mixture of solution A (1000 mL Water + 0.2 mL Trifluoro Acetic Acid) and solution B Water:acetonitrile:Trifluoro Acetic Acid,400:600:1, v/v/v) **Rt:-** 15.3min **ƛ max:-** 237nm **Linearity:-** 1-30µg/mL	[108]
65.	Tablet	Valsartan	**Mobile phase:-** acetonitrile-phosphate buffer (50/50, v/v) **Rt:-** 1.5 min **ƛ max:-** 254nm **Linearity:-** 2-20µg/mL	[109]
66.	Capsule	Valsartan	**Mobile phase:-** acetonitrile: phosphate buffer (0.02M, pH 3.0),(56:44 v/v) **Rt:-** 3.07min **ƛmax:-** 234nm **Linearity:-** 1- 40µg/ml	[110]
67.	Blood	beta-blockers, calcium-channel antagonists, angiotensin-II antagonists and antiarrhytmic	**Mobile phase:-** 0.05M Ammonium acetate pH 6.8and acetonitrile in the ratio of 40:60, v/v. **Rt:-** 3.94min **ƛmax:-** 239nm **Linearity:-** 16-112µg/mL	[111]
68.	Human serum	Prazosin hydrochloride and calcium channel blockers	**Mobile phase:-** acetonitrile:methanol:water: (10:55:35 v/v, pH2.65±0.02) **Rt:-** 4.92min **ƛ max:-** 240nm **Linearity:-** 15-300µg/mL	[112]
69.	Human plasma	1,4-dihydropyridines	**Mobile phase:-** acetonitrile–10 mM acetate buffer (72:28,v/v) **Rt:-** 10 min **Linearity:-** 4.5–15µg/ml	[113]
70.	Tablet	Atenolol	**Mobile phase:-** methylene chloroform:methanol:ammonia solution (25% NH3)(50:50:0.1; v/v) **Linearity:-** 229nm	[114]

2.1.1.3. HPTLC Techniques:

The stationary phase used is aluminium plates pre-coated with silica gel 60 F_{254}.

Table 2.1.3: HPTLC Techniques of AMLO

Sr no.	Matrix	Other analytes	Method	Description	Ref
71.				**Mobile phase:**-50volume of buffer(pH=3)+7ml TEA+35volume of methanol +15volume of Acetonitrile **ƛ max:-** 237nm	[22]
72.	Tablet	Benazepril	Simultaneous equation method	**Mobile phase:-** methylene chloride:methanol:ammonia solution (25% NH3) (8.8:1.3:0.1; v/v) **Rf:-** 0.75 **ƛ max:-** 230nm **Linearity:-** 10–500ng/mL	[115]
73.	Tablet	Nebivolol Hydrochloride	Simultaneous equation method	**Mobile phase:-** chloroform:toluene:methanol:glacialacetic acid, (5:2:3:0.1, v/v/v/v),N-0.48, 500–2500 ng /spot **Rf:-** 0.33 **ƛ max:-** 271nm **Linearity:-**250–1250 ng/spot	[116]
74.	Tablet	Nebivolol Hydrochloride	Simultaneous equation method	**Mobile phase:-** chloroform-methanoltolueneammonia (8+ 1.8+ 1+ 0.3, v/v/v/v/) **Rf:-** 0.27 **ƛmax:-** 273nm **Linearity:-**200– 800ng/spot	[117]
75.	Tablet	Nebivolol Hydrochloride	Stability method	**Mobile phase:-** Methylene Chloride : Methanol :Ammonia (8.5:1:0.5 v/v) **Rf:-** 0.19 **ƛmax:-** 285nm **Linearity:-** 250–1000ng/spot	[118]
76.	Tablet	Olmesartan Medoxomil	Simultaneous equation	**Mobile phase:-** Ethyl acetate: Methanol: Dilute	[119]

			method	ammonia (8.5:1:1, v/v/v). **Rf:-** 0.40 **ƛmax:-** 280nm **Linearity:-**500–2000 ng/spot	
77.	Human Plasma and Dosage Forms	Valsartan		**Mobile phase:-** Ethyl acetate: 1,4 Dioxane:Methanol: 25% Ammonia in the ratio of 15:1.5:3:1.5 v/v **Rf:-** 0.33 **ƛmax:-** 323nm **Linearity:-** 200-1000ng/ml	[120]
78.	Human Plasma	Amlodipine Besylate		**Mobile phase:-** Ethyl acetate/ methanol /ammonium hydroxide (55:45:5 by volume). **ƛmax:-** 237nm **Linearity:-** 0.5-4.0ng/spot	[121]
79.		Nebivolol Hydrochloride	Simultaneous equation method	**Mobile phase:-** ethyl acetate-methanol-ammonia solution (8.5:2.0:1.0, v/v/v). **Rf:-** 0.58 **ƛ max:-** 254nm **Linearity:-** 0.1-0.8ng/mL	[122]

2.2.2. NEBI:

For determination NEBI various techniques are avalaible alone and in combination with other drugs. HPTLC is official in I.P.2010.

2.2.2.1. UV-Visible Spectrometric Techniques:

Table 2.2.1: UV-visible Spectroscopic Techniques for NEBI.

Sr no	Matrix	Other Analytes	Method	Description	Ref
1.	Tablet		Absorption maxima	**Solvent:-** Methanol **λ max:-** 282 nm **Linearity:-** 5-50µg/mL	[123]
2.	Tablet		First Order Derivative Spectrophotometry	**Solvent:-** Methanol-Water (3:7) **λ max:-** 281 nm **Linearity:-** 10 - 70µg/mL	[124]
3.	Tablet		Absorption maxima	**Solvent:-** Tetrahydrofuran **λ max:-** 301 nm **Linearity:-** 5 – 60µg/mL	[125]
4.	Tablet		Second And Third Order Derivative Spectrophotometry	**Solvent:-** Methanol **λ max:-** 296 nm and 290nm **Linearity:-** 40-80 µg/ml and 10-60µg/ml	[126]
5.	Tablet		Absorption maxima	**Solvent:-** 5.0 M niacinamide solution as solubilizer and Distilled Water **λ max:-** 286.5nm **Linearity:-** 5-35µg/ml.	[127]
6.	Tablet	Hydrochlorothiazide	Absorption Correction Method	**Solvent:-** Aqueous Methanol (20 % v/v). **λ max:-** 281 nm **Linearity:-** 5-	[128]

				35µg/mL	
7.	Tablet	Hydrochlorothiazide	First-order derivative spectrophotometry	**Solvent:-** Methanol **λ max:-** 294.6nm **Linearity:-** 8-40µg/mL	[129]
8.	Tablet	Hydrochlorothiazide	Q-Analysis Method	**Solvent:-** Methanol **λ max:-** 290 nm **Linearity:-** 0-80µg/mL	[130]
9.	Tablet	Hydrochlorothiazide	Multicomponent Mode	**Solvent:-** Aqueous Methanol(20 % v/v) **λ max:-** 281 nm **Linearity:-** 5-35µg/mL	[131]
10.	Tablet	Hydrochlorothiazide	Simultaneous Equations and Multicomponent mode	**Solvent:-** Methanol **λ max:-** 290 nm **Linearity:-** 0-80µg/mL	[132]
11.	Capsule	Valsartan	Simultaneous Equation Method	**Solvent:-** Methanol **λ max:-** 281 nm **Linearity:-** 5-80µg/mL	[133]
12.	Capsule	Valsartan	Absorption Ratio Method	**Solvent:-** Methanol **λ max:-** 281 nm **Linearity:-** 4 - 80µg/mL	[134]
13.		valacyclovir, aceclofenac, racecadotril, valdecoxib, nebivolol and reboxetine	Absorption Maxima	**Solvent:-** Aqueous Methanol **λ max:-** 281 nm **Linearity:-** 10-60µg/ml	[135]

2.2.2.2. HPLC Techniques:

The column used in almost all techniques is C-18.

Table 2.2.2: RP-HPLC Techniques for NEBI.

Sr no.	Matrix	Other analytes	Description	Ref
14.	Tablet		**Mobile phase:-** Methanol-Water(70:30, v/v) **Retention Time:-** 3.3 min **λ max :-** 282 nm	[136]

			Linearity:- 0.25-8.0µg/mL	
15.	Tablet		**Mobile phase:-** Water and Methanol (40:60) **Retention Time:-** 3.15 min **λ max :-** 282 nm **Linearity:-** 5-100µg/mL	[137]
16.	Tablet		**Mobile phase:-** Methanol: Water (80:20v/v) **Retention Time:-** 3.175 min **λ max :-** 281 nm **Linearity:-** 1-400µg/mL	[138]
17.	Tablet		**Mobile phase:-** 50 mM KH_2PO_4 Buffer (pH 3.0±0.1):Acetonitrile:(45:55 v/v) **Retention Time:-** 3.76±0.02min **λ max :-** 282 nm **Linearity:-** 10-150µg/ml	[139]
18.	Tablet		**Mobile phase:-** Methanol: acetonitrile:0.02 M potassiumdihydrogen phosphate (60:30:10, v/v/v; pH 4.0) **Retention Time:-** 2.6 min **λ max :-** 280 nm **Linearity:-** 0.2-10mg/ml	[140]
19.	Human plasma		**Mobile phase:-** Water:Acetonitrile:formic acid (30:70:0.03, v/v) **Retention Time:-** 2 min **Linearity:-** 50-10,000pg/mL	[141]
20.	Tablet		**Mobile phase:-** Acetonitrile-pH 3.5 phosphate Buffer (35 +65, v/v) **λ max :-** 280 nm **Linearity:-** 40-160µg/mL	[142]
21.	Tablet		**Mobile phase:-** Water:Acetonitrile:formic acid (30:70:0.03, v/v) **Linearity:-** 50–10,000 pg/mL	[143]
22.	Tablet	S-Amlodipine besylate	**Mobile phase:-** Ammonium acetate Buffer (pH 4.5):Acetonitrile (50:50v/v) adjusted with TEA **Retention Time:-** 13.56min **λ max :-** 274 nm **Linearity:-** 120-320µg/ml	[144]
23.	Tablet	Hydrochlorothiazide	**Mobile phase:-** 0.05 M potassium dihydrogen phosphate-Acetonitrile-Methanol (30 + 20 + 50, v/v/v; pH 4) **Retention Time:-** 5.1 min **λ max :-** 220 nm **Linearity:-** 1-14microg/mL	[145]

24.	Tablet	Hydrochlorothiazide	**Mobile phase:-** Acetonitrile and potassium dihydrogen phosphate Buffer (pH 3.2 ± 0.1) in the ratio of 50:50 v/v **Retention Time:-** 3.57 min **λ max :-** 282 nm **Linearity:-** 8-32 µg/ml	[146]
25.	Tablet	Hydrochlorothiazide	**Mobile phase:-** 30 mM phosphate Buffer (K2HPO4), Acetonitrileand triethylamine (50:50:0.1% v/v) with pH 5.5 using orthophosphoric acid **Retention Time:-** 3.31 min	[147]
26.	Tablet	Hydrochlorothiazide	**Mobile phase:-** Methanol: Water (60:40 v/v) adjusted to pH 3:2 with o-phosphoric acid **Retention Time:-** 2.628 min **λ max :-** 281 nm **Linearity:-** 5 to 50µg/ml	[148]
27.	Tablet	Hydrochlorothiazide	**Mobile phase:-** Acetonitrile: 50mM ammonium acetate (adjusted to pH 3.5 using orthophosphoric acid)(70:30 v/v) **Retention Time:-** 3.32 min **λ max :-** 254 nm **Linearity:-** 2 to 10µg/ml	[149]
28.			**Mobile phase:-** Acetonitrile--0.03%Aqueous formic acid, pH 3.3 (65 + 35, v/v), **Retention Time:-** 2.133min **λ max :-** 217 nm	[150]
29.	Tablet	Valsartan	**Mobile phase:-** Methanol:Water (80:20 v/v) with addition of 0.1% 1-hexane sulfonic acid monohydrate sodium salt as an ion-pairing reagent **λ max :-** 289 nm **Linearity:-** 10-250µg/mL	[151]
30.	Human plasma	Valsartan	**Mobile phase:-** Acetonitrile and 0.05 mM Formic acid (50:50 v/v,pH 3.5) **Retention Time:-** m/z 406.1-->m/z 150.9 **Linearity:-** 0.01-50.0ng/ml	[152]
31.	Fixed-dose combination	Valsartan	**Mobile phase:-** Buffer-Acetonitrile (55 +45, v/v) **λ max :-** 230 nm **Linearity:-** 2-8 µg /mL	[153]
32.	Animal tissues	acebutalol,atenolol,bopindolol,bufurolol,carazolol,metaprolol, oxprenolol,practolol, propranolol,two	**Mobile phase:-** (85:15:0.1,v/v/v),(90:10:0.1, v/v/v), and (95:05:0.1, v/v/v)combinations of n-heptane,ethanol, and diethylamine solvents **λ max :-** 225 nm	[154]

		beta-adrenergic agonists clenbuterol		

2.2.2.3. HPTLC Techniques:

The stationary phase used was aluminium plates precoated with silica gel 60F_{254}.

Table 2.2.3: HPTLC Techniques for NEBI.

Sr no.	Matrix	Other analytes	Description	Ref
33.			**Mobile phase:-**28volume Acetonitrile+72volume buffer+0.3ml of diethylamine **λ max :-** 220nm	[22]
34.	Tablet		**Mobile phase:-** ethyl acetate: toluene: Methanol:ammonium hydroxide (1:6:2:0.1 v/v/v/v) **Rf:-** 0.33 **λ max :-** 282nm **Linearity:-** 150 -550ng/spot	[155]
36.	Tablet		**Mobile phase:-** toluene-Methanol-triethylamine (3.8:1.2:0.2 v/v/v) **Rf:-** 0.33±0.02 **λ max :-** 281nm **Linearity:-** 500-3000ng/spot	[156]
37.	Tablet		**Mobile phase:-** -ethyl acetate-Methanol-formic acid,8 + 6 + 4 + 1 (v / v), **λ max :-** 285nm	[157]
38.	Tablet	Amlodipine Besylate	**Mobile phase:-** Methylene Chloride : Methanol :Ammonia (8.5:1:0.5 v/v) **Rf:-** 0.41 **λ max :-** 285nm	[158]
39.	Tablet	Amlodipine Besylate	**Mobile phase:-** chloroform-Methanol toluene ammonia(8+ 1.8+ 1+ 0.3, v/v/v/v/) **Rf:-** 0.51 **λ max :-** 273nm **Linearity:-** 400-1000ng/spot	[159]
40.	Tablet	Amlodipine Besylate	**Mobile phase:-** Ethyl acetate: Methanol: Dilute ammonia (8.5:1:1,	[160]

			v/v/v). **Rf:-** 0.40 **λ max :-** 280nm **Linearity:-** 500–2000ng/ spot	
41.	Tablet	Amlodipine Besylate	**Mobile phase:-** chloroform:toluene:Methanol:glacialacetic acid, (5:2:3:0.1, v/v/v/v) **Rf:-** 0.48 **λ max :-** 271nm **Linearity:-** 500–2500ng /spot	[161]

Chapter 3
Aim and Objective

3.1 AIM OF PRESENT WORK

Pharmaceutical analytical procedures may be used for identification and quantitative analysis of the active moiety in the sample of drug substances or products. Multicomponent dosage forms are introduced as they are known to be beneficial. The development of an assay procedure for such dosage forms posses considerable challenges to the analytical chemist owing to complexity of these dosage forms as it contain multiple drug entities.

Several methods were reported for the individual estimation of AMLO and NEBI and their combination with other drugs but no chemometric determination method has been reported. Spectrophotometric, HPLC and HPTLC method has been reported for simultaneous estimation of these drugs in combined dosage forms.

3.2 OBJECTIVES OF PRESENT WORK

- To develop a suitable Chemometric method using UV-VIS Spectrophotometric estimation of Amlodipine Besylate and Nebivolol Hydrochloride in their combined dosage form.

Chapter 4
Identification of Drug

4.1.IDENTIFICATION OF DRUG:

Identification of drugs was carried out by Melting point, UV-VIS Spectrophotometry and IR spectroscopy.

4.4.1. Melting Point Determination:

Melting points of both the drugs AMLO and NEBI were determined by capillary method by using Melting Point Apparatus. The results obtained are as follows (Table 4.1):

Table 4.1: Melting Point of AMLO and NEBI.

Drugs	Reported Melting Point(°C) [20]	Observed Melting point(°C)
AMLO	178 -179	175-178
NEBI	140.7	138-139

4.1.2. UV spectra of AMLO and NEBI:

UV spectra of AMLO and NEBI, both 20µg/ml each, in methanol were taken on UV-VIS spectrophotometer. The absorption maxima of AMLO and NEBI were found to be 237.2 nm and 281.80 nm, respectively which matched with reported value (Table 4.2, Fig-4.1 and 4.2).

Table 4.2: Absorption Maxima of AMLO and NEBI, 20µg/ml, methanol.

Drugs	Reported λmax(nm)	Observed λmax(nm)
AMLO	239.00	237.2
NEBI	282.00	281.8

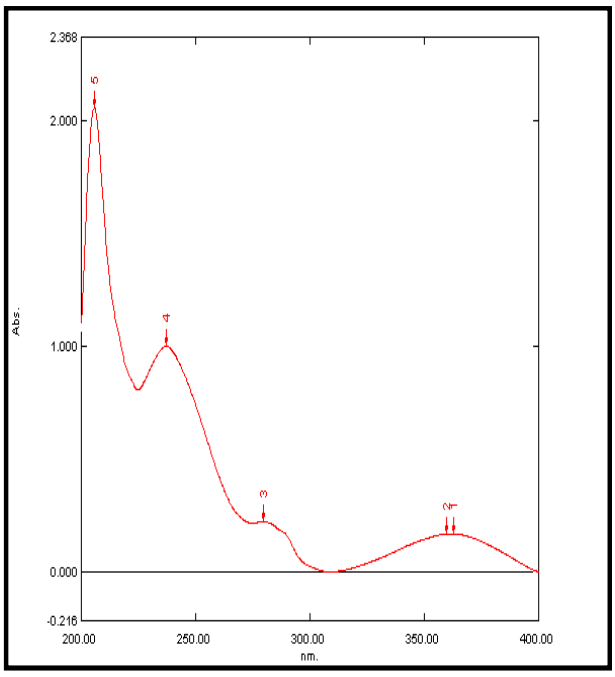


Figure 4.1.: UV spectra of 20µg/ml AMLO Solution in methanol.

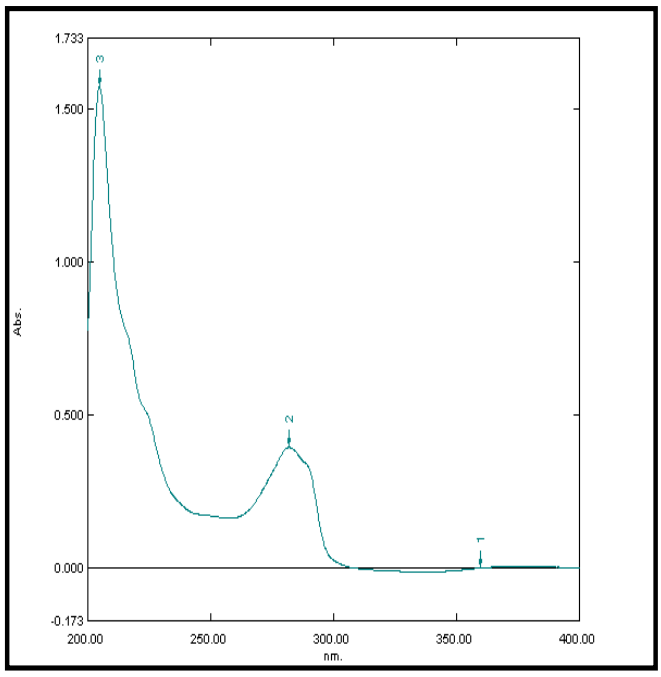


Figure 4.2.: UV spectra of 20µg/ml NEBI Solution in methanol.

4.1.3. FT-IR spectra of AMLO and NEBI [22]:

FTIR spectra of both the drugs AMLO and NEBI were recorded on FTIR Spectrophotometer, by mixing with dry KBr powder. Fig.4.3 (a) and (b) indicates identical FT-IR spectra of AMLO in fingerprint region. For NEBI (Fig.4.4) recorded peaks were matched with reported peaks.

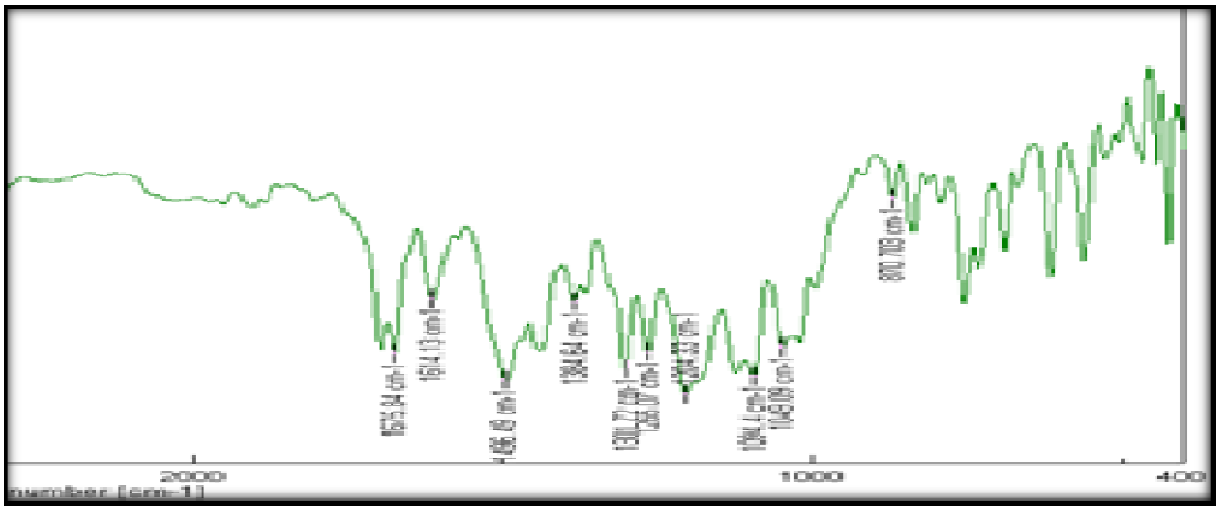


Figure 4.3(a): Recorded FTIR Spectra of AMLO.

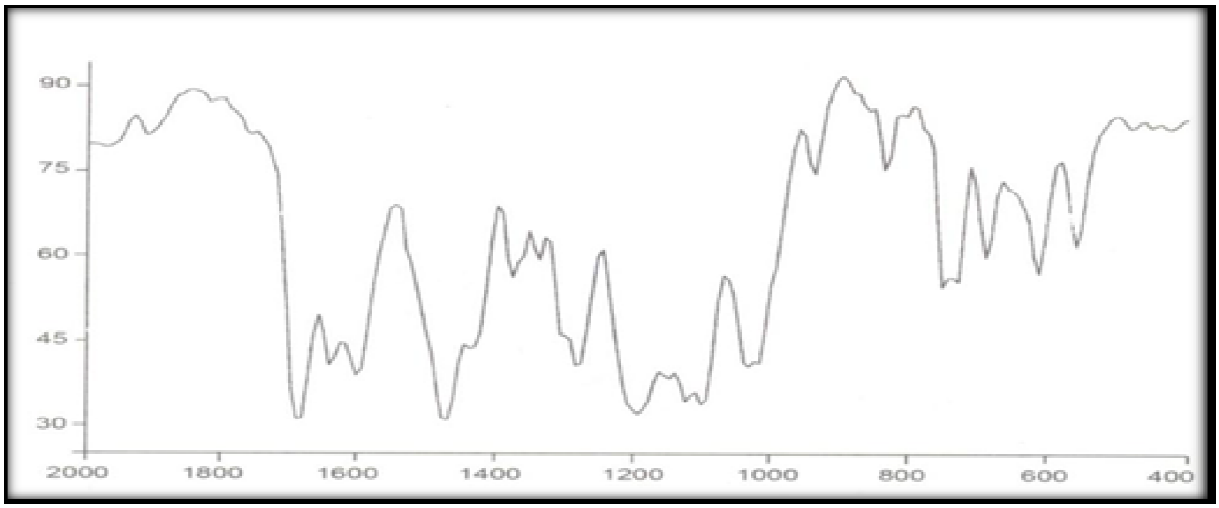


Figure 4.3(b): Reported FTIR Spectra of AMLO.

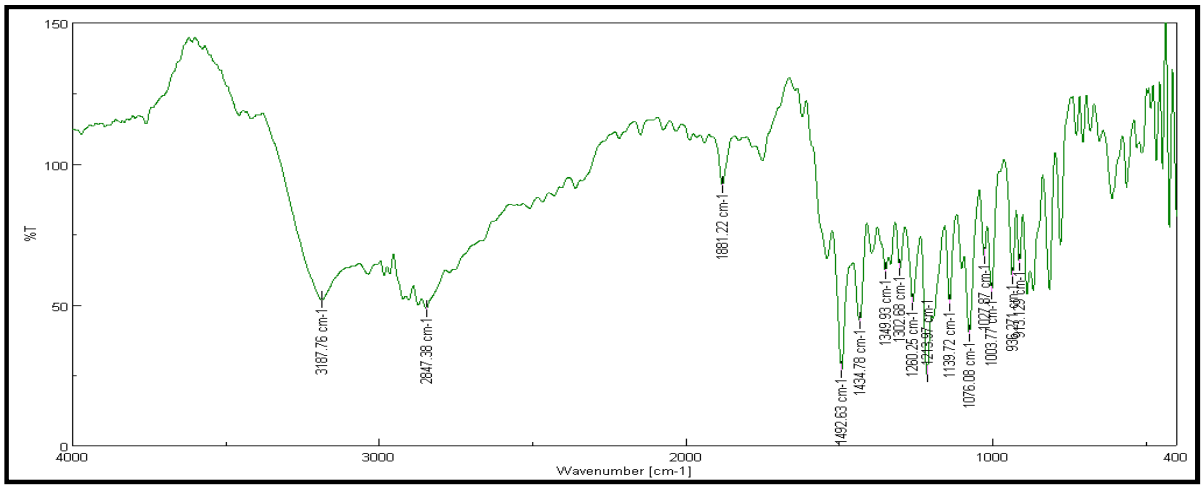


Figure 4.4: Recorded FTIR spectra of NEBI.

Table 4.3: Reported and Recorded IR peaks of NEBI.

Functional Group	**Reported IR Peaks(cm^{-1})**	**Recorded IR Peaks(cm^{-1})**
C-H stretching (aromatic)	3000-3100	3192
C=O stretching	1735-1750	1882
C=C stretching (aromatic)	1450-1600	1492
C=N vibrations	1000-1400	1214

Chapter 5
Experimental Work

5.1. EXPERIMENTAL WORK

AMLO is photosensitive drug, So all the solution were prepared in amber coloured volumetric flask.

5.1.1. Preparation of standard stock solution of AMLO (1000µg/ml) and NEBI (1000µg/ml):-

25mg each of AMLO and NEBI were accurately weighed and transferred to volumetric flask individually and dissolved in methanol. The solution was sonicated for 10min.The flask was shaken and made up the volume up to the mark with methanol to give solution containing 1000µg/ml .An aliquot of 5ml was pipetted out from stock solution of AMLO(1000µg/ml) and transferred to 50ml volumetric flask. The volume was made with methanol to obtain conc. of 100µg/ml of AMLO and NEBI respectively. From this diferrent six sets were prepared.

5.1.2. Preparation of different sets of AMLO and NEBI for Multivariate Calibration:-

5.1.2.1.Set-1(API AMLO):- From stock solution of AMLO(100µg/ml),an aliquot of 1,2,3,4,5,6ml was pipetted out and transferred to 10ml amber coloured volumetric flask individually. The volume was made up with methanol to obtain conc. of 10-60µg/ml of AMLO.

5.1.2.2.Set-2(API NEBI):- From stock solution of NEBI(100µg/ml),an aliquot of 1,2,3,4,5,6ml was pipetted out and transferred to 10ml amber coloured volumetric flask individually.The volume was made up with methanol to obtain conc. of 10-60µg/ml of NEBI.

5.1.2.3.Set-3(Binary mixture of AMLO and NEBI):- From stock solution of AMLO(100µg/ml) and NEBI(100µg/ml),an aliquot of 1,2,3,4,5,6ml was pipetted out and transferred to 10ml amber coloured volumetric flask individually. The volume was made up with methanol.

5.1.2.4.Set-4[Binary mixture of AMLO(10µg/ml) and NEBI(var)]:- Series of volumetric flasks were taken and aliquot of 1,2,3,4,5,6 ml of NEBI stock solution was pipetted out and

transferred in individual flask.In each flask,1ml of stock solution of AMLO was transferred with pipette and volume was made upto the mark with methanol.

5.1.2.5.Set-5[Binary mixture of AMLO(var) and NEBI(10µg/ml)]:- Series of volumetric flasks were taken and aliquot of 1,2,3,4,5,6 ml of AMLO stock solution was pipetted out and transferred in individual flask. In each flask,1ml of stock solution of NEBI was transferred with pipette and volume was made upto the mark with methanol.

5.1.2.6.Set-6[Binary mixture of AMLO(inc) and NEBI(dec)]:- Series of volumetric flasks were taken and aliquot of 1,2,3,4,5,6,3.5,2.5,2.5,3 ml of AMLO stock solution was pipetted out and transferred in individual flask. In each flask,6,5,4,3,2,1,2.5,3,2.5,3.5 ml of stock solution of NEBI was transferred with pipette and volume was made upto the mark with methanol.

5.1.3. Analysis of tablet sample:-

Ten tablets of marketed formulation were weighed accurately to determine avg.wt of tablet and powdered.An amount of tablet powder equivalent to 25mg of AMLO and equivalent of 25mg of NEBI was transferred to 25ml amber coloured volumetric flask.10ml of methanol was added to flask and sonicated for 10min.The solution was shaken and made up to the mark with methanol to give conc. of 1000µg/ml. The above solution was filtered through Whatmann filter paper(No.41). An aliquot of 5ml was pipetted out from this stock solution (1000µg/ml) and transferred to 50ml amber coloured volumetric flask(100µg/ml).An aliquot of 3ml was pipetted out from this stock solution (100µg/ml) and transfer to 10ml amber coloured volumetric flask(30µg/ml).

5.1.4. Multivariate Calibration:-

Training set was designed in 34 laboratory made sample mixtures in the concentration range of 10-60 µg/ml for each of drug. A validation set containing ten synthetic binary mixtures in the range of 10-60 µg/ml for both drugs was prepared using the above stock solutions for PLS, PCR methods as shown in table- 5.1.Computations were made using SPSS software. PLS, PCR models were applied to the UV absorption spectra using four latent variables (or principal components) for determination of the studied components.

Table 5.1.: UV-VIS spectrophotometer parameter:- (using UV probe 2.1 software)

Parameter	
Linearity	10-60µg/ml in methanol
Wavelength	240-380nm with 1nm interval
Path length	1cm

Table-5.2: Training set using PLS and PCR method for AMLO and NEBI

[Sr No:-1-24=Calibration set, 25-34=Validation set]

Sr No.	**Lab Mixtures (µg/mL)**		**Sr No.**	**Lab Mixtures (µg/mL)**	
	AMLO	**NEBI**		**AMLO**	**NEBI**
1	10.00	0.00	18	60.00	10.00
2	20.00	0.00	19	10.00	10.00
3	30.00	0.00	20	10.00	20.00
4	40.00	0.00	21	10.00	30.00
5	50.00	0.00	22	10.00	40.00
6	60.00	0.00	23	10.00	50.00
7	0.00	10.00	24	10.00	60.00
8	0.00	20.00	25	10.00	60.00
9	0.00	30.00	26	20.00	50.00
10	0.00	40.00	27	30.00	40.00
11	0.00	50.00	28	40.00	30.00
12	0.00	60.00	29	50.00	20.00
13	10.00	10.00	30	60.00	10.00
14	20.00	10.00	31	30.00	25.00
15	30.00	10.00	32	25.00	30.00
16	40.00	10.00	33	35.00	25.00
17	50.00	10.00	34	25.00	35.00

- Using UV-VIS parameter and training sets of AMLO and NEBI absorbance were recorded and put into SPSS software and various parameters are calculated.

5.2. COMPARISION OF THE DEVELOPED CHEMOMETRIC-ASSISTED UV-VIS SPECTROPHOTOMETRY TECHNIQUE WITH THE REPORTED RP-HPLC TECHNIQUE:

5.2.1. HPLC Condition:-

- **Stationary Phase** :-ZORBAX Rx C8 (250mm* 4.6mm , 5 µ particle size)
- **Mobile phase**:-20Mm Acetate buffer+1ml TEA (pH 2.5) with glacial acetic acid : ACN (50:50)
- **Detection wavelength**:- 291 nm
- **Flow rate** :- 1 ml/min
- **Retention time of AMLO:-** 6.517 min.
- **Retention time of NEBI** :- 7.458 min.
- **Diluent :-** ACN:Water (50:50)

5.2.2. Preparation of solutions:

5.2.2.1. Preparation of standard stock solution :

50 mg standard AMLO and Standard NEBI were weighed individually and transferred to 50ml amber coloured volumetric flask, dissolved in diluent ACN:Water (50:50).The solution was sonicated for 10min.The flask was shaken and volume was made up to the mark with diluent .From both stock ,an aliquot of 10 ml was taken and transferred to 50 ml amber coloured volumetric flask (200µg/ml).An aliquots of 1,2.5,4,5,6ml were taken from the stock solution(200µg/ml) and transfer to 10ml amber coloured volumetric flask to obtain concentration 20,50,80,100,120µg/ml of AMLO+NEBI respectively.

5.2.2.2. Preparation of Marketed formulation assay solution:

Ten tablets of marketed formulation were weighed accurately to determined avg.wt of tablet and powdered equivalent to 5 mg AMLO and 5 mg NEBI was dissolved in 25ml ACN:Water (50:50) (200µg/ml) .This solution is sonicated for 30 mins and then filtered through syringe filter paper. An aliquot 5ml was pipetted out and transferred to 10ml

amber coloured volumetric flask and volume was made up to the mark with ACN:Water (50:50) to obtain concentration 100µg/ml of AMLO and NEBI respectively.

5.2.2.3. Procedure :

Chromatograms of all the solutions were recorded using above mentioned method.% assay value was calculated and compared with developed method. Student t-test was applied and t-calculated value was compared with t-table value . The results of assay was compared to the standard mixtures were reported.

Chapter 7
Summary and Future Scope

7.1 SUMMARY

Accurate and precise chemometric-assisted UV-VIS spectrophotometry method was developed for Amlodipine Besylate and Nebivolol Hydrochloride in their combined dosage form.

The estimating wavelength for AMLO and NEBI was done at 239 nm and 281.1nm respectively. The linearity was obtained in the range of 10-60µg/ml each of AMLO an NEBI. The regression equation for AMLO was Y=0.99x+0.037 (r^2 = 0.999) (By PLS) and Y=0.995x-0.044(r^2 = 0.999) (By PCR) for NEBI it was Y=0.994x - 0.276(r^2 = 0.999) (By PLS) and Y=0.997x-0.229 (r^2 = 0.999) (By PCR) found. % Recovery was found to be 99.54±1.3 (By PLS) and 99.31±1.39 (By PCR) for AMLO and for NEBI 98.02±1.37 (By PLS) and 98.386±1.26 (By PCR).

Assay results obtained by developed method was compared with reported HPLC method was achieved on C_8 Zorbex column (250 mm length, 4.6 i.d, 5 um particle size) with 20mM Ammonium acetate buffer (pH 2.5) glacial acetic acid : Acetonitrile (50 : 50) with 1ml Triethyl amine as mobile phase. The flow rate was 1 ml/min .Detection was carried out at 291 nm with PDA detector. The linear concentration range of AMLO and NEBI was 20-120 µg/ml. The regression equation for AMLO was Y=2973 x - 16274 (r^2 = 0.999) and for NEBI it was Y= 10925 x + 35642 (r^2 =0.992). % Recovery was found to be 100.47±5278.67 for AMLO and for NEBI it was 98.26±4509.4.

The assay results of the developed method was compared by applying Student t-test and it was found that t-calculated value is less than t-tabulated value. Hence there is no significant difference between the developed method and all the methods can be successfully applied for the routine quality control laboratories for analysis of pharmaceutical formulation containing AMLO and NEBI in combined dosage form.

7.2 FUTURE SCOPE

- NIR and FTIR can be used for quantifying Amlodipine Besylate and Nebivolol in combine dosage form.
- Amlodipine Besylate show flourescence so spectroflourimetric methods can be tried for these combinations.
- Also capillary Electrophoretic methods can be developed for their simultaneous estimation.

Chapter 8
References

1.Saraf S.;Various U.V. Spectrophotometric simultaneous estimation methods, Pharmainfo.net.in, volume-2, issue 4, 2006.

2. Beckett A.H.,Stenlake J.B.,Practical pharmaceutical chemistry, CBS publishers and distributors,2002, 4th edition, part II.

3.Lewis C.,Patrick E.,Rourke,process analytical technology,Edited by Katherine A. Bakeev,Blackwell publishing,chapter 6,170-178.

4.Willard, H.O.,Merritt, L.L.,Dean J.A.,Settle, F.A.,Instrumental Methods of Analysis,6th Edition,Wadsworth Publishing Company,Belmont, 1981.

5. General Notices,USP 26,2003,6–7.

6. http://www.fda.gov/cder/OPS/PAT.htm.(Acessed on 15/05/2013)

7.Bakeev K.A.,Near-Infrared Spectroscopy as a Process Analytical Tool. Part I: Laboratory Applications; Spectroscopy 2003,18(11),32–35.

8.Ciurczak E.W.,Drennen, J.K.,Pharmaceutical and Medical Applications of Near-Infrared Spectroscopy, Practical Spectroscopy Series, Volume 31,Marcel Dekker,New York,2002.

9.Rhodes C.T.,Morissau K.,Tablet Evaluation Using Near Infrared spectroscopy,Encyclopedia Pharmaceutical Technology, 2nd Edition;Marcel Dekker,New York, 2002; pp. 2689–2700.

10.Workman, J.J.,Review of Process and Non-Invasive Near-Infrared and Infrared Spectroscopy;Appl. Spectrosc. Rev. 1999, 34, 1–89.

11.Colthrup N.B.,Daly L.H.,Wiberley S.E.,Introduction to Infrared and Raman Spectroscopy;Academic Press; San Diego, 1990.

12.Wang, X.,Soos, J.,Li Q.,Crystal J.,An Acousto-Optical Tunable Filter (AOTF) NIR Spectrometer for On-line Process Control; Process Contr. Qual. 1993,5,9–16.

13.Schlossberg H.R.,Kelley P.L.,Infrared Spectroscopy Using Tunable Lasers.,Spectrometric Techniques, Vol. II,Academic Press,New York, 1981,161–238.

14.Wright J.C.,Wirth, M.J., Lasers and Spectroscopy,Anal. Chem.,1980,52,988–996.

15.Prisant L. M.,Fixed low-dose combination in first line treatment of hypertension, Journal of Hypertension.,2002, 20,511-519.

16.Moser M.,Black H. R.,The role of combination therapy in the treatment of hypertension; American Journal of Hypertension,1998, 11,73-78.

17.Mettimano M.,Pichetti F.,Fazzari L.,Combination therapy with beta-adrenergic blockade and amlodipine as second line treatment in essential hypertension; International Journal of Clinical Practice,2000,54,424-428.

18.Florey K.,Analytical Profiles of Drug Substances,The squibb Institute for Medical Research New Brunswick, 8,1-10.

19.The Merck Index, An Encyclopedia of chemicals, Drugs and Biologicals,14th Edition.,2006, Page no : 143, 403, 140.

20. Clarke's Analysis of Drugs and Poisons in pharmaceuticals, body fluids and postmortem material,Pharmaceutical Press,3rd edition,2004, 654-656, 834-835, 651-655.

21.Martindale The Complete Drug Reference,Pharmaceutical Press,36th edition,2007,1218-1220, 1250-1253, 20-22, 1424-1425.

22. Indian Pharmacopeia,Government Of India,Ministry Of Health And Family Welfare, The Indian Pharmacopeial Commission,2010,Vol 1- 2,770, 1758,1859.

23.United States Pharmacopoeia,USP31-NF26,United States Pharmacopoeial Convention,Asian Edition,2008,Vol 1-2,1268,1400.

24.British Pharmacopoeia,British Pharmacopoeia Comission Office,The Department of Health,The Stationery Office on behalf of MHRA,2010,Vol 1,138,1454,1611.

25.Skoog, (2007) Principles of Instrumental Analysis, 6th edition, Thomson Brooks/Cole, 169-173.

26.Wiberg, K. (2004),Multivariate spectroscopic methods for the analysis of solutions,(http://www.anchem.su.se/downloads/diss_pdf/k_wiberg_ths 2004.pdf). (Accessed on 15/05/2013).

27. Varmuza, K. (2002),Chemometrics : Introduction to multivariate data analysis in chemistry, (http://www.lcm.tuwien.ac.at)(Accessed on 15/05/2013)

28. Esteban M.,Arino C.;Diaz J.M. -Cruz,Chemometrics for the analysis of voltammetric data, Trends Anaytical Chemistry. 25 (2006) 86-92.

29.Meyers R.A. (2000),Encyclopedia of Analytical chemistry,John Wiley and Sons .USA,V11, 9671.

30.Juan A.D.,Tauler R.,Chemometrics applied to unravel multicomponent processes and mixtures revisiting latest trends in multivariate resolution, Analytical. Chim. Acta 500(2003), 195–210.

31.Jimeneza R.,Capotea F.P.,Olmob J.G.,Castroa M.D.,Use of chemometrics and mid infrared spectroscopy for the selection of extraction alternatives to reference analytical methods for total fat isolation, Anal. Chim. Acta 525 (2004), 159–169.

32.Windig W.,Smith W.F.,Nichol W.F.,Fast interpretation of complex LC/MS data using chemometrics, Anal. Chim. Acta 446 (2001), 467–476.

33.Multivariate Analysis techniques explained, (http:/www.icmrsearch.co.uk/research_questions answered/multivariate_analysis.as). (Accessed on 15/05/2013).

34.Wehrens R,Gelde R.D.,Kemperman G.J.,Zwanenburg B., BuydenL.M.C. ,Molecular challenges in modern chemometrics, Anal. Chim. Acta 400 (1999) ,413–424.

35.Townshend A.,Encyclopedia of Analytical Science.,Academic press. London, Vol-1,1995,632-635.

36.Danzer K.,Otto M., Currie L.A. Guidelines for calibration in analytical chemistry.Part 2: Multicomponent calibration (IUPAC Technical Report),Pure and Applied Chemistry, 76 (2004), 1215– 1225.

37.Ghasemi J.,Nikrahi A.,Niazi A.,Extraction-spectrophotometric determination of trace amounts of barium and strontium by 18-crown-6 and rose Bengal using partial least squares, Turk. J. Chem. 29 (2005), 669 - 678.

38.Ghasemi J.,Saaidpour S.,Ensafi A.A.,Simultaneous kinetic spectrophotometric determination of periodate and iodate based on their reaction with pyrogallol red in acidic media by chemometrics methods, Anal. Chim. Acta, 508 (2004), 119–126.

39.Aktas A.H.,Yasarb S., Potentiometric titration of some hydroxylated benzoic acids and cinnamic acids by artificial neural network calibration, Acta Chim. Slov. 51 (2004) ,273−282.

40.Ferraro M.C.F.,Castellano P.M.,Kaufmana T.S.,Chemometric determination of Amiloride hydrochloride, Atenolol , Hydrochlorothiazide and Timolol maleate in synthetic mixtures and pharmaceutical formulations, Journal of Pharmaceutical and Biomedical Analysis 34 (2004),305-314.

41. Chemometrics, (http://web.chemistry.gatech.edu/class/6282/janata/Multivariate_methods Nutshell.pdf). (Accessed on 15/05/2013).

42.Dinc E.,Ustundag O.,Chemometric resolution of a mixture containing hydrochlorothiazide and amiloride by absorption and derivative spectrophotometry, Journal of Pharmaceutical and Biomedical Analysis 29 (2002), 371–379.

43. Tan H.,Brown S.D., Multivariate calibration of spectral data using dual-domain regression analysis, Anal. Chim. Acta 490 (2003), 291–301.

44.Algorithms Partial Least Squares, (http:www.thermo.com/com/cda/resources/resourcesdetail/1,2166,13429,00.html). (Accessed on 15/05/2013)

45.Hussain A.S.,The Subcommittee on Process Analytical Technologies (PAT):Overview and Objectives,FDA's Advisory Committee for Pharmaceutical Science,Panel Meeting in Gaithersburg, MD, February 25, 2002.

46.Chemometric Working Group Recommendations,The Subcommittee on Process Analytical Technologies (PAT),FDA's Advisory Committee for Pharmaceutical Science, Panel Meeting in Gaithersburg, MD, February 25, 2002.

47.Anandkumari P. H.;Girdhar R,A spectrophotometric method for the determination of amlodipine besylate in pure form and in tablets; Indian drug,2002,39(4),204-208.

48.Ayşegül G.,Cem Y,Colorimetric Determination of Amlodipine Besylate in Tablets,Journal of Science and Engineering,2006,9(2),52-55.

49.Basavaiah K.,Titrimetric and Modified Spectrophotometric Methods for the determination of Amlodipine Besylate Using Bromate-Bromide Mixture and Two Dyes,Science Asia,2006,32 ,271-278.

50.Ali A.,Visible spectro photometric determination of Amlodipine in pharmaceutical Formulation and bulk drug by using bromophenol blue,International Journal of Pharmacy and Pharmaceutical Sciences,2010,2(2),13-14.

51.Rahman N.,Azmi S. N. H.,Spectrophotometric Determination of Amlodipine Besylate by Charge-Transfer Complex Formation with p-Chloranilic Acid,Analytical science,2002,16,1353.

52.Rahman N.,Hoda M. N.,Validated spectrophotometric methods for the determination of amlodipine besylate in drug formulations using 2,3-dichloro 5,6-dicyano 1,4-benzoquinone and ascorbic acid,Journal of Pharmaceutical and Biomedical Analysis,2003,31,381-392.

53.Gohil K.;,Trivedi P.,Molvi K. I.,Sepectrophotometric Analysis Of Amlodipine Besylate In Bulk And In Tablet Dosage Forms,Indian Journal of Pharmaceutical Science, 2005,67(3),376-378.

54.Sridhar K.;,Sastry C. S. P.,Reddy M. N.;Sankar D. G.,Srinivas K. R.,Spectrophotometric Determination of Amlodipine Besylate in Pure Forms and Tablets,Analytical Letters,1997,30(1),121 – 133.

55.Reddy M.N.,Rani G. T.,Prasad Rao K. V. S.,Sankar D. G,Sreedhar K.,Extractive Spectrophotometric Determination Of Amlodipine Using Eriochrome Black-T and Indigo Carmine, Indian Journal of Pharmaceutical Science,1997,59 (4),188-189.

56.Singhvi I.;Chaturvedi S. C.,Visible Spectrophotometric Methods For Estimation Of Amlodipine Besylate Form Tablets,Indian Journal of Pharmaceutical Science,1998,60 (5),309-310.

57.Mishra P.;Shah K. ;Gupta A.,Spectrophotometric Methods For Simultaneous Estimation Of Nebivolol Hydrochloride And Amlodipine Besylate In Tablets; International Journal of Pharmacy and Pharmaceutical Sciences,2009,1(2),86-89.

58.Kasture A.V.;Ramteke M.,Simultaneous UV-spectrophotometric method for the estimation of atenolol and amlodipine besylate in combined dosage form, Indian Journal of Pharmaceutical Science, 2006,68 (3), 394-396.

59.Chandrashekar T., Simultaneous determination of amlodipine Besylate and atorvastatin calcium in tablet Dosage forms by spectrophotometric methods,International Journal of Biopharmaceutics,2010,1(2),62-66.

60.Ramesh D.,Ramakrishna S.,New Spectrophotometric methods for simultaneous determination of Amlodipine besylate and Atorvastatin calcium in tablet dosage forms; International Journal of Pharmacy and Pharmaceutical Sciences, Indian Journal of Pharmaceutical Science,2007, 2(4), 215-219.

61.Sahu R.,Patel V. B.,Simultaneous spectrophotometric determination of amlodipine besylate and atorvastatin calcium in binary mixture,Indian Journal of Pharmaceutical Science, 2007, 69(1),110-111.

62.Khan M.,Jain D.,Simultaneous spectrophotometric determination of atorvastatin calcium and amlodipine besylate in tablets, Simultaneous spectrophotometric determination of atorvastatin calcium and amlodipine besylate in tablets;; 2006; 68 (4); 546-548.

63.Kumar P.,Method development and its validation for simultaneous estimation of Atorvastatin and Amlodipine in combination in tablet dosage form by UV spectroscopy, using multi-component mode of analysis; Journal of Pharmacy Research,Indian Journal of Pharmaceutical Science, 2008,1(2),182-188.

64.Rao L.,Rajeswari K. R.,Sankar G. G,Spectrophotometric Method for Simultaneous Estimation of Atorvastatin and Amlodipine in Tablet Dosage Form,RJPBCS,2010,1(2), 66.

65.Mishra P.,Gupta A.,Shah K.,Simultaneous estimation of atorvastatin calcium and amlodipine besylate from tablets, Indian Journal of Pharmaceutical Science,2007, 69 (6),831-833.

66.Singhvi I.,Chaturvedi S. C.,Spectrophotometric Method For Estimation Of Amlodipine Besylate And Benidipine Hydrochloride From Tablets, Indian Journal of Pharmaceutical Science 1999,61(3),190-191.

67.Prasad C. V. N.,Saha R. N.,Parimoo P.,Simultaneous Determination of Amlodipine-Enalapril Maleate and Amlodipine-Lisinopril in Combined Tablet Preparations by Derivative Spectrophotometry; Pharmacy and Pharmacology Communications,1999,5,383–388.

.

68.Vichare V.,Tambe V.,Kashikar V.,Dhole S. N.,Spectrophotometric simultaneous determination of Amlodipine besylate and Hydrochlorothiazide in combined tablet dosage form by simultaneous Equation, absorption ratio and first order derivative spectroscopy methods; Int J Chem Res, 2011,2(1), 710.

69.Patil P.R.,,Rakesh S. U.,Dhabale P. N.,Burade K.B.,Simultaneous UV pectrophotometric Method for Estimation of Losartan Potssium and Amlodipine Besylate in Tablet Dosage Form,Asian J. Research Chem.,2009,2(1), 183-188.

70.Wankhede S. B.,Raka K. C.,Wadkar S. B.,Chitlange S. S.,Spectrophotometric and HPLC Methods for Simultaneous Estimation of Amlodipine Besilate, Losartan Potassium and Hydrochlorothiazide in Tablets;Indian J Pharm Sci, 2010,72(1), 136–140.

71.Nagavalli D.,Vaidhyalingam V.,Santha A.,Sankar A. S. K.,Divya O.,Simultaneous spectrophotometric determination of losartan potassium,amlodipine besilate and

hydrochlorothiazide in pharmaceuticals by chemometric methods,Acta pharmaceutica,2010,60(2), 141-152.

72.Giri C. H.,Simultaneous estimation of Nebivolol hydrochloride and amlodipine Besylate in combined tablet dosage form by Q-analysis method; IJPRD,2010,5(2),1-8.

73.Chandnani V. C.,Simultaneous uv-spectrophotometric determination of Amlodipine besylate and Nebivolol hydrochloride In tablet dosage form,International Journal of ChemTech Research,2010,2(1),69-73.

74.Wankhede S. B.,Wadkar S. B.;,Raka K. C.; Chitlange S. S., Simultaneous Estimation of Amlodipine Besilate and Olmesartan Medoxomil in Pharmaceutical Dosage Form, Indian J Pharm Sci.,2009, 71(5),563–567.

75.Patil P. S,More H. N.,Kumar R.,Singh H.,Spectrophotometric method for simultaneous determination of Olmesartan Medoxomil and Amlodipine Besylate from tablet dosage form,IJPRD,2011, 3(2), 16-18

76.Patil P.R.,Rakesh S. U.,Dhabale P. N.;,Burade K. B.,Simultaneous Estimation of Ramipril and Amlodipine by UV Spectrophotometric Method,Research J. Pharm. and Tech.,2009, 2 (2), 304-308.

77.Çelebier M.;Altınöz S; Dinç E.; Fractional Wavelet Transform and Chemometric Calibrations for the Simultaneous Determination of Amlodipine and Valsartan in Their Complex Mixture,New Trends in Nanotechnology and Fractional Calculus Applications,2010,4,333-340.

78.Wankhede S. B.;Wadkar S. B.;Raka K. C. Chitlange S. S.,Simultaneous Estimation of Amlodipine Besilate and Valsartan in Pharmaceutical Dosage Form; Indian J Pharm Sci.,2010,72(5),563–567.

79.Gupta K.R.,Mahapatra A.D.,Wadodkar A.R.;,Wadodkar S.G.,Simultaneous UV Spectrophotometric Determination of Valsartan and Amlodipine in Tablet, International Journal of ChemTech Research,2010,2(1),551-556.

80. Kamat K.,Chaturvedi S. C.,Stability Indicating Assay Method For Amlodipine Tablets, Indian Journal of Pharmaceutical Science,2005, 67(2),236-239.

81.Tatar S.,Atmaca S.,Determination of amlodipine in human plasma by highperformance liquid chromatography with fluorescence detection; Journal of Chromatography B: Biomedical Sciences and Applications,2001,758(2),305-310.

82.Pollen K.F.,Mosher S. J.,Pollak P. T.,Liquid chromatography assay for amlodipine: Chemical stability and pharmacokinetics in rabbits, Journal of Pharmaceutical and Biomedical Analysis,1991, 9(7),565-571.

83.Suchanova B.,Kostiainenc R.,Ketola R. A.,Characterization of the in vitro metabolic profile of amlodipine in rat using liquid chromatography–mass Spectrometry, European Journal of Pharmaceutical Sciences,2008,3(3),91–99.

84.Yuanyuan M.,Feng Q.,Xiaohong S.,Xiumei L.L.,Determination and pharmacokinetic study of amlodipine in human plasma by ultra performance liquid chromatography–electrospray ionization mass spectrometry; Journal of Pharmaceutical and Biomedical Analysis,2007,43,1540–1545.

85. Klinkenberg R.,Streel B.,Ceccato A.,Development and validation of a liquid chromatographic method for the determination of amlodipine residues on manufacturing equipment surfaces,Journal of Pharmaceutical and Biomedical Analysis,2003, 32,345-352.

86. Bahrami G. H.,Mirzaeei S. H.,Simple and rapid HPLC method for determination of amlodipine in human serum with fluorescence detection and its use in pharmacokinetic studies; Journal of Pharmaceutical and Biomedical Analysis,2004,36,163–168.

87.Pal T. K.,Simultaneous determination of metoprolol succinate and amlodipine besylate in human plasma by liquid chromatography–tandem mass spectrometry method and its application in bioequivalence study; Journal of Chromatography B,2008,873(1),77-85.

88.Barman R. K.,Simultaneous high-performance liquid chromatographic Determination of atenolol and amlodipine In pharmaceutical-dosage form; Pak.J. Pharm. Sci.,2007,20(4),274-279.

89. Shah D.A,Bhatt K.K.,Mehta R.S.,Baldania S.L.,Gandhi T.R,Stability indicating RP-HPLC estimation of atorvastatin calcium and amlodipine besylate in pharmaceutical formulations,Indian Journal Of Pharmaceutical Science,2008,70(6),754-760.

90. Shah D.A.,Bhatt K.K.,Shankar M.B.,Mehta R.S.,Gandhi T.R.,Baldania S.L,RPHPLC determination of atorvastatin calcium and amlodipine besylate combination in tablets,Indian Journal of opiate Sciences,2006,68(6),796-799.

91.Mohammadi A.,Rezanour N.,Ansari Dogaheh M.,Ghorbani B. F.,Hashem M.,Walker R.B.,A stability-indicating high performance liquid chromatographic assay for the simultaneous determination of atorvastatin and amlodipine in commercial tablets,Journal of Chromatography B,2007,846(1-2), 215-221.

92. Kasawar R.B.,Farooqui M. N.,Simultaneous Determination of Amlodipine Besylate and Benazepril Hydrochloride in Pharmaceutical Dosage Form by LC,Analytical sciences,2005,29(12), 1495-1498.

93.Kasawar G. B.,Farooqui M.N.,Simultaneous determination of amlodipine besylate and benazepril hydrochloride in pharmaceutical dosage form by LC,Anal Sci., 2009, 25(12),1495-8.

94.Naidu K. R.,Kale U. N.,Shingare M. S.,Stability indicating RP-HPLC method for simultaneous determination of amlodipine and benzapril hydrochloride from their combination drug product. J Pharm Biomed Anal.,2005, 39,147–55.

95.Joshi S.,Sharma A.,Rawat M. S.,Bal C. S.,Reversed phase liquid chromatographic conditions for simultaneous determination of antihypertensive Formulations,Asian Journal of Pharmaceutics, 2009, 3(4),274-277.

96.Ulavapally K.R.,Sriramulu J.,Reddy V.,Bobbarala V.,Single RP-HPLC method for the determination of Hydrochlorothiazide, Amlodipine besylate and Valsartan in pharmaceutical product, Journal of Pharmacy Research,2011,4(3),122.

97.Wankhede S. B.,Raka K. C.,Wadkar S. B.,Chitlange S. S.,Spectrophotometric and HPLC Methods for Simultaneous Estimation of Amlodipine Besilate, Losartan Potassium and Hydrochlorothiazide in Tablets;Indian J Pharm Sci,2010, 72(1),136–140.

98.Raman N.V.S.,Development and validation of RP-HPLC method for the determination of genotoxic alkyl benzenesulfonates in amlodipine besylate.,Journal of Pharmaceutical and Biomedical Analysis,2008,48,227–230.

99.Chitlange S.S.;Imran M.;Sakarkar D.M; RP-HPLC method for simultaneous estimation of amlodipine and metoprolol in tablet formulation,Asian J Pharm.,2008,2,232-4.

100.Pal T. K.,Simultaneous determination of metoprolol succinate and amlodipine besylate in human plasma by liquid chromatography–tandem mass spectrometry method and its application in bioequivalence study; Journal of Chromatography B, 2008,873(1), 77-85.

101.Sudhakar M.,Rao V. J.,Devika G. S.,Petchi R. R., A Validated RP-HPLC Method for Simultaneous Estimation of Nebivolol Hydrochloride and SAmlodipine Besylate in Tablet Dosage Forms; International Journal of Chemical and Pharmaceutical Sciences, 2010,1 (2),28-33.

102 Jain M.,Sukriti T.,Mishra V. K.,Shukla S.,Sheikh S.,Simultaneous estimation of amlodipine besylate and nebivolol hydrochloride in combined dosage form by RP-HPLC, 2010,IJPLS,1(7),428-432.

103.López J. A.,Martínez V.,Alonso R. M.,Limenez R. M.,Simultaneous determination of five 1,4-dihydropyridines in pharmaceutical formulations by high-performance liquid chromatography–amperometric detection; Journal of Chromatography A,2000, 870(1-2),105-114.

104.Patil K.R.,Rane V.P.,Sangshetti J.N.,Yeole R.D.,Shinde D.B,Stability Indicating LC Method For the Simultaneous Determination of Amlodipine and Olmesartan in Dosage Form,Journal of Chromatographic Science, 2010,48,601-606.

105.Wankhede S.B.,Wadkar S.B.,Raka K.C.,Chitlange S.S, Simultaneous Estimation of Amlodipine Besilate and Olmesartan Medoxomil in Pharmaceutical Dosage Form,Indian J Pharm Sci.,2009,71(5),563–567.

106.Thomas A. B.,A Validated Stability Indicating RP-HPLC Method for the Determination of Amlodipine Besylate and Telmisartan in Pharmaceutical Formulation; Ananbioanal,2010, 24, 34-36.

107.Abdallah ,Determination of Amlodipine and Valsartan in Binary Mixture Using Derivative- Ratio Spectrophotometric, Chemometric and High-Performance Liquid Chromatographic-UV Methods,Int. J. Ind. Chem.,24(3),32-35.

108.Chaudhari B.,Stability indicating RP-HPLC method for Simultaneous determination of Valsartan and Amlodipine from their combination drug product; Int. J. ChemTech Res.,2009,1(4), 1257-1268.

109.Al-Shaalan N. H., Simultaneous determination of Amlodipine Besilate and Valsartan in pharmaceutical formulation using high performance liquid chromatography, J. Chem. Pharm. Res.,2010,2(6),129-134.

110 Chitlange S. S.,Bagri K.,Sakarkar D.M.,Stability Indicating RP- HPLC Method for Simultaneous Estimation of Valsartan and Amlodipine in Capsule Formulation,Asian J. Research Chem.,2008,1(1),15-19.

111.Kristoffersen L.,Simultaneous determination of 6 beta-blockers, 3 calcium-channel antagonists, 4 angiotensin-II antagonists and 1 antiarrhytmic drug in post-mortem whole blood by automated solid phase extraction and liquid chromatography mass spectrometry: Method development and robustness testing by experimental design,Journal of Chromatography B,2007,850(1),147-160.

112.Naz S.,Sultana N.,Saeed M.,,Shafi N.,Simultaneous determination of prazosin hydrochloride and three calcium channel blockers; in raw material,pharmaceutical formulation and human serum by RP-HPLC,IJPRD,2010,2(9),31-35.

113. López J. A.,Martínez V.,Alonso R. M.,Jiménez R. M.,High-performance liquid chromatography with amperometric detection applied to the screening of 1,4-dihydropyridines in human plasma; Journal of Chromatography A,200,870(1-2), 105-114.

114. Meyyanathan S.N.,Suresh B,HPTLC method for the simultaneous determination of amlodipine and benazepril in their formulations., J ChromatogrySci,2005,43(2):73-5.

115.Sharma. A.,Simultaneous estimation of Nebivolol Hydrochloride and sAmlodipine Besylate by high performance thin layer Chromatography,International Journal of Pharma and Bio Sciences, 2010, 1(4), 339-348.

116.Patel S.R.,Patel S. M.,Patel J. I.,Patel P. U.,Development and Validation of High-Performance Thin-Layer Chromatography Method for Determination of Nebivolol and Amlodipine in Combined Dosage Forms, Journal of Pharmacy Research; 2010, 3(9),2273-2275.

117.Dhandapani B.,HPTLC Method Development and Validation for the Simultaneous Estimation of Amlodipine Besylate and Nebivolol Hydrochloride in tablet dosage form, Journal of Pharmacy Research,2010,29,30.

118. Dangi ,Stability Indicating HPTLC Method for Estimation of Nebivolol Hydrochloride and Amlodipine Besylate in Combination, Eurasian J. Anal.Chem. , 2010. 5(2): 161-169.

119. Kamble A. Y.,Validated HPLC and HPTLC Method for Simultaneous Quantitation of Amlodipine Besylate and Olmesartan Medoxomil in Bulk Drugand Formulation; Analytical Letters; 2010, 43(2); 251 – 258.

120. Ramadan N. R.,Mohame H. M..,Moustafa H. A.,Rapid and Highly Sensitive HPLC and TLC Methods for Quantitation of Amlodipine Besilate and Valsartan in Bulk Powder and in Pharmaceutical Dosage Forms and in Human Plasma,Analytical Letters,2010,43(4),570 – 581.

121. Beresford P.,Analysis of amlodipine in human plasma by gas chromatography, Journal of Chromatography, Biomedical Applications,1987,420,178-183.

122.Kamila M. M.,A validated UV spectrophotometric method for estimation of nebivolol hydrochloride in bulk and pharmaceutical formulation, Pharmazie, 2007,62(7):486-7.

123.Shirkhedkar A. A., First Order Derivative Spectrophotometric Determination of Nebivolol in Bulk and Tablets, The Pharma Review,2008,5(3),45-49.

124.ThomasD. G.,A validated UV- Spectrophotometric determination of An antihypertensive drug – nebivolol from tablet formulations, International Journal f Pharmaceutical Sciences Review and Research, 2010, 3(2),139-141.

125.Malipatil S. M.,Second and Third order derivative Spectrophotometric estimation of Nebivolol hydrochloride in bulk and pharmaceutical dosage forms,int j pharm pharm sci, 2020, 3(1), 1315.

126.Sharma S.,Spectrophotometric Analysis of Nebivolol Hydrochloride in Tablet Dosage form using 5.0M Niacinamide solution as hydrotropic solubilizing agent, Journal of Pharmacy Research, 2010, 3(5),1074-1076.

127.Tarte P. S.,Wate S. P.,Paunikar G. V.,Khedikar P. B.,Absorption Correction Method for Estimation of Nebivolol and Hydrochlorothiazide in Combined Tablet Dosage Form, Asian J. Research Chem, 2008, 1(2),74-76.

128.Shah D. A.,Bhatt K. K.,Mehta R. S., Baldania S. L.,Determination of nebivolol hydrochloride and hydrochlorothiazide in tablets by first-order derivative spectrophotometry and liquid chromatography, J AOAC Int., 2008 91(5):1075-82.

129.Shah K. V.,Thumber B. L.,Desai T. R.,Simultaneous Estimation of Nebivolol Hydrochloride and Hydrochlorothiazide in Bulk and Tablet Dosage Form by QAnalysis Method, International Journal of Pharmaceutical Science & Research (2009) 1(2) 80-85.

130.Tarte P. S.,Wate S. P.,Paunikar G. V.,Khedikar P. B. Simultaneous Estimation of Nebivolol and Hydrochlorothiazide in Combined Tablet Dosage Form by Multicomponent Mode of Analysis, Asian J. Research Chem.,2008, 1(2),77-79.

131.Shah K. V.,Tirgar P. R.,Sheth D. B.,Desai T. R., simultaneous estimation of nebivolol Hydrochloride and hydrochlorothiazide in bulk and in a tablet dosage form by multicomponent and simultaneous estimation method, Pharma Science Monitor: An International Journal of Pharmaceutical Sciences, 2011, 2(1),15-18.

132.Modiya J. S.,Pandya C. B.,Channabasavaraj K. P., Simultaneous Estimation of Nebivolol Hydrochloride and Valsartan in Bulk and Capsule Dosage Form by

Simultaneous Equation Method, International Journal of ChemTech Research,july-sep 2010, 2(3), 1387-1390.

133.Chaudhary A. B ,Estimation of Valsartan and Nebivolol in pharmaceutical dosage forms by absorption ratio method, Int. J. Res. Pharm. Sci. 2010, 1(2),108-112.

134.Rao A. L..,Rajeswari K. R.,Sankar G.G,Spectrophotometric Methods for the Determination of Selected Drugs in Pharmaceutical Formulations, J. Chem.Pharm. Res., 2010, 2(1): 280-282.

135.Yilmaz B.,Reverse Phase HPLC method for Determination of Nebivolol in pharmaceutical preparations, International Journal of Pharmaceutical Sciences Review and Research, 2010; 1 (2), 14-18.

136.Sastry B.S.,Srinivasalu D.,Ramana H., Reverse phase HPLC method for the analysis of nebivolol in pharmaceutical dosage forms, JPRHC, 2009, 1(1), 25-33.

137.Sahoo M. K.,Giri R. K.,Barik C. S.,Kanungo S. K.,Ravi Kumar B. V. V.,RP-HPLC Method for the Estimation of Nebivolol in Tablet Dosage Form, E.Journal of Chemistry, 2009, 6(3), 915-919.

138. Patel L. J.,Suhagia B. N.,Shah P. B.,RP-HPLC and HPTLC methods for the estimation of nebivolol hydrochloride in tablet dosage form. Indian J Pharm Sci 2007,69,594-6.

139.Shah D. A.,Bhatt K. K.,Mehta R. S.,Baldania S. L.,Gandhi T. R.,Stability indicating RP-HPLC estimation of nebivolol hydrochloride in pharmaceutical formulations. Indian J Pharm Sci,2008,70, 591-5.

140.Ramakrishna N. V.,Vishwottam K. N.,Koteshwara M.,Manoj S.,Santosh M.,Varma D. P., Rapid quantification of nebivolol in human plasma by liquid chromatography coupled with electrospray ionization tandem mass spectrometry, J Pharm Biomed Anal. 2005,39(5),1006-13.

141 Kachhadia P. K.,Doshi A. S.,Joshi H. S.,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.

142.Ramakrishna N. V. S.,Vishwottam K. N.,Koteshwara M.,Manoj S.,Santosh M., Varma D.P.,Rapid quantification of nebivolol in human plasma by liquid chromatography coupled with electrospray ionization tandem mass spectrometry, Journal of Pharmaceutical and Biomedical Analysis, 2005,39 (5),1006-1013.

143. Sudhakar M.,Venkateshwara rao J.,Devika G. S.,Petchi R. R.,A Validated RP-HPLC Method for Simultaneous Estimation of Nebivolol Hydrochloride and S-Amlodipine Besylate in Tablet Dosage Forms, International Journal of Chemical and Pharmaceutical Sciences 2010,1 (2), 28-33.

144.Shah D.A.,Bhatt K.K.,Mehta R.S,,Baldania S.L, Determination of nebivolol hydrochloride and hydrochlorothiazide in tablets by first-order derivative spectrophotometry and liquid chromatography, J AOAC Int. 2008,91(5),1075-82.

145.Gupta Y.,Shrivastava A,,Duggal D.,Patel A.,Agrawal S.,A new RP-HPLC method for simultaneous estimation of nebivolol hydrochloride and hydrochlorthiazide in dosage forms, Journal of Pharmaceutical Analysis,2009,1(3),264-269.

146.Dhandapani B.;Thirumoorthy N; D.Jose P., Development and Validation for the Simultaneous Quantification of Nebivolol Hydrochloride and Hydrochlorothiazide by UV Spectroscopy, RP-HPLC and HPTLC in Tablets,Eu. J. Chem., 2010, 7(2), 341- 348.

147. Tarte P. S.,Wate S. P.,Bondre. A. V.,Paunikar G. V.,Simultaneous Determination of Nebivolol and Hydrochlorothiazide in tablet dosage form by RP-HPLC, International Journal of PharmTech Research, 2009, 1(3), 720-724.

148. Meyyanathan S. N.,Rajan S.,Muralidharan S.,Birajdar A. S.,Suresh B.,A validated RP-HPLC method for simultaneous estimation of nebivolol and hydrochlorothiazide in tablets. Indian J Pharm Sci 2008;70:687-9.

149. Gowda N.,Panghal S.,Vipul K.,Rajshree M.,Validation and application of high-performance liquid chromatographic and spectrophotometric methods for simultaneous estimation of nebivolol and hydrochlorothiazide: a novel approach to multivariate calibrations by R-Software Environment, J AOAC Int.2009,92(5),1356-65.

150.Kokil S. U.,Bhatia M. S.,Simultaneous Estimation of Nebivolol Hydrochloride and Valsartan using RP HPLC, Indian J Pharm Sci.,2009,71(2),111-4.

151.Selvan P. S.,Gowda K. V.,Mandal U.,Solomon W. D.,Pal T. K.,Simultaneous determination of fixed dose combination of nebivolol and valsartan in human plasma by liquid chromatographic-tandem mass spectrometry and its application to pharmacokinetic study, J Chromatogr B Analyt Technol Biomed Life Sci.,2007,858(1-2),143-50.

152.Doshi A. S.,Bhagwan S. S.,Mehta T. N.,Gupta V. K.,Subaaiah G.,Determination of nebivolol and valsartan in a fixed-dose combination by liquid chromatography,J AOAC Int. 2009,91(2),292-8.

153. Zhang J.,Shao B.,Yin J.,Wu Y.,Duan H.,Simultaneous detection of residues of β-adrenergic receptor blockers and sedatives in animal tissues by high-performance liquid chromatography/tandem mass spectrometry, Journal of Chromatography B, 2009, 877, 20-21,1915-1922.

154. Patel L. J.,Suhagia B. N.,Shah P. B,RP-HPLC and HPTLC methods for the estimation of nebivolol hydrochloride in tablet dosage form. Indian J Pharm Sci 2007,69, 594-6.

155.Shirkhedkar A. A.,Bugdane P. M.,Surana S. J.,Stability-Indicating TLCDensitometric Determination of Nebivolol Hydrochloride in Bulk and Pharmaceutical Dosage Form, Journal of Chromatographic Science, 2010,48(2), 109-113.

156.Reddy T. S.,Devi P. S.,Validation of a high- performance thin-layer chromatographic method, with densitometric detection, for quantitative analysis of nebivolol hydrochloride in tablet formulations, JPC - Journal of Planar Chromatography - Modern TLC, 2007, 20(2),149-152.

157. Dhandapani B.; Anjaneyulu N.;Venkateshwarlu Y.; Rasheed S. H., HPTLC Method Development and Validation for the Simultaneous Estimation of Amlodipine Besylate and Nebivolol Hydrochloride in tablet dosage form,Journal of Pharmacy Research, 2010, 3 (2),35-38.

158. Patel S. R.,Patel S. M.,Patel J. I.,Patel P. U.,Development and Validation of High-Performance Thin-Layer Chromatography Method for Determination of Nebivolol and Amlodipine in Combined Dosage Forms, Journal of Pharmacy Research,2010,3(9),2273-2275.

159.Dangi M.,Chaudhari D.,Sinker M.,Racha V.,Damle M. C.,Stability Indicating HPTLC Method for Estimation of Nebivolol Hydrochloride and Amlodipine Besylate in Combination, Eurasian J. Anal. Chem.,2010, 5(2),161-169.

160. Sharma A.,Patel B.,Patel R.,Simultaneous estimation of nebivolol hydrochloride and (s)amlodipine besylate by high performance thin layer Chromatography, International Journal of Pharma and Bio Sciences, 2010, 1(4),339-347.

161.Dhandapani B.,Thirumoorthy N., Development and Validation for the Simultaneous Quantification of Nebivolol Hydrochloride and Hydrochlorothiazide by UV Spectroscopy, RP-HPLC and HPTLC in Tablets,Eu. J. Chem.,2010,7(2), 341- 348.