Development and validation of chromatographic and spectroscopic methods for estimation of some Antihypertensive agents and their combined dosage forms.

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy

by

Avani Harshadkumar Sheth

Enrollment No 119997290050

under supervision of

Dr Nehal Shah

समुचित ज्ञान समन्वय

ESTD - 2007

GUJARAT TECHNOLOGICAL UNIVERSITY
AHMEDABAD

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DECLARATION

I declare that the thesis entitled **Development and validation of chromatographic and spectroscopic methods for estimation of some antihypertensive agents and their combined dosage forms** submitted by me for the degree of Doctor of Philosophy is the record of research work carried out by me during the period from May 2011 to May 2018 under the supervision of Dr Nehal Shah and this has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institution of higher learning.

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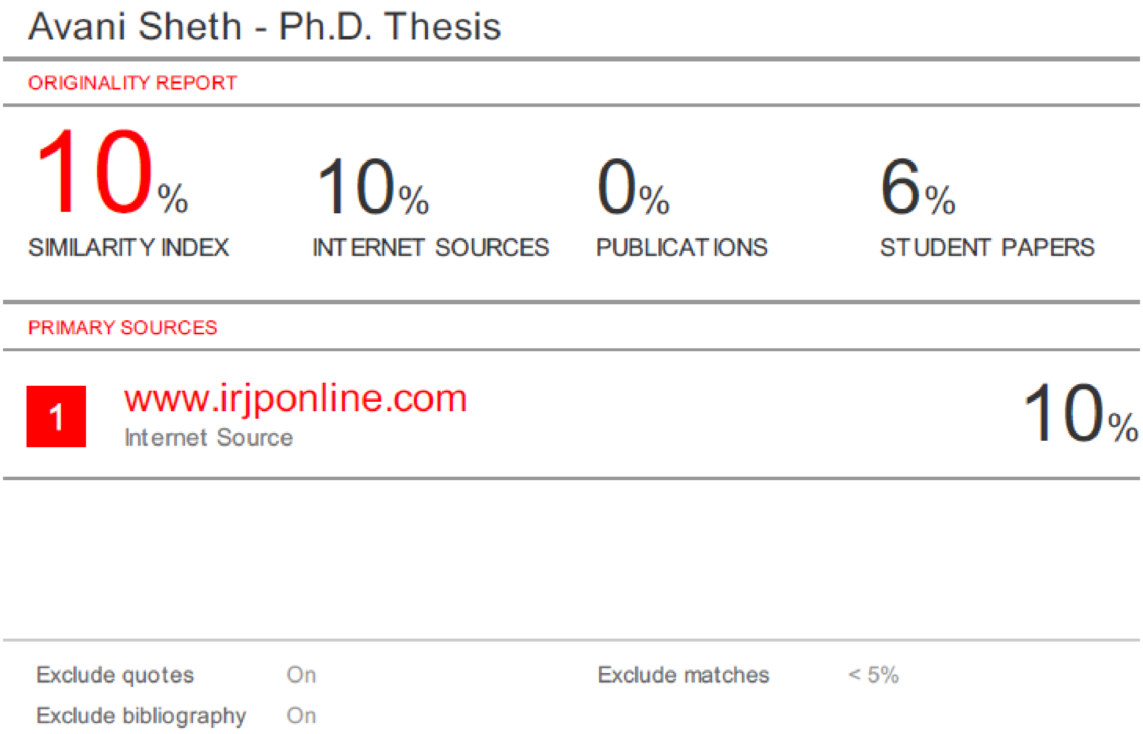

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ABSTRACT

There are numbers of newer antihypertensive drugs and their formulations are approved by FDA which is either new molecule or partial modification of existing molecule. These newer drugs and their combinations are not official in any pharmacopeias and are take more time to include in pharmacopeias. Because of continuous and longer use of these drugs individually newer side effects, toxicity, resistances are observed.

So present investigation was undertaken with a view to develop and validate new analytical methods (HPLC & UV/HPTLC) for simultaneous estimation of following combinations

1. Olmesartan medoxomil and Indapamide tablets
2. Olmesartan medoxomil and Chlorthalidone tablets
3. Nebivolol Hydrochloride and Chlorthalidone tablets
4. Rosuvastatin calcium and Hydrochlorthiazide tablets
5. Metoprolol succinate and Chlorthalidone tablets

Statistical comparison of developed HPLC methods & UV/HPTLC methods is done for proving equivalency of developed methods.

Acknowledgement

Completing a task is never one man effort, but it is often result of invaluable contribution of a number of individuals in direct or indirect manner. This suitably applies to my Dissertation. Without help, encouragement & blessings from several persons, I would never have been able to finish this work.

First of all, I would like to thank almighty, the God whose unseen guidance made work possible in a useful way.

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For Construction of any strong Building, we need strong foundation. That is why I would have an opportunity to owe my thanks to entire management of SAL Institute of Pharmacy for supporting me in my dissertation work in such a friendly and delightful environment.

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Avani Harshadkumar Sheth

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List of Abbreviations

IP	Indian Pharmacopoeia
BP	British Pharmacopoeia
USP	United states pharmacopoeia
Olme	Olmesartan medoxomil
ID	Internal diameter
Inda	Indapamide
HCTZ	Hydrochlorthiazide
ml	Milliliter
mg	Milligram
µg	Microgram
PDA	Photo Diode Array
µl	Microliter
IMP	Impurity
ng	Nanogram
RSD	Relative Standard Deviation
RP HPLC	Reverse Phase High Performance Liquid Chromatography
Rosu	Rosuvastatin calcium
HPTLC	High Performance Thin Liquid Chromatography
RRT	Relative Retention Time
ICH	International Conference of Harmonization
RT	Retention Time
nm	Nano Meter
AR	Analytical reagent
PVDF	Polyvinylidene diflouride
WHO	World Health organization
GMP	Good Manufacturing Practice
GLP	Good Laboratory Practice
FD	Forced Degradation
FDA	Food and Drug Administration
LOD	Limit of Detection
LOQ	Limit of Quantitation
pKa	Dissociation constant
SD	Standard Deviation
CV	Coefficient of Variation
HCl	Hydrochloric acid
NaOH	Sodium Hydroxide
NLT	Not Less Than
IS	Internal Standard
AU	Absorbance
H_2O	Water
Amt	Amount
Conc	Concentration
MeOH	Methanol
ACN	Acetonitrile
Ros	Rosuvastatin calcium
Nebi	Nebivolol HCl

Chlor	**Chlorthalidone**
Hrs	**Hours**
Meto	**Metoprolol succinate**
Soln	**Solution**
Vol	**Volume**
SST	**System suitability**
OPA	**Orthophosphoric acid**
TEA	**Triethylamine**
PDA	**Photodiode array**
NMT	**Not more than**
EP	**European pharmacopoeia**
JP	**Japanese pharmacopoeia**
WS	**Working standard**
UV	**Ultra violet**
BCS	**Biopharmaceutics classification system**
IJPS	**Indian journal of pharmaceutical sciences**
Org	**Organic phase**
Sys. Sui.	**System suitability**
wt	**weight**
Conc	**Concentration**
USFDA	**United states food and drug administration**
MHRA	**Medicine and healthcare products regulatory agency**
EU/EMEA	**European medicine evaluation agency**

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CHAPTER-1

Introduction

1.1 Introduction to Hypertension [1-3]

Hypertension is a disease of elevated systolic and /or diastolic blood pressure characterized by increase in blood pressure by 150 mm Hg & diastolic blood pressure by 100 mm Hg. Hypertension is one of the leading cause of mortality in the world. In hypertension blood pressure of arteries are constantly elevated. Hypertension leads to heart attack, stroke, peripheral vascular disease, loss of vision and kidney disease.

FIGURE 1.1: Causes of Hypertension

Increased blood pressure develops slowly over many years usually without any specific identifiable cause. However, some possible medical causes, such as particular medications, kidney disease, adrenal problems or thyroid problems, must first be excluded. High blood pressure that develops over time without a specific cause is considered benign or essential hypertension. Blood pressure also tends to increase as a person ages. A primary risk factor for prehypertension is being overweight. Other risk factors include a sedentary lifestyle, eating high sodium foods, smoking and excessive alcohol intake. Blood pressure levels appear to be familial, but there is no clear genetic pattern. Figure 1.1 describes causes of hypertension.

1.2 Introduction to drug analysis

a) Qualitative Analysis

The Qualitative analysis identifies the nature of substance, and if it is mixture, the nature of the components present.

b) Quantitative Analysis

The Quantitative analysis determines the elemental composition of the substance and the quantitative distribution of each component.

The pharmaceutical analysts play a major role in assuring the identity, safety, efficacy and quality of the drug product. Safety and efficacy studies require that drug substance and drug product meet two critical requirements are:

- Established identity and purity
- Established bioavailability and dissolution

In brief, the reasons for the development of newer methods of drug analysis are:

- The drug or drug combination may not be official in any pharmacopoeias.
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for the quantitation of the drug in biological fluids may not be available.
- Analytical methods for a drug in combination with other drugs may not be available. It may also involve cumbersome extraction and separation procedures and these may not be reliable.
- The existing analytical procedures may require expensive reagents and solvents.
- Analytical techniques that are generally used for drug analysis are biological and microbiological methods, radioactive methods, physical methods and miscellaneous techniques like conventional titrimetric, gravimetric and polarimetric methods.
- Analytical techniques that are generally used for drug analysis are biological and microbiological methods, radioactive methods, physical methods and miscellaneous techniques like conventional titrimetric, gravimetric and polarimetric methods.

1.3 Reverse phase High Performance Liquid Chromatographic technique [4-6]

Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are:

- Speed (analysis can be accomplished in 20 minutes or less)
- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phases)
- Reusable columns (expensive columns but can be used for many analysis)
- Ideal for the substances of low volatility
- Easy sample recovery, handling and maintenance
- Instrumentation tends itself to automation and quantitation (less time and less labor)
- Precise and reproducible
- Calculations are done by integrator itself
- Suitable for preparative liquid chromatography on a much larger scale

Modes of HPLC

- Mainly two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography.
- In Normal-phase chromatography the stationary bed is strongly polar in nature (e.g., silica gel), and the mobile phase is non polar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.
- Reverse-phase chromatography is the inverse of this. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained

Above mentioned types cover almost 90% of all chromatographic applications. Eluent polarity plays the highest role in all types of HPLC.

There are two elution mode types: isocratic and gradient. In the first type constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run.

Reversed phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is non polar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and non polar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octa decyl silane (ODS) or C_{18}, C_{8}, C_{4}, etc., (in the order of increasing polarity of the stationary phase).

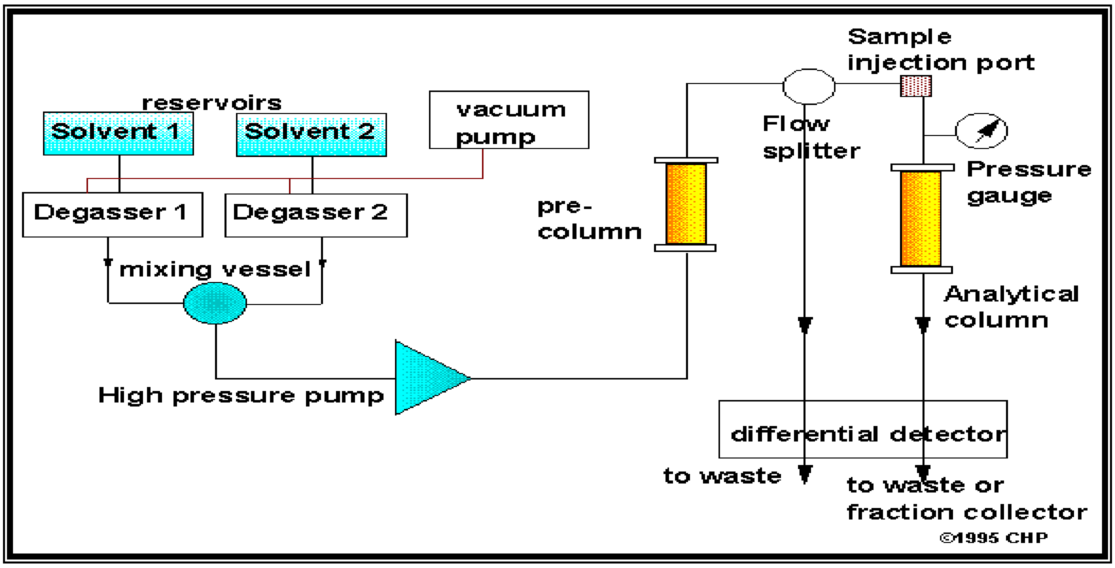


FIGURE 1.2: A schematic diagram of HPLC equipment

Various components of HPLC are

a) A solvent delivery system, including pump,
b) Sample injection system,
c) A chromatographic column,
d) A detector,
e) A strip chart recorder,
f) Data handling device and microprocessor control.

Methods for analyzing drugs in multi component dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble. Method development and design of separation method depends on selection of best mobile phase, detector, column length and diameter, buffer, pH of buffer, type of stationary phase, gradient programming etc.

In reverse phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, it retention decreases. When separating mixtures containing acid and/or bases by reversed phase HPLC, it is necessary to control the pH of mobile phase using appropriate buffer in order to achieve reproducible results.

When separating acids & bases a buffered mobile phase is recommended to maintain consistent retention and selectivity. A buffered mobile phase, resists changes in pH so that the analytes and silica will be consistently ionized, resulting in reproducible chromatography. For neutral sample, buffers are not required in the mobile phase. Acids or bases usually require the addition of a buffer to the mobile phase. For basic or cationic samples, “less acidic” reverse-phase columns are recommended and amine additives for the mobile phase may be beneficial. Optimum buffering capacity occurs at a pH equal to the p^{Ka} of the buffer. Beyond that, buffering capacity will be inadequate. Buffers play an additional role in the reproducibility of a separation. The buffer salts reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion-exchange interactions with unprotonated silanols (Figure 5). To be most effective, a buffer concentration range of 10 - 50 mM is recommended for most basic compounds.

TABLE 1.1: Commonly used buffers for reversed phase HPLC

Buffer	**P^{Ka} (25°C)**	**Maximum buffer range**	**UV cutoff (nm)**
Phosphate,p^{K}_{1} H_2PO_4	2.1	1.1-3.1	< 200
Phosphate,p^{K}_{2} HPO_4^{2-}	7.2	6.2-8.2	< 200
Phosphate,p^{K}_{3} PO_4^{3-}	12.3	11.3-13.3	< 200
Perchlorate $(HClO_4)^-$	9.0	7.0-11.0	< 200
Citrate, p^{K}_{1} $C_3H_5O(COOH)_2(COO)^{1-}$	3.1	2.1-4.1	230
Citrate, p^{K}_{2} $C_3H_5O(COOH)_1(COO)^{2-}$	4.7	3.7-5.7	230
Citrate, p^{K}_{3} $C_3H_5O(COO)^{3-}$	6.4	4.4-6.4	230
Carbonate, p^{K}_{1} HCO_3^{1-}	6.1	5.1-7.1	< 200
Carbonate, p^{K}_{2} CO_3^{2-}	10.3	9.3-11.3	< 200
Perchlorate $(HClO_4)^-$	9.0	7.0-11.0	< 200
Formate	3.8	2.8-4.8	210
Acetate	4.8	3.8-5.8	210
Ammonia	9.3	8.3-10.3	200
Borate	9.2	8.2-10.2	200

The pH range most often used for reversed-phase HPLC is 1 - 8 and can be divided into low pH (1 - 4) and intermediate pH (4 - 8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which peak tailing is minimized and method ruggedness is, maximized. For this reason, operating at low pH is recommended.

At a mobile phase pH greater than 7, dissolution of silica can severely shorten the lifetime of columns packed with silica-based stationary phases.

The pKa value [acid dissociation (ionization) constant] for a compound is the pH at which equal concentrations of the acidic and basic forms of the molecule are present in aqueous solutions. Analytes may sometimes appear as broad or tailing peaks when t-he mobile phase pH is at, or near, their pKa values. A more rugged mobile phase pH will be at least 1 pH unit different from the analyte pKa. This shifts the equilibrium so that 99% of the sample will be in one form. The result is consistent chromatography.

Dramatic changes in the retention and selectivity (peak spacing) of basic and acidic compounds can occur when the pH of the mobile phase is changed. This is often a result of different interactions between the column and the analytes when the ionization of these compounds changes. It is important to evaluate these changes when a method is developed in order to select the mobile phase pH that provides the most reproducible results.

1.4 Ultraviolet Spectrophotometric technique (UV) [7-11]

Spectroscopy deals with interaction of electromagnetic radiation with matter. Ultraviolet–visible spectroscopy refers to technique of absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. Source of light in this technique is UV and visible range. Molecules containing π-electrons or non-bonding electrons (n-electrons) can absorb the energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals. Based on the fact of four type of transition- π-π^*, n-π^*, σ-σ^*, and n-σ^*. The energy required for various transitions obey the following order σ-σ^*>n-σ^*>π-π^*>n-π^*. Easily excited electrons absorb longer wavelength and vice versa.

Simultaneous equation UV spectroscopy (Vierodt's method).

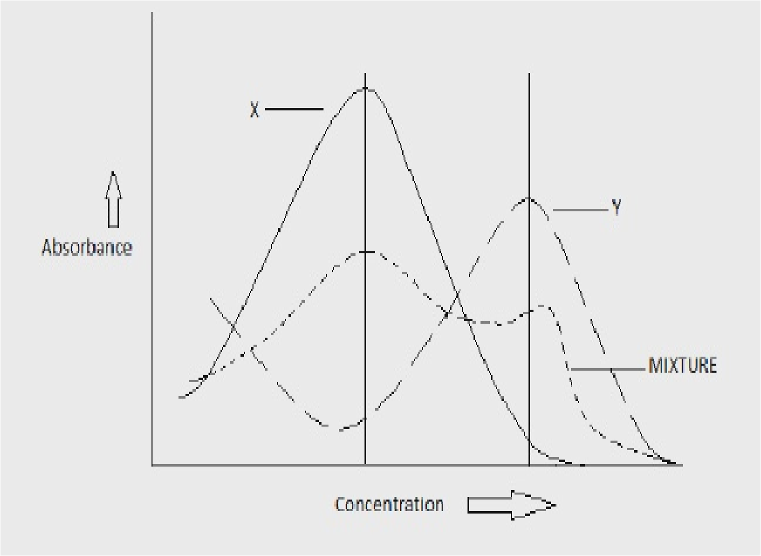
The spectrometric assay of drug generally involves the measurement of absorbance of samples containing more than one absorbing component. The analyst frequently encounters situation where concentration of one or more substance is required in samples known to contain other absorbing substances, which potentially interfere in the assay. If the formula of the sample is known, identity and concentration of interferents are known and extent of interference in the assay may be determined. The absorbance of a solution is the sum of absorbance of individual components. If a sample contain two absorbing drugs (X & Y) each of this absorbs at the λmax of each other i.e. λ1 and λ2, it may be possible to determine both drugs by technique of simultaneous equation method provided that some basic requirements are fulfilled.

Basic requirements for simultaneous estimation are as follows.

Absorbance of solution is sum of absorbance of individual components.

Excipients of formulation are not absorbing UV wavelength of experiment.

If sample contains two UV absorbing drugs (X and Y) each of which absorbs at λ_{max} of other, it may be possible to determine concentration of both drugs if both drugs don't react chemically with each other and λ_{max} of both drugs are reasonably dissimilar.



$$Cx = \frac{A2\ ay1 - A1ay2}{ax2\ ay1 - ax1ay2}$$

$$Cy = \frac{A1\ ax2 - A2\ ax1}{ax2\ ay1 - ax1ay2}$$

Using above equation we can determine concentration of unknown drug X and Y where ax1, ax2, ay1 and ay2 are molar absorptivities of drug at specific wavelength. A1 and A2 are UV absorbance at specific wavelength. Cx and Cy are concentration of unknown drugs. Two equations are constructed based upon the fact that at λ1 and λ2 the absorbance of the mixture is the sum of individual absorbance of X & Y.

1.5 High Performance Thin Layer Chromatography (HPTLC) [12-15]

High Performance Thin Layer Chromatography (HPTLC) is a chromatographic technique that uses the capillary action of a solvent and a stationary phase to separate compounds in a sample mixture. A thin layer of adsorbent material (the stationary phase) is coated on a sheet of glass, aluminum foil or even plastic. High performance thin layer chromatography (HPTLC) is an enhanced form of thin Layer Chromatography. A number of advances can be made to the basic thin layer chromatographic method to automate the different steps, in order to improve the resolution achieved and get more accurate quantitative measurements. Automation help to improve droplet size and position when the sample is applied to the TLC plate by hand. One recent approach to automation has been done by the use of piezoelectric devices and inkjet printers for applying the sample. The spot capacity (analogous to peak capacity in HPLC) can be improved by developing the plate with two different solvents, using 2D chromatography. The procedure begins with development of sample loaded plate with first solvent. After removing it, the plate is rotated 90° and developed with a second solvent.

Advantages of HPTLC

- HPTLC is technique which is simple to learn and operate.
- Many analysts work simultaneously on the system.
- Lower analysis time and less cost per analysis.
- Lower maintenance cost
- Visual detection possible – as it is an open system
- HPTLC has many ranges of stationary phases with unique selectivity for mixture components. Chromatographic layer (sorbent) requires no regeneration as TLC/HPTLC plates are disposable.

- Ability to choose solvents for the mobile phase is not restricted by low UV transparency or the need for ultra-high purity. Corrosive and UV-absorbing mobile phases can be employed.
- HPTLC does not require prior treatment for solvents like filtration and degassing.
- In HPTLC there is no possibility of interference from previous analysis as fresh stationary and mobile phases are used for each analysis. No carry over, hence no contamination.
- Repetition of densitometric evaluation of the same sample can be achieved under different conditions without repeating the chromatography to optimize quantification, since all sample fractions are stored on the TLC/HPTLC plate.
- Samples rarely require cleanup.
- Lower expenditure of solvent purchase and disposal since the required amount of mobile phase per sample is small. In addition, it minimizes exposure risks of toxic organic effluents and reduces possibilities of environment pollution.
- Accuracy and precision of quantification is high because samples and standards are chromatographed and measured under the identical experimental conditions on a single TLC/HPTLC plate.
- HPTLC sensitivity limits of analysis are typically at nanogram (ng) to pictogram (pg) levels.
- Use of different universal and selective detection methods.

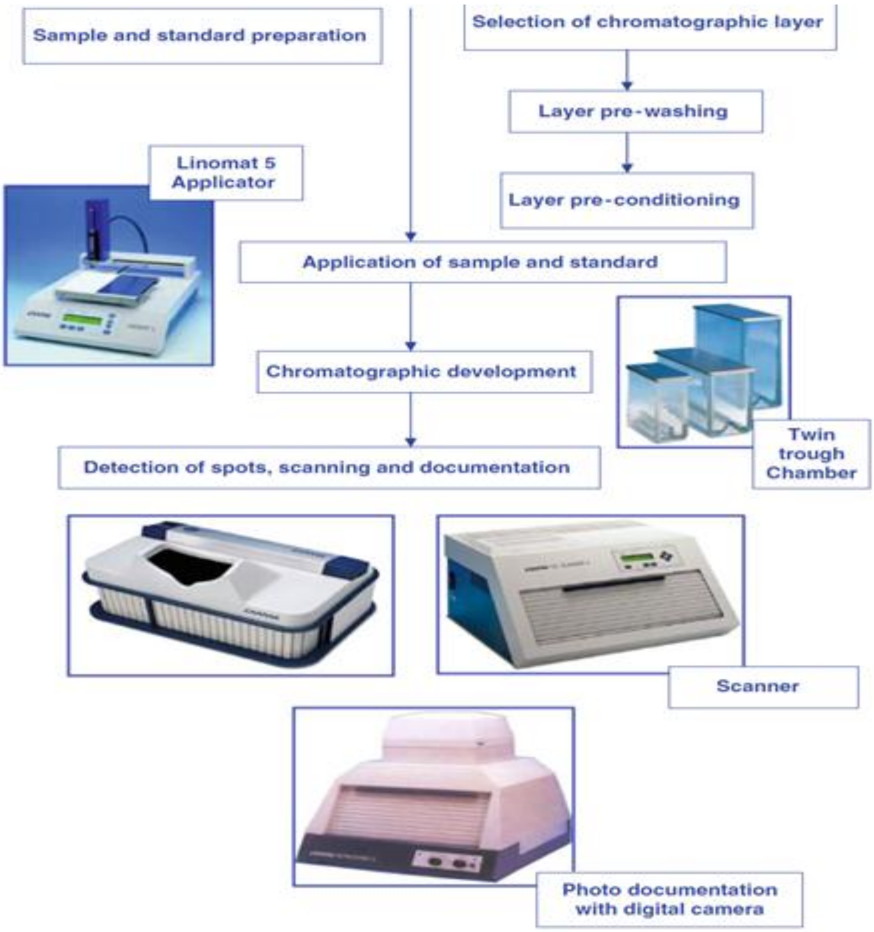


FIGURE 1.3: Schematic diagram of HPTLC

1.6 Analytical method validation parameters [16-20]

a) Definition

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

It is an act of proving that any procedure, process, equipment, material, activity and system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness, etc[31].

When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by same or different persons, in same or different laboratories, using different reagents, different equipments, etc.

Validation of newly developed methods is done by ICH Q(2) R1 guideline and checked for acceptance criteria as per guidelines

b) Benefits of Analytical Method Validation

- The biggest advantage of method validation is that it builds a high degree of confidence, not only for the developer but also to the user.
- Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end.
- Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

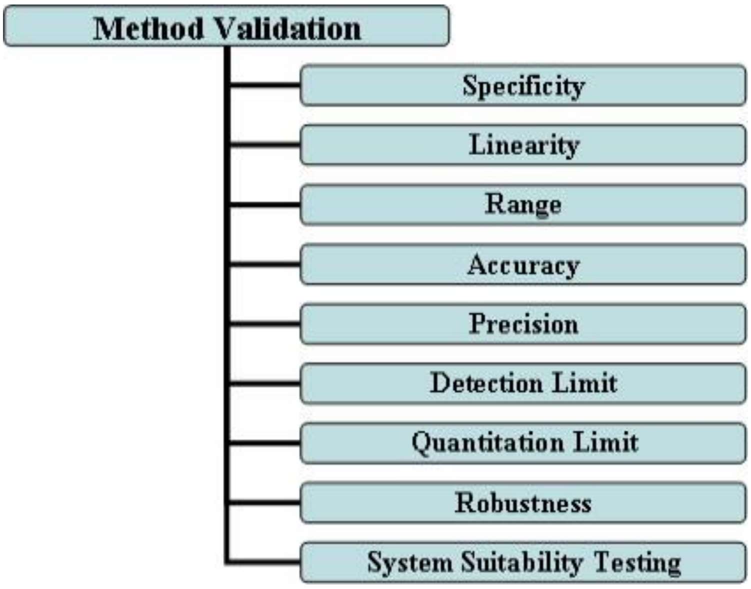


FIGURE 1.4: Schematic diagram of method validation parameters

➢ Selectivity and Specificity [21-23]

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix.

If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective. On the other hand, if the method determines or measures quantitatively the component of interest in the sample matrix without separation, it is said to be specific.

Hence one basic difference in the selectivity and specificity is that, while the former is restricted to qualitative detection of the components of a sample, the latter means quantitative measurement of one or more analytes.

Selectivity may be expressed in terms of the bias of the assay results obtained when the procedure is applied to the analyte in the presence of expected levels of other components, compared to the results obtained on the same analyte without a added substances. When the other components are all known and available, selectivity may be determined by comparing the test results obtained on the analyte with and without the addition of the potentially interfering materials. When such components are either unidentified or unavailable, a measure of selectivity can often be obtained by determining the recovery of a standard addition of pure analyte to a material containing a constant level of the other components.

➢ Linearity and Range

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well defined mathematical transformation) proportional to the analyte concentration in samples within a given range. Linearity usually expressed in terms of the variance around the slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte.

The linear range of detectability that obeys Beer’s law is dependent on the compound analyzed and the detector used. The working sample concentration and samples tested for accuracy should be in the linear range. The claim that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression. Data is processed by linear least square regression declaring the regression co-efficient and b of the linear equation $y= ax + b$ together with the correlation coefficient of determination r^2. For the method to be linear, the r^2 value should be close to 1.

The range of an analytical method is the interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

➢ Accuracy

The accuracy of an analytical method may be defined as the closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. Accuracy may often express as percent recovery by the assay of a known amount of analyte added.

Accuracy may be determined by applying the method to samples or mixtures of excipients to which known amount of analyte have been added, both above and below the normal levels expected in the samples. Accuracy is then calculated from the test results as the percentage of the analyte recovered by the assay.

The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three concentrations and three replicated of each concentration).

➢ Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation).

Precision is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances. Repeatability involves analysis of replicates by the analyst using the same equipment, method and conducting the precision study over short period of time while reproducibility involves precision study at

- Different Occasions
- Different Laboratories
- Different Batch of Reagent

  - ⅄ Different Analysts
  - ⅄ Different Equipments

- **Determination of Repeatability**

Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions like same reagents, equipments, settings and laboratory over a short interval of time.

It is normally expected that at least six replicates should be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n value. The RSD values are important for showing degree of variation expected when the analytical procedure is repeated several time in a standard situation. It should be below 2% for bulk drugs and below 2% for assay in finished product.

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e. three concentrations and three replicates of each concentration or using a minimum of six determinations at 100% of the test concentration).

- **Determination of Reproducibility**

Reproducibility means the precision of the procedure when it is carried out under different conditions, usually in different laboratories on separate, putatively identical samples taken from the same homogeneous batch of material. Comparisons of results obtained by different analysts, by the use of different equipments or by carrying out the analysis at different times can also provide valuable information.

- **Limit of Detection and Limit of Quantitation**

i) Limit of Detection

The limit of detection is the parameter of limit tests. It is the lowest level of analyte that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. The limit test thus merely substantiates that the analyte concentration is above or below a certain level.

The determination of the limit of detection of instrumental procedures is carried out by determining the signal-to-noise ratio by comparing test results from the samples with known concentration of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted.

The signal-to-noise ratio is determined by dividing the base peak by the standard deviation of all data points below a set threshold. Limit of detection is calculated by taking the concentration of the peak of interest divided by three times the signal-to-noise ratio.

For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (Sa) which may be related to LOD and the slope of the calibration curve, b, by:

$\mathrm{LOD} = 3.3\ \mathrm{Sa} / b$.

ii) Limit of Quantitation

Limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantitation is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied.

It is measured by analyzing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable. Where the final assessment is based on an instrumental reading, the magnitude of background response by analyzing a number of blank samples and calculating the standard deviation of this response. The standard deviation multiplied by a factor (usually 10) provides an estimate of the limit of quantitation. In many cases, the LOQ is approximately twice the limit of detection. Sa is the standard deviation of the intercept which may be related to LOQ and the slope of the calibration curve, b, by:

$\mathrm{LOQ} = 10\ \mathrm{Sa} / b$

➢ Robustness and Ruggedness

i) Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The determination of robustness requires that methods characteristic are assessed when one or more operating parameter varied.

ii) Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay.

The testing of ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness is normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method.

➢ Stability of Analytical Solution

Stability of the sample, standard and reagents is required for a reasonable time to generate reproducible and reliable results. For example, 24 hour stability is desired for solutions and reagents that need to be prepared for each analysis. System suitability test provide the added assurance that on a specific occasion the method is giving, accurate and precise results. System suitability test are run every time a method is used either before or during analysis.

➢ System Suitability

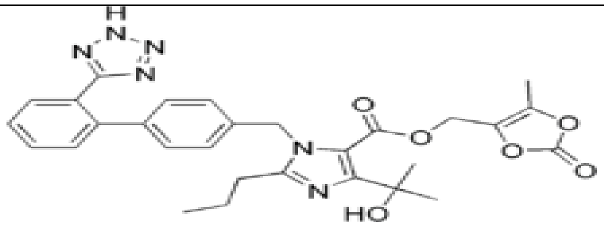
System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (% RSD, retention time and area for six repetitions) are determined and compared against the specifications set for the method. “System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The test are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.” List of the terms to be measured and their recommended limits of the system suitability samples are given in Table 1.2

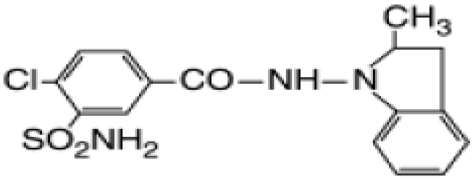
TABLE 1.2: System Suitability Parameters and Recommendations

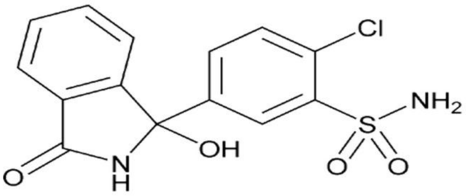
Parameters	**Recommendation**
Capacity Factor (k’)	The peak should be well-resolved from other peaks and the void volume, generally K’ > 2
Repeatability	RSD ≤ 1% N ≥ 5 is desirable
Relative retention	Not essential as the resolution stated
Resolution (R_s)	Rs of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation products, internal standard, etc.)
Tailing factor (T)	T ≤ 2
Theoretical plates (N)	In general should be > 2000

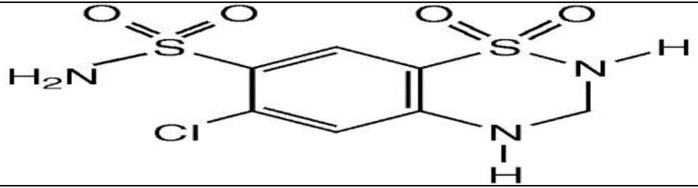
1.7 DRUG PROFILES [24-28]

Drug profile of drugs

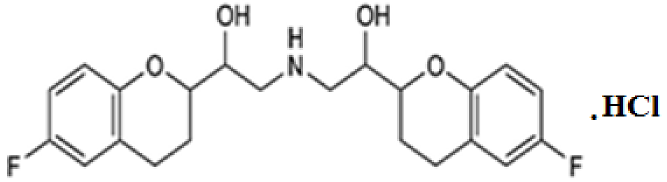
Drug profile of Olmesartan medoxomil	
Chemical Structure	
Chemical IUPAC Name	(5-methyl-2-oxo-2H-1,3-dioxol-4-yl)methyl4-(2-hydroxypropan-2-yl)-2-propyl-1-({4-[2-(2H-1,2,3,4-tetrazol-5-yl)phenyl]phenyl}methyl)-1H-imidazole-5-carboxylate
Chemical Formula	$C_{29}H_{30}N_6O_6$
Molecular Weight	558.5851 g/mol
BCS Class	BCS class II drug, low solubility, high permeability
Melting Point	175-180°C
pKa value	0.91 and 5.57
Log P value	5.9
Solubility	Slightly soluble in distill water, ethanol and freely soluble in methanol
State	Solid
Oral absorption	Less absorbed from GIT
Volume of distribution	13Liter
Plasma protein binding	99%
Metabolism	Hepatic
Excretion	Feces, urine
Plasma half life	13 hour
Pharmacology	Angiotensin II Blockers Olmesartan blocks the vasoconstrictor effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT receptor in vascular smooth muscle.
Dose	5 mg, 12.5mg, 20 mg, 40 mg

Drug profile of Indapamide	
Chemical Structure	
Chemical IUPAC Name	4-chloro-N-(2-methyl-2,3-dihydro-1H-indol-1-yl)-3-sulfamoylbenzamide
Chemical Formula	$C_{16}H_{16}ClN_3O_3S$
Molecular Weight	365.835
pKa value	8.8 and 0.097
Log P value	8.8
BCS Class	BCS class III drug, high solubility, low permeability
Melting Point	185°C
Solubility	Methanol, Ethanol, Acetic acid, Acetonitrile, Slightly in water
State	Solid
Oral absorption	Rapidly absorbed from gastrointestinal tract.
Volume of distribution	20 liter
Plasma protein binding	71-79%
Metabolism	Hepatic.
Excretion	Indapamide is an extensively metabolized drug, with only about 7% of the total dose administered, recovered in the urine as unchanged drug during the first 48 hours after administration.
Plasma half life	14 hours
Pharmacology	Thiazide like diuretic which inhibits sodium chloride co transporters and causes diuretic effect
Dose	1.25mg, 1.5mg, 2 mg, 2.5mg, 5 mg

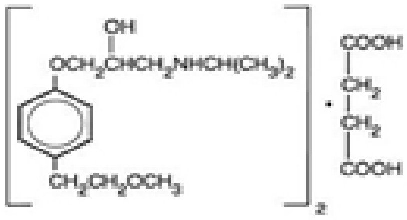
Drug profile of Chlorthalidone	
Chemical Structure	
Chemical IUPAC Name	(*RS*)-2-chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1*H*-isoindol-1-yl)benzene-1-sulfonamide
CAS Number	77-36-1
Chemical Formula	$C_{14}H_{11}ClN_2O_4S$
Molecular Weight	338.766 g/mol
BCS Class	BCS class II drug, low solubility, high permeability
Log P value	0.85 and 1.6
Melting Point	239°C
pKa	8.58 and 2.6
Solubility	Slightly soluble in distill water, ethanol and freely soluble in methanol
State	Solid
Oral absorption	Less absorbed from GIT
Volume of distribution	18 Liter
Plasma protein binding	75%
Metabolism	Hepatic
Excretion	Feces, urine
Plasma half life	40 hour
Pharmacology	It is considered a thiazide-like diuretic.
Dose	12.5mg, 25 mg, 50 mg

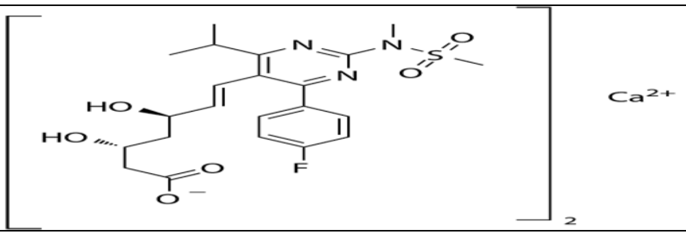
Drug profile of Hydrochlorthiazide	
Chemical Structure	
Chemical IUPAC Name	6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide
Chemical Formula	$C_7H_8ClN_3O_4S_2$
Molecular Weight	297.97 g/mol
pKa Value	7.9 and -2.7
Log P Value	-0.07
BCS Class	BCS class II drug, low solubility, high permeability
Melting Point	274°c

Solubility	Slightly soluble in distill water, and soluble in methanol, Acetonitrile.
State	Solid
Oral absorption	Less absorbed from GIT
Volume of distribution	28 Liter
Plasma protein binding	68%
Metabolism	Not Metabolized
Excretion	urine
Plasma half life	15 hour
Pharmacology	Hydrochlorothiazide, a thiazide diuretic, inhibits water reabsorption in the nephron by inhibiting the sodium-chloride symporter in the distal convoluted tubule, and henceforth reduces the osmotic gradient and water reabsorption throughout the nephron
Dose	2.5mg , 6.25mg , 12.5mg, 20 mg, 50 mg

Drug profile of Nebivolol HCl	
Chemical Structure	
Chemical IUPAC Name	1-(6-fluorochroman-2-yl)-{[2-(6-fluorochroman-2-yl)-2-hydroxy-ethyl] amino} ethanol.
CAS Number	152520-56-4
Chemical Formula	$C_{22}H_{25}F_{2}NO_{4}$•HCl
Molecular Weight	441.90 g/mol
BCS Class	BCS Class-II drug
pKa Value	8.4 for amino group
Log P value	3.21
Melting Point	219-222 °C
Solubility	soluble in methanol, dimethylsulfoxide, and N,N-dimethylformamide
State	Solid : White powder
Oral absorption	The absorption of nebivolol is rapid and not affected by food
Volume of distribution	10 Liters
Plasma protein binding	98 %
Metabolism	Metabolized in the hepatic (CYP2D6 mediated)

Excretion	Renal and fecal
Plasma half life	10 hrs
Pharmacology	selective β_1 receptor blocker with nitric oxide-potentiating vasodilatory effect
Dose	2.5 mg , 5.0mg, 10.0 mg, 20.0 mg

Drug profile of Metoprolol Succinate	
Chemical Structure	
Chemical IUPAC Name	1-(iso-propylamino)-3-[4′(2-methoxyethyl)phenoxy]-2-propanol.
Chemical Formula	$(C_{15}H_{25}NO_3)_2\cdot C_4H_6O_4$
Molecular Weight	652.81 g/mol
BCS Class	BCS Class-I drug which is freely soluble in water, high permeability
pKa Value	9.68
Log P value	1.76
Melting Point	136-138°C
Solubility	freely soluble in water, soluble in methanol
State	Solid
Oral absorption	Rapid and complete, 50%
Volume of distribution	Not Available
Plasma protein binding	12.0%
Metabolism	Metabolized in the liver to inactive metabolite
Excretion	Urine
Plasma half life	3-7 hrs
Pharmacology	selective β_1 receptor blocker used in treatment of several diseases, especially hypertension.
Dose	25 mg , 50mg, 100 mg

Drug profile of Rosuvastatin calcium	
Chemical Structure	
Chemical IUPAC Name	bis[(E)-7-[4(4-fluorophenyl)-6-isopropyl-2[methyl (methylsulfonyl) amino] pyrimidin-5-yl](3R,5S)3,5-dihydroxyhept-6-enoic acid] calcium salt
Chemical Formula	$(C_{22}H_{27}FN_3O_6S)_2Ca$
CAS number	147098-20-2
Molecular Weight	1001.14 g/mol
pKa value	4 and -2.8
Log P value	0.13
BCS Class	BCS class II drug, low solubility, high permeability
Melting Point	122-123°C
Solubility	Slightly in water, Freely in Methanol and Acetonitrile.
State	Solid
Oral absorption	gastrointestinal tract
Volume of distribution	134 liters
Plasma protein binding	88%
Metabolism	Hepatic
Excretion	Urine
Plasma half life	19 hrs
Pharmacology	Competitive inhibitor of HMG-Co A reductase, which affects rate limiting step in hepatic cholesterol synthesis. It lowers LDL levels in body
Dose	5 – 40 mg

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CHAPTER-2

Review of Literature

2.1 Reported selected Literature review methods of Olmesartan medoxomil, Hydrochlorthiazide, Indapamide, Chlorthalidone, Rosuvastatin calcium, Nebivolol HCl, Metoprolol Succinate for HPLC and UV [1-81]

Sr No	Method	Description	Ref No
Olmesartan Medoxomil HPLC			
1	Estimation of Olmesartan in Tablet dosage Form. (Eurasian Journal of Analytical Chemistry 2010)	Column: Hypersil BDS C18 25 cm x 4.6 mm, 5 µm Mobile phase: buffer (3 g of sodium acetate & 3ml T.E.A (triethylamine) in 1000ml water at pH 3 with Orthophosphoric acid) : Acetonitrile (55:45, v/v) Flow rate: 1 ml/min Diluent: Methanol Wavelength: 250 nm Injection volume: 10 µl Retention time: 6.9 min Run time: 15 min	1
2	RP- HPLC method for simultaneous estimation of Amlodipine besylate and Olmesartan medoxomil from tablet (International Journal Of Pharmacy And Pharmaceutical Sciences)	Column: HIQ SII C_{18} column -10 (4.5 mm x 250mm). Mobile Phase: ACN: Water (60:40, v/v). Flow rate: 1ml/min Wavelength: 248 nm Injection Volume: 20 µL Run Time: 08 min Retention Time: Olmesartan 4.24 min Amlodipine besylate 1.82 min Temperature: 55 ˚ C	2
3	Simultaneous estimation of Amlodipine besylate and Olmesartan medoxomil in pharmaceutical dosage form (IJPS Journal 2009)	Column: Kromasil C_{18} 25 cm x 4.6 mm, 5 µm Mobile phase: 0.05M Potassium dihydrogen phosphate buffer pH 3 with Orthophosphoric acid) : acetonitrile (50:50, v/v) Flow rate: 1 ml/min Diluent: Mobile Phase	3

		Wavelength: 238 nm Injection volume: 20 µl Retention time: Amlodipine besylate 3.69 & Olmesartan medoxomil 5.36 min Run time: 15 min	
4	Validated reversed phase HPLC-method for Determination of Olmesartan medoxomil and Hydrochlorothiazide. (Saudi pharmaceutical journal 2009)	Column: (25 cm x 4.6 mm ID, 5µm) Qualisil BDS Octadeyl silane C_{18} Mobile phase: buffer(5.5g Sodium dihydrogen phosphate +1 ml T.E.A) : Acetonitrile pH 3.5 with Orthophosphoric acid) (50:50, v/v) Flow rate : 1ml/min Diluent: Mobile phase Wavelength: 225 nm Injection volume: 20 µl Retention time: 5 min for Olmesartan medoxomil and 7 min for Hydrochlorthiazide Run time: 15 min	4
Olmesartan Medoxomil UV			
5	Development of the UV spectrophotometric method of Olmesartan medoxomil in bulk drug and pharmaceutical formulation and stress degradation studies (Pharmaceutical Methods)	UV Spectrophotometric Method Detection: 257 nm Diluent: Methanol	5
6	Rapid and selective UV Spectrophotometric method for the analysis of Olmesartan medoxomil in bulk and dosage form (International Journal of Drug Delivery)	UV Spectrophotometric Method Detection: 256 nm Diluent : Methanol	6
7	Spectrophotometric Determination of Hydrochlorothiazide and Olmesartan Medoxomil in Pharmaceutical Formulation (E-Journal of Chemistry)	Simultaneous Equation Method Detection: 271.5 nm for Hydrochlorthiazide and 257.0 nm for Olmesartan medoxomil Absorbance Ratio Method Detection: 261 nm for Hydrochlorthiazide & 257 nm Olmesartan medoxomil	7

8	Spectrophotometric estimation of Olmesartan medoxomil in tablet dosage form with stability studies (International Journal of Chem Tech Research)	UV Spectrophotometric Method Diluent used: Methanol Wavelength : 257 nm UV	8
9	Simultaneous Estimation of Amlodipine Besylate and Olmesartan Medoxomil in Pharmaceutical Dosage Form (Indian Journal of Pharmaceutical Sciences)	Simultaneous Equation Method Detection: 237.5 nm for Amlodipine besylate and 255.5 nm for Olmesartan medoxomil Concentration Range : 10-50 µg/ml and 10-50 µg/ml for amlodipine besylate and olmesartan medoxomil	9
Indapamide HPLC			
10	Estimation of Indapamide in Tablet dosage Form (British Pharmacopoeia 2010)	Column: Octa Decyl Silane C_{18} 25 cm x 4.6 mm, 5 µ Mobile phase: Glacial Acetic Acid: Acetonitrile: Methanol: 0.2g/l Sodium edetate (0.1:17.5:17.5:65, v/v/v/v) Flow rate: 2 ml/min Wavelength: 254 nm Injection volume: 10 µl Run time: 15 min Retention time: 8 min	10
11	Estimation of Indapamide in Tablet dosage Form (Indian Pharmacopoeia 2014)	Column: Octa Decyl Silane C_{18} 15 cm x 4.6 mm, 5 µ Mobile phase: 0.154 % w/v solution of Sodium 1-octanesulphonate: Glacial Acetic Acid: ACN (70:1:30, v/v/v) Flow rate: 1.0 ml/min Wavelength: 242 nm Injection volume: 20 µl Retention time: 7 min Run time: 15 min	11
12	A Validated RP-HPLC Method for Simultaneous Estimation of Atenolol and Indapamide in Pharmaceutical Formulations (E Journal net 2011)	Column: 25 cm x 4.6 mm, 5 µ Waters C_{18} Mobile phase: Methanol: water (adjusted to pH 2.7 with 1% Orthophosphoric acid) (80:20, v/v) Flow rate: 1 ml/min Wavelength: 230 nm Injection volume: 10 µl Run time: 10 min Retention time: 3 min Atenolol and 5 min Indapamide	12

13	Simultaneous estimation of Atenolol and Indapamide in combined tablet dosage form using RP-HPLC. (International journal of biomedical and Pharmaceutical Sciences)	Column: Phenomenex Luna C_{18} (25 cm x 4.6 mm, 5 µm) Mobile phase: Methanol: ACN: Water (45:25:30, v/v/v) pH 3.5 adjusted with OPA Flow rate: 1.2 ml/min Wavelength: 226 nm Injection volume: 20 µl Run time: 10 min Retention time: 2. 3 min for Atenolol and 4.3 min for Indapamide	13
Indapamide UV			
14	UV-Spectrophotometric methods for estimation of Indapamide in bulk and tablet dosage (Der Pharma Chemica)	UV spectrophotometric method Detection: 240 nm for simple method and 223 nm for derivative method Solvent: phosphate buffer 7.4	14
15	Development & Validation of Spectrophotometric Method for Simultaneous Estimation of Perindopril and Indapamide in combined dosage form by absorbance correction method (International Journal of PharmTech Research)	Absorbance Correction Method Detection: 210 nm for Perindopril and 285 nm for Indapamide Solvent: methanol	15
16	Simultaneous Estimation of Perindopril Erbumine and Indapamide in Combined Dosage form using Derivative Spectrophotometric Method (International Journal of Pharma Research & Review)	First-Order Derivative Spectrophotometric Method (Zero crossing point) Solvent: methanol Detection: 215 nm for Perindopril and Erbumine and 241 nm for indapamide	16
17	Development and Validation of Spectrophotometric Method for Simultaneous Estimation of Atenolol and Indapamide in Tablet Dosage Form (Int. J. Pharm. Sci. Rev. Res)	Simultaneous method Detection: 225 nm for Atenolol and 240nm for Indapamide Absorbance Ratio Method Detection: 227 nm for Atenolol and 225 nm for Indapamide Concentration Range: 5-20 µg/ml for Atenolol and 5-20µg/ml Indapamide for both methods Solvent: methanol	17

18	Development and validation of spectrophotometric methods for simultaneous estimation of Amlodipine besylate and Indapamide in combined pharmaceutical formulation. (International Journal of Pharmacy and Pharmaceutical Sciences)	Zero Crossing First Derivative Spectrophotometry Detection: 240.6 nm for Amlodipine besylate and 236.8 nm for Indapamide Absorbance Correction Method, Detection: 360.2 nm for Amlodipine besylate and 241.2 nm for Indapamide Ratio Derivative Spectrophotometry, Detection: 332.8 nm for Amlodipine besylate and 299.4 nm for Indapamide Concentration Range: 5 to 30 µg/ml for Amlodipine besylate and 1.5 to 9 µg/ml for Indapamide all methods Solvent: Methanol	18
19	Estimation of Indapamide Tablets (USP NF 2009)	Column: Octa Decyl Silane C_{18} 15 cm x 4.6 mm, 5 µ with packing L1 Mobile phase: Water: ACN: Methanol: Glacial acetic acid (650:175:175:1, v/v/v/v) Flow rate: 1.5 ml/min Wavelength: 242 nm Injection volume: 50 µl Run time: 15 min Retention time: 8 min	19
Hydrochlorthiazide HPLC			
20	Estimation of Hydrochlorthiazide (IP 2014)	Column: ODS 10 cm x 4.6 mm ODS bonded to porous silica (3 µm) Mobile phase: A: 940 ml (0.5 m M Sodium phosphate buffer pH 3.2) : 60 ml of Methanol: 10 ml of THF (v/v/v) B: 500 ml Chloroform : 500 ml Sodium phosphate buffer pH 3.2 : 50 ml THF v/v Mobile Phase A: Mobile Phase B (65:35 v/v) Flow: 0.8 ml/min Wavelength: 224 nm Injection volume: 10 µl	20
21	A Validated RP-HPLC Method for Simultaneous Estimation of Nebivolol and Hydrochlorothiazide in Tablets (Indian Journal of Pharmaceutical sciences)	Column: Phenomenex Gemini C18 25 cm x 4.6 mm, 5 µm Mobile Phase: : Acetonitrile & 50mM ammonium acetate (adjusted pH 3.5 using Orthophosphoric acid) (70:30, v/v) Flow: 1.0 ml/min Wavelength: 254 nm Injection volume: 50 µl	21

<table>
<tr><td>22</td><td>Simultaneous estimation of Bisoprolol fumarate and Hydrochlorothiazide in tablet dosage form by RP-HPLC method
(Indian Journal of Pharmaceutical sciences)</td><td>Column: lichrosphere 100 C18 20 cm x 4.6 mm, 5 µm
Mobile Phase: Water: Acetonitrile: THF (80:20:5, v/v/v)
Flow: 1.0 ml/min
Wavelength: 225 nm
Injection volume: 20 µl</td><td>22</td></tr>
<tr><td>23</td><td>Estimation of Hydrochlorthiazide (IP 2007)</td><td>Stationary phase: silica gel GF 254
Mobile phase: ethyl acetate
UV light Wavelength: 254 nm
(TLC method mobile phase was not applied to HPLC analytical trials)</td><td>23</td></tr>
<tr><td>24</td><td>Estimation of Hydrochlorthiazide
(USP NF 2009)</td><td>Column: Hypersil BDS C18 5 cm x 4.6 mm, 3.5 µm
Mobile phase:
A: ACN : Methanol (30:10, v/v)
B: Prepare and degas a solution of anhydrous formic acid in water (0.5% v/v)
Flow: 1 ml/min
Wavelength: 275 nm
Injection volume: 10 µl
Diluent: Sodium phosphate buffer : ACN (70:30, v/v)
Buffer preparation:
Weighed 2.76 g of monobasic sodium phosphate in 1000 ml volumetric flask & add 990 ml of water, adjust pH with H_3PO_4 (2.7)
Column Temp: 35 ˚ C
Retention time: 5.961 min Hydrochlorthiazide peak.
<table>
<tr><th>Time (Minute)</th><th>Solution A</th><th>Solution B</th></tr>
<tr><td>0</td><td>3</td><td>97</td></tr>
<tr><td>5</td><td>3</td><td>97</td></tr>
<tr><td>14</td><td>36</td><td>64</td></tr>
<tr><td>18</td><td>3</td><td>97</td></tr>
<tr><td>20</td><td>3</td><td>97</td></tr>
</table></td><td>24</td></tr>
<tr><td>25</td><td>Simultaneous Determination of Valsartan and Hydrochlorothiazide in Tablets by RP-HPLC
(Indian Journal of pharmaceutical sciences)</td><td>Column: Diamonsil TM C18 20 cm x 4.6 mm, 5 µm
Mobile Phase: Methanol: ACN : Water : Isopropylalcohol (22:18:68:2, v/v/v/v) adjusted pH 8 with triethylamine
Flow rate: 1.0 ml/min
Wave length: 270 nm
Injection volume: 20 µl
Temp: 30 ˚ C</td><td>25</td></tr>
</table>

26	Simultaneous determination of Losartan potassium and Hydrochlorothiazide from tablets and human serum by RP-HPLC (Journal of liquid chromatography & related technologies)	Column: C 18 25 cm x 4.6 mm, 5 µm Mobile Phase: 0.01 M Potassium dihydrogen phosphate : ACN (65:35, v/v) adjusted pH 3.1 with H_3PO_4 Flow: 1.0 ml/min Wavelength: 232 nm Injection volume: 10 µl	26
Hydrochlorthiazide UV			
27	Spectrophotometric method for simultaneous estimation of Atenolol in combination with Losartan potassium and Hydrochlorothiazide in bulk and tablet formulation (Journal of Pharmacy and Bio allied sciences)	Diluent: Methanol:Water (50:50, v/v) Wavelength : 224 and 271nm	27
28	Novel UV spectrophotometric methods for estimation of Ramipril and Hydrochlorothiazide by simultaneous equation and area under curve Method (International Journal of applied pharmaceutics)	Diluent : Methanol Wavelength: 210 and 270 nm	28
Rosuvastatin Calcium HPLC			
29	Estimation of Rosuvastatin calcium (IP 2014)	Column: S.S ODS C18 25 cm x 4.6 mm, 5 µm Mobile Phase: 0.2 % v/v acetic acid in water : ACN: methanol (50:25:25, v/v/v) Flow: 1.0 ml/min Wavelength: 248 nm Injection volume: 20 µl	29
30	Estimation of Rosuvastatin calcium tablets (IP 2014)	Column: S.S ODS C18 25 cm x 4.6 mm, 5 µm Mobile Phase: 1.54 g of ammonium acetate in 900 ml water, adjust pH 4.0 with glacial acetic acid & dilute to 1000 ml with water: ACN: THF (585 : 360: 50, v/v/v) Flow: 1.5 ml/min Wavelength: 248 nm Injection volume: 20 µl Temp: 30 ˚ C	30

31	Estimation of Rosuvastatin calcium tablets (IP 2007)	Column: S.S ODS C18 25 cm x 4.6 mm, 5 µm Mobile Phase: 1.54 g of ammonium acetate in 900 ml water, adjust pH 4.0 with glcial acetic acid & dilute to 1000 ml with water: ACN: THF (585 : 360: 50, v/v/v) Flow: 1.5 ml/min Wavelength: 248 nm Injection volume: 20 µl Temp: 30 ˚ C	31
32	Validated RP-HPLC method for simultaneous estimation of Rosuvastatin and Telmisartan in bulk and pharmaceutical dosage form (International journal of advances in pharmaceutical sciences)	Column: Thermo C18 25 cm x 4.6 mm, 5 µm Mobile Phase: ACN : 1.36 g of potassium dihydrogen phosphate buffer in the ratio of (60: 40, v/v) pH adjusted to 4.5 with dil.ortho phosphoric acid solution Flow: 1 ml/min Wavelength: 231 nm Injection volume: 20 µl	32
33	Simultaneous determination of rosuvastatin and amlodipine in human plasma using tandem mass spectrometry: Application to disposition kinetics (Journal of advanced Research)	Column: Zorbax SB C18 50 cm x 4.6 mm, 3.5 µm Mobile Phase: Mixture of 0.1 % formic acid in 5M ammonium acetate: methanol: ACN (20:20:60, v/v/v) Flow: 1.5 ml/min Injection volume: 20 µl Temp: 30 ˚ C	33
34	RP-HPLC method for simultaneous estimation of Rosuvastatin and Ezetimibe from their combination tablet dosage form (International journal of chemical and analytical science)	Column: Water AF C18 25 cm x 4.6 mm, 5 µm Mobile Phase: Mixture of ACN : water: 0.02 M phosphate buffer in the ratio of (40: 10: 50 v/v/v) pH adjusted to 8 TEA Flow: 1.2 ml/min Wavelength: 230 nm Injection volume: 20 µl Temp: 30 ˚ C	34
Rosuvastatin Calcium UV			
35	Simultaneous Estimation of Rosuvastatin Calcium and Ezetimibe in Tablet Dosage Form by UV-Spectrophotometry (International Journal of Pharmacy and Pharmaceutical sciences)	Diluent : Methanol Wavelength: 232 and 237 nm	35

36	UV Spectrophotometric estimation of Rosuvastatin Calcium and Fenofibrate in bulk Drug and Dosage Form using Simultaneous Equation Method (International Journal of ChemTech Research)	Diluent : Methanol Wavelength: 244 and 286 nm	36
Chlorthalidone HPLC			
37	Estimation of Chlorthalidone (EP 5.0)	Dissolve 0.200 g in 50 ml of acetone. Titrate with 0.1 M tetrabutyl ammonium hydroxide, determining the end-point potentiometrically. 1 ml of 0.1 M tetrabutyl ammonium hydroxide is equivalent to 33.88 mg of $C_{34}H_{56}N_2O_{10}$.	37
38	Estimation of Chlorthalidone (IP 2014)	Dissolve 0.200 g in 50 ml of acetone. Titrate with 0.1 M tetrabutyl ammonium hydroxide, determining the end-point potentiometrically. 1 ml of 0.1 M tetrabutyl ammonium hydroxide is equivalent to 33.88 mg of $C_{34}H_{56}N_2O_{10}$.	38
39	Estimation of Chlorthalidone (USP 32 NF 27 2009)	Column: Packing L7 25 cm x 4.6 mm Mobile phase: buffer (0.01 M dibasic ammonium phosphate: Methanol (3:2 v/v) at pH 5.5 with Orthophosphoric acid) Flow: 1 ml/min Diluent: Methanol Wavelength: 254 nm Injection volume: 25 µl	39
40	Estimation of Chlorthalidone (BP 2001)	Stationary phase: silica gel GF 254 Mobile phase: Water: Ethyl acetate (1.5: 98.5, v/v) UV light Wavelength: 254 nm	40
41	Estimation of Chlorthalidone (IP 2007)	Stationary phase: silica gel GF 254 Mobile phase: Dioxane : 2-propanol : Toluene : Ammonia (30:30:30:20, v/v/v/v) UV light Wavelength: 254 nm	41
42	Estimation of Chlorthalidone in Tablet dosage Form (USP 35 NF 30)	Column: Hypersil BDS C8 25 cm x 4.6 mm, 5 µm Mobile phase: buffer (0.01 M dibasic ammonium phosphate: Methanol (3:2, v/v) at pH 5.5 with Orthophosphoric acid)	42

		Flow: 1 ml/min Diluent: Methanol Wavelength: 254 nm Injection volume: 25 µl	
43	Estimation of Chlorthalidone in Tablet dosage Form (International Journal of Pharmacy & Pharmaceutical sciences)	Column: Hypersil BDS cyano C18 15 cm x 4.6 mm, 5 µm Mobile phase: buffer (50mM disodium hydrogen phosphate: Methanol: Acetonitrile (70:25:5, v/v/v) at pH 3.5 with Orthophosphoric acid) Flow: 1 ml/min Wavelength: 220 nm Injection volume: 10 µl Diluent: Mobile Phase	43
Chlorthalidone UV			
44	Standard Absorbance method & Q absorbance method for Chlorthalidone (Indo American Journal of Pharmaceutical Research)	Chlorthalidone λ_{max} 275 nm (Standard absorbance method) Diluent Methanol: Water (10:90 v/v)	44
45	Spectroscopic method for Chlorthalidone (Arabian journal of chemistry, Science Direct)	Chlorthalidone λ_{max} 276nm Diluent: Methanol	45
46	Simulaneous estimation method for Azilsartan medoxomil & Chlorthalidone (Anal Chem Insights)	Azilasartan 286 nm Chlorthalidone 257nm Solvent : Methanol	46
47	Derivative UV-spectrophotometric Method-Metoprolol succinate & Chlorthalidone (International Journal of Analytical & Bio analytical Chemistry)	Metoprolol 227nm & Chlorthalidone 229 nm Diluent: Water : Acetonitrile (50:50, v/v)	47
48	UV Absorption Correction Method Olmesartan medoxomil and chlorthalidone (The Pharma Innovation Journal)	Olmesartan 260 nm & Chlorthalidone 215 nm. Diluent : Methanol	48
49	First order derivative UV Spectrophotometric Method Metoprolol and Chlorthalidone (Pharmagene Journal)	Metoprolol succinate 267 nm & Chlorthalidone 243 nm Diluent : Methanol	49

50	Simultaneaous estimation of Metoprolol tartrate and Chlorthaldione. (Research J. Pharm. and Tech)	Metoprolol 270 nm & Chlorthalidone 280 nm. Diluent Methanol	50
Chlorthalidone HPTLC			
51	Validated HPTLC Method For The Simultaneous Estimation Of Losartan Potassium And Chlorthalidone In Combined Tablet Dosage Form (International journal of pharmacy & biological sciences)	Stationary Phase: Silica Gel G60 F254 TLC plate Mobile Phase: Chloroform: Methanol: Ammonia (9: 2: 0.2, v/v/v) Wave length: 254 nm. R_f values: 0.43 for Losartan potassium and 0.82 for Chlorthalidone	51
52	Simultaneous Estimation of Telmisartan & Chlorthalidone in the tablet dosage form by HPTLC (Int. Journal of Pharmacy & Pharmaceutical sciences)	Stationary Phase: Silica Gel 60 F254 TLC plate Mobile Phase: ACN: Toluene: Glacial acetic acid (7.5:2.5:0.05%, v/v/v/v) Wave length: 242 nm. R_f values: 0.26 for Telmisartan and 0.67 for Chlorthalidone	52
53	Validated stability-indicating methods for the simultaneous determination of Amiloride hydrochloride, Atenolol, and Chlorthalidone using HPTLC and HPLC with photodiode array detector. AOAC Journal (Journal of the Association of Official Analytical Chemists)	Stationary Phase: Silica Gel 60 F254 TLC plate Mobile Phase: Chloroform: Methanol: Ammonia 27% (9:2:0.3%, v/v/v) Wave length: 4: 2 nm. R_f values: 0.26 for Amiloride hydrochloride, 0.40 Atenolol and 0.67 for Chlorthalidone	53
Nebivolol HCl HPLC			
54	Estimation of Nebivolol HCl (IP 2014)	Column: S.S C18 (Phenyl group) 25 cm x 4.6 mm, 5 µm Buffer Prepartion: buffer (3.4 g tetrabutyl ammonium hydrogen Phosphate in 1000 ml water and 0.3 ml diethylamine) Mobile Phase: Buffer: Acetonitrile (72:28, v/v) pH 3 with Orthophosphoric acid Flow: 1.0 ml/min Diluent: Mobile phase Wavelength: 220 nm Injection volume: 20 µl	54
55	Estimation of Nebivolol HCl by HPLC (International Journal of Chem Tech research)	Column: Hypersil BDS C18 250 cm x 4.6 mm, 5 µm Buffer Prepartion: buffer (40.8 g Potassium dihydrogen Phosphate in 1000 ml water)	55

		Mobile Phase: Buffer: Acetonitrile (50:50, v/v) pH 3.2 with Orthophosphoric acid Flow: 1.2 ml/min Diluent: Mobile phase Wavelength: 278 nm Injection volume: 10 µl	
56	Estimation of Nebivolol by HPLC (International Journal of Pharma science review & research)	Column: ACE C18 250 cm x 4.6 mm, 5 µm Mobile Phase: Water: Methanol (30:70, v/v) Flow: 1.0 ml/min Diluent: Mobile phase Wavelength: 282 nm Injection volume: 10 µl	56
57	Estimation of Nebivolol HCl tablets (IP 2014)	Column: S.S C18 25 cm x 4.6 mm, 5 µm Buffer Prepartion: buffer (6.8 g potassium dihydrogen orthophosphate in 1000 ml water and 2 ml of TEA adjusting pH 3 with 10% v/v OPA) Mobile Phase: Buffer: Acetonitrile: Methanol (45:25:30, v/v/v) pH 3 with Orthophosphoric acid Flow: 1.0 ml/min Diluent: Mobile phase Wavelength: 281 nm Injection volume: 20 µl	57
58	Simultaneous Estimation Of Nebivolol HCl And S-Amlodipine Besylate By HPTLC (International Journal of Pharma and Bio Sciences)	HPTLC plates : 10 cm × 10 cm aluminum coated with 0.2 mm layers of silica gel 60 F 254 Mobile Phase: Chloroform:Toluene:Methanol: Glacial acetic acid (5:3:2:0.1, v/v/v/v) Wavelength: 271 nm	58
Nebivolol HCl UV			
59	Simultaneous Estimation of Nebivolol hydrochloride and Valsartan in Bulk and Capsule Dosage Form by Simultaneous Equation Method (International Journal of ChemTech Research)	A Shimadzu UV-1800 UV/VIS spectrophotometer was used with 1 cm matched quartzcell λ_{max} for Nebivolol hydrochloride: 281nm λ_{max} for Valsartan : 251nm	59
60	Simultaneous quantification of Nebivolol HCl and Hydrochlorothiazide by first derivative UV- Spectroscopy (Scholar Research Library)	Shimadzu (Japan) UV-Visible spectrophotometer (UV-1800) with 1cm matched quartz cells was used for spectrophotometric analysis, λ_{max} for Nebivolol hydrochloride: 272nm λ_{max} for Hydrochlorthiazide : 280 nm	60
61	Spectrophotometric methods for simultaneous estimation of	A GBC Cintra 10 double beam UV-Visible spectrophotometer equipped with 10 mm	61

	Nebivolol HCl and Amlodipine besylate in tablets.(International Journal of Pharmacy and Pharmaceutical Sciences,)	matched quartz cells was used in the present investigation. λ_{max} for Nebivolol hydrochloride: 281nm λ_{max} for Amlodipine besylate : 238 nm	
62	Simultaneous Estimation of Nebivolol HCl and Valsartan in Bulk and Capsule Dosage Form by Simultaneous Equation Method (International Journal of Chem Tech Research)	A Shimadzu UV-1800 UV/VIS spectrophotometer was used with 1 cm matched quartzcell λ_{max} for Nebivolol hydrochloride: 281nm λ_{max} for Valsartan : 251nm	62
63	Development and validation of UV spectrophotometric method for determination of Nebivolol HCl (Asian Journal of Pharmaceutical & Clinical Research)	λ_{max} for Nebivolol hydrochloride: 281nm Diluent: Methanol: Water (10:90, v/v)	63
64	Development and validation of UV spectrophotometric method for determination of Nebivolol HCl (International Journal of Pharma Research & Bio-Science)	λ_{max} for Nebivolol hydrochloride: 284nm Diluent: DMSO	64
65	Simultaneous estimation of Nebivolol HCl & Valsartan in bulk & capsule dosage form by simultaneous equation method (Ind. j. pharm. edu res)	λ_{max} for Nebivolol hydrochloride: 281nm λ_{max} for Valsartan : 251nm Diluent: Methanol	65
66	Simultaneous quantification of Nebivolol HCl & Hydrochlorothiazide by first derivative UV- Spectroscopy (Der pharm. Letter)	Nebivolol hydrochloride λ_{max} : 272nm Hydrochlorthiazide λ_{max} : 280 nm Diluent: Methanol	66
67	Spectrophotometric methods for simultaneous estimation of Nebivolol HCl and Amlodipine besylate in tablets. (International journal of pharma & pharmaceutical sciences)	Nebivolol hydrochloride λ_{max} : 281nm Amlodipine besylate λ_{max} :238 nm Diluent: Methanol	67
68	Spectrophotometric methods for simultaneous estimation of Nebivolol HCl and Amlodipine besylate in tablets. (Current trends in technology & science Journal)	Nebivolol hydrochloride λ_{max} : 218nm Amlodipine besylate λ_{max} :238 nm Diluent: Methanol	68

69	Simultaneous Estimation of Nebivolol HCl & Cilnidipine in Bulk dosage & tablets by simultaneous equation method (International journal of pharma sciences review & research)	Nebivolol hydrochloride λ_{max} : 221 nm Cilnidipine λ_{max} : 249 nm Diluent: Methanol	69
Metoprolol Succinate HPLC			
70	Estimation of Metoprolol succinate (USP 32 NF 27)	Column: Hypersil BDS C8 12.5 cm x 4.0 mm, 4 µm Mobile phase: buffer (1.3 g Sodium Do decyl sulphate in 0.1% Phosphoric acid buffer) Mobile Phase: Buffer: Acetonitrile (60:40, v/v) Flow: 0.9 ml/min Diluent: Methanol Wavelength: 223 nm Injection volume: 10 µl Diluent: Mobile Phase	70
71	Estimation of Metoprolol Succinate (BP 2008)	Dissolve 0.250 g Metoprolol succinate in 40 ml of anhydrous acetic acid. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically. 1 ml of 0.1 M perchloric acid is equivalent to 32.64 mg of $C_{34}H_{56}N_2O_{10}$.	71
72	Estimation of Metoprolol succinate in ER Tablets (International journal of pharmacy and Pharmaceutical sciences 2014)	Column: Zorbax SB C8 250 cm x 4.6 mm, 5 µm Buffer (0.05 M Phosphate buffer pH 3 with Orthophosphoric acid) Mobile Phase: Buffer: Acetonitrile (60:40, v/v) Flow: 1.0 ml/min Diluent: Methanol Wavelength: 225 nm Injection volume: 10 µl Diluent: Methanol	72
73	Estimation of Metoprolol succinate ER Tablets (USP 32 NF 27)	Column: X Bridge C8 Column 12.5 cm x 4.0 mm, 4 µm Buffer pH 3.0 Phosphate buffer— Mix 50 mL of 1 M monobasic sodium phosphate and 8.0 mL of 1 M phosphoric acid, and dilute with water to 1000 mL. If necessary, adjust with 1 M monobasic potassium phosphate or 1 M phosphoric acid to a pH of 3.0. Mobile Phase: Buffer: Acetonitrile (375:125, v/v) Flow: 1.0 ml/min Diluent: Methanol Wavelength: 280 nm	73

		Injection volume: 10 µl	
Metoprolol Succinate HPTLC			
74	Simultaneous Estimation of Metoprolol Succinate & Cilnidipine and Metoprolol Succinate in the tablet dosage form by HPTLC (Pharm Methods)	Stationary Phase: Silica Gel G60 F254 TLC plate Mobile Phase: Toluene: Chloroform: Methanol: Glacial acetic acid (45: 25: 25: 5, v/v/v/v) Wave length: 231 nm. R_f values: 0.70 for Cilnidipine and 0.34 for Metoprolol succinate	74
75	Simultaneous Estimation of Metoprolol Succinate & Atorvastatin in the tablet dosage form by HPTLC (Int. Res J of Pharmacy)	Stationary Phase: Silica Gel 60 F254 TLC plate Mobile Phase: ACN: Methanol: Ethyl acetate: Glacial acetic acid (2:4:4:0.06%, v/v/v/v) Wave length: 223 nm. R_f values: 0.32 for Metoprolol succinate and 0.77 for Atorvastatin	75
76	Simultaneous Estimation of Metoprolol Succinate & Telmisartan in the tablet dosage form by HPTLC (International Scholarly Research)	Stationary Phase: with aluminium plates precoated with silica gel 60F254 Mobile phase: Toluene : Propanol : Methanol : Triethylamine (8 : 1 : 1 : 0.5, v/v/v) R_f values : 0.45 Telmisartan and 0.70 Metoprolol succinate Wavelength 242 nm	76
Metoprolol Succinate UV			
77	Simultaneous Estimation of Metoprolol Succinate & Olmesartan Medoxomil in pharmaceutical dosage form by UV spectroscopy (International Journal of Research in Pharmaceutical and Biomedical Sciences)	Metoprolol succinate 223 nm and Olmesartan medoxomil 256 nm Diluent: Methanol	77
78	Simultaneous estimation of Telmisartan & Metoprolol in tablet dosage form (Int Res J Pharm)	Telmisartan 299 nm & Metoprolol 224 nm, Diluent : Methanol	78

79	Analytical study of UV-Spectrophotometric methods for simultaneously determination of Metoprolol and Hydrochlorthiazide in fixed dosage Combinations (J Chem and Pharm Res)	Hydrochlorthiazide 250 nm Metoprolol 224 nm Solvent : Methanol	79
80	Derivative UV-spectrophotometric Method-Metoprolol succinate & Chlorthalidone (International Journal of Analytical & Bio analytical Chemistry)	Metoprolol succinate 227nm & Chlorthalidone 229 nm Diluent Water : Acetonitrile (50:50, v/v)	80
81	Development and validation of the simultaneous UV spectrophotometric method for estimation of Metoprolol succinate and Olmesartan medoxomil in the tablet dosage form (Pharm Methods)	Olmesartan medoxomil 257 nm & Metoprolol succinate 221 nm. Diluent: Methanol	81

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CHAPTER-3

Aim, objectives and rationale of work

3.1 Objectives and rationale of work

- To develop and validate analytical methods for simultaneous estimation of anti-hypertensive drugs in pharmaceutical dosage form.
- There are numbers of newer antihypertensive drugs and their formulations are approved by FDA which is either new molecule or partial modification of existing molecule.
- These newer drugs and their combinations are not official in any pharmacopeias and it takes more time to include these drugs and their combinations, in pharmacopoeia due to unavailability of proper analytical methods for their estimation.
- There is continuous and longer use of these drugs individually newer side effects, toxicity, resistances are observed.
- Development & validation of methods as per ICH Q2 (R1) for newer combinations help to detect drug substances present in diluent and excipients.
- Also as per latest DCGI guidelines assay studies for newer drugs combinations are mandatory for launching of drugs in market and for proper patient use.
- So present investigation was undertaken with a view to develop and validate new analytical method for simultaneous estimation of Olmesartan medoxomil & Indapamide, Olmesartan medoxomil & Chlorthalidone, Rosuvastatin calcium & Hydrochlorthiazide, Nebivolol HCl & Chlorthalidone, Metoprolol succinate and Chlorthalidone.
- Newly developed methods & validated method as per ICH Q2 (R1) may be applied for routine analysis of drug samples in analytical development laboratories and quality control laboratories (USFDA, MHRA, EU, Brazil, and Canada, WHO approved).

3.2 Aim of work

- To develop RP-HPLC methods for simultaneous estimation of Olmesartan medoxomil and Indapamide, Olmesartan medoxomil and Chlorthalidone, Nebivolol HCl and Chlorthalidone, Rosuvastatin calcium and Hydrochlorthiazide, Metoprolol succinate and Chlorthalidone in combination drug products.
- To develop UV or HPTLC methods (wherever UV method is not feasible) for simultaneous estimation of Olmesartan medoxomil and Indapamide, Olmesartan medoxomil and Chlorthalidone, Nebivolol HCl and Chlorthalidone, Rosuvastatin calcium and Hydrochlorthiazide, Metoprolol succinate and Chlorthalidone in combination drug products.
- All developed methods to be validated for specificity, linearity, accuracy, repeatability (precision), and ruggedness, limit of detection and limit of quantification, robustness and system suitability as per ICH Q(2) R1 guidelines.
- To perform statistical comparison of developed methods.

CHAPTER-4

Chemicals and instruments of research work

4.1 Materials used in research work

Drug	Company
Olmesartan medoxomil	Cadila Healthcare ltd, Ankleshwar, Gujarat
Indapamide	Glenmark pharmaceuticals ltd, Ankleshwar Gujarat
Rosuvastatin calcium	Intas pharmaceuticals ltd, Matoda, Ahmedabad, Gujarat
Hydrochlorthiazide	Intas pharmaceuticals ltd, Matoda, Ahmedabad, Gujarat
Nebivolol HCl	Cadila Healthcare ltd, Moraiya, Ahmedabad, Gujarat
Metoprolol succinate	Torrent pharmaceuticals ltd, Gandhinagar, Gujarat
Chlorthalidone	Cadila Healthcare ltd, Ankleshwar, Gujarat

4.2 Marketed formulations

Drug name	Marketed formulation-I	Marketed formulation-II
Olmesartan medoxomil 20 mg + Indapamide 1.5 mg	Olmy I tablets (Zydus Cadila Healthcare limited, Moraiya, Ahmedabad)	Olmesat ID tablets (Biocon limited, Hosur, Banglore.)
Olmesartan medoxomil 20 mg and Chlorthalidone 12.5 mg	Olmesar-CH tablets (Macleods, Vapi, India)	Olbet CT tablets (Zuventus Healthcare, Mumbai, India)
Nebivolol HCl 5 mg and Chlorthalidone 12.5 mg	Nebula C tablets (Zydus Cadila Healthcare limited, Moraiya Ahmedabad)	Nodon CH tablets (Cadila Pharma limited, Dholka Ahmedabad.)

Rosuvastatin calcium 10 mg & Hydrochlorthiazide 12.5 mg	Zyrova H tablets (Zydus Cadila Healthcare limited, Moraiya, Ahmedabad	Rozustat H tablets (Macleods, Vapi, Gujarat)
Metoprolol succinate 50 mg & Chlorthalidone 12.5 mg	Vinicor D tablets (IPCA laboratories ltd) Kandivali (West), Mumbai, Maharashtra	Promet D tablets (Grownbury Pharma Pvt. ltd) Krishna nagar, New Delhi.

4.3 Chemicals used in research work

Name of chemical	**Grade**	**Company**
Water (Milli Q)	HPLC	In house
Triethylamine	HPLC	Merck
Acetonitrile	HPLC	Merck
Sodium perchlorate	Analytical reagent	Spectrochem
Ammonium acetate	Analytical reagent	Merck
Potassium dihydrogen phosphate	Analytical reagent	Merck
Sodium Hydroxide	Analytical reagent	Merck
Orthophosphoric acid	HPLC	Merck
Chloroform	HPLC	Merck
Toluene	HPLC	Merck
Glacial acetic acid	HPLC	Merck
Methanol	HPLC	Merck
Sodium dihydrogen Phosphate	Analytical reagent	Merck
Ammonia	Analytical reagent	Merck
Ethyl acetate	HPLC	Merck

4.4 Instruments used in research work

HPLC Used : LC 2010C, Shimadzu (Shimadzu Corporation, Japan)

Analytical Balance : AX 205, METTLER TOLEDO

pH Meter : Thermo Orion, model 420

Sonicator : Oscar Ultra Sonics, OU- 72 (SPL)

UV Used : UV 1800, Shimadzu (Shimadzu Corporation, Japan)

4.5 Apparatus and instruments for HPTLC:

Precoated silica gel aluminium plate 60 F-254 (10 x 10 cm with 250 μm thickness)

Applicator Linomat 5 of Camag, Software winCATS

Desaga-250 μl dosing syringe (Hamilton)

Desaga-100 μl dosing syringe (Hamilton)

Desaga-Twin through chamber (200 x 100) with stainless steel lid

Desaga TLC scanner, Proquant

Desaga photo chamber, Povidoc with canon powershot G5 digital camera

UV cabinet with dual wavelength UV Lamp (254 nm and 366 nm)

HPTLC plates precoated silica gel aluminium plate 60 F-254 (10 cm x 10 cm and 20 cm x 10 cm) with 250 μm thickness), Merck India ltd

CHAPTER-5

RP-HPLC and UV method for Olmesartan medoxomil and Indapamide

5.1 RP-HPLC method for Olmesartan medoxomil and Indapamide [1-3]

TABLE 5.1 Drug & marketed formulation details of Olmesartan medoxomil and Indapamide

Drug	Company	
Olmesartan Medoxomil	Cadila healthcare ltd, Ankleshwar, Gujarat	
Indapamide	Glenmark pharmaceuticals ltd, Ankleshwar Gujarat	
Drug name	**marketed formulation-I**	**marketed formulation-II**
Olmesartan medoxomil 20 mg + Indapamide 1.5 mg	Olmy I tablets (Zydus Cadila healthcare limited, Moraiya, Ahmedabad)	Olmesat ID tablets (Biocon limited, Hosur, Banglore.)

Experimental work

Chromatographic condition

Column : ACE C_{18} AR Column (250 X 4.6 mm), 5µ

Detector : 280 nm

Injection volume: 10 µl

Flow Rate : 1.0 mL min^{-1}

Temperature : 30º C

Run time : 9 minutes

Mobile phase : Sodium perchlorate & triethylamine buffer (at pH 3): acetonitrile (60:40, v/v)

Diluent : Water: Acetonitrile (50:50, v/v)

Buffer preparation

Accurately weighed 4.3 g sodium perchlorate was dissolved in to 1000 mL milli-Q water and 1ml triethylamine was added to this buffer solution, than pH was adjusted to 3.0 with ortho-phosphoric acid.

Standard Preparation (Olmesartan medoxomil (200 µg/ml) & Indapamide (15 µg/ml)

Preparation of standard solution

The standard stock solution Olmesartan medoxomil (1000 µg/ml) and Indapamide (75 µg/ml) were prepared by weighing Olmesartan medoxomil 200 mg and Indapamide 15 mg in 200ml volumetric flask respectively and making volume up to mark with diluent. Then 10 ml of standard stock solution was diluted to 50 ml with diluent to make final standard concentration of Olmesartan medoxomil (200 µg/ml) and Indapamide (75 µg/ml), respectively.

Preparation of test solution (Olmesartan medoxomil 20 mg & Indapamide 1.5 mg Tablets)

Accurately 20 intact tablets were weighed and average weight of tablet was calculated. Then tablets were finely crushed, powdered and sample powder about 5000 mg (Tablet powder Equivalent to 200 mg Olmesartan medoxomil and 15 mg Indapamide) was transferred into 200 ml volumetric flask. Then about 100.0 ml diluent was added and sonicated for 40 minutes with intermittent shaking. The volume was made up to mark with diluent. Then 10 ml of standard stock solution was diluted to 50 ml with diluent to make final standard concentration of Olmesartan medoxomil (200 µg/ml) and Indapamide (15 µg/ml), respectively. The test solution was filtered through 0.45µ (PVDF Millipore Filter) and analyzed by using HPLC.

The isocratic program throughout HPLC method was adopted to analyze both components in a single run as shown in Figure 5.1.

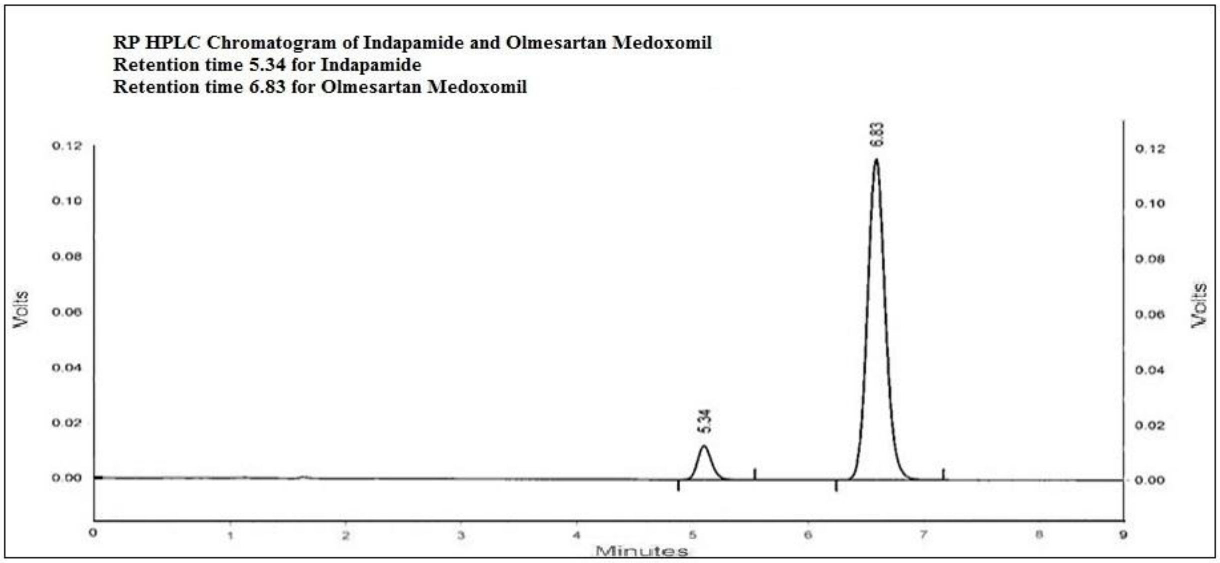

FIGURE 5.1: RP-HPLC chromatogram of Olmesartan medoxomil and Indapamide.

Method validation [4-6]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1). The developed method validated with respect to parameters such as system suitability, solution stability, specificity, linearity, repeatability, accuracy, intermediate precision, robustness, limit of detection and limit of quantitation.

System suitability and system precision

System suitability test were performed to check repeatability of system for particular analysis performed. The results for system suitability parameters were found satisfactory. The results of system suitability and system precision are presented in Table 5.2.

TABLE 5.2: System suitability parameters of RP-HPLC method for Olmesartan medoxomil and Indapamide.

Compound	Retention time ± SD (min) (N=5)	Theoretical plates ± SD (N=5)	Asymmetry ± SD (N=5)	% RSD (N=5)	Resolution ± SD (N=5)
Indapamide	5.34 ± 0.0022	8361 ± 85	1.12 ± 0.026	0.4	-
Olmesartan medoxomil	6.83 ± 0.0031	8919 ± 120	1.09 ± 0.012	0.5	4.35± 0.05

Solution stability

Standard and sample solutions were kept at room temperature (25°C) for 24 hours and solution stability data after 0 hours, 8 hours and 24 hours were calculated. The change in % RSD was calculated. Standard solution and sample solution of concentration of Olmesartan medoxomil (200 μg/ml) and Indapamide (75 μg/ml) were taken to test solution stability. It was found that change in % RSD for standard and sample solution was not more than 2%. The results of standard and sample solution stability are summarized in Table 5.3 and Table 5.4, respectively.

TABLE 5.3: Standard solution stability data of RP-HPLC method for Olmesartan medoxomil and Indapamide

Standard solution stability				
Time (Hours)	Area		Difference (% RSD)	
	Olmesartan medoxomil	Indapamide	Olmesartan medoxomil	Indapamide
0	1254187	102536	==	==
8	1258362	102563	0.17	0.07
24	1299652	102942	1.50	0.17
% Mean RSD			0.44	0.09

TABLE 5.4: Sample solution stability data of RP-HPLC method for Olmesartan medoxomil and Indapamide

Sample solution stability				
Time (Hours)	Area		% difference	
	Olmesartan medoxomil	Indapamide	Olmesartan medoxomil	Indapamide
0	1248253	102489	==	==
8	1251321	102599	0.12	0.05
24	1292544	102234	1.48	0.13
% Mean RSD			0.54	0.08

Specificity [7-10]

Specificity of analytical method is ability to measure analyte accurately and specifically in presence of component that may be expected to be present in the sample matrix. Chromatograms of standard, sample, blank and placebo were overlaid for specificity check in sample. Peak purity of standard and sample were obtained using photodiode array detector. The results of peak purity showed peak purity index greater than 0.99. The results are summarized in Table 5.5.

TABLE 5.5 Peak purity data of RP-HPLC method for Olmesartan medoxomil and Indapamide

Sample	3 point purity	
	Olmesartan medoxomil	Indapamide
Standard solution	0.9999	0.9998
Test solution	0.9998	0.9999

Peak purity

Peak purity curve of Olmesartan medoxomil and Indapamide for standard and sample is shown in Figure 5.2 & 5.3.

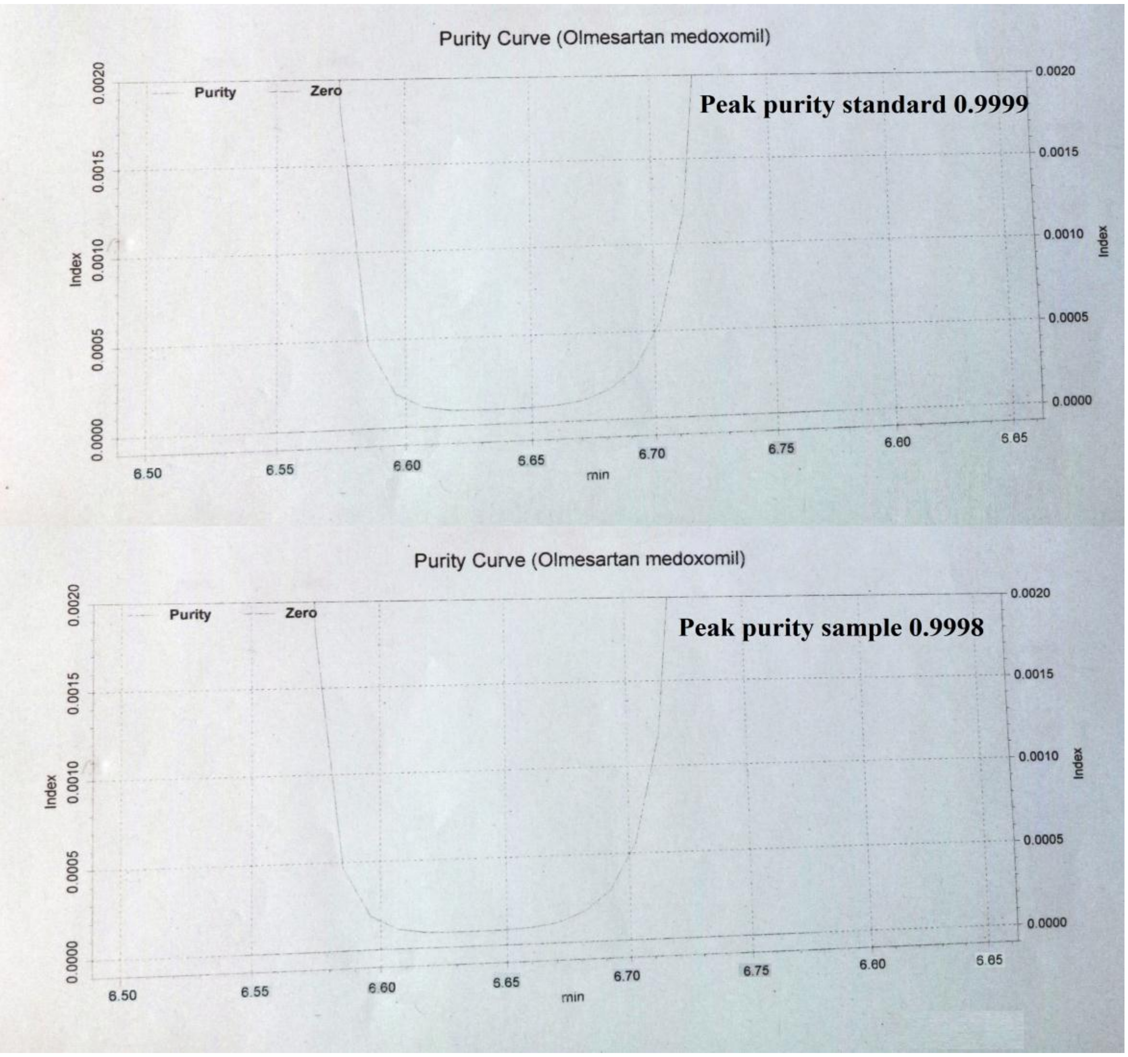

FIGURE 5.2: Peak purity curve of Olmesartan medoxomil

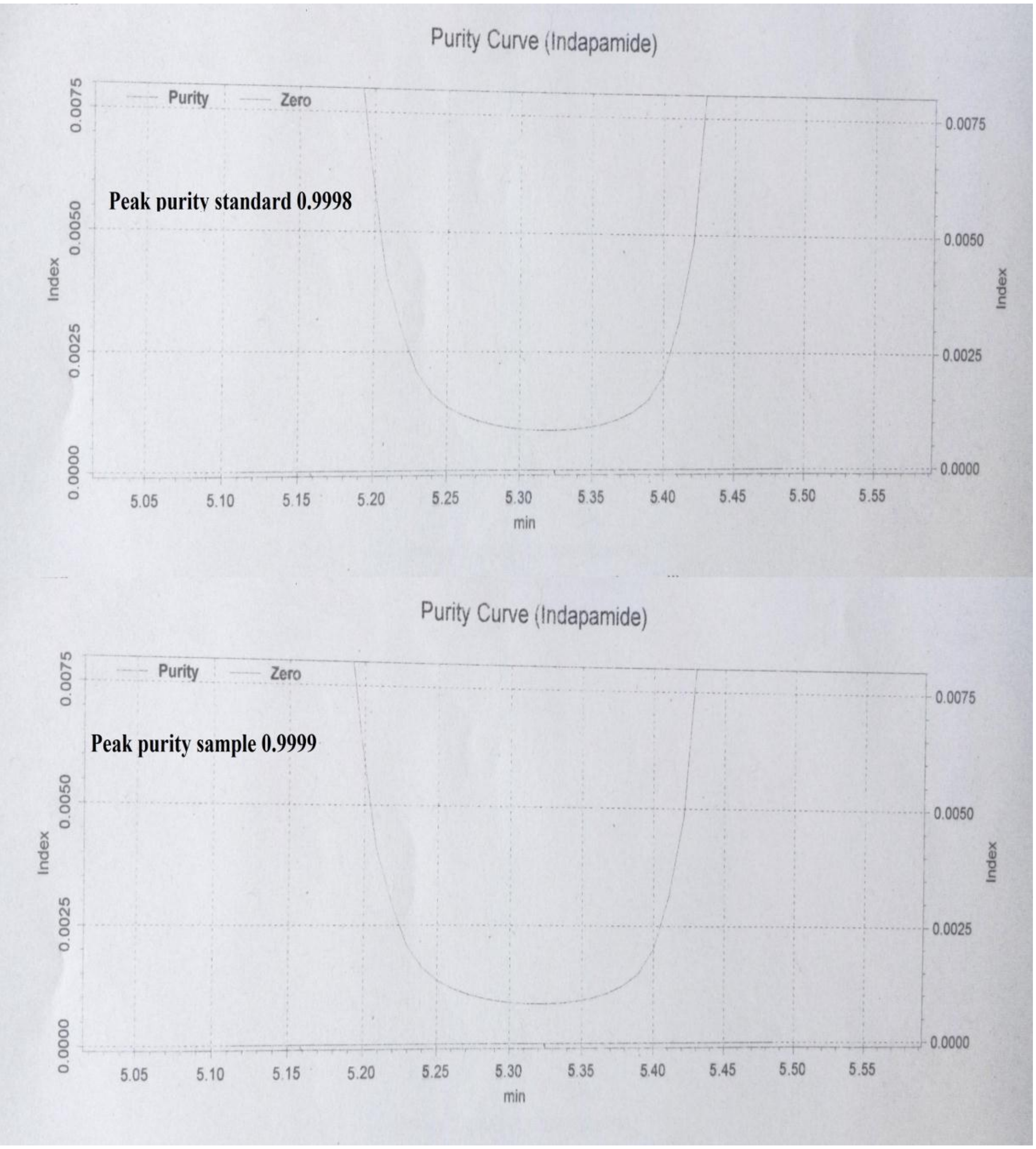

FIGURE 5.3: Peak purity curve of Indapamide

Linearity

To achieve linearity and range, stock solution containing Olmesartan medoxomil (1000 µg/ml) and Indapamide (75 µg/ml) were prepared. Olmesartan medoxomil and Indapamide stock solutions were diluted to yield solution in the concentration range of 50-300 µg mL^{-1} and

3.75-22.5 µg mL^{-1}, respectively. The solutions were analyzed by using HPLC. Overlaid chromatograms of linearity for both drugs are shown in Figure 5.4. Calibration curve for Olmesartan medoxomil and Indapamide are shown in Figure 5.5 and Figure 5.6, respectively. The results of linearity are presented in Table 5.6 and Table 5.7, respectively.

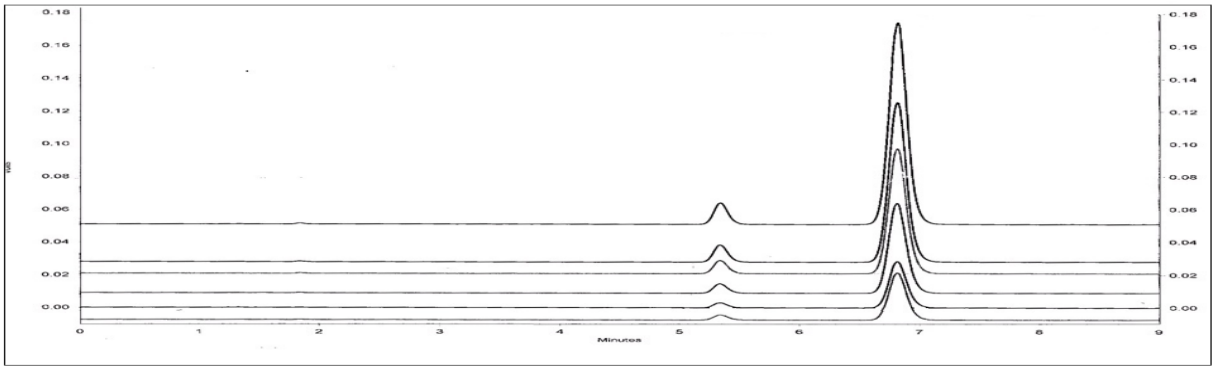

FIGURE 5.4: Overlaid linearity RP-HPLC chromatogram of Olmesartan medoxomil & Indapamide

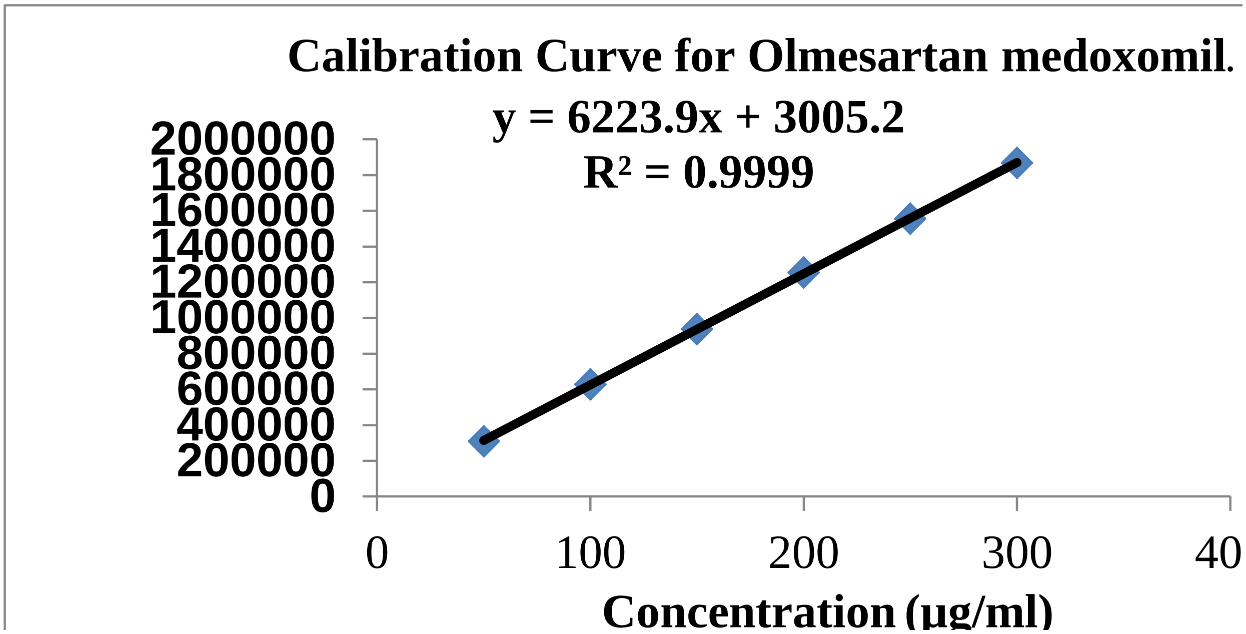

FIGURE 5.5: Calibration curve for Olmesartan medoxomil

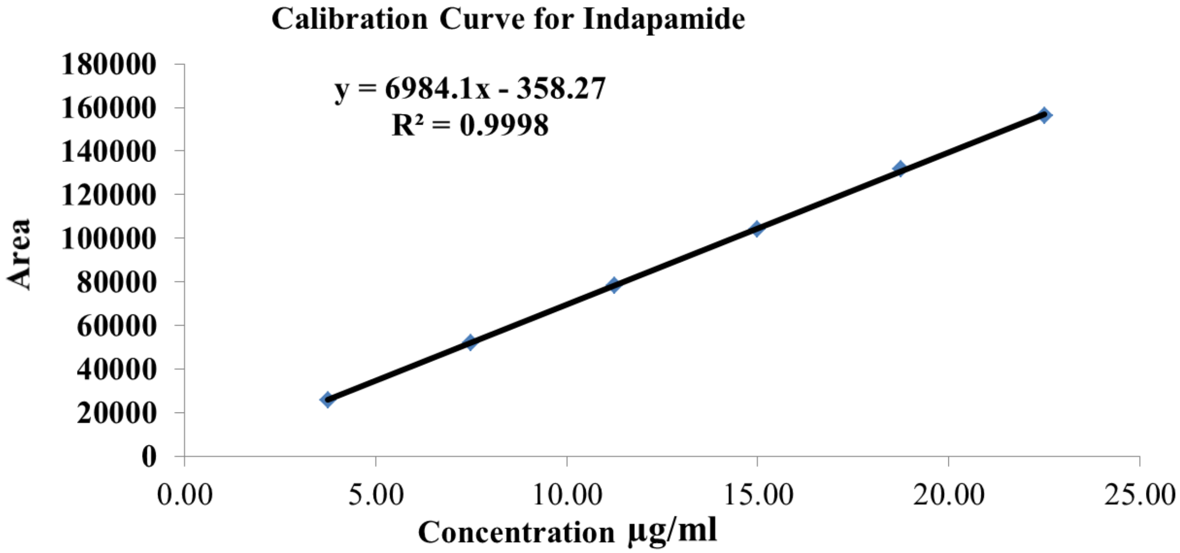

FIGURE 5.6: Calibration curve for Indapamide

Table 5.6: Linearity RP-HPLC data of Olmesartan medoxomil

Linearity data for Olmesartan medoxomil				
Linearity range	**Stock solution taken in ml**	**Diluted to volume ml**	**Final conc (µg/ml)**	**Area ±SD (N=3)**
25%	2.50ml	50 ml	50	309360 ± 2253
50%	5.00ml	50 ml	100	628320 ± 4046
75%	7.50ml	50 ml	150	937246 ± 8526
100%	10.0 ml	50 ml	200	1254142 ± 9246
125%	12.5 ml	50 ml	250	1556739 ±10523
150%	15.0 ml	50 ml	300	1867282 ± 11261

Table 5.7: Linearity RP-HPLC data for Indapamide

Linearity data for Indapamide				
Linearity range	**Stock solution taken in ml**	**Diluted to volume ml**	**Final conc (µg/ml)**	**Area ± SD (N=3)**
25%	2.50ml	50 ml	3.75	25853 ± 329
50%	5.00ml	50 ml	7.50	51919 ± 498
75%	7.50ml	50 ml	11.25	78245 ± 619
100%	10.0 ml	50 ml	15.00	103944 ± 946
125%	12.5 ml	50 ml	18.75	131723 ± 1562
150%	15.0 ml	50 ml	22.50	156163 ± 1956

Repeatability [11-14]

The method repeatability was done by preparing six different sample preparations by analyst. The results obtained were within 2% RSD. The results are presented in Table 5.8. Repeatability is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances. Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions like same reagents, equipments, settings and laboratory over a short interval of time. It is normally expected that at least six replicates should be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n value. The RSD values are important for showing degree of variation expected when the analytical procedure is repeated several time in a standard situation. Repeatability should be below 2% for bulk drugs and below 2% for assay in finished product.

Intermediate precision

Intermediate precision was determined between different analyst, instrument and column. The value of percentage RSD was below 2.0%, showed intermediate precision of developed analytical method. The results are presented in Table 5.8.

TABLE 5.8: Repeatability and intermediate precision RP-HPLC data for Olmesartan medoxomil and Indapamide

Repeatability									
Inj no.	**Sample wt (mg)**	**Olmesartan medoxomil (200 µg/ml)**				**Indapamide (75 µg/ml)**			
		Area	% Assay	% Mean	% RSD	Area	% Assay	% Mean	% RSD
1	5000.50	1255488	99.9	99.9	0.2	104118	101.1	101.2	0.3
2	5006.56	1254493	99.7			104472	101.3		
3	4985.15	1254035	100.1			104041	101.4		
4	5000.70	1253058	99.8			103971	101		
5	5009.52	1254753	99.7			103896	100.7		
6	4983.60	1254344	100.2			104417	101.7		
Intermediate precision									
1	5015.62	1258563	100.0	100.5	1.0	1002536	99.4	100.4	1.1
2	5010.53	1278564	101.7			1023252	101.5		
3	5032.65	1285642	101.8			1020999	100.9		
4	5008.32	1254624	99.8			1002543	99.5		
5	5014.15	1254856	99.7			1024108	101.6		
6	5003.5	1256347	100.0			1002563	99.6		

Accuracy

The difference between theoretical added amount and practically achieved amount is called accuracy of analytical method. Accuracy of method was performed by spiking API to placebo sample at specified levels. Accuracy was determined at three different level 50%, 100% and

150% of the target concentration in triplicate. The results are presented in Table 5.9 and Table 5.10.

TABLE 5.9: RP-HPLC data for accuracy of Olmesartan medoxomil

	Accuracy data of Olmesartan medoxomil							
Level	**Placebo (mg)**	**Conc µg/ml**	**Amount of drug added (mg)**	**Amount of drug recovered (mg)**	**Area**	**Recovery ± SD (%) (N=3)**	**Mean ± SD (%)**	**% RSD**
50%	4785.15	100 µg/ml	100.10	100 .09	627682	100.1±0.1	99.9±0.2	0.2
	4785.22		100.20	99.95	626788	99.9±0.1		
	4784.06		100.60	100.06	627451	99.8±0.1		
100%	4785.65	200 µg/ml	200.10	199.92	1253701	100.0±0.2	100.0 ± 0.1	0.1
	4785.70		200.30	199.97	1254001	100.0±0.1		
	4785.62		200.60	200.03	1254357	99.9±0.2		
150%	4785.12	300 µg/ml	300.30	297.77	1867325	99.3±0.2	99.3± 0.1	0.1
	4785.25		300.10	298.11	1869400	99.4±0.1		
	4785.68		300.50	297.72	1866986	99.2±0.1		

Table 5.10: RP-HPLC data for accuracy of Indapamide.

Level	Placebo (mg)	Conc µg/ml	Amount of drug added (mg)	Amount of drug recovered (mg)	Area	Recovery ± SD (%) (N=3)	Mean ± SD (N=3) (%)	% RSD
50%	4785.15	7.5 µg/ml	8.20	8.11	51961	99.0±0.2	101.1 ±0.9	0.9
	4785.22		8.10	8.14	52146	100.6±0.3		
	4784.06		8.00	8.04	51546	100.6±0.2		
100%	4785.65	15 µg/ml	16.50	16.50	104205	98.7±0.1	99.0 ± 0.7	1.0
	4785.70		16.70	16.70	105145	98.4±0.5		
	4785.62		16.30	16.30	104059	99.8±0.3		
150%	4785.12	22.5 µg/ml	24.00	24.32	155843	101.4±0.2	100.7 ±0.9	0.9
	4785.25		24.10	24.41	155832	101.0±0.1		
	4785.68		24.50	24.50	156409	99.7±0.1		

Robustness

Robustness of the method was carried out by deliberately made small changes in the flow rate, pH, and organic phase ratio and column oven temperature. Results were presented in Table 5.11

TABLE 5.11: RP-HPLC data of robustness for Olmesartan medoxomil and Indapamide

	Sys. suit.	Temp. -5°C	Temp. +5°C	Flow -10%	Flow +10%	Org. -2%	Org. +2%	pH = 3.2	pH = 2.8
Olmesartan medoxomil %RSD N=5	0.1	0.2	0.4	0.6	0.5	0.7	0.3	0.9	0.3
Mean %RSD	0.4								
Indapamide %RSD N=5	0.2	0.6	0.1	0.1	0.3	0.1	0.8	0.1	0.6
Mean %RSD	0.3								

Limit of detection and Limit of quantitation:

Limit of detection (LOD) and Limit of quantitation (LOQ) were calculated using the standard deviation of y intercept of calibration curve of linear lower concentrations (N) and slope (S) of the calibration curve. LOD and LOQ results are mentioned in Table 5.12.

LOD=3.3 x N/S

LOQ=10 x N/S

TABLE 5.12: LOD and LOQ values of Olmesartan medoxomil and Indapamide by RP-HPLC

Parameters	Olmesartan medoxomil	Indapamide
LOD	5 μg/ml	0.375 μg/ml
LOQ	16.5 μg/ml	1.2375 μg/ml

Results and discussion for HPLC method

Literature review reveals only individual methods for estimation of Olmesartan medoxomil and Indapamide individually but no methods were reported for simultaneous estimation of Olmesartan medoxomil and Indapamide was reported till date. So, method was developed method more superior to previously published methods of individual estimation of all drugs. The detection wavelength of 280 nm was chosen in order to achieve a good sensitivity for quantitative determination of Olmesartan medoxomil and Indapamide in solid dosage form. The retention time for Indapamide was 5.3 min and for Olmesartan medoxomil was 6.8 min.

TABLE 5.13: Summary of validation parameters of RP-HPLC method for simultaneous estimation of Olmesartan medoxomil and Indapamide

Parameter	**Acceptance criteria**	**Olmesartan Medoxomil**	**Indapamide**
Range of Linearity	Range following Beer Lambert's law	50 – 300 μg/ml	3.75 – 22.5 μg/ ml
Correlation coefficient	Correlation coefficient R> 0.995	0.9999	0.9999
Regression coefficient	$R^2 > 0.995$	0.9999	0.9998
LOD	S/N > 2 or 3	5.0000 μg/ml	0.3750 μg/ml
LOQ	S/N > 10	16.5000 μg/ml	1.2375 μg/ml
Repeatability	RSD < 2%	0.2%	0.3%
Intermediate precision	RSD < 2%	1.0%	1.1%
Accuracy	Recovery 98% - 102%	99.3% to 100.0%	99.0% to 100.7%
Specificity	1) No interference from blank, placebo	No interference	No interference
Solution Stability	> 12 hour	Stable up to 24 hr %RSD = 0.7%	Stable up to 24 hr %RSD =0.9%
Robustness **Flow rate (+ & -),** **Buffer pH (+ & -),** **Column temperature(+ & -),** **& Organic ratio (+ & -), in mobile phase**	RSD NMT 2% in modified condition	Complies % RSD 0.5% & 0.6% % RSD 0.9% & 0.3% % RSD 0.4% & 0.2 % % RSD 0.3 % & 0.7%	Complies % RSD 0.3% & 0.1% % RSD 0.1% & 0.6% % RSD 0.1% & 0.6 % % RSD 0.8 % & 0.1%

5.2 UV spectroscopic method for Olmesartan medoxomil and Indapamide

METHOD OF ANALYSIS [15-18]

Diluent: methanol

Wavelength: 240 nm and 256 nm at UV Spectrophotometer

Proper wavelength selection for estimation of both drugs depends on nature of drugs and their solubility. Many trials for solubility of both drugs in different diluents like acetonitrile, water, water: acetonitrile (50:50), ethanol, isopropanol, methanol etc. were conducted and methanol as diluent was finalized for method. Method employs solving of simultaneous equations based on the measurement of absorbance at two wavelengths 240 nm and 256 nm which are the λmax values of Olmesartan medoxomil and Indapamide in methanol. Spiking solution concentration is subtracted from the total concentration of Indapamide in sample for assay for actual concentration determination of Indapamide. Known amount of Indapamide solution is spiked to sample in order to get proper absorbance of Indapamide. Calculation for both drugs are done as per below formula.

$$\text{At } \lambda_1 \qquad A_1 = a X_1 b\, Cx + aY_1 b\, Cy \text{ ---------------------- } (1)$$

$$\text{At } \lambda_2 \qquad A_2 = a X_2 b\, Cx + aY_2 b\, Cy \text{----------------------- } (2)$$

For measurements in 1 cm cells b=1

Rearrange eq. (2)

$$Cy = \frac{A_2 - a X_2 b\, Cx}{aY_2}$$

Substituting for Cy in eq. (1) and rearranging

$$Cx = \frac{A_2\, aY_1 - A_1\, aY_2}{a X_2\, aY_1 - a X_1 aY_2} \text{ --------------------------- } (3)$$

$$Cy = \frac{A_1\, a X_2 - A_2\, a X_1}{a X_2\, aY_1 - a X_1 aY_2} \text{ --------------------------- } (4)$$

A1= Absorption of mixture at 256 nm.

A2= Absorption of mixture at 240 nm.

ay1=Molar absorptivity of Indapamide at 256 nm.

ay2=Molar absorptivity of Indapamide at 240nm.

Cy=Concentration of Olmesartan medoxomil (μg/ml).

Cx=Concentration of Indapamide (μg/ml).

ax1=Molar absorptivity of Olmesartan medoxomil at 256 nm.

ax2=Molar absorptivity of Olmesartan medoxomil at 240nm.

Preparation of solutions

Preparation of Indapamide spiking solution

The spiking stock solution Indapamide (300 μg/ml) were prepared by weighing Indapamide 30 mg in 100 ml volumetric flask respectively. Add 60 ml diluent to it and sonicate well for 5 minutes and making volume up to mark with diluent. The final spiking standard concentration of Indapamide is 300 μg/ml.

Preparation of standard solution

The standard stock solution Olmesartan medoxomil (1000 μg/ml) and Indapamide (75 μg/ml) were prepared by weighing Olmesartan medoxomil 200 mg and Indapamide 15 mg in 200 ml volumetric flask, respectively. Add 120 ml diluent to it and sonicate well for 5 minutes and making volume up to mark with diluent. Then add 4 ml of standard stock solution & 4 ml of Indapamide spiking solution to 200 ml volumetric flask. Make up to mark with diluent to get standard concentration of Olmesartan medoxomil (20 μg/ml) and Indapamide (7.5 μg/ml), respectively.

Preparation of test solution

Accurately 20 intact tablets were weighed and average weight of tablet was calculated. Then tablets were finely crushed, powdered and sample powder about 5000 mg (Tablet powder Equivalent to 200 mg Olmesartan medoxomil and 15 mg Indapamide, i.e. 10 tablets powder) was transferred into 200 ml volumetric flask. Then add about 120.0 ml diluent was added and sonicate for 30 minutes with intermittent shaking. Then volume was made up to mark with diluent. Then 4 ml of sample stock solution was added to 200 ml volumetric flask. Add 4 ml of Indapamide spiking solution to it. Make up sample solution with diluent to make final concentration of Olmesartan medoxomil (20 µg/ml) and Indapamide (7.5 µg/ml) respectively. The test solution was filtered through 0.45µ (PVDF Millipore Filter) and analyzed by using UV Spectrophotometer.

UV spectra for Olmesartan medoxomil (15 µg/ml) and Indapamide (15 µg/ml) for wavelength maxima selection is shown in (Figure 5.7) and (Figure 5.8). Absorbance at both wavelength 240 nm and 256 nm for both drugs are shown in Table 5.14

TABLE 5.14: Absorbance data of Olmesartan medoxomil and Indapamide at both wavelengths

Drug	Wavelength 240 nm	Wavelength 256 nm
Olmesartan medoxomil	0.7520	0.8309
Indapamide	1.4418	0.6408

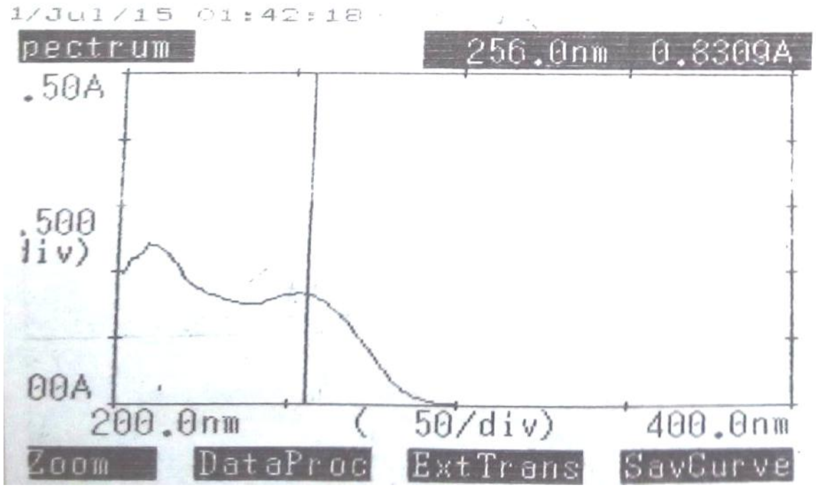

FIGURE 5.7: UV Spectrum of Olmesartan medoxomil (15 µg/ml).

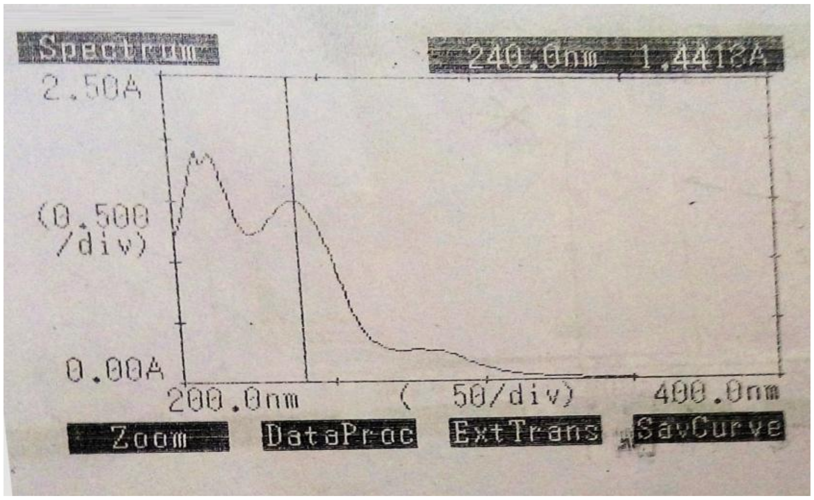

FIGURE 5.8: UV Spectrum of Indapamide (15 μg/ml).

Method validation [19-21]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1). The developed method validated with respect to parameters such as linearity, repeatability, accuracy, specificity, intermediate precision and solution stability.

Specificity

Diluent and placebo spectra are scanned over a range from 200 nm and 400 nm. Specificity of standard and sample are shown in Figure 5.9 and Figure 5.10

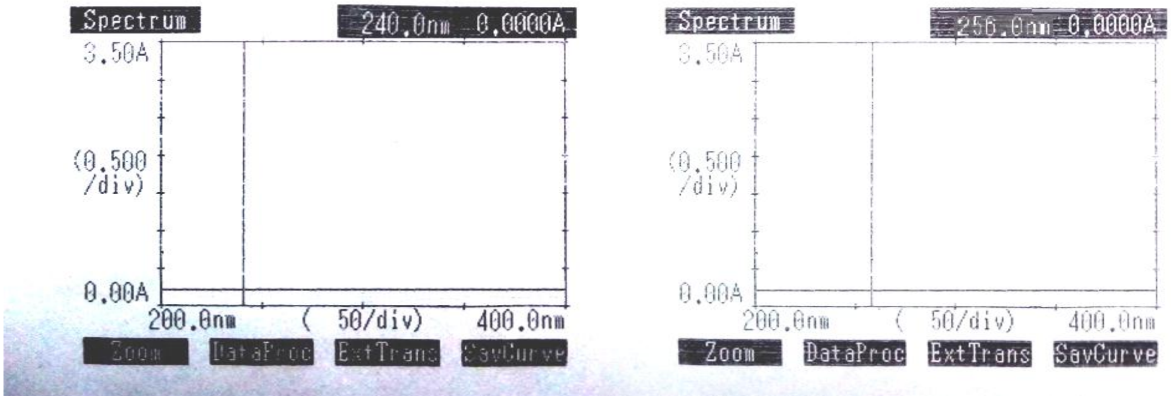

FIGURE 5.9: **Specificity study for diluent at both wavelengths**

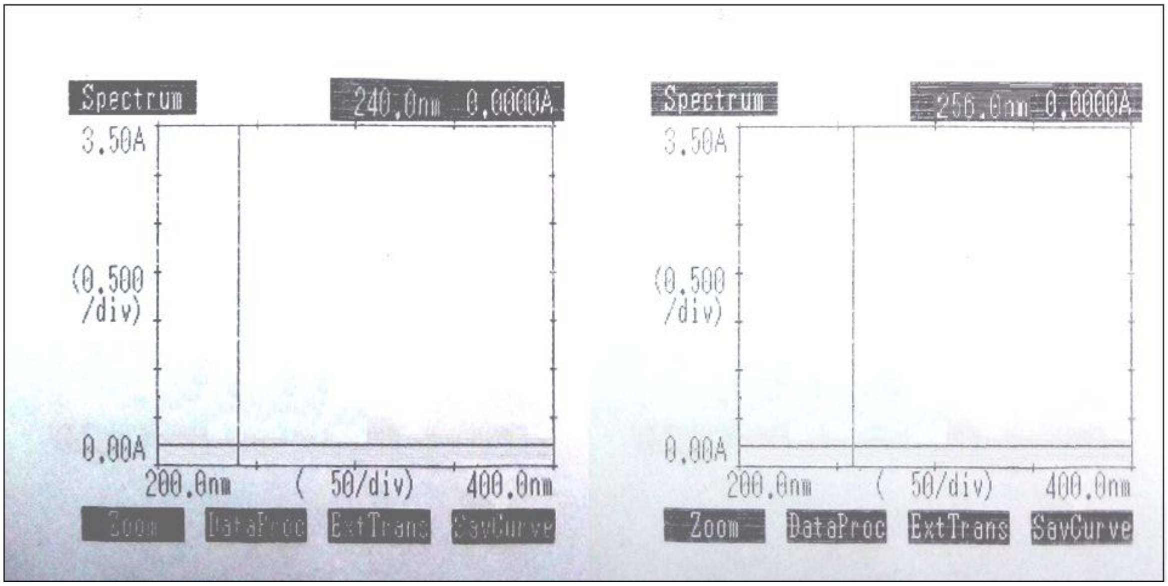

FIGURE 5.10: Specificity study for placebo at both wavelengths

Linearity

To achieve linearity and range, stock solution containing Olmesartan medoxomil (500 μg/ml) and Indapamide (500 μg/ml) were separately prepared. Olmesartan medoxomil and Indapamide Stock solutions were diluted to yield solutions in the concentration range of 5-25 μg mL^{-1} and 5-25 μg mL^{-1}, respectively. The solutions were analyzed by using UV. Overlain linearity spectra for Olmesartan medoxomil and Indapamide are shown in Figure 5.11 and Figure 5.12 Calibration curve for both the drugs are shown in Figure 5.13, Figure 5.14, Figure 5.15 and Figure 5.16. The results of linearity are presented in Table 5.15. Molar extinction coefficient for wavelength 240 nm and 256 nm for both drugs are obtained using data from linearity table.

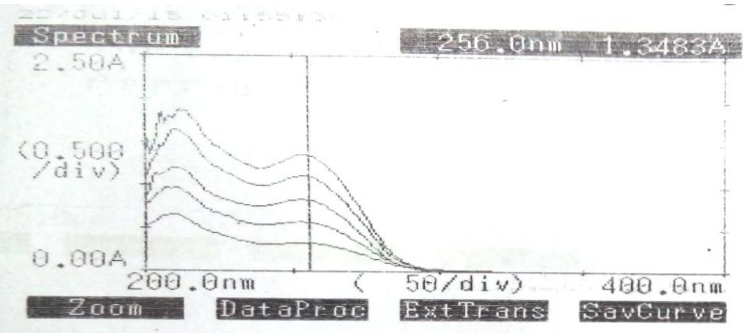

FIGURE 5.11: Overlain spectra of linearity for Olmesartan medoxomil

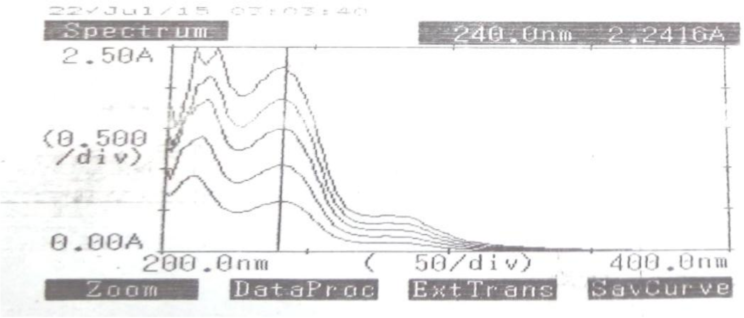

FIGURE 5.12: Overlain spectra of linearity for Indapamide

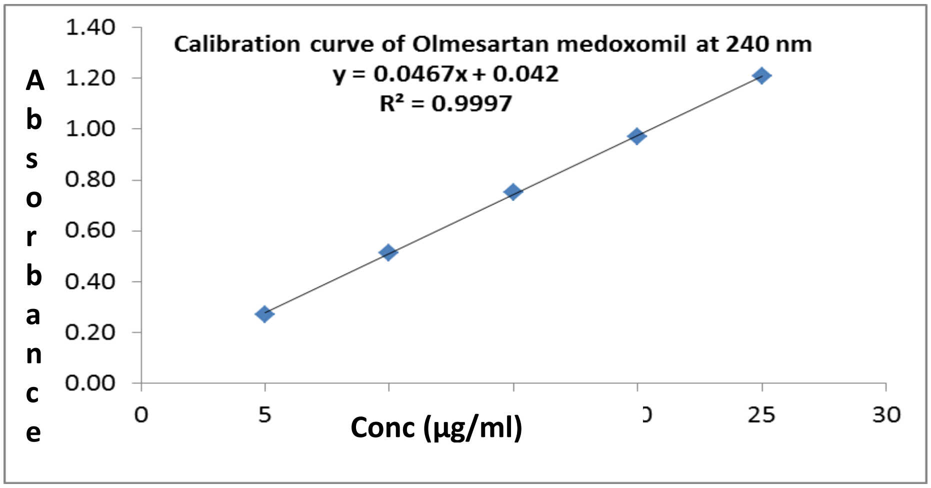

FIGURE 5.13: Calibration curve for Olmesartan medoxomil at 240 nm

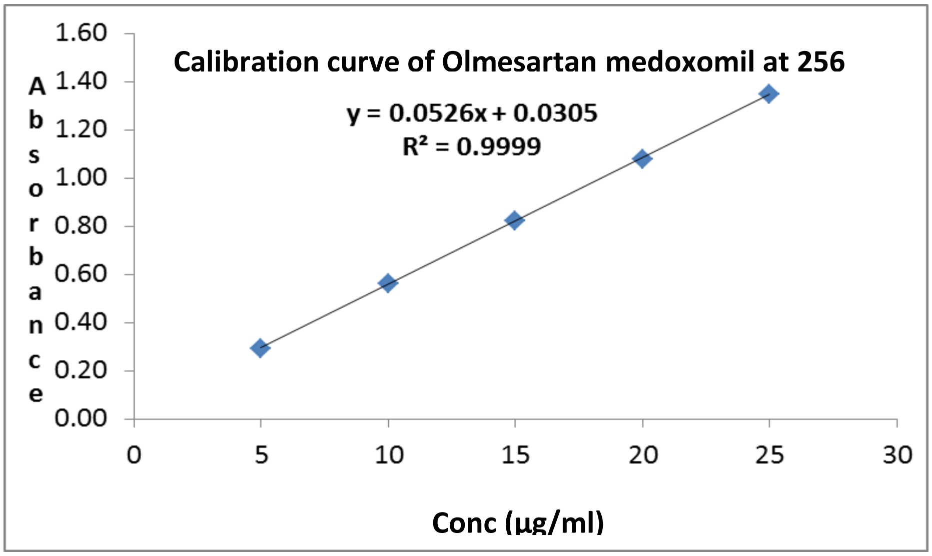

FIGURE 5.14: Calibration curve for Olmesartan medoxomil at 256 nm

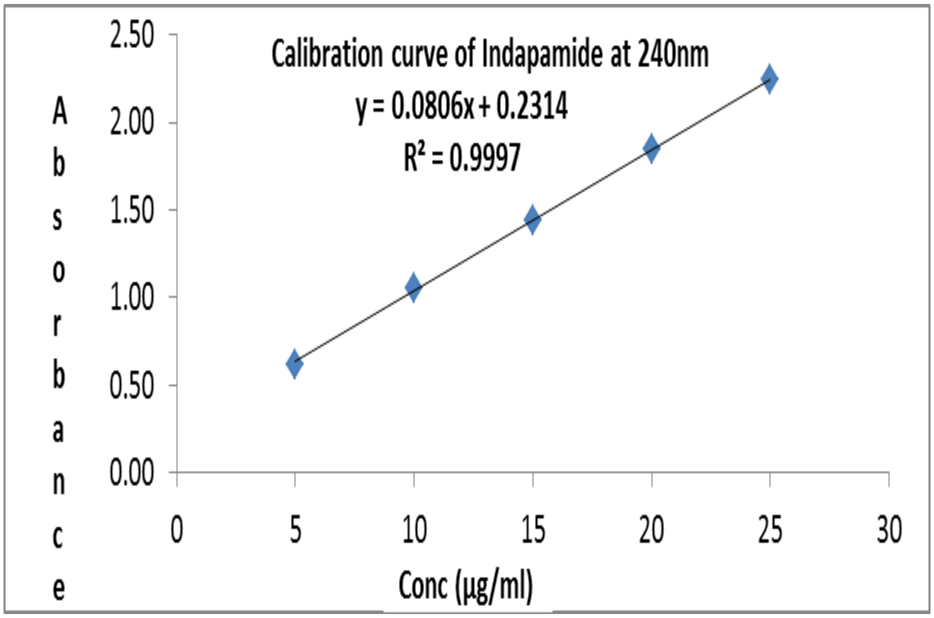

FIGURE 5.15: Calibration curve for Indapamide at 240 nm

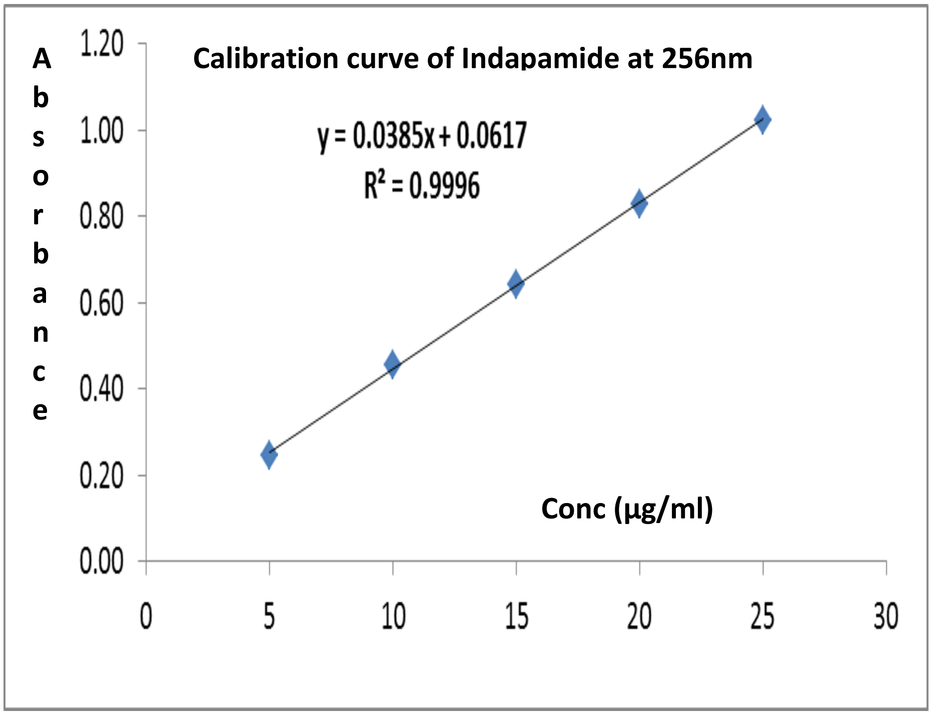

FIGURE 5.16: Calibration curve for Indapamide at 256 nm

TABLE 5.15: Linearity UV data of Olmesartan medoxomil and Indapamide.

Stock soln ml	**Diluted to volume ml**	**Final conc**	**Olmesartan medoxomil**	
		Olmesartan medoxomil µg/ml	**Absorbance ± SD (N=3)**	
			λ 240 nm	**λ 256 nm**
2.0	200	5.00	0.2699±0.0001	0.2912±0.0001
4.0	200	10.00	0.5115±0.0002	0.5598±0.0002
6.0	200	15.00	0.7520±0.0003	0.8222±0.0004
8.0	200	20.00	0.9689±0.0003	1.0747±0.0003
10.0	200	25.00	1.2079±0.0005	1.3483±0.0004
Stock soln ml	**Diluted to volume ml**	**Final conc**	**Indapamide**	
		Indapamide µg/ml	**Absorbance ± SD (N=3)**	
			λ 240 nm	**λ 256 nm**
2.0	200	5.00	0.6207±0.0001	0.2473 ±0.0001
4.0	200	10.00	1.0546±0.0004	0.4567 ± 0.0003
6.0	200	15.00	1.4418±0.0003	0.6408 ± 0.0002
8.0	200	20.00	1.8418±0.0005	0.8289 ± 0.0001
10.0	200	25.00	2.2416±0.0006	1.0244 ± 0.0003

Repeatability

The method repeatability was done by preparing six different sample preparations by one analyst. The results are presented in Table 5.16. The results obtained were within 2% RSD.

Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions like same reagents, equipments, settings and laboratory over a short interval of time. Repeatability should be below 2% for assay of bulk drugs and finished products.

TABLE 5.16: Repeatability data of Olmesartan medoxomil and Indapamide

Sr. No	% Assay repeatability					
	Olmesartan medoxomil (20 µg/ml)					
	Absorbance			**% Assay**	**% Mean**	**% RSD**
	wt (mg)	240 nm	256 nm			
1	5000.50	1.4309	1.7616	99.6	99.8	0.6
2	5009.56	1.4341	1.7514	99.8		
3	4998.51	1.4316	1.7495	98.9		
4	4997.89	1.4310	1.7529	99.9		
5	4999.71	1.4319	1.7610	99.7		
6	5002.63	1.4326	1.7618	100.7		
Indapamide (7.5µg/ml)						
Sr. No	**Absorbance**			**% Assay**	**% Mean**	**% RSD**
	wt (mg)	240 nm	256 nm			
1	5000.50	1.4309	1.7616	99.4	99.9	0.7
2	5009.56	1.4341	1.7514	101.4		
3	4998.51	1.4316	1.7495	99.9		
4	4997.89	1.4310	1.7529	99.5		
5	4999.71	1.4319	1.7610	99.6		
6	5002.63	1.4326	1.7618	99.8		

Intermediate precision

Intermediate precision test was determined between different analysts. The value of percentage RSD was below 2.0%, showed intermediate precision of developed analytical method. The results are presented in Table 5.17.

TABLE 5.17: Intermediate precision data for Olmesartan medoxomil and Indapamide.

Sr. No	**% Assay intermediate precision**					
	Olmesartan medoxomil (20 µg/ml)					
	Absorbance			**% Assay**	**% Mean**	**% RSD**
	wt (mg)	240 nm	256 nm			
1	5001.60	1.4347	1.7640	100.1	99.6	0.5
2	5000.61	1.4341	0.3739	99.3		
3	4999.81	1.4356	0.3754	98.9		
4	4994.29	1.4319	0.3782	100.4		
5	5000.71	1.4315	0.3759	99.7		
6	5005.63	1.4334	0.3761	99.6		
Indapamide (1.5µg/ml)						
Sr. No	**Absorbance**			**% Assay**	**% Mean**	**% RSD**
	Wt (mg)	240 nm	256 nm			
1	5001.60	1.4347	1.7640	100.1	99.9	0.6
2	5000.61	1.4341	0.3739	100.0		
3	4999.81	1.4356	0.3754	100.7		
4	4994.29	1.4319	0.3782	99.2		
5	5000.71	1.4315	0.3759	99.5		
6	5005.63	1.4334	0.3761	100.4		

Accuracy [22-23]

The difference between theoretical added sample amount to the placebo and practically achieved sample amount from placebo (after UV analysis) is called accuracy of analytical method. Accuracy was determined at three different level 80%, 100% and 120% of the target concentration in triplicate. The results are presented in Table 5.18 and Table 5.19.

Table 5.18: UV accuracy data of Olmesartan medoxomil

Accuracy data of Olmesartan medoxomil								
Level	**Placebo (mg)**	**Absorbance**		**Amount of drug added (µg/ml)**	**Amount of drug recovered (µg/ml)**	**Recovery ± SD (%) (N=3)**	**Mean ± SD (%)**	**% RSD**
		240 nm	**256 nm**					
80%	4785.62	1.1447	1.4092	16.05	16.10	100.3±0.1	99.8±0.5	0.5
	4786.52	1.1442	1.4085	16.12	16.01	99.3±0.2		
	4788.56	1.1438	1.4088	16.14	16.12	99.9±0.1		
100%	4785.42	1.4315	1.7608	20.15	20.10	99.7±0.2	99.9±0.5	0.4
	4785.19	1.4309	1.7616	20.18	20.25	100.4±0.3		
	4789.56	1.4326	1.7611	20.20	20.14	99.7±0.1		
120%	4786.19	1.7190	2.1096	24.20	24.10	99.5±0.2	99.7±1.6	1.5
	4789.12	1.7184	2.1124	24.60	24.15	98.2±0.1		
	4787.23	1.7170	2.1139	24.50	24.80	101.2±0.2		

Table 5.19: UV accuracy data for Indapamide

Accuracy data of Indapamide								
Level	**Placebo (mg)**	**Absorbance**		**Amount of drug added (µg/ml)**	**Amount of drug recovered (µg/ml)**	**Recovery ± SD (%) (N=3)**	**Mean ± SD (%)**	**% RSD**
		240 nm	**256 nm**					
80%	4785.62	1.1447	1.4092	6.08	6.10	100.3±0.2	100.9±0.7	0.7
	4786.52	1.1442	1.4085	6.10	6.20	101.6±0.1		
	4788.56	1.1438	1.4088	6.01	6.05	100.6±0.3		
100%	4785.42	1.4315	1.7608	7.50	7.60	101.3±0.2	99.8±1.5	1.4
	4785.19	1.4309	1.7616	7.52	7.41	98.5±0.1		
	4789.56	1.4326	1.7611	7.56	7.52	99.4±0.3		
120%	4786.19	1.7190	2.1096	9.04	9.01	99.6±0.4	100.8±1.0	1.0
	4789.12	1.7184	2.1124	9.08	9.20	101.3±0.2		
	4787.23	1.7170	2.1139	9.10	9.24	101.5±0.2		

Solution stability

The standard and sample solutions were found stable up to 24 hours at room temperature. The results are presented in Table 5.20 and Table 5.21

TABLE 5.20: Solution stability data for standard solution.

Time (Hr)	**Absorbance**		**% Difference**	
	240 nm	**256 nm**	**Olmesartan medoxomil**	**Indapamide**
0	1.4310	1.7618	==	==
8	1.4349	1.7684	0.26	0.19
24	1.4398	1.7784	0.47	0.31

TABLE 5.21: Solution stability data for sample solution.

Time (Hr)	**Absorbance**		**% Difference**	
	240 nm	**256 nm**	**Olmesartan medoxomil**	**Indapamide**
0	1.4308	1.7616	==	==
8	1.4328	1.7689	0.29	0.10
24	1.4346	1.7756	0.40	0.13

LOD (Limit of detection and LOQ (Limit of quantitation)

The results of LOD and LOQ are mentioned in Table 5.22

Table 5.22: LOD and LOQ data for sample solution.

Parameters	**Olmesartan medoxomil**	**Indapamide**
LOD	1.3670 μg/ml	0.5114 μg/ml
LOQ	4.5000 μg/ml	1.6875 μg/ml

Results and discussion for UV method

No interference in spectra of blank and placebo implies that method is specific for use. The linearity of method was investigated in range of 5-25 µg/ml for Olmesartan medoxomil and 5-25 µg/ml for Indapamide respectively. Repeatability and intermediate precision values for six replicate spectral analysis has % RSD less than 2% which implies method is precise and rugged. Accuracy of method was confirmed by doing a recovery study at three different spectral levels (n=3). The results of accuracy are in range of 98 % to 102% as per ICH norms. Low LOD and LOQ values show sensitivity of method. Solution stability for standard and sample are for 24 hours.

Table 5.23: Summary of validation parameters of UV method for simultaneous estimation of Olmesartan medoxomil and Indapamide

Parameters of validation	Acceptance criteria	Olmesartan medoxomil		Indapamide	
Range of Linearity	Follows Beer Lambert's law	5 – 25 µg/ml		5-25 µg/ ml	
Correlation coefficient	Correlation coefficient R > 0.990 or 0.995	240 nm	256 nm	240 nm	256 nm
		0.9998	0.9999	0.9998	0.9998
Regression coefficient	Correlation coefficient R^2 > 0.990 or 0.995	0.9997	0.9999	0.9997	0.9996
Regression equation	-	Y= 0.0467x+ 0.042	Y = 0.0526x + 0.0305	Y = 0.0806x + 0.2314	Y= 0.0385x + 0.0617
LOD	-	1.3670 µg/ml		0.5114 µg/ml	
LOQ	-	4.5000 µg/ml		1.6875 µg/ml	
Repeatability	RSD < 2%	0.60%		0.70%	
Intermediate precision	RSD < 2%	0.50%		0.60%	
Accuracy	98% - 102%	99.7% to 99.9%		99.8% to 100.9 %	
Specificity	No interference of blank, Placebo	No interference of blank, placebo		No interference of blank, placebo	
Solution Stability	> 12 hour	Stable for 24 hr %RSD = 0.40%		Stable for 24 hr %RSD =0.13%	

Table 5.24: Marketed formulation assay comparison for Olmesartan medoxomil and Indapamide

Assay by HPLC						
Brand name	**Label claim (mg)**		**Amount Found**		**% Assay ± % CV**	
	Olmesartan medoxomil	**Indapamide**	**Olmesartan medoxomil**	**Indapamide**	**Olmesartan medoxomil (N=3)**	**Indapamide (N=3)**
Olmy I	20	1.5	19.95	1.48	99.75 ± 0.81	98.66 ± 0.21
Olmesat ID	20	1.5	20.02	1.52	100.10 ± 0.29	99.33 ± 0.27

Assay by UV						
Brand name	**Label claim (mg)**		**Amount Found**		**% Assay ± % CV**	
	Olmesartan medoxomil	**Indapamide**	**Olmesartan medoxomil**	**Indapamide**	**Olmesartan medoxomil (N=3)**	**Indapamide (N=3)**
Olmy I	20	1.5	19.98	1.51	99.90 ± 0.75	100.77 ± 0.57
Olmesat ID	20	1.5	20.28	1.52	101.40 ± 0.35	101.33 ± 0.17

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CHAPTER-6

RP-HPLC and UV method for Chlorthalidone and Olmesartan medoxomil

6.1 RP-HPLC method for Chlorthalidone and Olmesartan medoxomil [1-4]

TABLE 6.1 Drug & marketed formulation details of Chlorthalidone and Olmesartan medoxomil

Drug	Company	
Chlorthalidone	Cadila Healthcare ltd, Ankleshwar, Gujarat	
Olmesartan Medoxomil	Cadila Healthcare ltd, Ankleshwar, Gujarat	
Drug name	**Marketed formulation-I**	**Marketed formulation-II**
Olmesartan medoxomil 20 mg and Chlorthalidone 12.5 mg tablets	Olmesar-CH tablets (Macleods, Vapi, India)	Olbet CT tablets (Zuventus Healthcare, Mumbai, India)

Experimental work

Chromatographic condition

Column : Inertsil ODS 3V C_{18} (4.6mm, i.d. x 250 mm) 5 µ

Detector : 270 nm

Injection Volume : 5 µl

Flow Rate : 1.0 mL min^{-1}

Temperature : 30º C

Run Time : 9 minutes

Mobile Phase : Sodium dihydrogen phosphate buffer (at pH 3): acetonitrile (48:52, v/v)

Diluent I : Methanol: Acetonitrile (50:50, v/v)

Diluent II : Mobile phase

Buffer preparation

Accurately weighed 2.8 g sodium dihydrogen phosphate was dissolved in to 1000 mL milli-Q water, than pH was adjusted to 3.0 with ortho-phosphoric acid.

Preparation of standard solution (Chlorthalidone (25 µg/ml) & Olmesartan medoxomil (40 µg/ml)

The standard stock solution Chlorthalidone (250 μg/ml) and Olmesartan medoxomil (400 μg/ml) were prepared by weighing Chlorthalidone 25 mg and Olmesartan medoxomil 40 mg in 100 ml volumetric flask respectively and volume was made up to mark with diluent I. Then 10 ml of standard stock solution was diluted to 100 ml with diluent II to make final standard concentration of Chlorthalidone (25 μg/ml) and Olmesartan medoxomil (40 μg/ml), respectively.

Preparation of test solution (Chlorthalidone 12.5 mg & Olmesartan medoxomil 20 mg and tablets)

Accurately, 20 intact tablets were weighed and average weight of tablet was calculated. Then tablets were finely crushed, powdered and sample powder about 1575 mg (Tablet powder equivalent to 62.5 mg Chlorthalidone and 100 mg Olmesartan medoxomil. i.e. five tablets' powder was added in flask for assay of tablets.) was transferred into 250 ml volumetric flask. Diluent I (150.0 ml) was added and sonicated for 40 minutes with intermittent shaking. Then volume was made up to mark with diluent I to make sample stock solution of concentration of Chlorthalidone (250 μg/ml) and Olmesartan medoxomil (400 μg/ml), respectively. Then 10 ml of standard stock solution was diluted to 100 ml with diluent II to make final standard concentration of Chlorthalidone (25 μg/ml) and Olmesartan medoxomil (40 μg/ml), respectively. The test solution was filtered through 0.45µ (PVDF Millipore filter) and analyzed by using HPLC system. Figure 6.1 shows optimized RP HPLC chromatogram of Olmesartan medoxomil and Chlorthalidone.

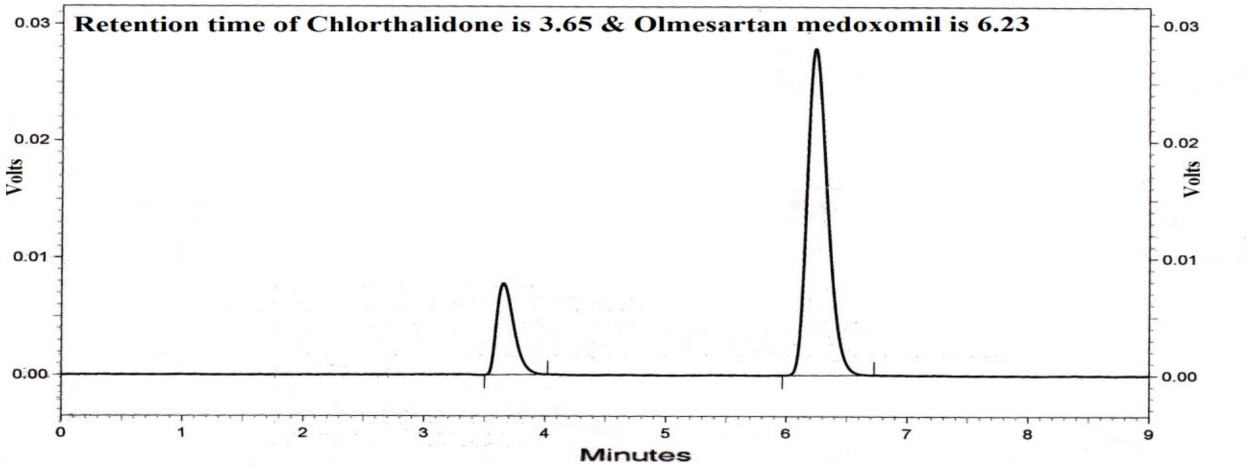

FIGURE 6.1: RP-HPLC chromatogram of Chlorthalidone & Olmesartan medoxomil

Method validation [5-7]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1). The developed method validated with respect to parameters such as system suitability, solution stability, specificity, linearity, repeatability, accuracy, intermediate precision, robustness, limit of detection and limit of quantitation.

System suitability and system precision

System suitability test were performed to check repeatability of system for particular analysis performed. The results for system suitability parameters were found satisfactory. The results of system suitability and system precision are presented in Table 6.2.

TABLE 6.2: System suitability parameters of RP-HPLC method for Chlorthalidone and Olmesartan medoxomil

Compound	Retention Time ± SD (min) (N=5)	Theoretical Plates ± SD (N=5)	Asymmetry± SD (N=5)	%RSD (N=5)	Resolution ± SD (N=5)
Chlorthalidone	3.65 ± 0.0031	3085± 152	1.38 ± 0.025	0.5	-
Olmesartan medoxomil	6.23 ± 0.0022	6521± 130	1.15 ± 0.019	0.4	8.91± 0.06

Solution stability

Standard and sample solutions were kept at room temperature (25°C) for 24 hours and solution stability data after 0 hours, 8 hours and 24 hours were calculated. The change in % RSD was calculated. Standard solution and sample solution of concentration of Olmesartan medoxomil (40 µg/ml) and Chlorthalidone (25 µg/ml) were taken to test solution stability. It was found that change in % RSD for standard and sample solution was not more than 2%. The results of solution stability are summarized in Table 6.3 and Table 6.4.

TABLE 6.3: Standard solution stability data of Chlorthalidone and Olmesartan medoxomil

Standard solution stability				
Time (Hrs)	**Area**		**Difference (% RSD)**	
	Chlorthalidone	**Olmesartan medoxomil**	**Chlorthalidone**	**Olmesartan medoxomil**
0	77871	328465	==	==
8	77451	332415	0.47	0.85
24	77046	334555	0.53	0.93
% Mean RSD			0.50	0.89

TABLE 6.4: Sample solution stability data of Chlorthalidone and Olmesartan medoxomil

Sample solution stability				
Time (Hrs)	**Area**		**Difference (% RSD)**	
	Chlorthalidone	**Olmesartan medoxomil**	**Chlorthalidone**	**Olmesartan medoxomil**
0	77845	329564	==	==
8	77564	331252	0.26	0.36
24	77049	332521	0.49	0.45
% Mean RSD			0.83	0.41

Specificity

Specificity of analytical method is ability to measure analyte accurately and specifically in presence of component that may be expected to be present in the sample matrix. Chromatograms of standard, sample, blank and placebo were overlaid for specificity check in sample. Peak purity of standard and sample were obtained using photodiode array detector. The results of peak purity showed peak purity index greater than 0.99. The results are summarized in Table 6.5.

TABLE 6.5 Peak purity data of RPHPLC method for **Chlorthalidone and Olmesartan medoxomil**

Sample	**3 point purity**	
	Chlorthalidone	**Olmesartan medoxomil**
Standard Solution	0.9999	0.9998
Test Solution	0.9998	0.9999

Peak Purity

Peak purity curve of Chlorthalidone and Olmesartan medoxomil for standard and sample is shown in Figure 6.2 & 6.3.

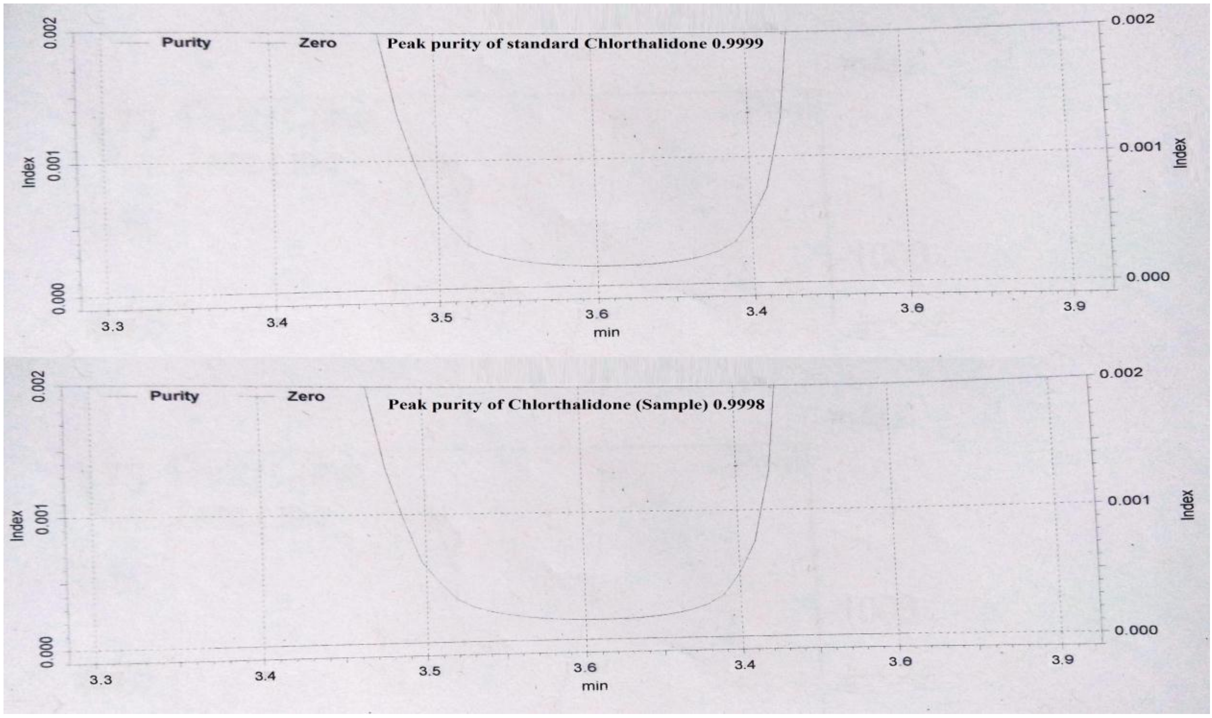

FIGURE 6.2: Peak purity curve of Chlorthalidone

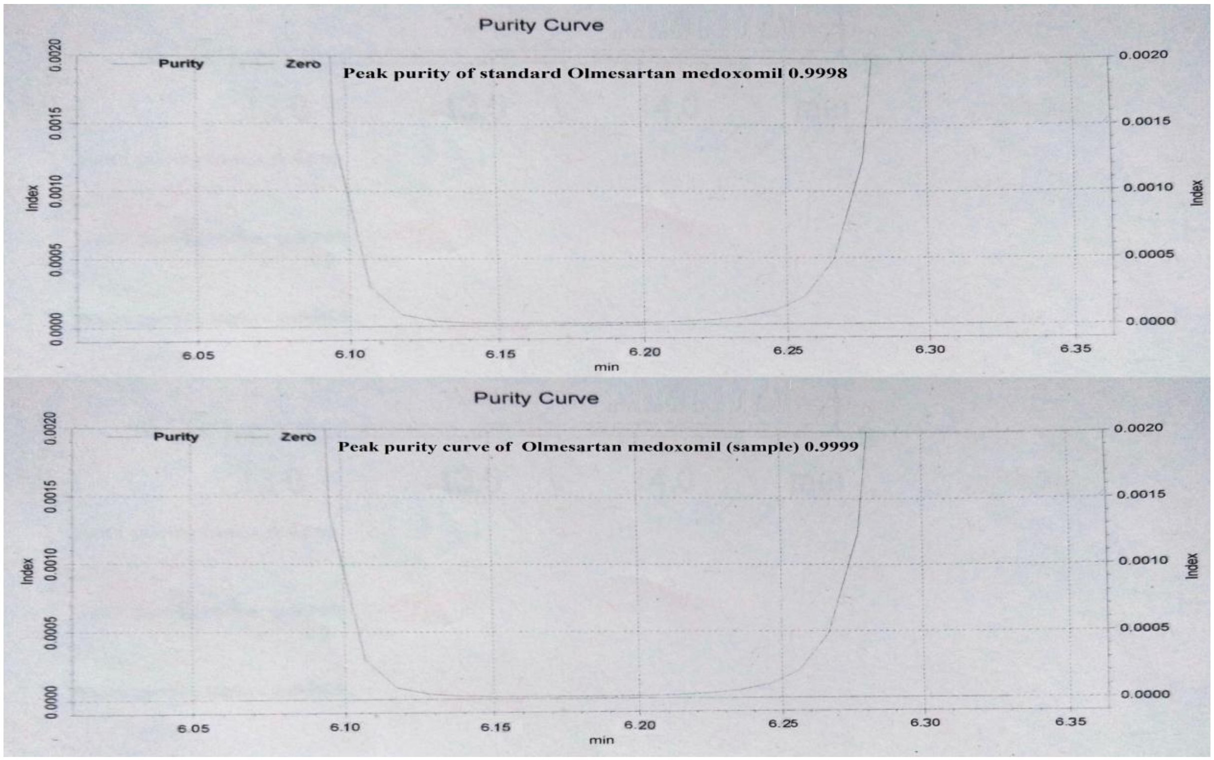

FIGURE 6.3: Peak purity curve of Olmesartan medoxomil

Linearity

To achieve linearity range, stock solution containing Chlorthalidone (250 µg/ml) and Olmesartan medoxomil (400 µg/ml) were prepared. Chlorthalidone and Olmesartan medoxomil Stock solutions were diluted to yield solutions in the concentration range of 6.25 - 37.50 µg mL^{-1} and 10-60 µg mL^{-1} , respectively. The solutions were analyzed by using HPLC. Calibration curve for both the drugs are shown in Figure 6.4 and Figure 6.5, respectively. The results of linearity are presented in Table 6.6 and Table 6.7, respectively.

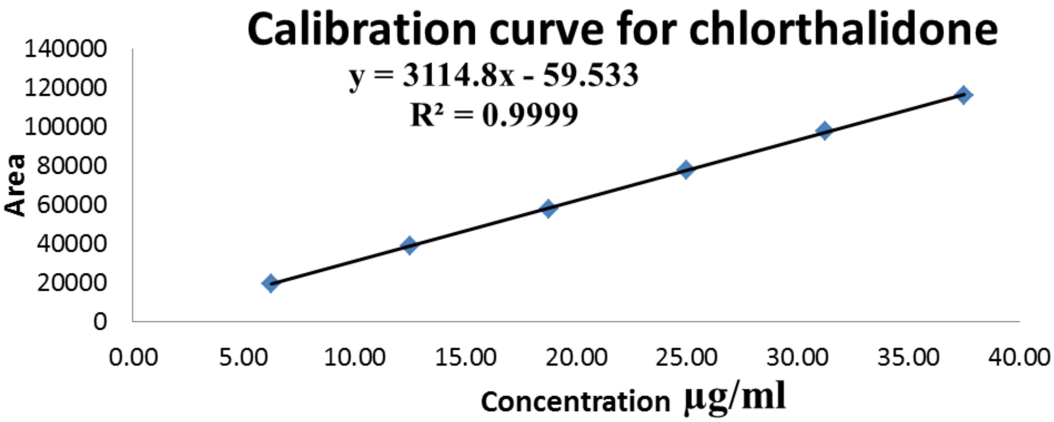

FIGURE 6.4: Calibration curve of Chlorthalidone by RP-HPLC analysis

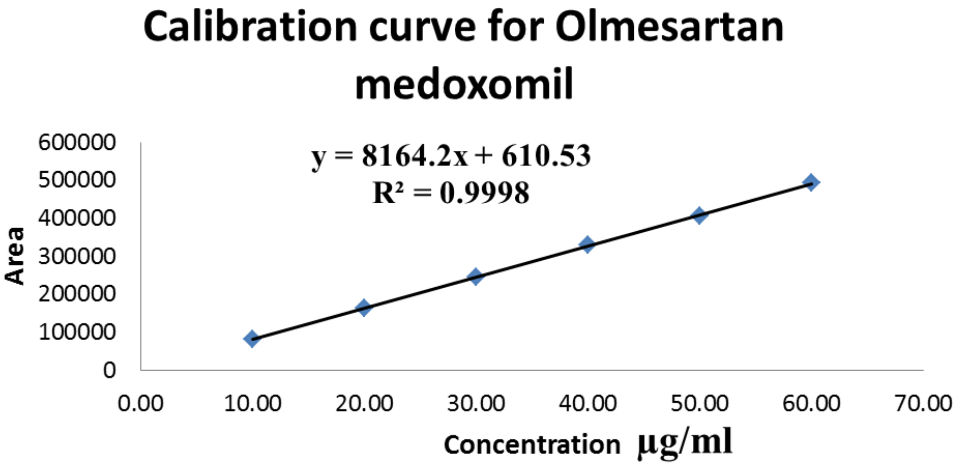

FIGURE 6.5: Calibration curve of Olmesartan medoxomil by RP-HPLC analysis

TABLE 6.6: Linearity data of Chlorthalidone by RP-HPLC analysis

Linearity data for Chlorthalidone				
Linearity range	**Stock solution taken in ml**	**Diluted to volume ml**	**Final conc (µg/ml)**	**Area ±SD (N=3)**
25%	2.50	100	6.25	19412±123
50%	5.00	100	12.50	38939±251
75%	7.50	100	18.75	57962±523
100%	10.0	100	25.00	77871±752
125%	12.5	100	31.25	98025±819
150%	15.0	100	37.50	116251±951

TABLE 6.7: Linearity data for Olmesartan medoxomil for RP-HPLC analysis

Linearity data for Olmesartan medoxomil				
Linearity range	**Stock solution taken in ml**	**Diluted to volume ml**	**Final conc (µg/ml)**	**Area ±SD (N=3)**
25%	2.5	100	10.00	82190±235
50%	5.00	100	20.00	164380±562
75%	7.50	100	30.00	245132±672
100%	10.0	100	40.00	328759±785
125%	12.5	100	50.00	405264±852
150%	15.0	100	60.00	492431±923

Repeatability [8-10]

The method repeatability was done by preparing six different sample preparations by one analyst. The results were presented in Table 6.8. The results obtained were within 2% RSD.

Intermediate precision

Intermediate precision test was determined between different analyst, instrument and column. The value of percentage RSD was below 2.0%, showed intermediate precision of developed analytical method. The results were presented in Table 6.8.

TABLE 6.8: Results of method repeatability and intermediate precision for Chlorthalidone and Olmesartan medoxomil

Repeatability									
Inj no.	**Sample wt (mg)**	**Chlorthalidone (25 µg/ml)**				**Olmesartan medoxomil (40 µg/ml)**			
		Area	% Assay	% Mean	% RSD	Area	% Assay	% Mean	% RSD
1	1575.5	77546	99.5	99.4	0.4	325641	98.9	99.2	0.6
2	1576.6	77652	99.6			328646	99.7		
3	1578.3	77845	99.7			329652	99.9		
4	1589.6	77561	98.7			329451	99.1		
5	1585.1	77854	99.3			328216	99.1		
6	1582.9	77985	99.6			325161	98.3		
Intermediate precision									
1	1572.5	78546	100	100.0	0.6	329564	100.2	99.6	0.6
2	1561.5	78892	101.1			326521	99.9		
3	1590.5	79485	100			329546	99.0		
4	1554.5	77492	99.8			325965	100.2		
5	1580.5	78485	99.2			328954	99.5		
6	1593.5	79546	99.7			329564	98.8		

Accuracy

The difference between theoretical added sample amount to the placebo and practically achieved sample amount from placebo (after HPLC analysis) is called accuracy of analytical method. Accuracy was determined at three different level 80%, 100% and 120% of the target concentration in triplicate. The results were presented in Table 6.9 and Table 6.10

TABLE 6.9: Results of accuracy of Chlorthalidone performed by RP-HPLC.

Accuracy data of Chlorthalidone								
Level	**Placebo (mg)**	**Conc (µg/ml)**	**Amount of drug added (mg)**	**Amount of drug recovered (mg)**	**Area**	**Recovery ± SD (%) (N=3)**	**Mean ± SD (%)**	**% RSD**
80%	1412.25	20 µg/ml	49.95	50.12	62512	100.3±0.1	100.1±0.2	0.2
	1412.62		50.15	50.14	62531	100.0±0.2		
	1412.14		50.25	50.23	62642	100.0±0.1		
100%	1412.87	25 µg/ml	62.44	62.42	77854	100.0±0.1	99.7±0.3	0.3
	1412.54		62.54	62.19	77562	99.4±0.1		
	1412.32		62.74	62.51	77965	99.6±0.1		
120%	1412.57	30 µg/ml	74.93	74.05	92351	98.8±0.1	99.5±0.5	0.6
	1412.46		75.12	74.99	93521	99.8±0.1		
	1412.35		75.52	75.54	94206	100.0±0.2		

RSD - Relative standard deviation

Table 6.10: Results of accuracy of Olmesartan medoxomil performed by RP-HPLC

Accuracy data of Olmesartan medoxomil								
Level	**Placebo (mg)**	**Conc (µg/ml)**	**Amount of drug added (mg)**	**Amount of drug recovered (mg)**	**Area**	**Recovery ± SD (%) (N=3)**	**Mean ± SD (%)**	**% RSD**
80%	1412.25	32 µg/ml	80.42	80.57	265135	100.2±0.1	100.0±0.5	0.5
	1412.62		80.22	80.57	265132	100.4±0.1		
	1412.14		80.42	79.99	263254	99.5±0.1		
100%	1412.87	40 µg/ml	99.90	100.34	330212	100.4±0.2	100.4±0.1	0.1
	1412.54		100.10	100.41	330451	100.3±0.2		
	1412.32		100.40	100.93	332152	100.5±0.1		
120%	1412.57	48 µg/ml	120.38	119.30	392611	99.1±0.1	99.1±0.6	0.7
	1412.46		120.68	118.88	391213	98.5±0.1		
	1412.35		120.48	120.22	395621	99.8±0.2		

Robustness

Robustness of the method was carried out by deliberately made small changes in the flow rate, pH, organic phase ratio and column oven temperature. Results were presented in Table 6.11

TABLE 6.11: RP-HPLC robustness data of Chlorthalidone & Olmesartan medoxomil

Name of Drug	**Sys Sui**	**Temp -5°C**	**Temp +5°C**	**Flow rate -10%**	**Flow rate +10 %**	**Org phase -2%**	**Org phase +2%**	**pH = 3.2**	**pH = 2.8**
Chlorthalidone %RSD (N=5)	0.6	0.5	0.7	0.5	0.6	0.6	0.1	0.4	0.8
Mean %RSD	0.5								
Olmesartan medoxomil	0.8	0.8	0.5	0.2	0.3	0.8	0.7	0.9	0.6
Mean %RSD	0.6								

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

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ) values, the blank sample was injected six times and the peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level while ten times the noise value gave the LOQ. The results of LOD and LOQ are mentioned in Table 6.12

TABLE 6.12: LOD and LOQ values of Chlorthalidone and Olmesartan medoxomil by RP-HPLC

Parameters	Chlorthalidone	Olmesartan medoxomil
LOD	0.1875 μg/ml	0.3 μg/ml
LOQ	0.6188 μg/ml	0.99 μg/ml

Result and discussion of HPLC method

The mobile phase was optimized after several trials with methanol, acetonitrile, and buffer solutions in various proportions and at different pH values. The values of relative standard deviation are satisfactorily low and recovery was close to 100% which indicated accuracy and reproducibility of methods. Literature review reveals only individual methods for estimation of Chlorthalidone and Olmesartan medoxomil, but no methods for simultaneous estimation of Chlorthalidone and Olmesartan medoxomil was reported till date. So, method much superior to previously published methods of individual estimation of all drugs was developed. The detection wavelength of 270 nm was chosen in order to achieve a good sensitivity for quantitative determination of Chlorthalidone and Olmesartan medoxomil in solid dosage form. Summary of validation parameters are shown in Table 6.13.

TABLE 6.13: Summary of validation parameters of RP-HPLC method for simultaneous estimation of Chlorthalidone and Olmesartan medoxomil

Parameter	Acceptance criteria	Chlorthalidone	Olmesartan medoxomil
Range of Linearity	Follows Beer Lambert's law	6.25 - 37.50 µg/ml	10-60 µg/ ml
Correlation Coefficient	Correlation coefficient R > 0.999 or 0.995	0.9998	0.9999
Regression Coefficient	Regression coefficient R^2 > 0.999 or 0.995	0.9999	0.9999
LOD	S/N > 2 or 3	0.1875 µg/ml	0.3000 µg/ml
LOQ	S/N > 10	0.6188 µg/ml	0.9900 µg/ml
Repeatability	RSD < 2%	0.4%	0.6%
Intermediate precision	RSD < 2%	0.6%	0.6%
Accuracy	98% - 102%	99.5% to 100.1%	99.1% to 100.4%
Specificity (Blank and placebo)	No interference	No interference of placebo, blank	No interference of placebo, blank
Solution Stability	> 12 hour	Stable for 24 hr %RSD = 0.50%	Stable for 24 hr %RSD =0.89%
Robustness **Flow rate (+ & -),** **Buffer pH (+ & -),** **Column temperature(+ & -),** **& Organic ratio (+ & -), in mobile phase**	RSD NMT 2% in modified condition	Complies % RSD 0.6% & 0.5% % RSD 0.4% & 0.8% % RSD 0.5% & 0.7 % % RSD 0.1 % & 0.6%	Complies % RSD 0.3% & 0.2% % RSD 0.9% & 0.6% % RSD 0.5% & 0.8 % % RSD 0.7 % & 0.8%

6.2 UV spectroscopic method for Chlorthalidone and Olmesartan medoxomil [11-13]

METHOD OF ANALYSIS

Diluent: Methanol

Wavelength: 220nm and 254 nm at UV Spectrophotometer

Proper wavelength selection for estimation of both drugs depends on nature of drugs and their solubility. Many trials for solubility of both drugs in different diluents like Acetonitrile, Water, and Water: Acetonitrile (50:50, v/v), ethanol, isopropanol, methanol etc were conducted and methanol as diluent was finalized for method. Method employs solving of simultaneous equations based on the measurement of absorbance at two wavelengths 220 nm and 254 nm which are the λmax values of Olmesartan medoxomil and Chlorthalidone in methanol. Calculation for both drugs are done as per below formula

At λ_1 $A_1 = a X_1 b\, Cx + aY_1 b\, Cy$ ---------------------- (1)

At λ_2 $A_2 = a X_2 b\, Cx + aY_2 b\, Cy$---------------------- (2)

For measurements in 1 cm cells b=1

Rearrange eq. (2)

$$Cy = \frac{A_2 - a X_2 b\, Cx}{aY_2}$$

Substituting for Cy in eq. (1) and rearranging

$$Cx = \frac{A_2\, aY_1 - A_1\, aY_2}{a X_2\, aY_1 - a X_1 aY_2} \quad \text{--------------------------}(3)$$

$$Cy = \frac{A_1\, a X_2 - A_2\, a X_1}{a X_2\, aY_1 - a X_1 aY_2} \quad \text{--------------------------}(4)$$

A1= Absorption of mixture at 220 nm.

A2= Absorption of mixture at 254 nm.

ay1=Molar absorptivity of Olmesartan medoxomil at 220 nm.

ay2=Molar absorptivity of Olmesartan medoxomil at 254nm.

Cy=Concentration of Olmesartan medoxomil (µg/ml).

Cx=Concentration of Chlorthalidone (µg/ml).

ax1=Molar absorptivity of Chlorthalidone at 220 nm.

ax2=Molar absorptivity of Chlorthalidone at 254nm.

Experimental work

Preparation of standard solution

The standard stock solution Chlorthalidone (250 μg/ml) and Olmesartan medoxomil (400 μg/ml) were prepared by weighing Chlorthalidone 40 mg and Olmesartan medoxomil 25 mg in 100 ml volumetric flask respectively and making volume up to mark with diluent. Then 10 ml of standard stock solution was diluted to 200 ml with diluent to make final standard concentration of Chlorthalidone (12.5 μg/ml) and Olmesartan medoxomil (20 μg/ml) respectively.

Preparation of test solution

Accurately 20 intact tablets were weighed and average weight of tablet was calculated. Then tablets were finely crushed, powdered and sample powder about 1600 mg (tablet powder equivalent to 62.5 mg Chlorthalidone and 100 mg Olmesartan medoxomil i.e. five tablets' powder was added in flask for assay of tablets.) was transferred into 250 ml volumetric flask. Then about 100.0 ml diluent was added and sonicated for 40 minutes with intermittent shaking. Then volume was made up to mark with diluent to make final standard concentration of Chlorthalidone (250 μg/ml) and Olmesartan medoxomil (400 μg/ml) respectively. The test solution was filtered through 0.45μ PVDF millipore filter. Then 10 ml of sample stock solution was diluted to 200 ml with diluent to make final standard concentration of Chlorthalidone (12.5 μg/ml) and Olmesartan medoxomil (20 μg/ml) respectively. The sample solution was analyzed by using UV spectrophotometer. A UV spectrum of Chlorthalidone and Olmesartan medoxomil for wavelength selection is shown in Figure 6.6. Absorbance at both wavelength 220 nm and 254 nm for both drugs are shown in Table 6.14

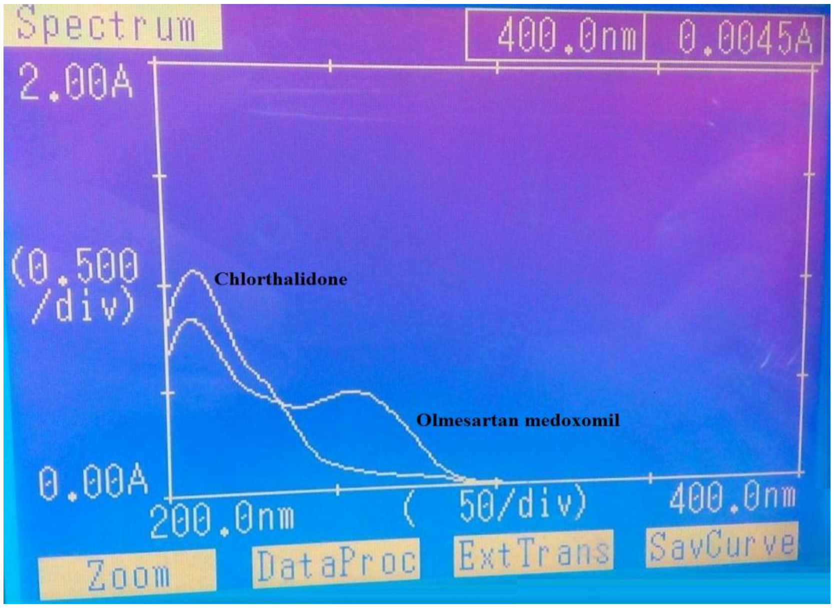

FIGURE 6.6: UV Spectra of Chlorthalidone (20 µg/ml) & Olmesartan medoxomil (15 µg/ml).

TABLE 6.14: Absorbance data of Chlorthalidone and Olmesartan medoxomil at both wavelengths.

Drug	wavelength 220 nm	wavelength 254 nm
Chlorthalidone (20 µg/ml)	1.2906	0.2202
Olmesartan medoxomil (15 µg/ml)	0.5665	0.4387

Method validation [14-18]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1). The developed method validated with respect to parameters such as linearity, repeatability, accuracy, specificity, intermediate precision and solution stability.

Specificity

Diluent and Placebo spectra are scanned over a range from 200 nm and 400 nm. Specificity of standard and sample are shown in Figure 6.7, Figure 6.8.

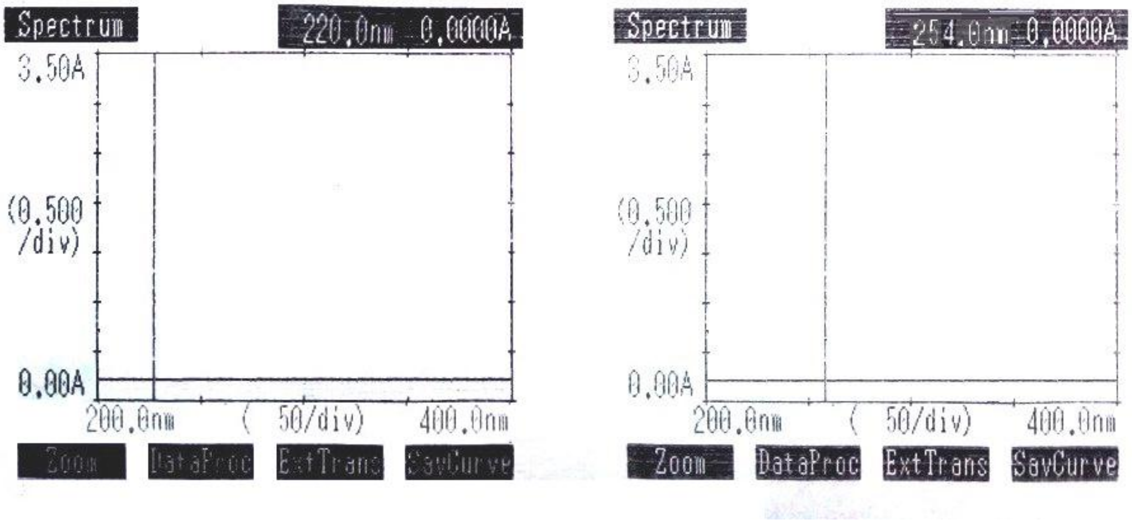

FIGURE 6.7: Specificity study for diluent at both wavelengths.

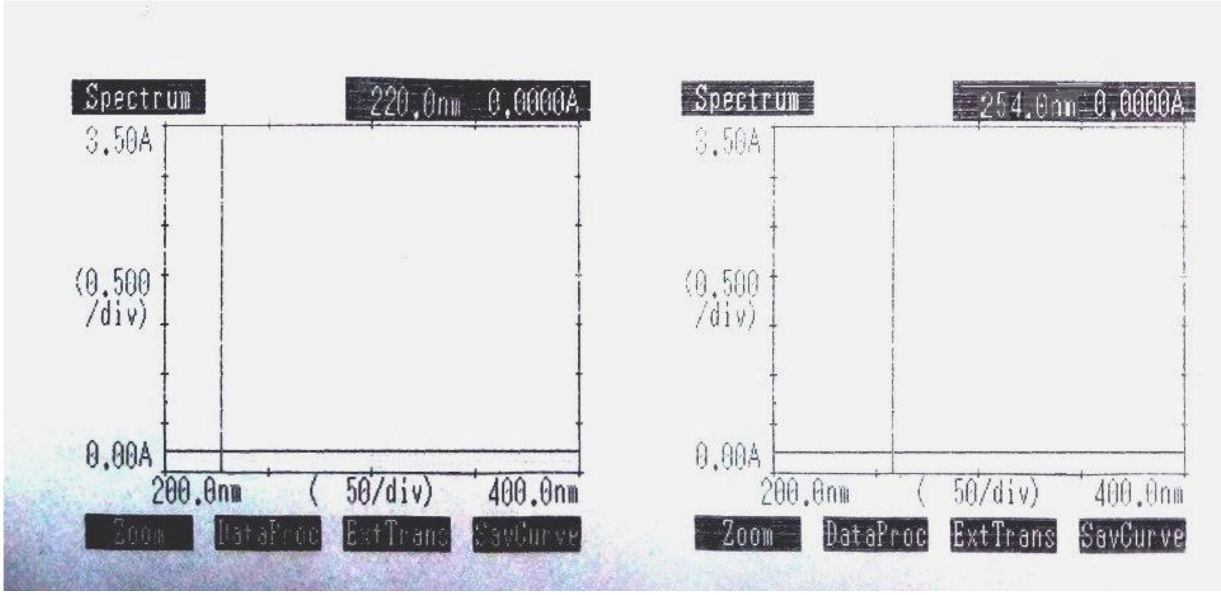

FIGURE 6.8: Specificity study for placebo at both wavelengths.

Linearity

For linearity and range, stock solution containing Olmesartan medoxomil (400 µg/ml) and Chlorthalidone (250 µg/ml) were separately prepared. Olmesartan medoxomil and Chlorthalidone stock solutions were diluted to yield solutions in the concentration range of 5-

25 µg mL^{-1} and 5-25 µg mL^{-1}, respectively. The solutions were analyzed by using UV. Overlain linearity spectra for Chlorthalidone and Olmesartan medoxomil are shown in Figure 6.9 and Figure 6.10 Calibration curve for both the drugs are shown in Figure 6.11, Figure 6.12, Figure 6.13 and Figure 6.14. The results of linearity are presented in Table 6.15 Molar extinction coefficient for wavelength 220 nm and 254 nm for both drugs are obtained using data from linearity table.

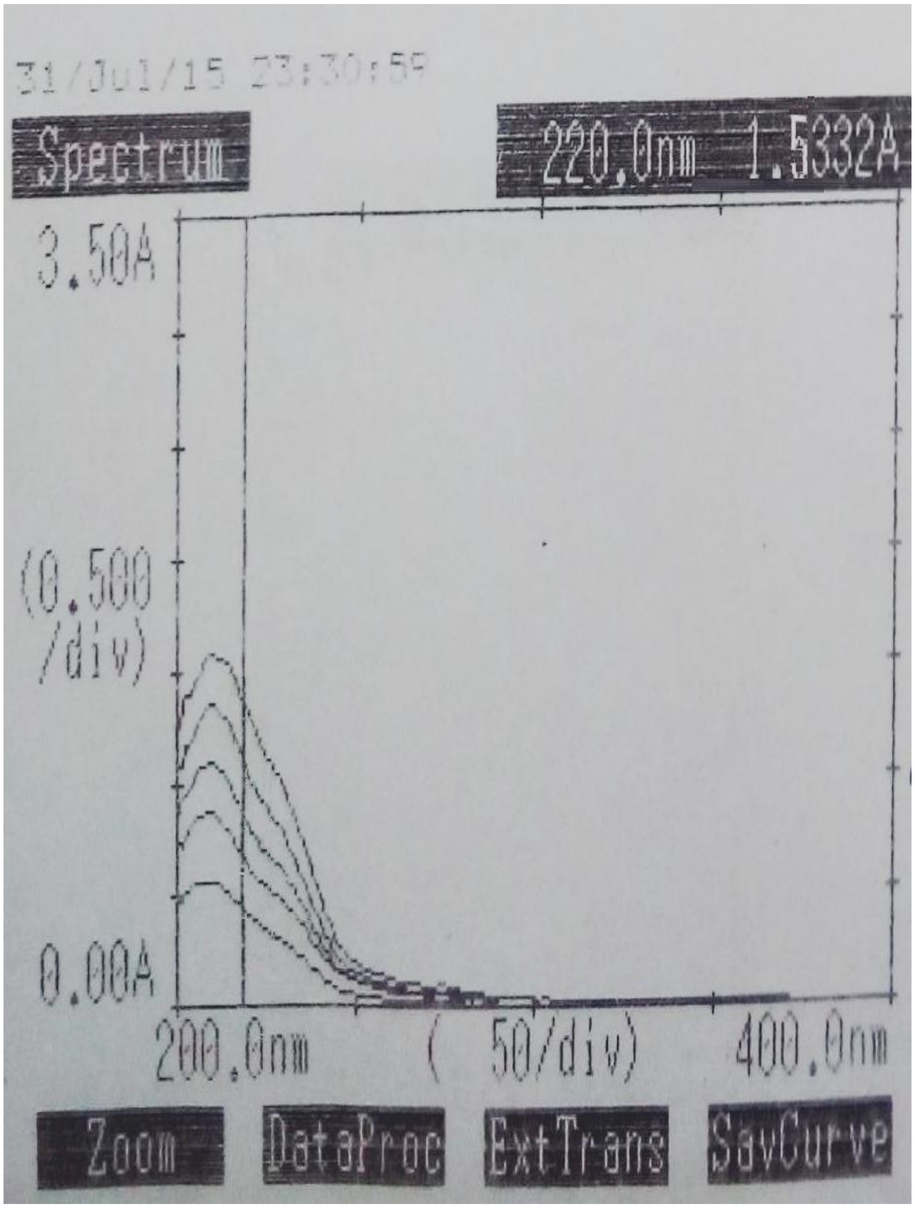

FIGURE 6.9: Overlain spectra of linearity for Chlorthalidone.

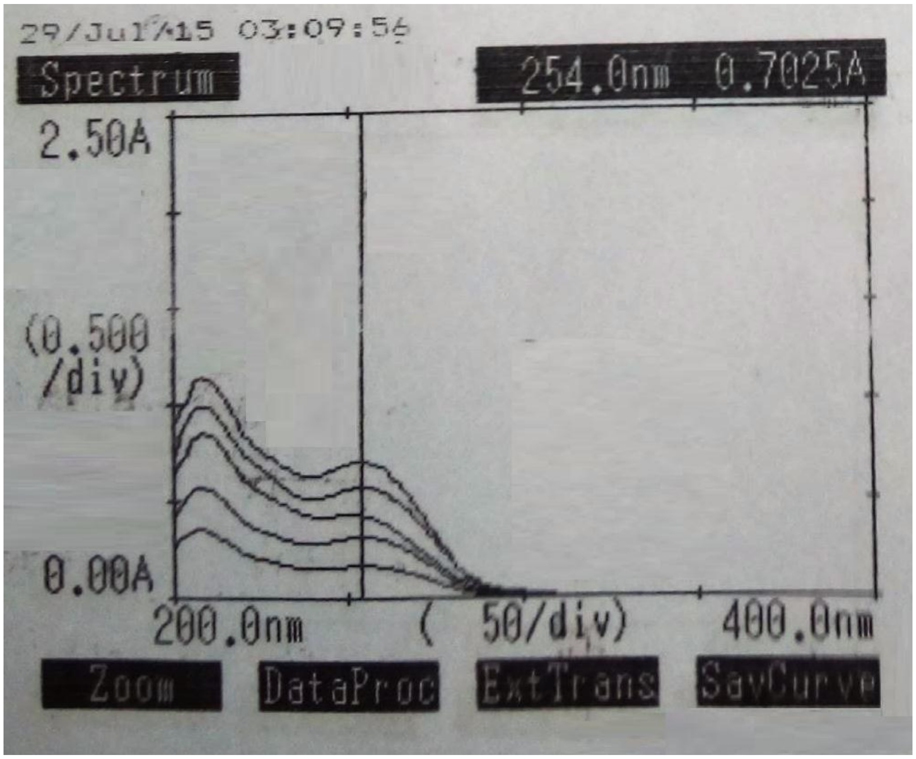

FIGURE 6.10: Overlain spectra of Linearity for Olmesartan medoxomil

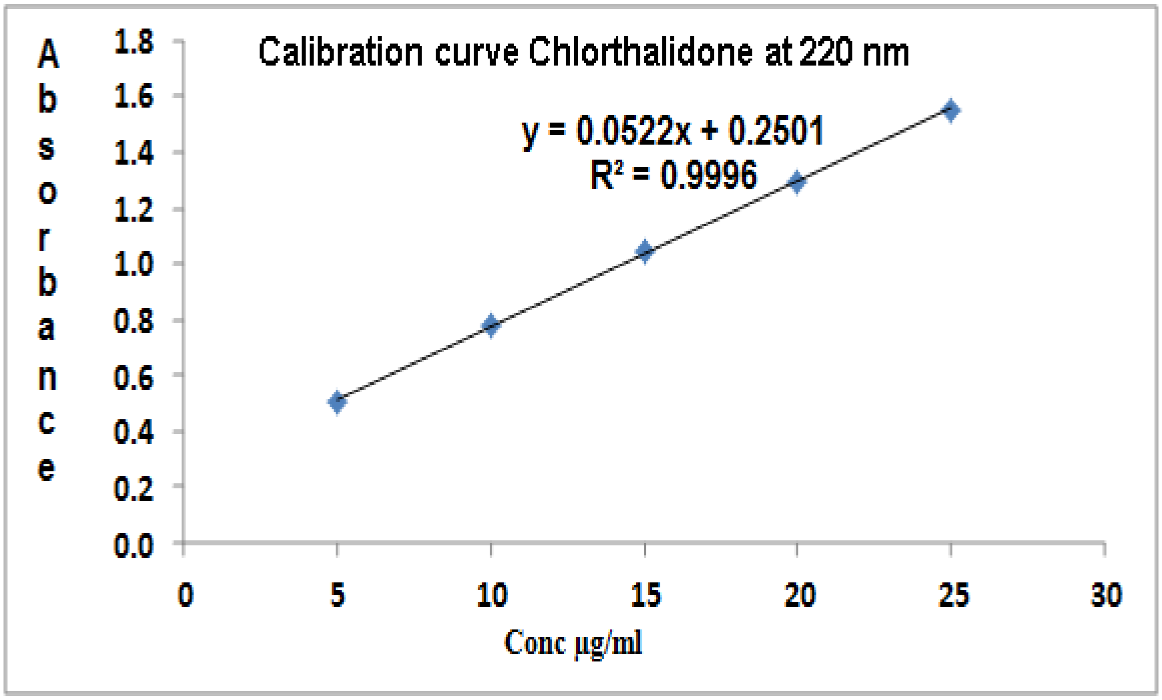

FIGURE 6.11: Calibration curve of Chlorthalidone at 220 nm

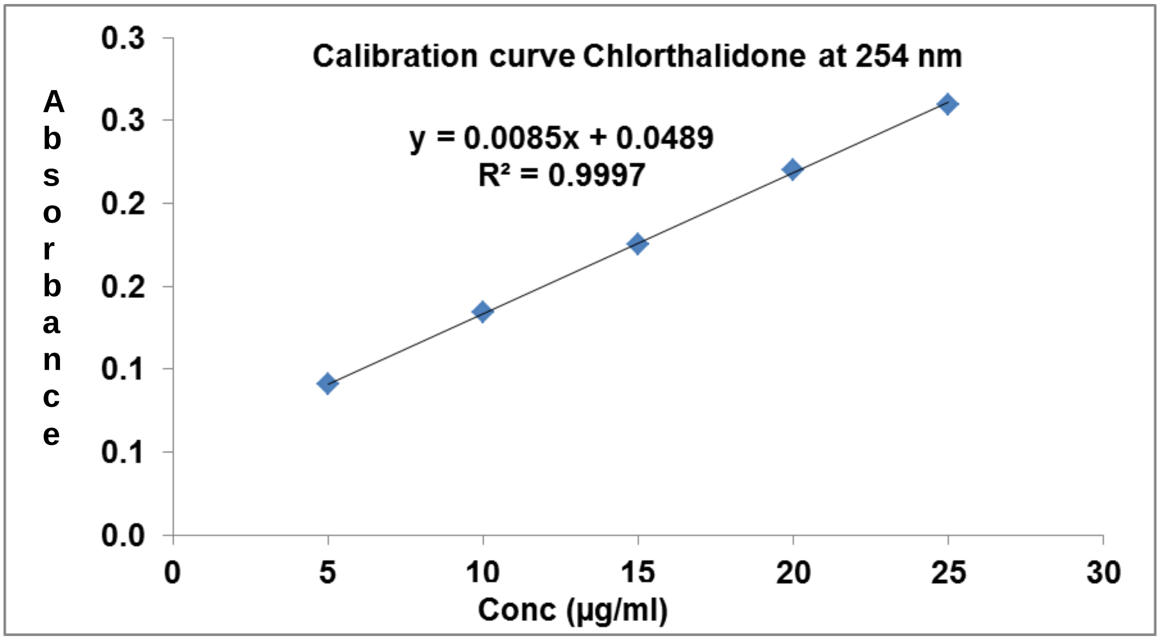

FIGURE 6.12: Calibration curve of Chlorthalidone at 254 nm

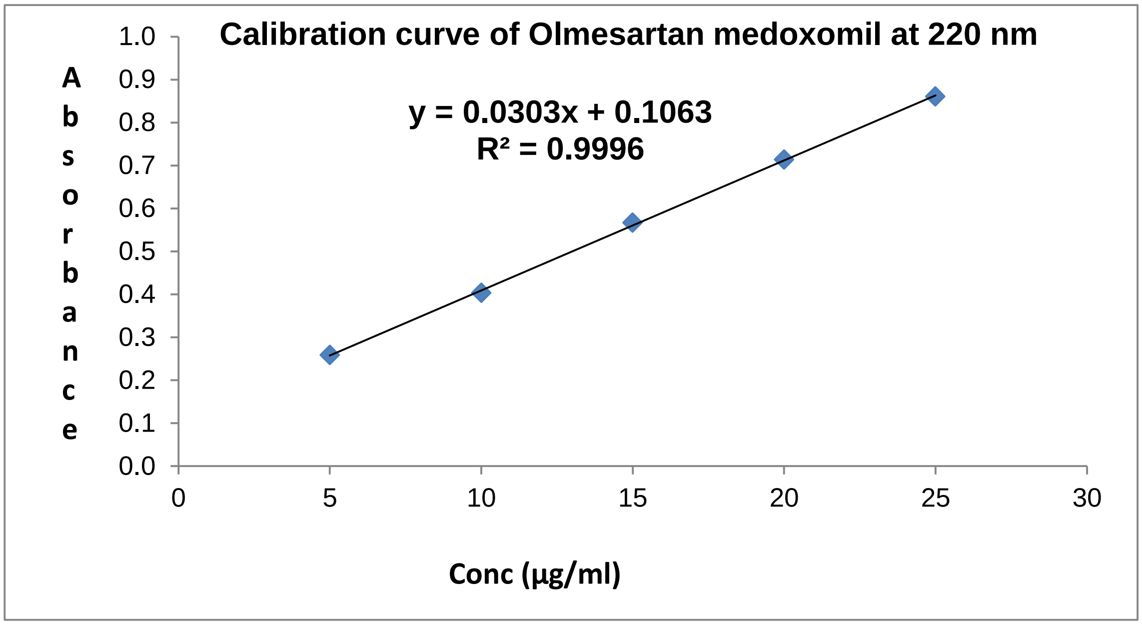

FIGURE 6.13: Calibration curve of Olmesartan medoxomil at 220 nm.

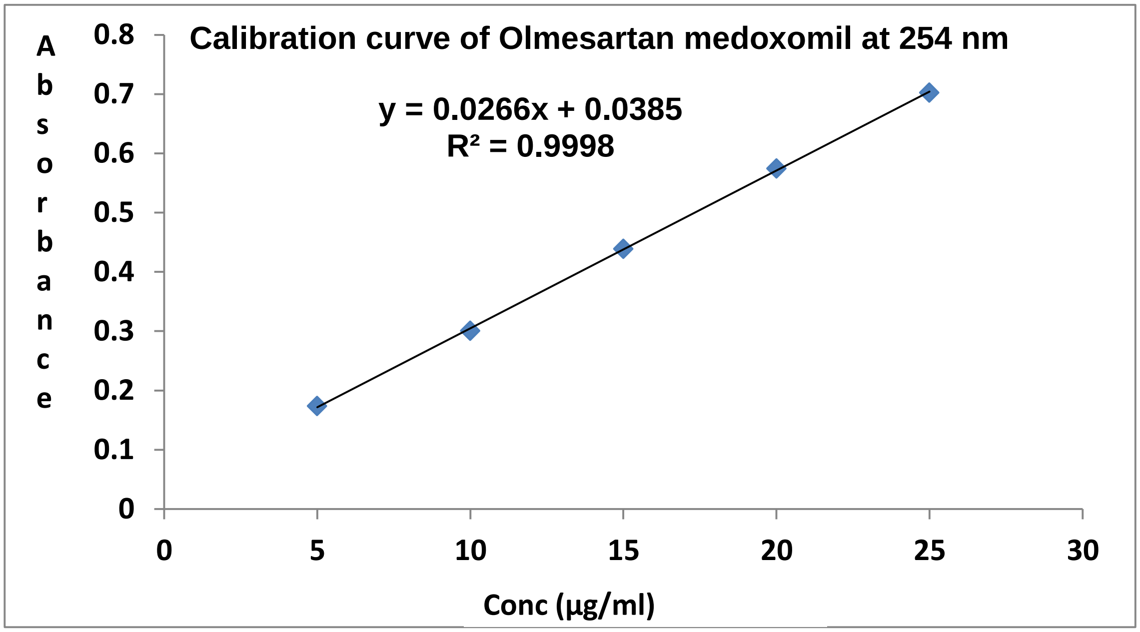

FIGURE 6.14: Calibration curve of Olmesartan medoxomil at 254 nm

TABLE 6.15: UV Linearity data of Chlorthalidone & Olmesartan medoxomil

Linearity stock soln ml	Linearity range	Diluted to volume ml	Final Conc Chlorthalidone (µg/ml)	Chlorthalidone Absorbance ± SD (N=3) λ 220 nm	Chlorthalidone Absorbance ± SD (N=3) λ 254 nm
5.0	50%	200	5.00	0.5005±0.0003	0.0909±0.0001
10.0	80%	200	10.00	0.7805±0.0004	0.1342±0.0001
15.0	100%	200	15.00	1.0421±0.0005	0.1752±0.0001
20.0	120%	200	20.00	1.2906±0.0006	0.2202±0.0002
25.0	150%	200	25.00	1.5332±0.0008	0.2598±0.0002

Linearity stock solution ml	**Linearity range**	**Diluted to volume ml**	**Final conc**	**Olmesartan medoxomil**	
			Olmesartan medoxomil (µg/ml)	**Absorbance ± SD (N=3)**	
				λ 220 nm	**λ 254 nm**
5.0	50%	200	5.00	0.2586±0.0002	0.1730±0.0001
10.0	80%	200	10.00	0.4034±0.0003	0.3004±0.0002
15.0	100%	200	15.00	0.5665±0.0004	0.4387±0.0003
20.0	120%	200	20.00	0.7139±0.0004	0.5742±0.0004
25.0	150%	200	25.00	0.8060±0.0006	0.7025±0.0005

Repeatability

The method repeatability was performed by preparing six different sample preparations by one analyst. The results are presented in Table 6.16. The results obtained were within 2% RSD.

Repeatability is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances. Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions like same reagents, equipments, settings and laboratory over a short interval of time. It is normally expected that at least six replicates should be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n value. The RSD values are important for showing degree of variation expected when the analytical procedure is repeated several time in a standard situation. Repeatability should be below 2% for bulk drugs and below 2% for assay in finished product.

TABLE 6.16: UV repeatability data of Chlorthalidone and Olmesartan medoxomil

Sr. No	**% Assay repeatability**					
	Chlorthalidone (12.5µg/ml)					
	Absorbance			**% Assay**	**% Mean**	**% RSD**
	Wt (mg)	220 nm	254nm			
1	1574.45	1.7299	0.7281	100.3	99.9	1.1
2	1575.90	1.7280	0.7274	100.5		
3	1580.95	1.7294	0.7284	101.6		
4	1572.63	1.7287	0.7286	98.9		
5	1571.92	1.7305	0.7289	98.7		
6	1575.45	1.7289	0.7279	99.6		
Olmesartan medoxomil (20 µg/ml)						
Sr. No	**Absorbance**			**% Assay**	**% Mean**	**% RSD**
	Wt (mg)	220 nm	254 nm			
1	1574.45	1.7299	0.7281	99.5	99.4	0.4
2	1575.90	1.7280	0.7274	99.8		
3	1580.95	1.7294	0.7284	99.1		
4	1572.63	1.7287	0.7286	100.2		
5	1571.92	1.7305	0.7289	99.1		
6	1575.45	1.7289	0.7279	99.5		

Intermediate precision

Intermediate precision test was determined between different analysts. The value of percentage RSD was below 2.0%, showed intermediate precision of developed analytical method. The results are presented in Table 6.17.

TABLE 6.17: UV intermediate precision data for Chlorthalidone and Olmesartan medoxomil.

Sr. No	% Assay intermediate precision					
	Chlorthalidone (12.5µg/ml)					
	Absorbance			**% Assay**	**% Mean**	**% RSD**
	wt (mg)	220 nm	254nm			
1	1581.45	1.7281	0.7289	101.4	100.3	1.4
2	1584.90	1.7291	0.7281	101.8		
3	1580.95	1.7295	0.7285	101.4		
4	1577.63	1.7294	0.7287	98.7		
5	1574.92	1.7298	0.7290	98.9		
6	1575.45	1.7290	0.7296	99.8		
Olmesartan medoxomil (20 µg/ml)						
Sr. No	**Absorbance**			**% Assay**	**% Mean**	**% RSD**
	wt (mg)	220 nm	254 nm			
1	1581.45	1.7281	0.7289	99.9	100.2	0.5
2	1584.90	1.7291	0.7281	100.8		
3	1580.95	1.7295	0.7285	100.4		
4	1577.63	1.7294	0.7287	99.7		
5	1574.92	1.7298	0.7290	100.9		
6	1575.45	1.7290	0.7296	99.7		

Accuracy

The difference between theoretical added sample amount to the placebo and practically achieved sample amount from placebo (after UV analysis) is called accuracy of analytical method. Accuracy was determined at three different level 80%, 100% and 120% of the target concentration in triplicate. The results are presented in Table 6.18 and Table 6.19

TABLE 6.18: UV accuracy data of Chlorthalidone

Accuracy data of Chlorthalidone								
Level	Placebo (mg)	Absorbance		Amount of drug added (μg/ml)	Amount of drug recovered (μg/ml)	Recovery ± SD (%) (N=3)	Mean ± SD (%)	% RSD
		220 nm	254 nm					
80%	1438.65	1.4200	0.5945	10.20	10.40	100.9±0.1	99.8±1.0	1.0
	1437.61	1.4228	0.5960	10.30	10.50	99.6±0.2		
	1438.14	1.4214	0.5952	10.50	10.40	99.0±0.1		
100%	1438.73	1.7299	0.7280	12.30	12.45	98.6±0.2	99.5±1.3	1.3
	1438.62	1.7284	0.7264	12.60	12.45	98.8±0.1		
	1439.14	1.7288	0.7284	12.42	12.52	101.0±0.1		
120%	1438.79	1.9444	0.8596	15.25	15.35	100.3±0.3	100.5±0.3	0.3
	1438.14	1.9462	0.8594	15.35	15.45	100.3±0.2		
	1438.84	1.9472	0.8591	15.22	15.25	100.9±0.1		

TABLE 6.19: UV accuracy data of Olmesartan medoxomil

Accuracy data of Olmesartan medoxomil								
Level	Placebo (mg)	Absorbance		Amount of drug added (μg/ml)	Amount of drug recovered (μg/ml)	Recovery ± SD (%) (N=3)	Mean ± SD (%)	% RSD
		220 nm	254 nm					
80%	1438.65	1.4200	0.5945	16.1	16.2	99.4±0.1	99.2±0.3	0.3
	1437.61	1.4228	0.5960	16.3	16.4	99.4±0.2		
	1438.14	1.4214	0.5952	16.2	16.4	98.8±0.1		
100%	1438.73	1.7299	0.7280	20.2	20.3	99.8±0.1	99.4±0.4	0.4
	1438.62	1.7284	0.7264	20.4	20.5	99.5±0.1		
	1439.14	1.7288	0.7284	20.1	20.3	99.0±0.1		

120%	1438.79	1.9444	0.8596	24.1	24.2	99.6±0.2	99.9±0.9	0.9
	1438.14	1.9462	0.8594	24.3	24.5	99.2±0.2		
	1438.84	1.9472	0.8591	24.5	24.3	100.9±0.1		

Solution stability [19]

The standard and sample solutions were found stable up to 24 hours at room temperature. The results are presented in Table 6.20

TABLE 6.20: UV solution stability data for standard solution and sample solution

Standard solution				
Time (Hr)	**Absorbance**		**% Difference**	
	220 nm	**254 nm**	**Chlorthalidone**	**Olmesartan medoxomil**
0	1.7285	0.7280	==	==
8	1.7254	0.7260	0.19	0.10
24	1.7237	0.7234	0.32	0.14
Sample solution				
Time (Hr)	**Absorbance**		**% Difference**	
	220 nm	**254 nm**	**Chlorthalidone**	**Olmesartan medoxomil**
0	1.7289	0.7287	==	==
8	1.7260	0.7266	0.20	0.12
24	1.7234	0.7245	0.29	0.16

LOD (LIMIT OF DETECTION AND LOQ (LIMIT OF QUANTITATION)

The results of LOD and LOQ are mentioned in Table 6.21

TABLE 6.21: LOD and LOQ data for sample solution

PARAMETERS	Chlorthalidone	Olmesartan medoxomil
LOD	0.875 μg/ml	1.4 μg/ml
LOQ	2.8875 μg/ml	4.62 μg/ml

TABLE 6.22: Summary of validation parameters of UV spectrophotometric method for simultaneous estimation of Chlorthalidone and Olmesartan medoxomil

Parameters of validation	Acceptance criteria	Chlorthalidone		Olmesartan medoxomil	
Range of Linearity	Follows Beer Lambert's law	5 – 25 µg/ ml		5-25 µg/ ml	
Correlation Coefficient	Correlation coefficient R > 0.990 or 0.995	220 nm	254 nm	220 nm	254 nm
		0.9997	0.9998	0.9997	0.9999
Regression equation	-	Y= 0. 522x+ 0.2501	Y= 0.0085x + 0.0489	Y = 0 0303x + 0.1063	Y= 0.0266x + 0.0385
Regression coefficient	Regression coefficient R^2>0.990 or 0.995	0.9996	0.9997	0.9996	0.9998
LOD	-	0.875 µg/ml		1.4 µg/ml	
LOQ	-	2.8875 µg/ml		4.62 µg/ml	
Repeatability	RSD < 2%	1.1		0.4%	
Intermediate precision	RSD < 2%	1.4%		0.5%	
Accuracy	98- 102%	99.5% to 100.5%		99.2% to 99.9	
specificity	No interference of blank, Placebo	Complies		Complies	
Solution Stability	12 hour	Stable for 24 hr %RSD = 0.25%		Stable for 24 hr %RSD =0.16%	

TABLE 6.23: Marketed formulation assay comparison for Chlorthalidone and Olmesartan medoxomil

Assay by HPLC						
Brand Name	**Label claim (mg)**		**Amount Found**		**% Assay ± % CV**	
	Chlor-thalidone	**Olmesartan medoxomil**	**Chlor-thalidone**	**Olmesartan medoxomil**	**Chlorthalidone (N=3)**	**Olmesartan medoxomil (N=3)**
Olmy CH	12.5	20	12.38	19.91	99.04 ± 0.27	99.55 ± 0.49
Olbet CT	12.5	20	12.52	19.69	100.16 ± 0.39	98.45 ± 0.31
Assay by UV						
Brand Name	**Label claim (mg)**		**Amount Found**		**% Assay ± % CV**	
	Chlor-thalidone	**Olmesartan medoxomil**	**Chlor-thalidone**	**Olmesartan medoxomil**	**Chlorthalidone (N=3)**	**Olmesartan medoxomil (N=3)**
Olmy CH	12.5	20	12.42	19.98	99.37 ± 0.57	99.90 ± 0.45
Olbet CT	12.5	20	12.45	19.87	99.57 ± 0.69	99.37 ± 0.41

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CHAPTER-7

RP-HPLC and UV method for Nebivolol HCl and Chlorthalidone

7.1 RP-HPLC method for Nebivolol HCl and Chlorthalidone [1-4]

TABLE 7.1: Drug & marketed formulation details of Nebivolol HCl and Chlorthalidone.

<table>
<tr><th>Drug</th><th colspan="2">Company</th></tr>
<tr><td>Nebivolol hydrochloride</td><td colspan="2">Glenmark pharmaceuticals ltd., Ankleshwar, Gujarat.</td></tr>
<tr><td>Chlorthalidone</td><td colspan="2">Cadila healthcare ltd, Ankleshwar, Gujarat</td></tr>
<tr><th>Drug name</th><th>Marketed formulation-I</th><th>Marketed formulation-II</th></tr>
<tr><td>Nebivolol hydrochloride 5 mg and Chlorthalidone 12.5 mg tablets</td><td>Nebula C tablets
Zydus Cadila healthcare limited, Moraiya Ahmedabad</td><td>Nodon CH tablets
Cadila pharmaceuticals limited, Dholka, Ahmedabad</td></tr>
</table>

Chromatographic condition

Column : Zorbax eclipse XDB C_8 (4.6mm, i.d. x 250 mm) 5 μ

Detector : 280 nm

Injection Volume : 5 µl

Flow Rate : 1.0 mL min^{-1}

Temperature : 30º C

Run Time : 12 minutes

Mobile Phase : Potassium dihydrogen phosphate & Triethylamine buffer (at pH 3): Acetonitrile (65:35, v/v)

Diluent : Mobile Phase

Experimental work

Buffer preparation

Accurately weighed 1.5 g potassium dihydrogen phosphate and 1.5 ml triethylamine was dissolved in to 1000 mL milli-Q water and 1ml triethylamine was added to this buffer solution, than pH was adjusted to 3.0 with ortho-phosphoric acid.

Preparation of standard solution (Nebivolol HCl (50 µg/ml) and Chlorthalidone (125 µg/ml))

The standard stock solution Chlorthalidone (1250 µg/ml) and Nebivolol hydrochloride (500 µg/ml) were prepared by weighing Chlorthalidone 125 mg and Nebivolol hydrochloride 50 mg in 100 ml volumetric flask respectively and making volume up to mark with diluent. Then 10 ml of standard stock solution was diluted to 100 ml with diluent to make final standard concentration of Chlorthalidone (125 µg/ml) and Nebivolol hydrochloride (50 µg/ml), respectively.

Preparation of test solution

Accurately 20 intact tablets were weighed and average weight of tablet was calculated. Then tablets were finely crushed, powdered and sample powder about 1600 mg (Tablet powder Equivalent to 62.5 mg Chlorthalidone and 25 mg nebivolol hydrochloride. i.e. five tablets' powder was added in flask for assay of tablets) was transferred into 500 ml volumetric flask. 200 ml diluent was added and sonicated for 40 minutes with intermittent shaking. Then volume was made up to mark with diluent to make final standard concentration of Chlorthalidone (125 µg/ml) and Nebivolol hydrochloride (50 µg/ml), respectively. The test solution was filtered through 0.45µ (PVDF Millipore filter) and analyzed by using HPLC system. RP-HPLC chromatogram of Chlorthalidone and Nebivolol hydrochloride is shown in Figure 7.1

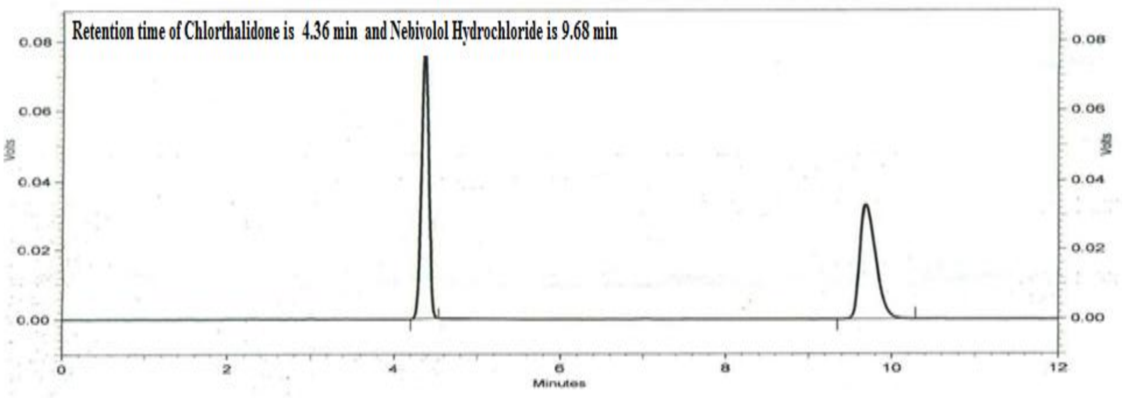

FIGURE 7.1: **RP-HPLC chromatogram of Chlorthalidone and Nebivolol hydrochloride**.

Method validation [5-8]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1). The developed method validated with respect to parameters such as system suitability, solution stability, specificity, linearity, repeatability, accuracy, intermediate precision, robustness, limit of detection and limit of quantitation.

System suitability and system precision

System suitability test were performed to check repeatability of system for particular analysis performed. The results for system suitability parameters were found satisfactory. The results of system suitability and system precision are presented in Table 7.2.

TABLE 7.2: RP HPLC system suitability data of Nebivolol HCl and Chlorthalidone analysis.

Compound	Retention Time ± SD (min) (N=5)	Theoretical plates ± SD (N=5)	Asymmetry± SD (N=5)	%RSD (N=5)	Resolution ± SD (N=5)
Nebivolol hydrochloride	4.36 ± 0.0022	9865±595	1.10±0.015	0.2	-
Chlorthalidone	9.68 ± 0.0031	12086±845	1.14±0.011	0.5	9.56 ± 0.03

Solution stability

Standard and sample solutions were kept at room temperature (25°C) for 24 hours and solution stability data after 0 hours, 8 hours and 24 hours were calculated. The change in % RSD was calculated. Standard solution and sample solution of concentration of Nebivolol hydrochloride (50 µg/ml) and Chlorthalidone (125 µg/ml) were taken to test solution stability. It was found that change in % RSD for standard and sample solution was not more than 2%. The results of solution stability are summarized in Table 7.3 and Table 7.4.

TABLE 7.3: Standard solution stability data of RP-HPLC method for Nebivolol hydrochloride and Chlorthalidone

Standard solution stability				
Time (hrs)	**Area**		**Difference (% RSD)**	
	Nebivolol HCl	**Chlorthalidone**	**Nebivolol HCl**	**Chlorthalidone**
0	437761	497214	==	==
8	435051	490512	0.33	0.48
24	432152	488525	0.58	0.85
% Mean RSD			0.46	0.67

TABLE 7.4: Sample solution stability data of RP-HPLC method for Nebivolol hydrochloride and Chlorthalidone

Sample solution stability				
Time (hrs)	**Area**		**Difference (% RSD)**	
	Nebivolol HCl	**Chlorthalidone**	**Nebivolol HCl**	**Chlorthalidone**
0	437856	498561	==	==
8	435161	491551	0.34	0.51
24	432519	487919	0.57	1.14
	% Mean RSD		0.83	0.46

Specificity

Specificity of analytical method is ability to measure analyte accurately and specifically in presence of component that may be expected to be present in the sample matrix. Chromatograms of standard, sample, blank and placebo were overlaid for specificity check in sample. Peak purity of standard and sample were obtained using photodiode array detector. The results of peak purity showed peak purity index greater than 0.99. The results are summarized in Table 7.5.

TABLE 7.5: Peak purity data of RP-HPLC method for Nebivolol HCl and Chlorthalidone

Sample	**3 point purity**	
	Nebivolol HCl	**Chlorthalidone**
Standard solution	0.9998	0.9999
Sample solution	0.9998	0.9999

Peak Purity

Peak purity curve of Nebivolol Hydrochloride and Chlorthalidone for standard and sample is shown in Figure 7.2 & 7.3.

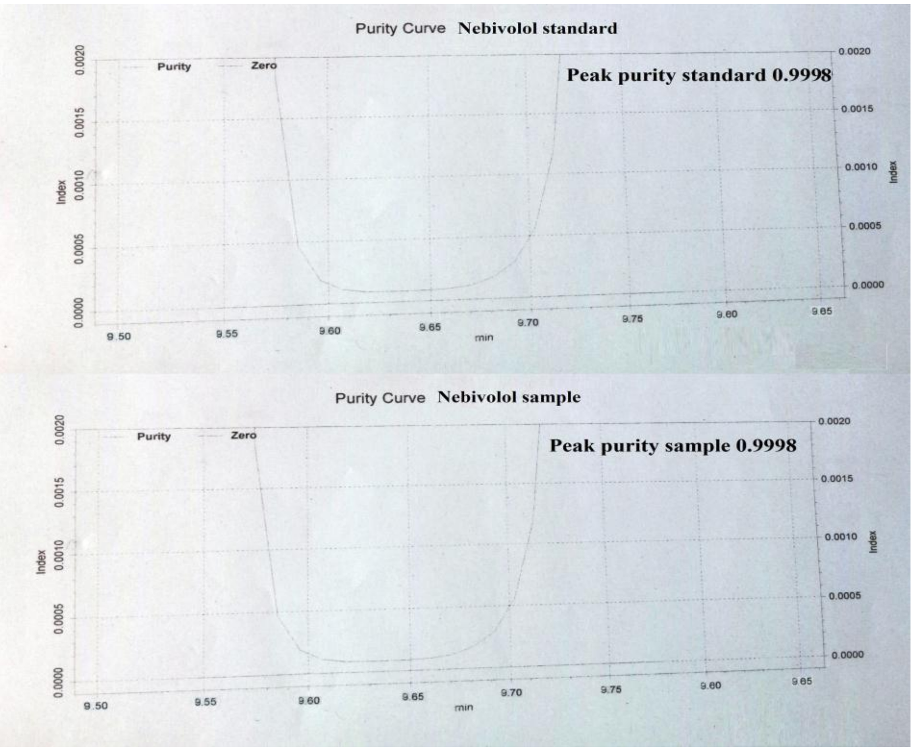

FIGURE 7.2: **Peak purity curve of Nebivolol Hydrochloride**

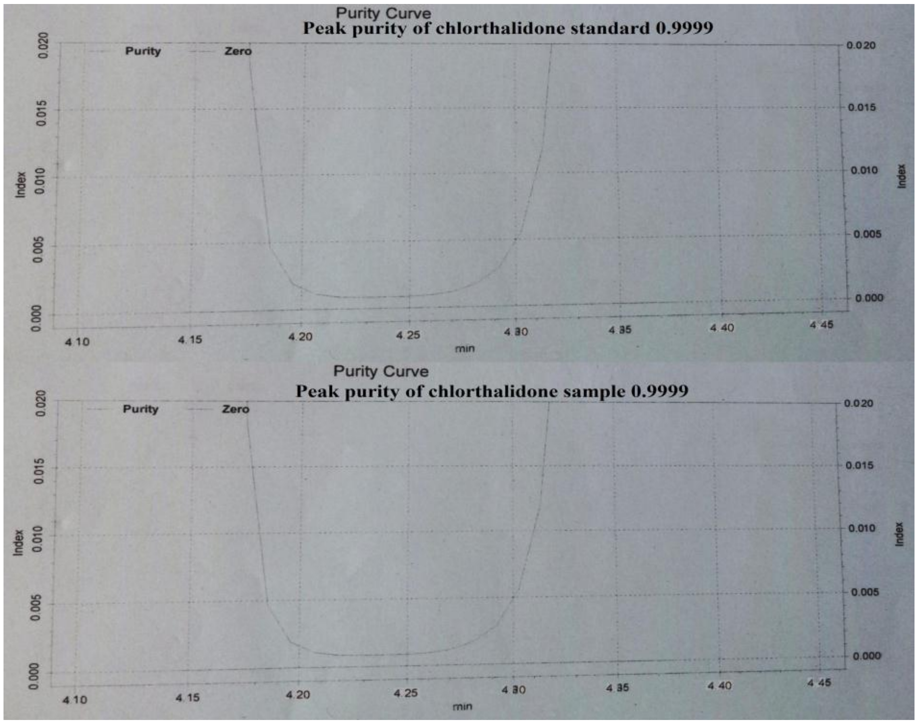

FIGURE 7.3: **Peak purity curve of Chlorthalidone**

Linearity

To achieve linearity and range, stock solution containing Chlorthalidone (1250 μg/ml) and Nebivolol hydrochloride (500 μg/ml) were prepared. Chlorthalidone and Nebivolol hydrochloride Stock solutions were diluted to yield solutions in the concentration range of 31.25-187.50 μg mL^{-1} and 12.50-75 μg mL^{-1}, respectively. The solutions were analyzed by using HPLC. Calibration curve for both the drugs are shown in Figure 7.4 and Figure 7.5. The results of linearity were presented in Table 7.6 and Table 7.7

TABLE 7.6: Linearity RP-HPLC data of Nebivolol hydrochloride

Linearity range	Stock solution taken in ml	Diluted to volume ml	Final conc (μg/ml)	Area +SD (N=3)
25%	2.5	100	12.50	109440±440
50%	5.00	100	25.00	218881±846
75%	7.50	100	37.50	326523±954
100%	10.0	100	50.00	437761±1021
125%	12.5	100	62.50	551201±1212
150%	15.0 ml	100	75.00	656642±1531

Table 7.7: Linearity RP-HPLC data of Chlorthalidone

Linearity range	Stock solution taken in ml	Diluted to volume ml	Final conc (μg/ml)	Area +SD (N=3)
25%	2.50	100	31.25	124304±122
50%	5.00	100	62.50	248607±265
75%	7.50	100	93.75	372912±390
100%	10.0	100	125.00	497214±492
125%	12.5	100	156.25	611900±865
150%	15.0	100	187.50	745821±1090

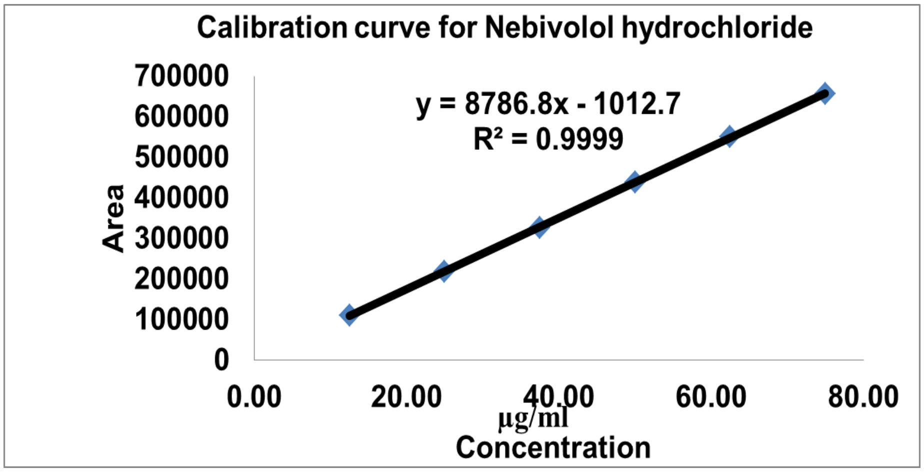

FIGURE 7.4: Calibration curve of Nebivolol HCl for RP-HPLC analysis

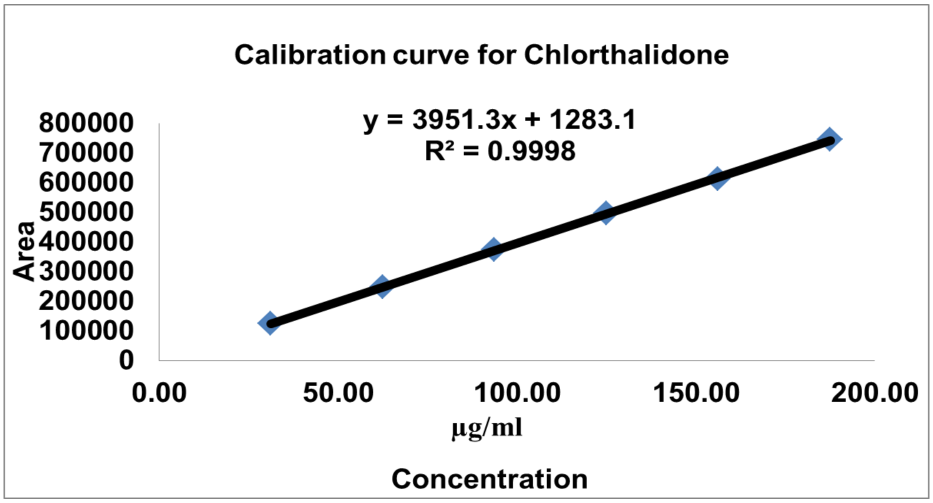

FIGURE 7.5: Calibration curve of Chlorthalidone for RP-HPLC analysis

Repeatability

The method repeatability was done by preparing six different sample preparations by one analyst. The results were presented in Table 7.8. The results obtained were within 2% RSD.

Intermediate precision

Intermediate precision test was determined between different analyst, instrument and Column. The value of percentage RSD was below 2.0%, showed intermediate precision of developed analytical method. The results were presented in Table 7.8.

TABLE 7.8: Repeatability and intermediate precision data for RP-HPLC analysis of Nebivolol hydrochloride and Chlorthalidone

Inj no.	Sample wt (mg)	Nebivolol hydrochloride (50 µg/ml)				Chlorthalidone (125 µg/ml)			
		Area	% Assay	% Mean	% RSD	Area	% Assay	% Mean	% RSD
Repeatability									
1	1600.00	437515	99.5	99.3	0.3	498561	99.9	99.3	0.4
2	1602.00	434761	98.8			494526	98.9		
3	1601.00	437761	99.5			495561	99.2		
4	1604.00	437749	99.4			495624	99.0		
5	1605.00	437660	99.3			496521	99.2		
6	1600.50	435762	99.1			496525	99.4		
Intermediate precision									
1	1601.30	443261	100.8	99.1	0.9	492561	98.6	99.4	0.7
2	1602.40	435162	98.9			495145	99.0		
3	1603.10	433261	98.5			496321	99.2		
4	1599.20	433216	98.4			495412	99.3		
5	1601.20	433198	98.6			501243	100.3		
6	1603.20	439951	99.6			501423	100.2		

Accuracy

The difference between theoretical added sample amount to the placebo and practically achieved sample amount from placebo (after HPLC analysis) is called accuracy of analytical method. Accuracy was determined at three different level 50%, 100% and 150% of the target concentration in triplicate. The results were presented in Table 7.9 and Table 7.10

TABLE 7.9: Accuracy data of Nebivolol hydrochloride for RP-HPLC analysis

Level	Placebo (mg)	Conc (µg/ml)	Amount of drug added (mg)	Amount of drug recovered (mg)	Area	Recovery ± SD (%) (N=3)	Mean ± SD (%)	% RSD
50%	1512.44	25 µg/ml	25.00	24.90	218858	99.6±0.1	99.6±0.9	0.9
	1512.54		25.80	25.92	227831	100.5±0.2		
	1512.58		25.20	24.91	218965	98.8±0.2		
100%	1512.47	50 µg/ml	50.00	49.79	437413	99.6±0.2	99.4±0.2	0.2
	1512.36		50.10	49.81	437851	99.4±0.3		
	1512.28		50.20	49.82	437951	99.2±0.1		
150%	1512.68	75 µg/ml	74.90	74.69	656574	99.7±0.2	99.8±0.7	0.7
	1512.47		75.30	75.73	665682	100.6±0.2		
	1512.34		75.60	74.96	658945	99.2±0.1		

TABLE 7.10: Accuracy data of Chlorthalidone for RP-HPLC analysis

Level	Placebo (mg)	Conc (µg/ml)	Amount of drug added (mg)	Amount of drug recovered (mg)	Area	Recovery ± SD (%) (N=3)	Mean ± SD (%) (N=3)	% RSD
50%	1512.44	62.5 µg/ml	62.25	61.69	246357	99.1±0.2	100.5±1.3	1.3
	1512.54		62.35	63.39	253145	101.7±0.2		
	1512.58		62.55	62.91	251243	100.6±0.1		
100%	1512.47	125 µg/ml	124.50	124.50	497982	100.0±0.1	99.4±0.6	0.6
	1512.36		125.50	124.76	498254	99.4±0.1		
	1512.28		126.49	125.08	499531	98.9±0.2		
150%	1512.68	187.5 µg/ml	186.75	186.19	743571	99.7±0.2	100.3±0.5	0.5
	1512.47		187.45	188.34	752162	100.5±0.1		
	1512.34		187.65	188.83	754123	100.6±0.1		

Robustness

Robustness of the method was carried out by deliberately made small changes in the flow rate, pH, and organic phase ratio and column oven temperature. Results were presented in Table 7.11.

TABLE 7.11 Robustness data for Nebivolol HCl and Chlorthalidone of RP-HPLC analysis

Name of Drug	Sys. Suit.	Temp. -5°C	Temp. +5°C	Flow -10%	Flow +10%	Org. -2%	Org. +2%	pH = 3.2	pH = 2.8
Nebivolol HCl %RSD (N=5)	0.6	0.8	0.5	0.6	0.7	0.5	0.9	0.5	0.6
Mean %RSD	0.3								
Chlor-thalidone %RSD (N=5)	0.3	0.2	0.1	0.4	0.6	0.2	0.5	0.7	0.8
Mean %RSD	0.2								

LOD (Limit of detection) and LOQ (Limit of quantitation)

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ) values, the blank sample was injected six times and the peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level while ten times the noise value gave the LOQ. The results of LOD and LOQ are mentioned in Table 7.12.

TABLE 7.12: LOD and LOQ values of Nebivolol HCl and Chlorthalidone by RP-HPLC

Parameters	Nebivolol HCl	Chlorthalidone
LOD	0.2500 μg/ml	0.1000 μg/ml
LOQ	0.8250 μg/ml	0.3300 μg/ml

Results and discussion of HPLC method

The mobile phase was optimized after several trials with methanol, acetonitrile, and buffer solutions in various proportions and at different pH values. The ion pair reagent triethylamine used in method helps to improve peak shape and enhances retention time of Chlorthalidone. The values of relative standard deviation are satisfactorily low and recovery was close to 100% which indicated accuracy and reproducibility of methods. Literature review reveals only individual methods for estimation of Chlorthalidone and Nebivolol hydrochloride, but no methods were reported for simultaneous estimation of Chlorthalidone and Nebivolol hydrochloride was reported till date. So, method much superior to previously published methods of individual estimation of all drugs was developed. The detection wavelength of 280 nm was chosen in order to achieve a good sensitivity for quantitative determination of Chlorthalidone and Nebivolol hydrochloride in solid dosage form. Summary of validation parameters is shown in Table 7.13.

TABLE 7.13: Summary of validation parameters of RP-HPLC method for simultaneous estimation of Nebivolol hydrochloride and Chlorthalidone

Parameter	**Acceptance criteria**	**Nebivolol hydrochloride**	**Chlorthalidone**
Range of Linearity	Follows Beer Lambert's law	12.50 – 75 µg/ ml	31.25-187.5 µg/ml
Correlation coefficient	R>0.999 or 0.995	1.0000	0.9999
Regression coefficient	R^2 >0.999 or 0.995	0.9999	0.9998
LOD	S/N > 2 or 3	0.2500 µg/ml	0.1000 µg/ml
LOQ	S/N > 10	0.8250 µg/ml	0.3300 µg/ml
Repeatability	RSD < 2%	0.3%	0.4%
Intermediate precision	RSD < 2%	0.9%	0.7%
Accuracy	98- 102%	99.4% to 99.8%	99.4% to 100.5%
Specificity (placebo, blank)	No interference	No interference of placebo, blank	No interference of placebo, blank
Solution Stability	> 12 hour	Stable for 24 hr %RSD =0.46%	Stable for 24 hr %RSD = 0.67%
Robustness Flow rate (+ & -), Buffer pH (+ & -), Column temperature(+ & -), & Organic ratio (+ & -), in mobile phase	RSD NMT 2% in modified condition	Complies % RSD 0.2% & 0.3% % RSD 0.2% & 0.3% % RSD 0.3% & 0.3 % % RSD 0.5 % & 0.1%	Complies %RSD 0.2% & 0.3% % RSD 0.1% & 0.1% % RSD 0.2% & 0.1 % % RSD 0.1 % & 0.3%

7.2 UV spectroscopic method for Nebivolol HCl and Chlorthalidone [9-12]

METHOD OF ANALYSIS

Diluent: Methanol

Wavelength: 226nm and 282 nm at UV Spectrophotometer

Proper wavelength selection for estimation of both drugs depends on nature of drugs and their solubility. Many trials for solubility of both drugs in different diluents like acetonitrile, water, water: acetonitrile (50:50), ethanol, isopropanol, methanol etc. were conducted and methanol as diluent was finalized for method. Method employs solving of simultaneous equations based on the measurement of absorbance at two wavelengths 226 nm and 282 nm which are the λmax values of Nebivolol hydrochloride and Chlorthalidone in methanol. Calculation for both drugs are done as per below formula

At λ_1 $$A_1 = a X_1 b\, Cx + aY_1 b\, Cy \text{ ---------------------- } (1)$$

At λ_2 $$A_2 = a X_2 b\, Cx + aY_2 b\, Cy \text{ ---------------------- } (2)$$

For measurements in 1 cm cells b=1

Rearrange eq. (2)

$$Cy = \frac{A_2 - a X_2 b\, Cx}{aY_2}$$

Substituting for Cy in eq. (1) and rearranging

$$Cx = \frac{A_2\, aY_1 - A_1\, aY_2}{a X_2\, aY_1 - a X_1 aY_2} \text{ --------------------------- } (3)$$

$$Cy = \frac{A_1\, a X_2 - A_2\, a X_1}{a X_2\, aY_1 - a X_1 aY_2} \text{ --------------------------- } (4)$$

A1= Absorption of mixture at 226 nm.

A2= Absorption of mixture at 282 nm.

ay1=Molar absorptivity of Nebivolol hydrochloride at 226 nm.

ay2=Molar absorptivity of Nebivolol hydrochloride at 282nm.

Cy=Concentration of Nebivolol hydrochloride (µg/ml).

Cx=Concentration of Chlorthalidone (µg/ml).

ax1=Molar absorptivity of Chlorthalidone at 226 nm.

ax2=Molar absorptivity of Chlorthalidone at 282nm.

Experimental work

Preparation of standard solution

The standard stock solution Chlorthalidone (250 µg/ml) and Nebivolol hydrochloride (100 µg/ml) were prepared by weighing Chlorthalidone 50 mg and Nebivolol hydrochloride 20 mg in 200 ml volumetric flask respectively and making volume up to mark with diluent. Then 5 ml of standard stock solution was diluted to 100 ml with diluent to make final standard concentration of Chlorthalidone (12.5 µg/ml) and Nebivolol hydrochloride (5 µg/ml), respectively.

Preparation of test solution

Accurately 20 intact tablets were weighed and average weight of tablet was calculated. Then tablets were finely crushed, powdered and sample powder about 1280 mg (Tablet powder Equivalent to 50 mg Chlorthalidone and 20 mg Nebivolol hydrochloride. i.e. four tablets' powder was added in flask for assay of tablets) was transferred into 200 ml volumetric flask. 100 ml diluent was added and sonicated for 40 minutes with intermittent shaking. Then volume was made up to mark with diluent to make final standard concentration of Chlorthalidone (250 µg/ml) and Nebivolol hydrochloride (100 µg/ml), respectively. The test solution was filtered through 0.45µ PVDF Millipore filter. Then 5 ml of sample stock solution was diluted to 100 ml with diluent to make final standard concentration of Chlorthalidone (12.5 µg/ml) and Nebivolol hydrochloride (5 µg/ml), respectively. The sample solution was analyzed by using UV Spectrophotometer.

UV spectra for Chlorthalidone (12.5 µg/ml) and Nebivolol hydrochloride (5 µg/ml) for wavelength maxima selection is shown in Figure 7.6 and Figure 7.7. Absorbance at both wavelength 226 nm and 282 nm for both drugs are shown in Table 7.14

TABLE 7.14: UV absorbance data of Nebivolol hydrochloride and Chlorthalidone at both wavelengths.

Drug	Wavelength 226 nm	Wavelength 282 nm
Nebivolol hydrochloride (5 µg/ml)	0.0493	0.0432
Chlorthalidone (12.5 µg/ml)	0.6400	0.7510

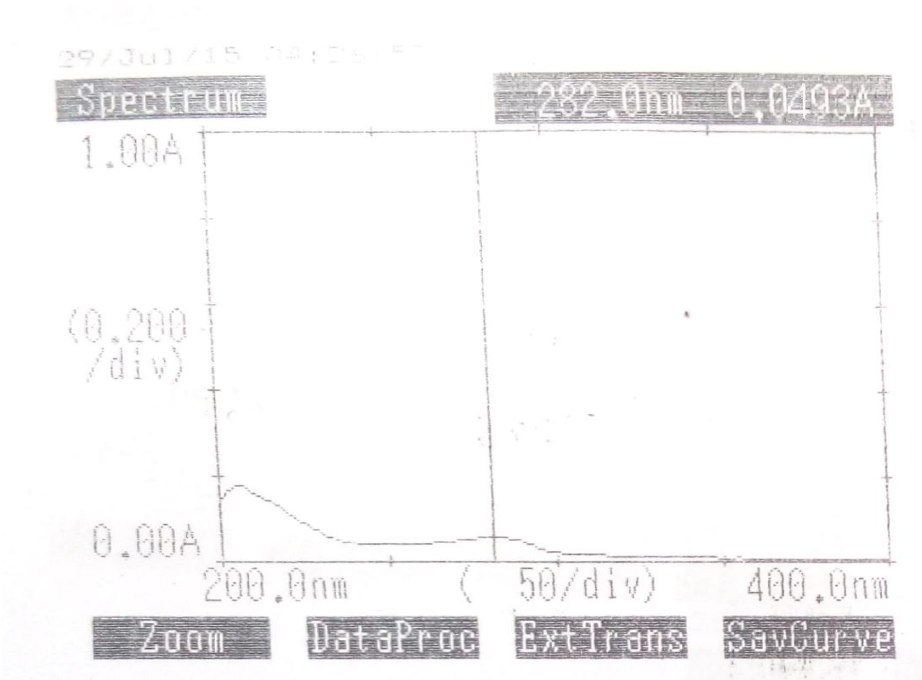

FIGURE 7.6: UV spectrum of Nebivolol hydrochloride (5 µg/ml)

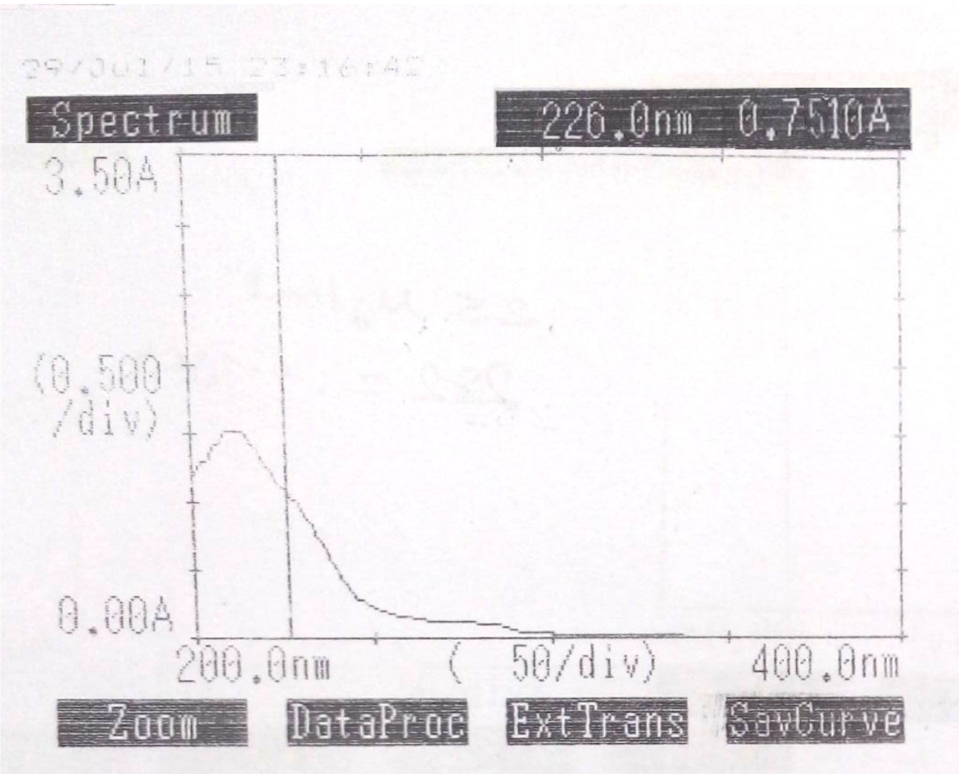

FIGURE 7.7: UV spectrum of Chlorthalidone (12.5 µg/ml).

Method validation [13-15]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1). The developed method validated with respect to parameters such as linearity, repeatability, accuracy, specificity, intermediate precision and solution stability.

Specificity

Diluent and placebo spectra are scanned over a range from 200 nm and 400 nm. Specificity of standard and sample are shown in (Figure 7.8), (Figure 7.9).

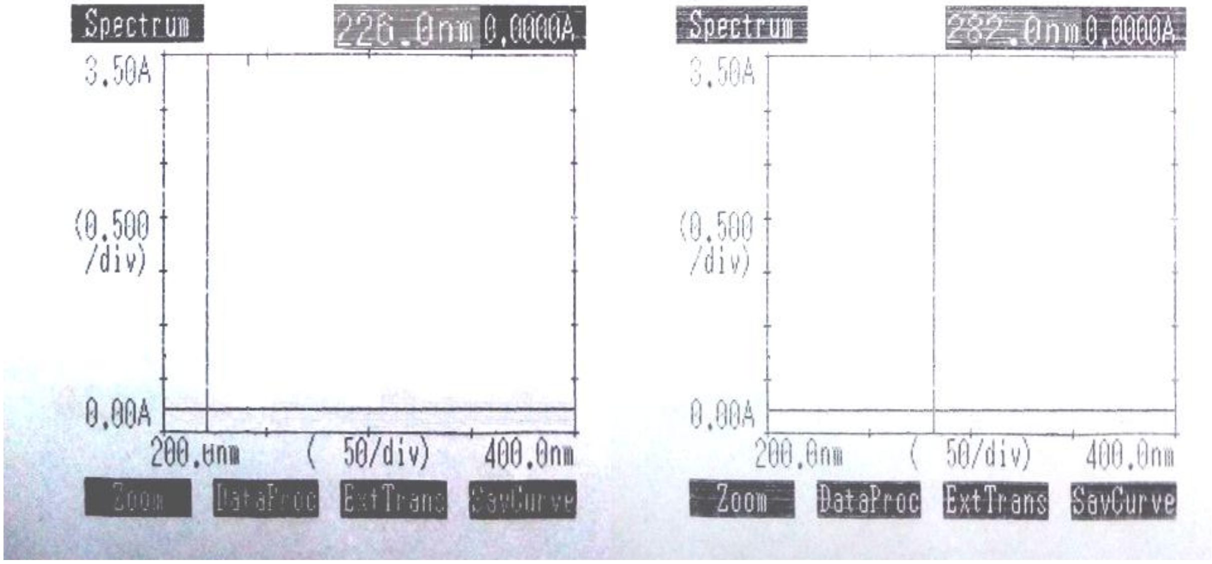

FIGURE 7.8: Specificity study for diluent at both wavelengths.

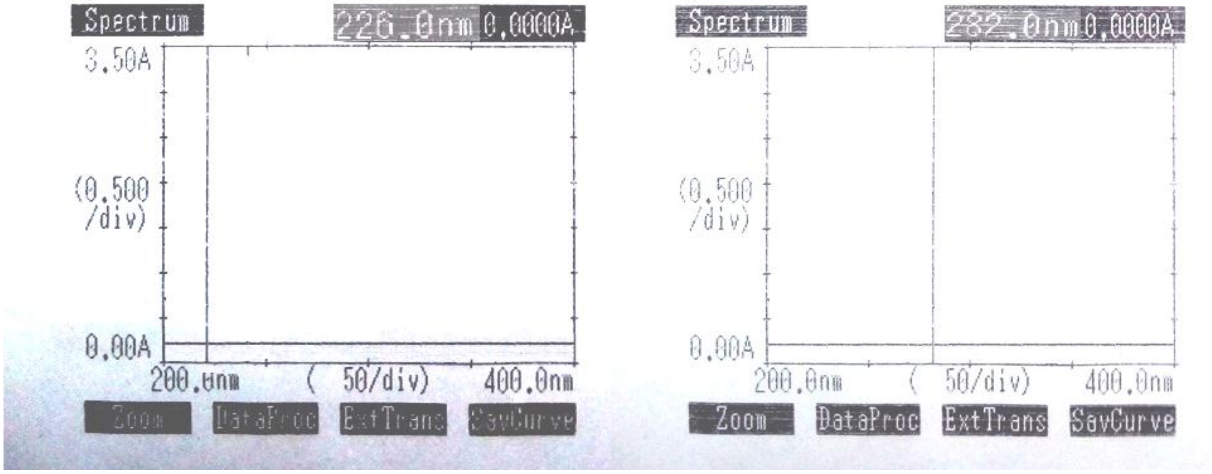

FIGURE 7.9: Specificity study for placebo at both wavelengths.

Linearity

To achieve linearity and range, stock solution containing Nebivolol hydrochloride (100 µg/ml) and Chlorthalidone (100 µg/ml) were separately prepared. Nebivolol hydrochloride and Chlorthalidone stock solutions were diluted to yield solutions in the concentration range of 5-25 µg mL^{-1} and 5-25 µg mL^{-1}, respectively. The solutions were analyzed by using UV. Overlain linearity spectra for Nebivolol hydrochloride and Chlorthalidone are shown in Figure 7.10 and Figure 7.11. Calibration curve for both the drugs are shown in Figure 7.12, Figure 7.13, Figure 7.14 and Figure 7.15. The results of linearity are presented in Table 7.15. Molar extinction coefficient for wavelength 226 nm and 282 nm for both drugs are obtained using data from linearity table.

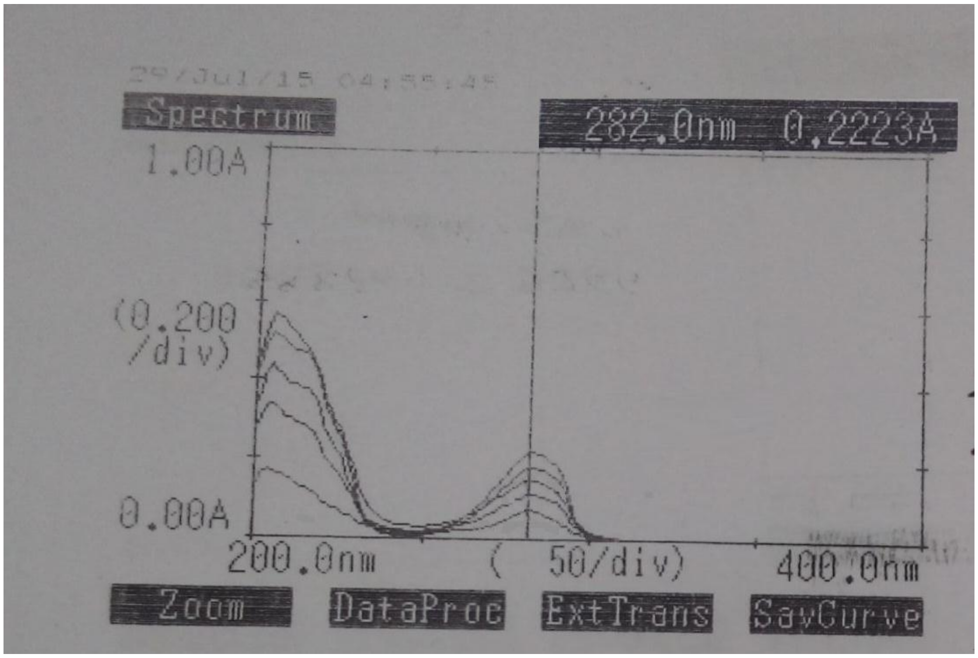

FIGURE 7.10: Overlain spectra of Linearity for Nebivolol Hydrochloride.

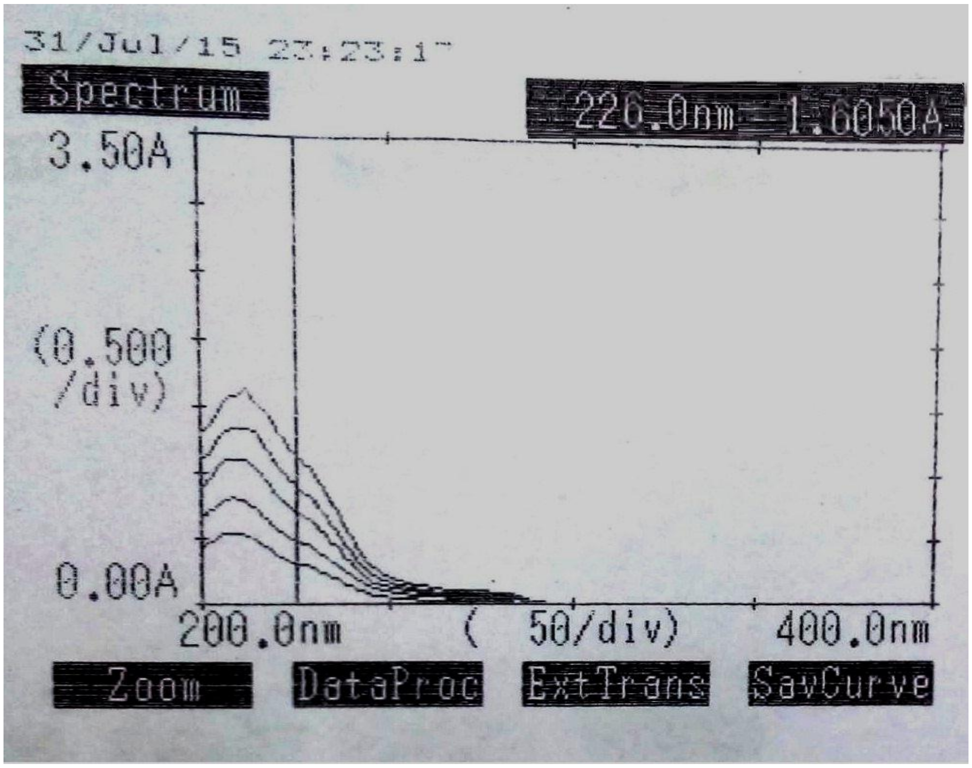

FIGURE 7.11: Overlain spectra of Linearity for Chlorthalidone.

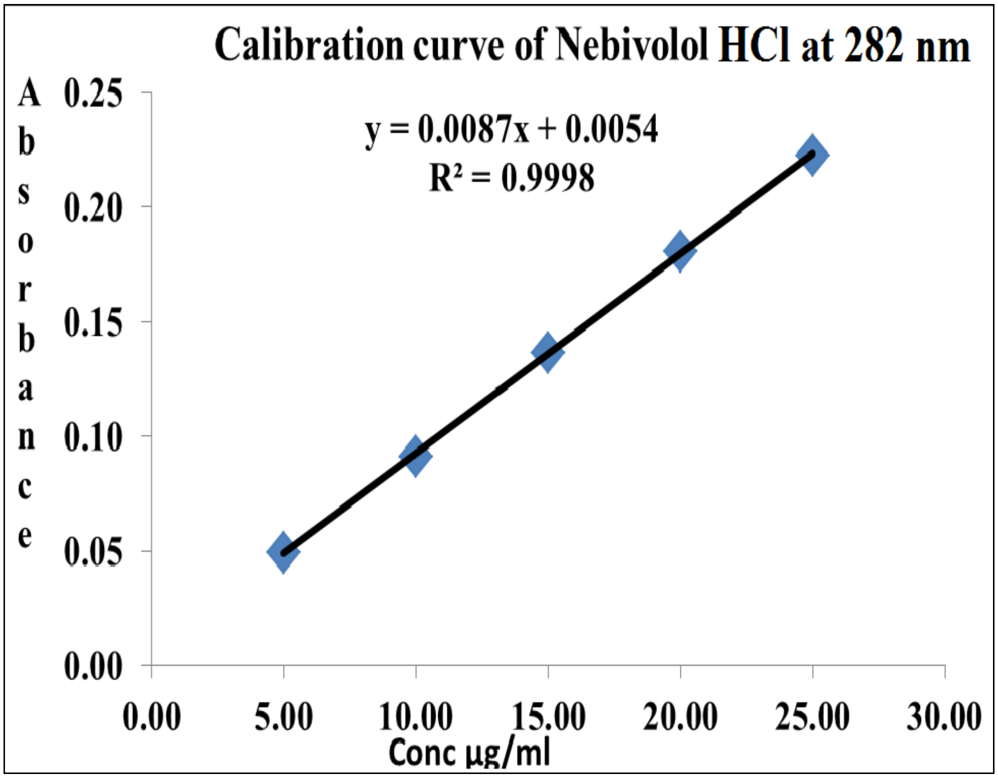

FIGURE 7.12: Calibration Curve of Nebivolol hydrochloride at 282 nm.

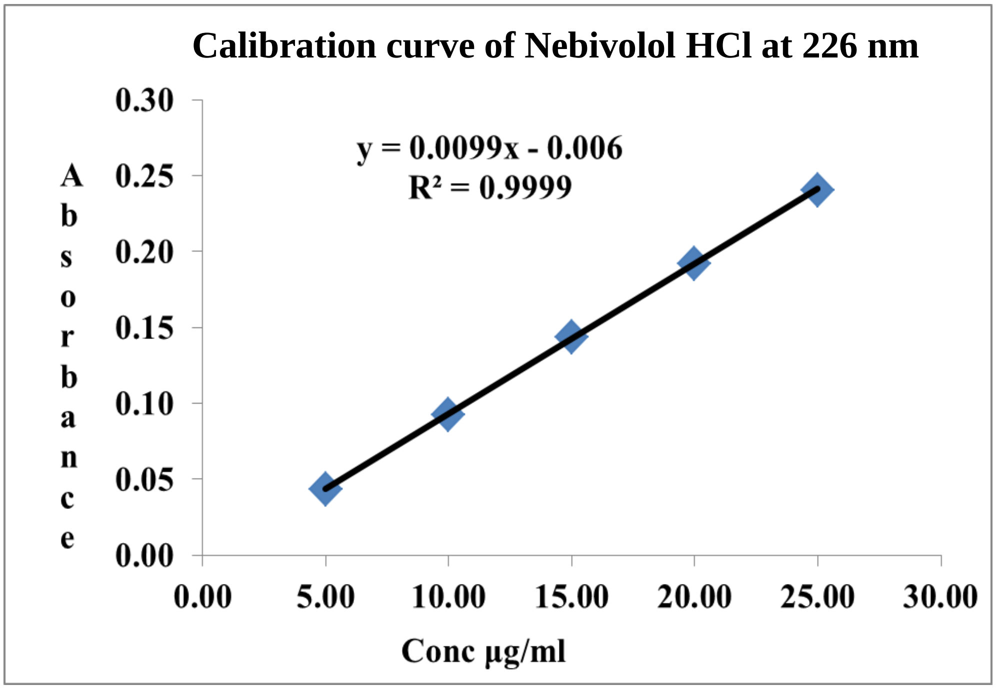

Figure 7.13: Calibration Curve of Nebivolol hydrochloride at 226 nm.

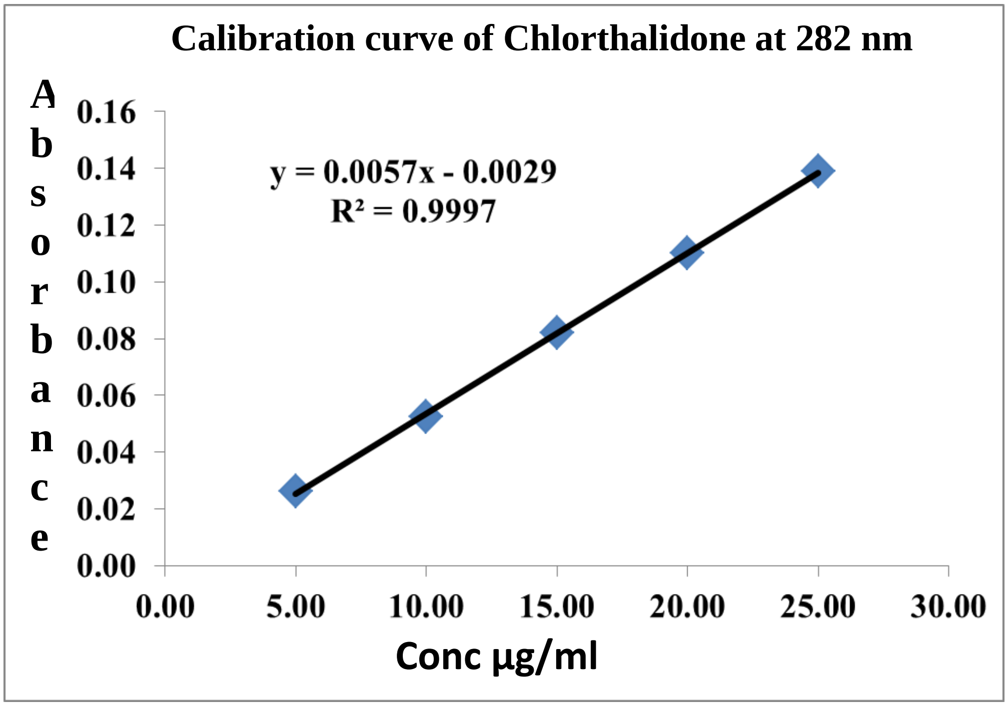

FIGURE 7.14: Calibration Curve of Chlorthalidone at 282 nm

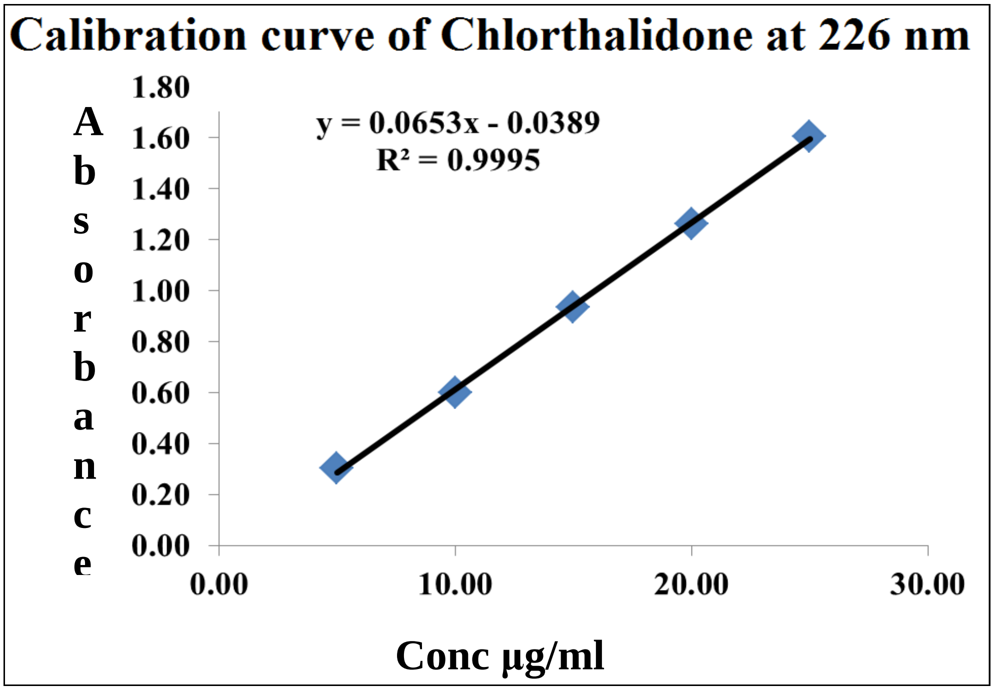

FIGURE 7.15: Calibration Curve of Chlorthalidone at 226 nm.

TABLE 7.15: Linearity UV data of Nebivolol Hydrochloride and Chlorthalidone

Linearity stock soln ml	Diluted to volume ml	Final conc	Nebivolol HCl	
		Nebivolol HCl (µg/ml)	Absorbance ± SD (N=3)	
			λ 282 nm	λ 226 nm
5.0	200	5.00	0.0490±0.0001	0.0432±0.0001
10.0	200	10.00	0.0913±0.0001	0.09252±0.0001
15.0	200	15.00	0.1365±0.0001	0.1436±0.0001
20.0	200	20.00	0.1807±0.0001	0.1923±0.0001
25.0	200	25.00	0.2223±0.0002	0.2407±0.0002
Linearity stock soln ml	**Diluted to volume ml**	**Final conc**	**Chlorthalidone**	
		Chlorthalidone (µg/ml)	**Absorbance ± SD (N=3)**	
			λ 282 nm	**λ 226 nm**
5.0	200	5.00	0.0263±0.0001	0.3021±0.0002
10.0	200	10.00	0.0526±0.0001	0.6008±0.0003
15.0	200	15.00	0.0820±0.0002	0.9362±0.0005
20.0	200	20.00	0.1101±0.0001	1.2623±0.0008
25.0	200	25.00	0.1391±0.0001	1.6050±0.0009

Repeatability

The method repeatability was done by preparing six different sample preparations by one analyst. The results are presented in Table 7.16. The results obtained were within 2% RSD.

TABLE 7.16: Repeatability UV data for Nebivolol hydrochloride and Chlorthalidone

Sr. no	**% Assay Repeatability**					
	Nebivolol hydrochloride (5 µg/ml)					
	Absorbance			**% Assay**	**% Mean**	**% RSD**
	wt (mg)	226 nm	282 nm			
1	1278.4	0.9053	0.1215	101.2	101.7	0.1
2	1278.9	0.9050	0.1210	101.8		
3	1281.5	0.9058	0.1219	101.9		
4	1279.2	0.9054	0.1226	101.1		
5	1278.5	0.9061	0.1229	101.3		
6	1281.5	0.9079	0.1228	101.4		
Chlorthalidone (12.5µg/ml)						
Sr. no	**Absorbance**			**% Assay**	**% Mean**	**% RSD**
	wt (mg)	226 nm	282 nm			
1	1278.4	0.9053	0.1215	99.9	99.4	0.4
2	1278.9	0.9050	0.1210	99.6		
3	1281.5	0.9058	0.1219	99.8		
4	1279.2	0.9054	0.1226	99.5		
5	1278.5	0.9061	0.1229	99.1		
6	1281.5	0.9079	0.1228	100.2		

Intermediate precision

Intermediate precision test was determined between different analysts. The value of percentage RSD was below 2.0%, showed intermediate precision of developed analytical method. The results are presented in Table 7.17.

TABLE 7.17: Intermediate precision UV data for Nebivolol hydrochloride and Chlorthalidone

Sr. no	% Assay intermediate precision					
	Nebivolol hydrochloride (5 µg/ml)					
	Absorbance			% Assay	% Mean	% RSD
	wt (mg)	226 nm	282 nm			
1	1281.5	0.9063	0.1205	100.9	101.6	0.6
2	1280.2	0.9060	0.1209	101.4		
3	1278.4	0.9051	0.1229	101.6		
4	1279.1	0.9052	0.1221	101.9		
5	1279.9	0.9064	0.1224	100.3		
6	1278.5	0.9071	0.1220	101.4		
Chlorthalidone (12.5µg/ml)						
Sr. no	Absorbance			% Assay	% Mean	% RSD
	wt (mg)	226 nm	282 nm			
1	1281.5	0.9063	0.1205	100.2	99.8	0.5
2	1280.2	0.9060	0.1209	99.9		
3	1278.4	0.9051	0.1229	99.1		
4	1279.1	0.9052	0.1221	99.4		
5	1279.9	0.9064	0.1224	99.9		
6	1278.5	0.9071	0.1220	100.5		

Accuracy

The difference between theoretical added sample amount to the placebo and practically achieved sample amount from placebo (after UV analysis) is called accuracy of analytical method. Accuracy was determined at three different level 80%, 100% and 120% of the target concentration in triplicate. The results are presented in Table 7.18 and Table 7.19.

TABLE 7.18: UV accuracy data of Nebivolol hydrochloride

Accuracy data of Nebivolol Hydrochloride								
Level	**Placebo (mg)**	**Absorbance**		**Amount of drug added (µg/ml)**	**Amount of drug recovered (µg/ml)**	**Recovery ± SD (%) (N=3)**	**Mean ± SD (%)**	**% RSD**
		226 nm	**282 nm**					
80%	1230.10	0.7242	0.0927	4.15	4.20	101.2±0.1	101.2±0.1	0.1
	1232.16	0.7245	0.0921	4.25	4.30	101.2±0.2		
	1230.14	0.7248	0.0923	4.55	4.60	101.1±0.2		
100%	1230.46	0.9051	0.1215	5.13	5.21	101.6±0.1	99.4±1.2	1.2
	1230.63	0.9054	0.1210	5.14	5.11	99.4±0.2		
	1230.16	0.9056	0.1265	5.15	5.12	99.3±0.3		
120%	1230.89	1.0862	0.1458	6.23	6.21	99.7±0.4	100.4±0.6	0.6
	1231.16	1.0865	0.1464	6.15	6.19	100.7±0.2		
	1235.61	1.0870	0.1473	6.19	6.25	101.0±0.2		

TABLE 7.19: UV accuracy Data of Chlorthalidone

Accuracy data of Chlorthalidone								
Level	**Placebo (mg)**	**Absorbance**		**Amount of drug added (μg/ml)**	**Amount of drug recovered (μg/ml)**	**Recovery ± SD (%) (N=3)**	**Mean ± SD (%)**	**% RSD**
		226 nm	**282 nm**					
80%	1230.10	0.7242	0.0927	10.21	10.30	100.9±0.1	99.8±1.0	1.0
	1232.16	0.7245	0.0921	10.35	10.31	99.6±0.2		
	1230.14	0.7248	0.0923	10.10	10.00	99.0±0.1		
100%	1230.46	0.9051	0.1215	12.50	12.22	98.6±0.2	99.5±1.3	1.3
	1230.63	0.9054	0.1210	12.60	12.45	98.8±0.1		
	1230.16	0.9056	0.1265	12.40	12.52	100.9±0.2		
120%	1230.89	1.0862	0.1458	15.20	15.25	100.3±0.3	100.5±0.3	0.3
	1231.16	1.0865	0.1464	15.40	15.45	100.3±0.2		
	1235.61	1.0870	0.1473	15.12	15.25	100.9±0.1		

Solution stability

The standard and sample solutions of Nebivolol HCl and Chlorthalidone were found stable up to 24 hours at room temperature. The results are presented in Table 7.20

TABLE 7.20: Solution stability UV data of Nebivolol HCl and Chlorthalidone.

Standard solution				
Time (Hr)	**Absorbance**		**% Difference**	
	226 nm	282 nm	**Nebivolol Hydrochloride**	**Chlorthalidone**
0	0.9051	0.1214	==	==
8	0.9045	0.1210	0.10	0.23
24	0.9040	0.1208	0.22	0.25

Sample solution				
Time (Hr)	**Absorbance**		**% Difference**	
	226 nm	282 nm	Nebivolol Hydrochloride	Chlorthalidone
0	0.9055	0.1218	==	==
8	0.9050	0.1210	0.10	0.21
24	0.9.45	0.1204	0.24	0.26

LOD (LIMIT OF DETECTION AND LOQ (LIMIT OF QUANTITATION) [16-20]

The results of LOD and LOQ are mentioned in Table 7.21

TABLE7.21: LOD and LOQ UV data of Nebivolol hydrochloride and Chlorthalidone

PARAMETERS	**Nebivolol HCl**	**Chlorthalidone**
LOD	0.5000 µg/ml	1.2500 µg/ml
LOQ	1.6500 µg/ml	4.125 µg/ml

TABLE 7.22: Summary of validation parameters of UV method for simultaneous estimation of Nebivolol hydrochloride and Chlorthalidone

Parameters of validation	Acceptance criteria	Nebivolol Hydrochloride		Chlorthalidone	
Range of linearity	Follows Beer Lambert’s law	5-25 µg/ml		5 – 25 µg/ml	
Correlation coefficient	R > 0.990 or 0.995	226 nm	282 nm	226 nm	282 nm
		1.0000	0.9999	0.9997	0.9999
Regression coefficient	$R^2 > 0.990$ or 0.995	0.9999	0.9998	0.9995	0.9997
Regression equation	-	Y = 0.0099x + 0.0066	Y= 0.0087x - 0.0054	Y= 0.0653x+ 0.0389	Y= 0.0057x - 0.0029
LOD	-	0.5000 µg/ml		1.2500 µg/ml	
LOQ	-	1.6500 µg/ml		4.1250 µg/ml	
Repeatability	RSD < 2%	0.10%		0.40%	
Intermediate precision	RSD < 2%	0.60%		0.50%	
Accuracy	98- 102%	99.4% to 101.2%		99.5% to 100.6%	
Specificity	No interference of blank, Placebo	No interference of blank, Placebo		No interference of blank, Placebo	
Solution stability	> 12 hour	Stable for 24 hr %RSD =0.2%		Stable for 24 hr %RSD = 0.3%	

TABLE 7.23: Marketed formulation assay comparison for Nebivolol hydrochloride and Chlorthalidone

Assay by HPLC						
Brand name	**Label claim (mg)**		**Amount Found**		**% Assay ± % CV**	
	Nebivolol HCl	**Chlor-thalidone**	**Nebivolol HCl**	**Chlor-thalidone**	**Nebivolol HCl (N=3)**	**Chlorthalidone (N=3)**
Nebula C	5	12.5	4.98	12.46	99.6 ± 0.19	99.36 ± 0.47
Nodon CH	5	12.5	4.95	12.43	99.0 ± 0.35	99.44 ± 0.22
Assay by UV						
Brand name	**Label claim (mg)**		**Amount Found**		**% Assay ± % CV**	
	Nebivolol HCl	**Chlor-thalidone**	**Nebivolol HCl**	**Chlor-thalidone**	**Nebivolol HCl (N=3)**	**Chlorthalidone (N=3)**
Nebula C	5	12.5	5.07	12.49	101.40 ± 0.19	99.92 ± 0.37
Nodon CH	5	12.5	4.93	12.56	98.60 ± 0.15	100.48 ± 0.62

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CHAPTER-8

RP-HPLC and UV method for Rosuvastatin calcium and Hydrochlorthiazide

8.1 RP-HPLC method for Rosuvastatin calcium and Hydrochlorthiazide [1-4]

TABLE 8.1 Drug & marketed formulation details of Rosuvastatin calcium and Hydrochlorthiazide.

Drug	Company	
Rosuvastatin calcium	Intas pharmaceuticals ltd., Ahmedabad, Gujarat.	
Hydrochlorthiazide	Intas pharmaceuticals ltd., Ahmedabad, Gujarat.	
Drug name	**Marketed formulation-I**	**Marketed formulation-II**
Rosuvastatin calcium 10 mg and Hydrochlorthiazide 12.5 mg tablets	Zyrova H tablets Zydus Cadila healthcare limited, Moraiya Ahmedabad	Rozustat H tablets Macleods pharma, Vapi Gujarat

Experimental work

Chromatographic condition

Column	: ACE C_{18} AR 4.6 X 250 mm, 5µm.
Detector	: 280 nm
Injection Volume	: 10 µl
Flow Rate	: 1.0 mL min^{-1}
Temperature	: 30° C
Run Time	: 12 minutes
Mobile Phase	: Sodium perchlorate Buffer (at pH 3.0): Acetonitrile (60:40, v/v)
Diluent	: Methanol

.

Buffer preparation

Accurately weighed 4.3 g sodium perchlorate was dissolved in to 1000 mL milli-q water and 1ml triethylamine was added to this buffer solution, than pH was adjusted to 3.0 with ortho-phosphoric acid.

Preparation of standard solution

The standard stock solution Rosuvastatin calcium (200 μg/ml) and Hydrochlorthiazide (250 μg/ml) were prepared by weighing Rosuvastatin calcium 40 mg and Hydrochlorthiazide 50 mg in 200 ml volumetric flask respectively. Add 80 ml diluent to it and sonicate well for 10 minutes and making volume up to mark with diluent. Then 10 ml of standard stock solution was diluted to 100 ml with diluent to make final standard concentration of Rosuvastatin calcium (20 μg/ml) and Hydrochlorthiazide (25 μg/ml), respectively.

Preparation of test solution

Accurately 20 intact tablets were weighed and average weight of tablet was calculated. Then tablets were finely crushed, powdered and sample powder about 1400 mg (Tablet powder Equivalent to 40 mg Rosuvastatin calcium and 50 mg Hydrochlorthiazide or four tablets powder) was transferred into 200 ml volumetric flask. Then add about 100.0 ml diluent was added and sonicated for 40 minutes with intermittent shaking. Then volume was made up to mark with diluent. Then 10 ml of standard stock solution was diluted to 100 ml with diluent to make final standard concentration of Rosuvastatin calcium (20 μg/ml) and Hydrochlorthiazide (25 μg/ml), respectively. The test solution was filtered through 0.45μ (PVDF Millipore Filter) and analyzed by using HPLC. RP-HPLC chromatogram of Rosuvastatin calcium and Hydrochlorthiazide is shown in Figure 8.1.

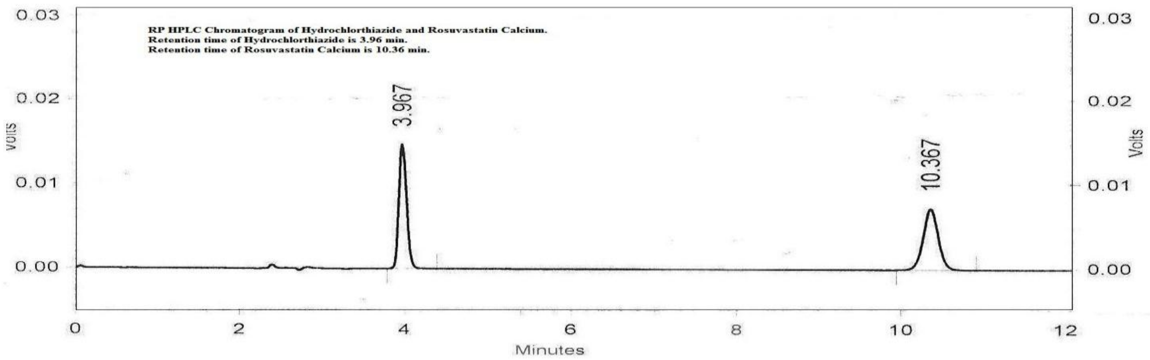

FIGURE 8.1: **RP-HPLC chromatogram of Rosuvastatin calcium and Hydrochlorthiazide.**

Method validation [5-9]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1). The developed method validated with respect to parameters such as system suitability, solution stability, specificity, linearity, repeatability, accuracy, intermediate precision, robustness, limit of detection and limit of quantitation.

System suitability and system precision

System suitability test were performed to check repeatability of system for particular analysis performed. The results for system suitability parameters were found satisfactory. The results of system suitability and system precision are presented in Table 8.2.

TABLE 8.2: System suitability parameters of RP-HPLC analysis for Rosuvastatin calcium and Hydrochlorthiazide.

Compound	Retention Time ± SD (min) (N=5)	Theoretical plates ± SD (N=5)	Asymmetry± SD (N=5)	%RSD (N=5)	Resolution ± SD (N=5)
Rosuvastatin calcium	10.3 ± 0.12	9619±414	1.12±0.12	0.5	11.54 ± 0.03
Hydro-chlorthiazide	3.9 ± 0.15	10351±552	1.09±0.10	0.2	-

Solution stability

Standard and sample solutions were kept at room temperature (25°C) for 24 hours and solution stability data after 0 hours, 8 hours and 24 hours were calculated. The change in % RSD was calculated. Standard solution and sample solution of concentration of Rosuvastatin calcium (20 µg/ml) and Hydrochlorthiazide (25 µg/ml) were taken to test solution stability. It was found that change in % RSD for standard and sample solution was not more than 2%. The results of solution stability are summarized in Table 8.3 and Table 8.4. The standard and sample solutions were found stable up to 24 hours at room temperature.

TABLE 8.3: RP-HPLC data of standard solution stability for Rosuvastatin calcium and Hydrochlorthiazide

Standard solution stability				
Time (hr)	**Area**		**% Difference**	
	Rosuvastatin calcium	**Hydrochlorthiazide**	**Rosuvastatin calcium**	**Hydrochlorthiazide**
0	205652	405236	==	==
8	204156	404562	0.5	0.1
24	202523	402781	0.8	0.3
% Mean RSD			07	0.2

TABLE 8.4: RP-HPLC data of sample solution stability for Rosuvastatin calcium and Hydrochlorthiazide

Sample solution stability				
Time (hr)	**Area**		**% Difference**	
	Rosuvastatin calcium	**Hydrochlorthiazide**	**Rosuvastatin calcium**	**Hydrochlorthiazide**
0	205146	405178	==	==
8	203589	401523	0.5	0.6
24	201415	397526	0.9	1.0
% Mean RSD			0.8	0.8

Specificity

Specificity of analytical method is ability to measure analyte accurately and specifically in presence of component that may be expected to be present in the sample matrix. Chromatograms of standard, sample, blank and placebo were overlaid for specificity check in sample. Peak purity of standard and sample were obtained using photodiode array detector. The results of peak purity showed peak purity index greater than 0.99. The results of specificity of Rosuvastatin calcium and Hydrochlorthiazide are summarized in Table 8.5.

TABLE 8.5 Peak purity data of RP-HPLC method for Rosuvastatin calcium and Hydrochlorthiazide

Sample	3 point purity	
	Rosuvastatin calcium	**Hydrochlorthiazide**
Standard solution	0.9999	0.9998
Test solution	0.9999	0.9997

Peak purity

Peak purity curve of Rosuvastatin calcium and Hydrochlorthiazide for standard and sample is shown in Figure 8.2 & 8.3.

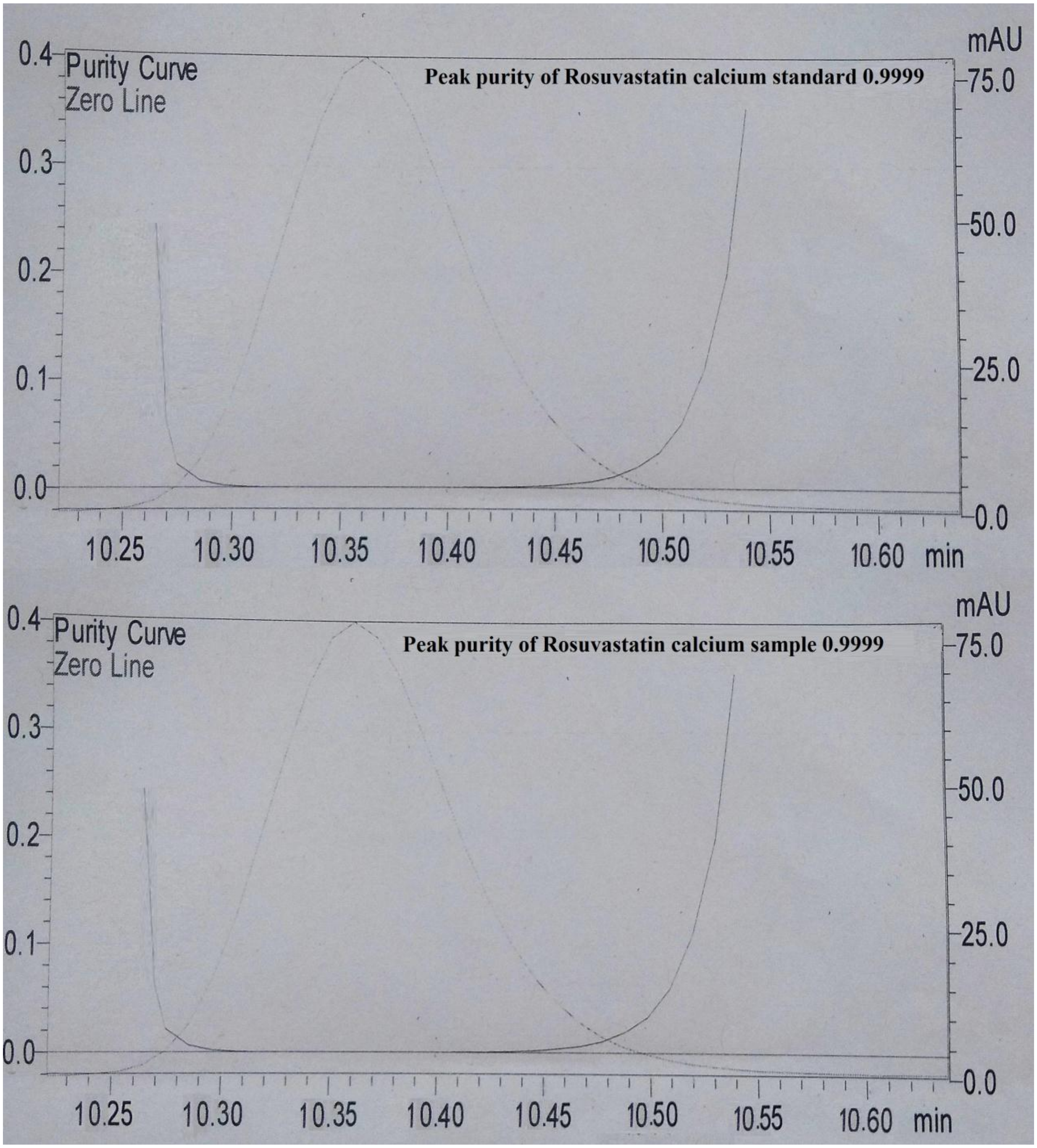

FIGURE 8.2: **Peak purity curve of Rosuvastatin calcium**

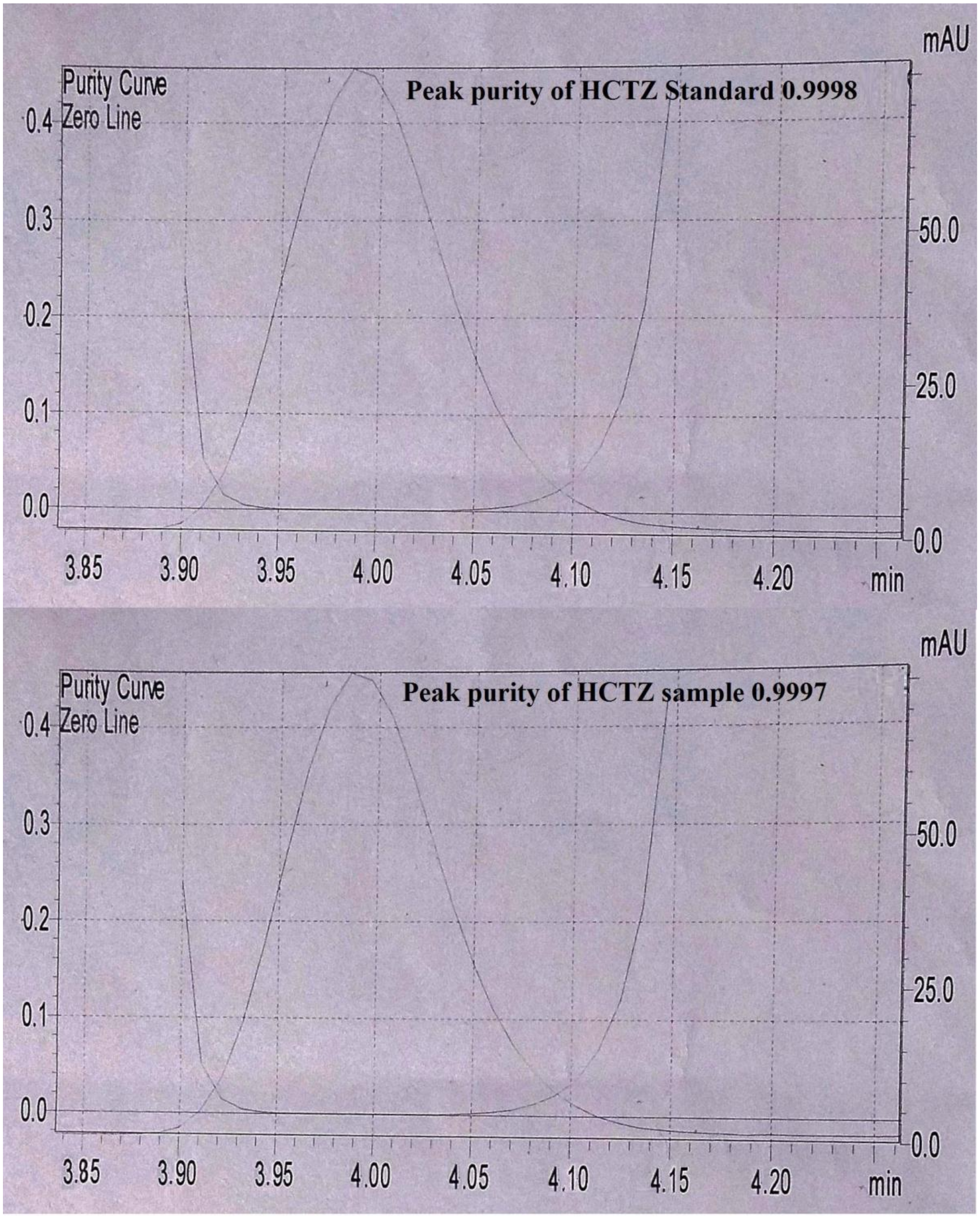

FIGURE 8.3: **Peak purity curve of Hydrochlorthiazide**

Linearity [10-13]

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well-defined mathematical transformation) proportional to the analyte concentration in samples within a given range. Linearity usually expressed in terms of the variance around the slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte.

To achieve linearity range, stock solution containing Rosuvastatin calcium (200 μg/ml) and Hydrochlorthiazide (250 μg/ml) were prepared. Rosuvastatin calcium and Hydrochlorthiazide stock solutions were diluted to yield solutions in the concentration range of 5-30 µg mL^{-1} and 6.25-37.5 µg mL^{-1}, respectively. The solutions were analyzed by using HPLC. Calibration curve for both the drugs are shown in Figure 8.4 and Figure 8.5. The results of linearity are presented in Table 8.6.

TABLE 8.6: RP-HPLC linearity data of Rosuvastatin calcium and Hydrochlorthiazide.

Linearity range	**Stock solution of linearity**	**Diluted to volume ml**	**Final conc Rosuvastatin calcium (µg/ml)**	**Rosuvastatin calcium Area ± SD**
25%	2.50ml	100 ml	5	46519±95
50%	5.00ml	100 ml	10	98026±123
75%	7.50ml	100 ml	15	150543±181
100%	10.0 ml	100 ml	20	205653±216
125%	12.5 ml	100 ml	25	260541±345
150%	15.0 ml	100 ml	30	315623±424
Linearity range	**Stock solution of linearity**	**Diluted to volume ml**	**Final conc Hydrochlorthiazide (µg/ml)**	**Hydrochlorthiazide Area ± SD**
25%	2.50ml	100 ml	6.25	101236±101

50%	5.00ml	100 ml	12.50	202513±214
75%	7.50ml	100 ml	18.75	303845±321
100%	10.0 ml	100 ml	25.00	405236±429
125%	12.5 ml	100 ml	31.25	505236±524
150%	15.0 ml	100 ml	37.50	612361±608

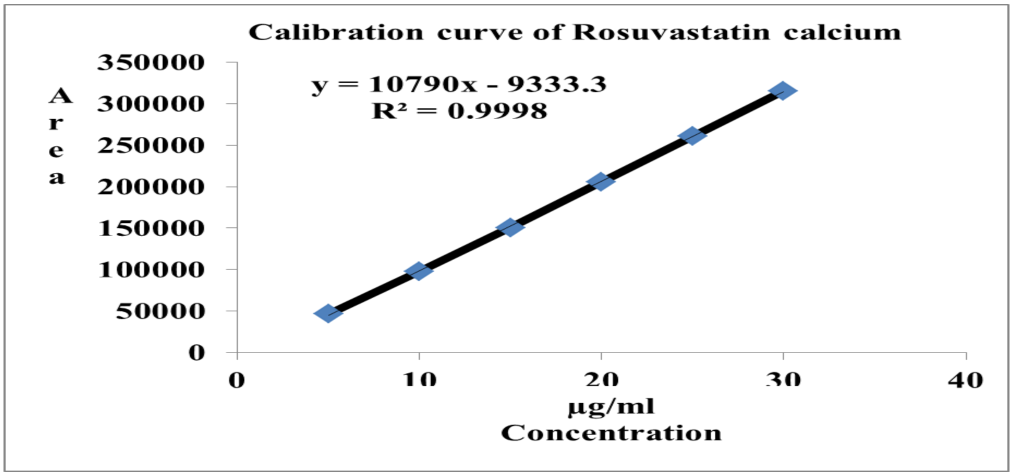

FIGURE 8.4: Calibration curve of Rosuvastatin calcium by RP-HPLC analysis

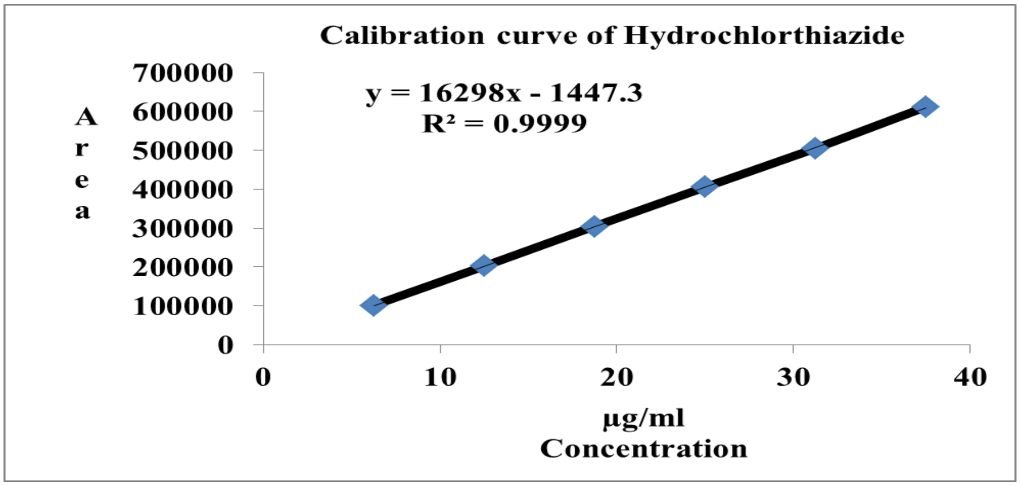

FIGURE 8.5: Calibration curve of Hydrochlorthiazide by RP-HPLC analysis

Repeatability

The method repeatability was done by preparing six different sample preparations by one analyst. The results are presented in Table 8.7. The results obtained were within 2% RSD.

Intermediate precision

Intermediate precision test was determined between different analyst, instrument and column. The value of percentage RSD was below 2.0%, showed Intermediate precision of developed analytical method. The results are presented in Table 8.7.

TABLE 8.7: RP-HPLC repeatability and intermediate precision data of Rosuvastatin calcium and Hydrochlorthiazide

Inj no.	**Sample wt (mg)**	**Rosuvastatin calcium (20 µg/ml)**				**Hydrochlorthiazide (25 µg/ml)**			
		Area	% Assay	% Mean	% RSD	Area	% Assay	% Mean	% RSD
Repeatability									
1	1403.50	205423	99.7	99.8	0.5	409536	100.6	100.1	0.8
2	1404.50	204632	99.2			400853	98.4		
3	1405.50	205846	99.8			409021	100.4		
4	1408.50	205952	99.6			409425	100.2		
5	1390.50	203654	99.8			405151	100.5		
6	1402.60	207645	100.8			408814	100.5		
Intermediate precision									
1	1408.00	205516	99.6	99.5	0.8	401456	99.3	100.0	0.9
2	1407.50	205412	99.6			401595	99.4		
3	1405.00	206547	100.3			401456	99.5		
4	1403.50	206651	100.4			401485	99.6		
5	1404.50	202456	98.3			405262	100.5		
6	1404.20	203561	98.9			409478	101.5		

Accuracy [14-17]

The difference between theoretical added sample amount to the placebo and practically achieved sample amount from placebo (after HPLC analysis) is called accuracy of analytical method. Accuracy was determined at three different level 50%, 100% and 150% of the target concentration in triplicate. The results are presented in Table 8.8 and Table 8.9.

TABLE 8.8: RP-HPLC accuracy data of Rosuvastatin calcium

Level	Placebo (mg)	Conc (µg/ ml)	Amount of drug added (µg/ml)	Amount of drug recovered (µg/ml)	Area	Recovery (%) ± SD (N=3)	Mean (%) ± SD	% RSD
50%	1312.50	10	20.66	20.88	107562	101.1±0.2	100.4±0.7	0.7
	1312.56		20.76	20.86	107456	100.5±0.3		
	1312.98		20.86	20.80	107145	99.7±0.2		
100%	1312.14	20	40.12	39.94	205765	99.6±0.1	99.3±0.6	0.6
	1312.50		40.02	39.88	205471	99.7±0.2		
	1312.81		40.42	39.90	205562	98.7±0.1		
150%	1312.94	30	59.98	60.59	312135	101.0±0.2	100.4±0.5	0.5
	1312.86		60.38	60.42	311254	100.1±0.3		
	1311.52		60.78	60.88	313616	100.2±0.2		

TABLE 8.9: RP-HPLC accuracy data of Hydrochlorthiazide

Level	Placebo (mg)	Conc (µg/ ml)	Amount of drug added (µg/ml)	Amount of drug recovered (µg/ml)	Area	Recovery (%) ± SD (N=3)	Mean (%) ± SD	% RSD
50%	1312.50	12.5	24.95	24.89	202152	99.8±0.2	99.2±0.6	0.6
	1312.56		25.05	24.79	201362	99.0±0.1		
	1312.98		25.15	24.82	201542	98.7±0.2		
100%	1312.14	25	49.90	49.89	405162	100.0±0.3	99.5±0.5	0.5
	1312.50		50.20	49.90	405231	99.4±0.2		

	1312.81		50.40	49.91	405316	99.0±0.4		
150%	1312.94	37.5	74.95	75.42	612513	100.6±0.2	100.4±0.2	0.2
	1312.86		75.15	75.47	612956	100.4±0.3		
	1311.52		75.25	75.42	612512	100.2±0.3		

Robustness

Robustness of the method was carried out by deliberately made small changes in the flow rate, pH, organic phase ratio and column oven temperature. Results are presented in Table 8.10.

TABLE 8.10: Robustness data for Rosuvastatin calcium and Hydrochlorthiazide

<table>
<tr><th colspan="9">Rosuvastatin calcium robustness study. (%RSD For n=5 injections)</th></tr>
<tr><th>Sys. Suit.</th><th>Temp. -5°C</th><th>Temp. +5°C</th><th>Flow -10%</th><th>Flow +10%</th><th>Org. -2%</th><th>Org. +2%</th><th>pH = 3.2</th><th>pH = 2.8</th></tr>
<tr><td>0.1</td><td>0.1</td><td>0.1</td><td>0.8</td><td>0.1</td><td>0.2</td><td>0.7</td><td>0.7</td><td>0.6</td></tr>
<tr><td>Mean %RSD</td><td colspan="8">0.3</td></tr>
<tr><th colspan="9">Hydrochlorthiazide robustness study. (%RSD For n=5 injections)</th></tr>
<tr><th>Sys. Suit.</th><th>Temp. -5°C</th><th>Temp. +5°C</th><th>Flow -10%</th><th>Flow +10%</th><th>Org. -2%</th><th>Org. +2%</th><th>pH = 3.2</th><th>pH = 2.8</th></tr>
<tr><td>0.1</td><td>0.9</td><td>0.5</td><td>0.9</td><td>0.7</td><td>0.3</td><td>0.1</td><td>0.3</td><td>0.2</td></tr>
<tr><td>Mean %RSD</td><td colspan="8">0.4</td></tr>
</table>

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

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ) values, the blank sample was injected six times and the peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level while ten times the noise value gave the LOQ. The results of LOD and LOQ are mentioned in Table 8.11

TABLE 8.11: LOD and LOQ RP-HPLC data for Rosuvastatin calcium and Hydrochlorthiazide

Parameters	Rosuvastatin calcium	Hydrochlorthiazide
LOD	0.2500 µg/ml	0.2000 µg/ml
LOQ	0.8250 µg/ml	0.6600 µg/ml

Result and discussions

The proposed method was found to be simple, accurate, precise and rapid for simultaneous estimation of Rosuvastatin calcium and Hydrochlorthiazide in all dosage forms. The values of relative standard deviation are satisfactorily low and recovery was close to 100% which indicated accuracy and reproducibility of methods.

Table 8.12: Summary of validation parameters of RP-HPLC method for simultaneous estimation of Rosuvastatin calcium and Hydrochlorthiazide

Parameters of validation	Acceptance criteria	Rosuvastatin calcium	Hydrochlorthiazide
Range of linearity	Follows Beer Lambert's law	5 – 30 μg/ml	6.25-37.5 μg/ ml
Correlation coefficient	r>0.999 or 0.995	0.9999	0.9999
Regression coefficient	$r^2 > 0.999$ or 0.995	0.9998	0.9999
LOD	S/N > 2 or 3	0.2500 μg/ml	0.2000 μg/ml
LOQ	S/N > 10	0.8250 μg/ml	0.6600 μg/ml
Repeatability	RSD < 2%	0.5%	0.8%
Intermediate precision	RSD < 2%	0.8%	0.9%
Accuracy	98- 102%	99.3% to 100.4%	99.2% to 100.4%
Specificity of blank, placebo	No interference	No interference of blank, placebo	No interference of blank, placebo
Solution stability	> 12 hour	Stable for 24 hr %RSD = 0.7%	Stable for 24 hr %RSD =0.9%
Robustness **Flow rate (+ & -),** **Buffer pH (+ & -),** **Column temperature(+ & -), &** **Organic ratio (+ & -), in mobile phase**	RSD NMT 2% in given condition	complies % RSD 0.7% & 0.9% % RSD 0.3% & 0.2% % RSD 0.5% & 0.9 % % RSD 0.1% & 0.3%	complies % RSD 0.1% & 0.8% % RSD 0.7% & 0.6% % RSD 0.1% & 0.1 % % RSD 0.7 % & 0.2%

8.2 UV spectroscopic method for Rosuvastatin calcium and Hydrochlorthiazide [14-18]

METHOD OF ANALYSIS

Diluent: Methanol

Wavelength: 243nm and 270 nm

Proper wavelength selection for estimation of both drugs depends on nature of drugs and their solubility. Many trials for solubility of both drugs in different diluents like acetonitrile, water, water: acetonitrile (50:50, v/v) ethanol, isopropanol, methanol etc were conducted and methanol as diluent was finalized for method. Method employs solving of simultaneous equations based on the measurement of absorbance at two wavelengths 243 nm and 270 nm which are the λmax values of Rosuvastatin calcium and Hydrochlorthiazide in methanol. Calculation for both drugs are done as per below formula.

At λ_1 $$A_1 = aX_1 b\, Cx + aY_1 b\, Cy \text{ ---------------------- } (1)$$

At λ_2 $$A_2 = aX_2 b\, Cx + aY_2 b\, Cy \text{ ---------------------- } (2)$$

For measurements in 1 cm cells b=1

Rearrange eq. (2)

$$Cy = \frac{A_2 - aX_2 b\, Cx}{aY_2}$$

Substituting for Cy in eq. (1) and rearranging

$$Cx = \frac{A_2\, aY_1 - A_1\, aY_2}{aX_2\, aY_1 - aX_1 aY_2} \text{ ---------------------------- } (3)$$

$$Cy = \frac{A_1\, aX_2 - A_2\, aX_1}{aX_2\, aY_1 - aX_1 aY_2} \text{ ---------------------------- } (4)$$

A1= Absorption of mixture at 243 nm.

A2= Absorption of mixture at 270 nm.

ay1=Molar absorptivity of Rosuvastatin calcium at 243 nm.

ay2=Molar absorptivity of Rosuvastatin calcium at 270nm.

C_Y=Concentration of Rosuvastatin calcium (μg/ml).

Cx=Concentration of Hydrochlorthiazide (μg/ml).

ax1=Molar absorptivity of hydrochlorothiazide at 243 nm.

ax2=Molar absorptivity of Hydrochlorthiazide at 270nm.

Preparation of standard solution

The standard stock solution Rosuvastatin calcium (200 µg/ml) and Hydrochlorthiazide (250 µg/ml) were prepared by weighing Rosuvastatin calcium 40 mg and Hydrochlorthiazide 50 mg in 200ml volumetric flask respectively. Add 120 ml diluent to it and sonicate well for 5 minutes and making volume up to mark with diluent. Then 10 ml of standard stock solution was diluted to 200 ml with diluent to make final standard concentration of Rosuvastatin calcium (10 µg/ml) and Hydrochlorthiazide (12.5 µg/ml), respectively.

Preparation of test solution

Accurately 20 intact tablets were weighed and average weight of tablet was calculated. Then tablets were finely crushed, powdered and sample powder about 1400 mg (Tablet powder Equivalent to 40 mg Rosuvastatin calcium and 50 mg Hydrochlorthiazide or four tablets powder) was transferred into 200 ml volumetric flask. Then add about 120.0 ml diluent was added and sonicate for 30 minutes with intermittent shaking. Then volume was made up to mark with diluent. Then 10 ml of standard stock solution was diluted to 200 ml with diluent to make final standard concentration of Rosuvastatin calcium (10 µg/ml) and Hydrochlorthiazide (12.5 µg/ml), respectively. The test solution was filtered through 0.45µ (PVDF Millipore Filter) and analyzed by using UV Spectrophotometer.

UV spectra for Rosuvastatin calcium (25 µg/ml) and Hydrochlorthiazide (25 µg/ml) for wavelength maxima selection is shown in (Figure 8.6) and (Figure 8.7). Absorbance at both wavelength 243 nm and 270 nm for both drugs are shown in Table 8.13

TABLE 8.13: UV absorbance data of Rosuvastatin calcium and Hydrochlorthiazide at both wavelengths.

Drug	wavelength 243 nm	wavelength 270 nm
Rosuvastatin calcium	0.8781	0.4110
Hydrochlorthiazide	0.1086	0.6400

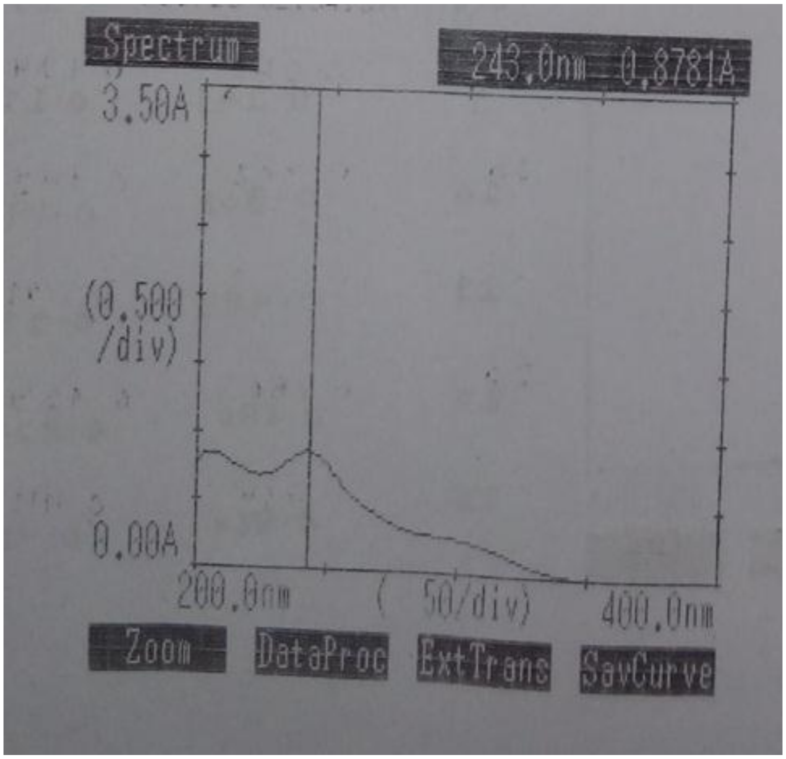

FIGURE 8.6: **UV spectrum of Rosuvastatin calcium (25 µg/ml)**

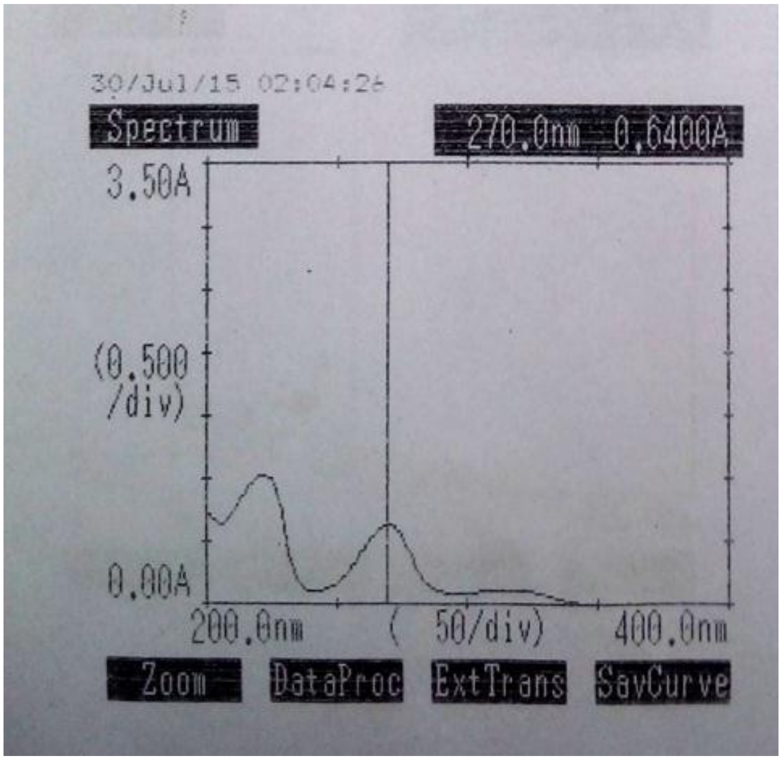

FIGURE 8.7: UV spectrum of Hydrochlorthiazide (25 µg/ml).

Method validation [19-21]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1). The developed method validated with respect to parameters such as linearity, Repeatability, accuracy, specificity, Intermediate precision and solution stability.

Specificity

Diluent and placebo spectra are scanned over a range from 200 nm and 400 nm. Specificity of standard and sample are shown in Figure 8.8 and Figure 8.9 respectively.

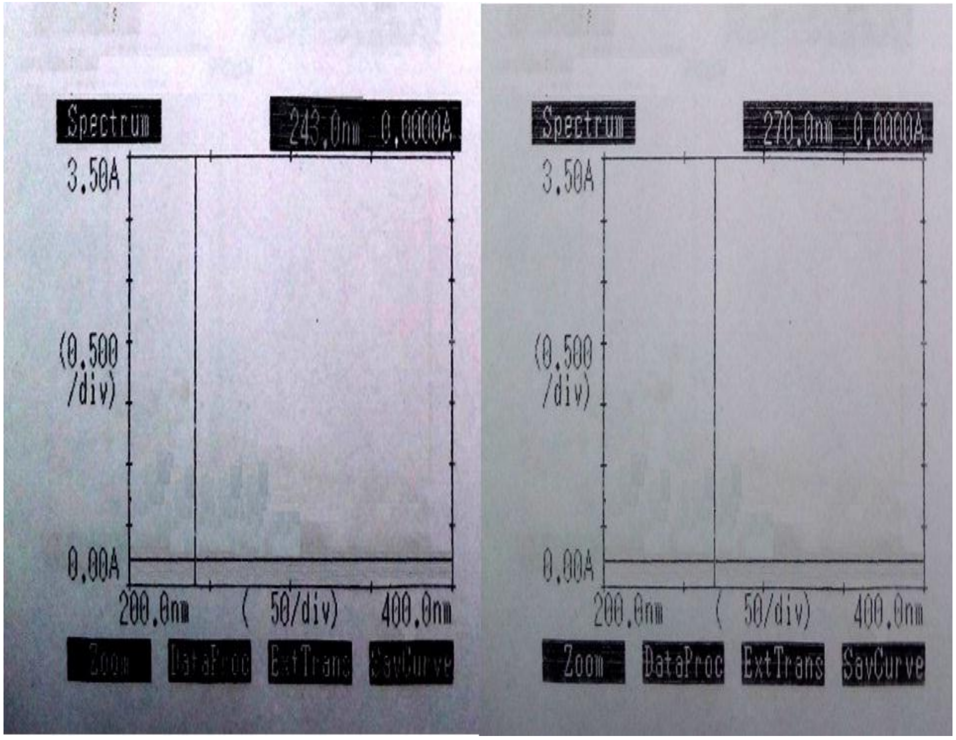

FIGURE 8.8: Specificity study for diluent at both wavelengths

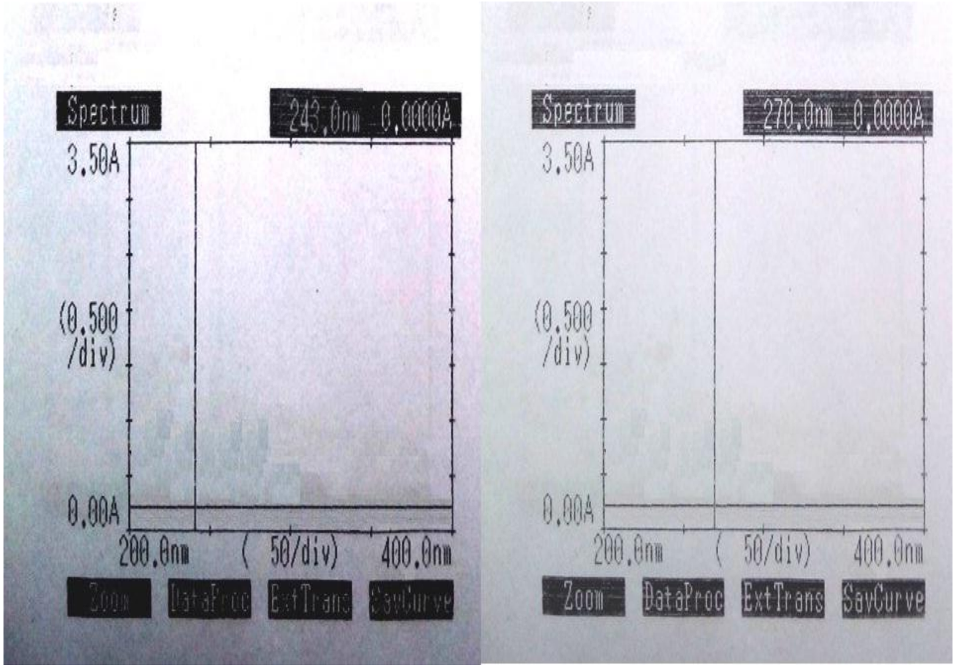

FIGURE 8.9: Specificity study for placebo at both wavelengths

Linearity

To achieve linearity and range, stock solution containing Rosuvastatin calcium (200 μg/ml) and Hydrochlorthiazide (250 μg/ml) were separately prepared. Rosuvastatin calcium and Hydrochlorthiazide Stock solutions were diluted to yield solutions in the concentration range of 5-15 µg mL^{-1} and 6.25-18.75 µg mL^{-1}, respectively. The solutions were analyzed by using UV. Overlain linearity spectra for Rosuvastatin calcium and Hydrochlorthiazide are shown in Figure 8.10 and Figure 8.11. Calibration curve for both the drugs are shown in Figure 8.12, Figure 8.13, Figure 8.14 and Figure 8.15. The results of linearity are presented in Table 8.14. Molar extinction coefficient for wavelength 243 nm and 270 nm for both drugs are obtained using data from linearity table.

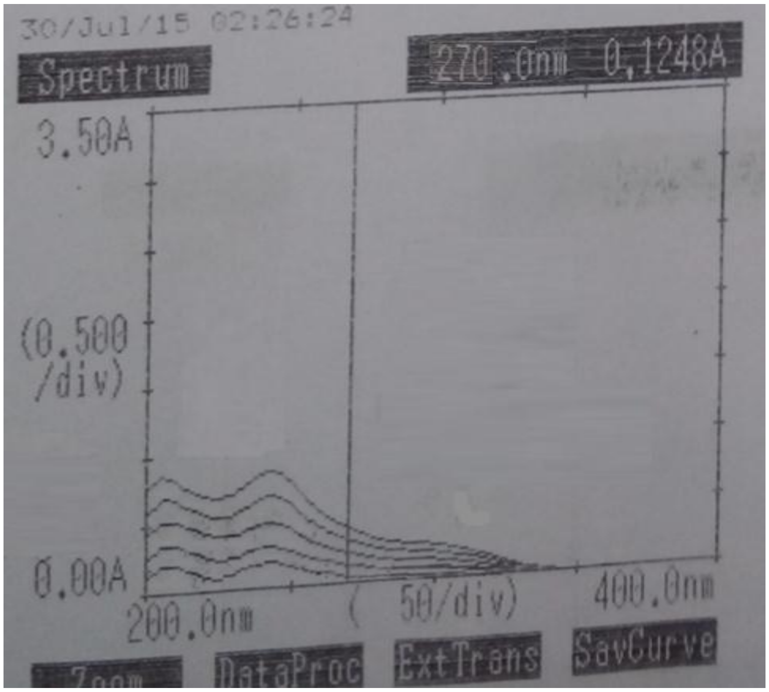

FIGURE 8.10: Overlain Spectra of linearity for Rosuvastatin calcium

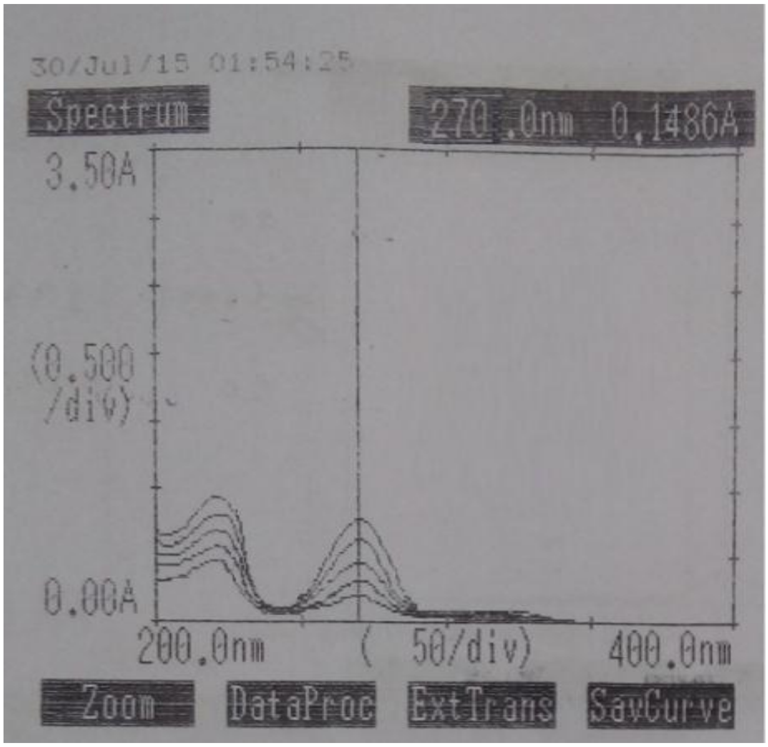

FIGURE 8.11: Overlain spectra of linearity for Hydrochlorthiazide

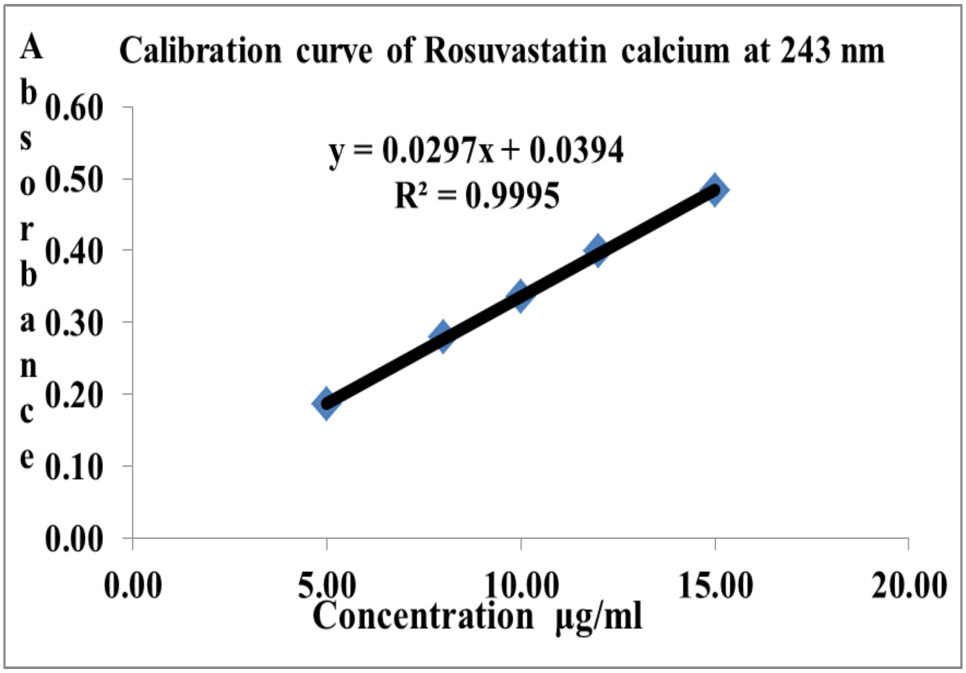

FIGURE 8.12: Calibration curve for Rosuvastatin calcium at 243 nm

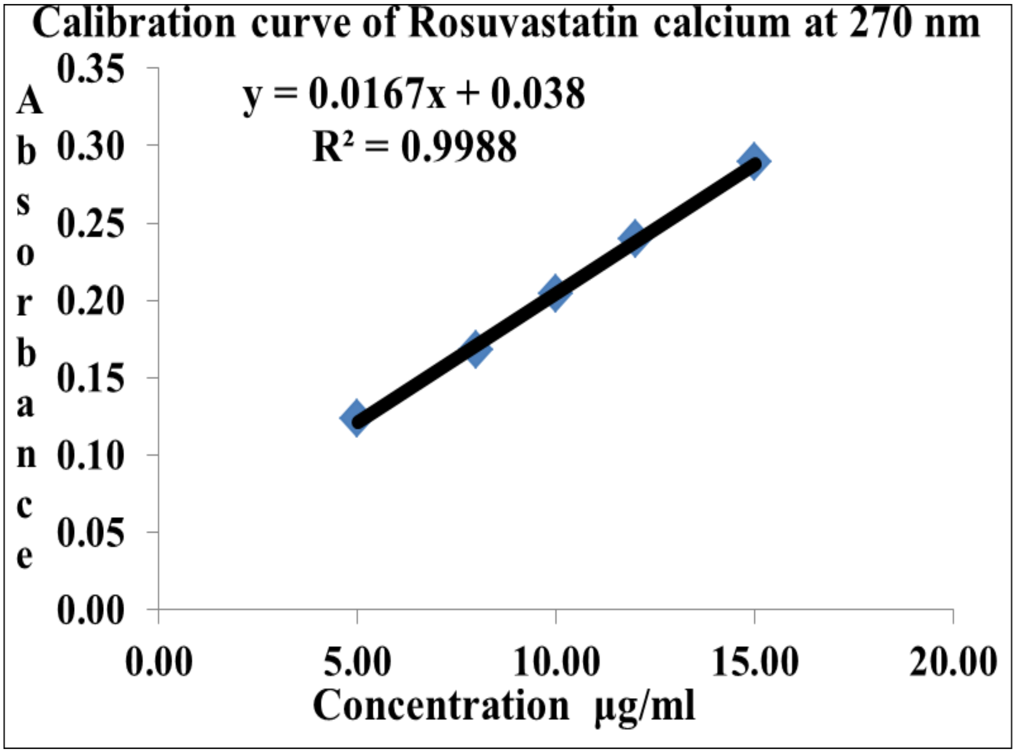

FIGURE 8.13: Calibration curve for Rosuvastatin calcium at 270 nm

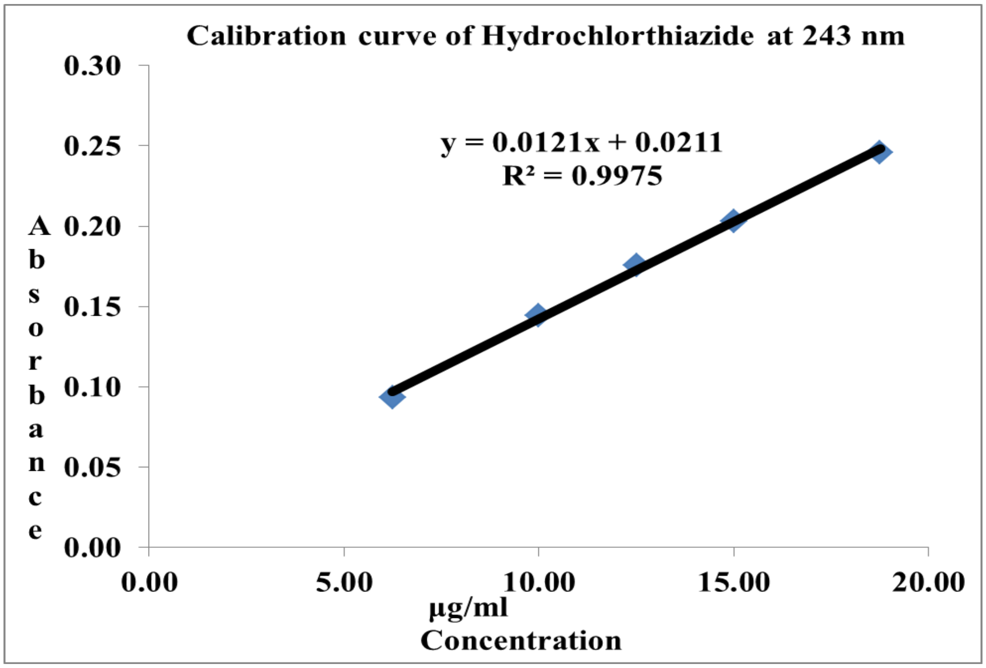

FIGURE 8.14: Calibration curve for Hydrochlorthiazide at 243 nm

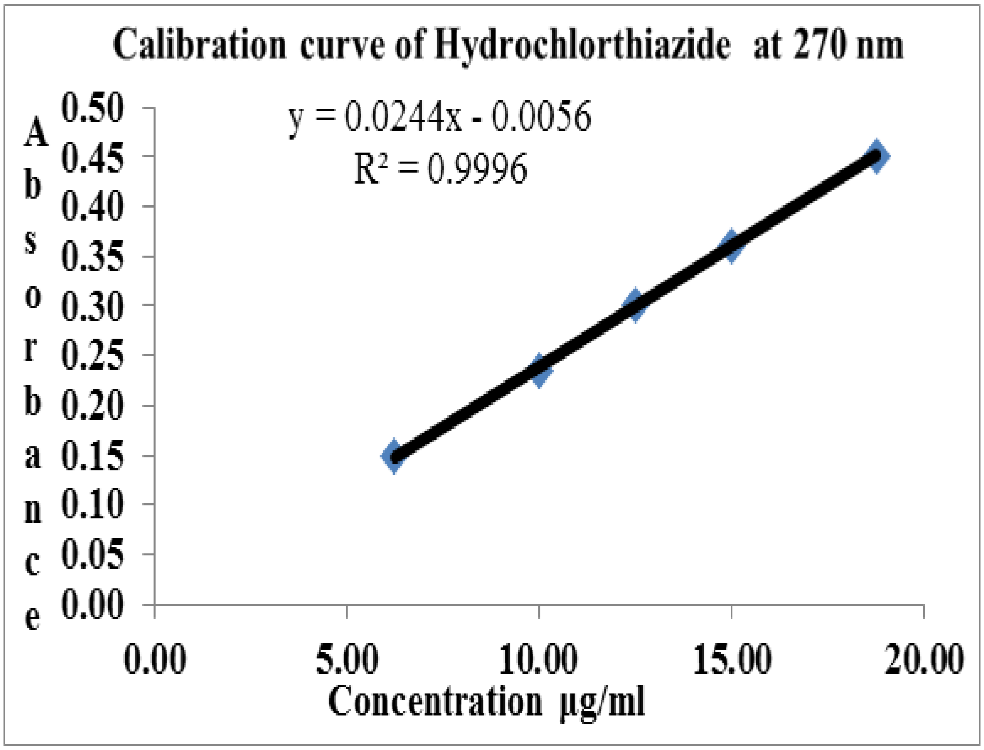

FIGURE 8.15: Calibration curve for Hydrochlorthiazide at 270 nm

TABLE 8.14: Linearity data of Rosuvastatin calcium and Hydrochlorthiazide

Linearity Range	**Stock soln ml**	**Diluted to volume ml**	**Final Conc Rosuvastatin calcium (µg/ml)**	**Rosuvastatin calcium Absorbance ± SD (N=3) λ 243 nm**	**Rosuvastatin calcium Absorbance ± SD (N=3) λ 270 nm**
50%	5.00	200	5.00	0.1860±0.0001	0.1248±0.0001
80%	8.0	200	8.00	0.2796±0.0001	0.1684±0.0002
100%	10.0	200	10.00	0.3350±0.0003	0.2040±0.0002
120%	12.0	200	12.00	0.3985±0.0004	0.2394±0.0001
150%	15.0	200	15.00	0.4830±0.0003	0.2893±0.0002
Linearity Range	**Stock soln ml**	**Diluted to volume ml**	**Final Conc Hydro-chlorthiazide (µg/ml)**	**Hydrochlorthiazide Absorbance ± SD (N=3) λ 243 nm**	**Hydrochlorthiazide Absorbance ± SD (N=3) λ 270 nm**
50%	5.00	200	6.25	0.0935±0.0001	0.1486±0.0001
80%	8.0	200	10.00	0.1445±0.0001	0.2342±0.0002
100%	10.0	200	12.50	0.1756±0.0002	0.301±0.0001
120%	12.0	200	15.00	0.2033±0.0003	0.3612±0.0002
150%	15.0	200	18.75	0.2456±0.0004	0.4515±0.0001

REPEATABILITY [22-25]

The method repeatability was done by preparing six different sample preparations by one analyst. The results are presented in Table 8.15. The results obtained were within 2% RSD.

TABLE 8.15: Repeatability UV data of Rosuvastatin calcium and Hydrochlorthiazide

Sr. No	% Assay repeatability					
	Rosuvastatin calcium (10 µg/ml)					
	Absorbance			**% Assay**	**% Mean**	**% RSD**
	wt (mg)	243 nm	270 nm			
1	1403.20	0.4344	0.3768	98.1	98.5	0.3
2	1403.10	0.4341	0.3732	98.6		
3	1403.50	0.4366	0.3762	98.5		
4	1402.52	0.4319	0.3792	98.9		
5	1401.90	0.4329	0.3781	98.3		
6	1400.50	0.4346	0.3774	98.7		
Hydrochlorthiazide (12.5 µg/ml)						
Sr. No	**Absorbance**			**% Assay**	**% Mean**	**% RSD**
	wt (mg)	243 nm	270 nm			
1	1403.20	0.4344	0.3768	101.6	101.1	0.6
2	1403.10	0.4341	0.3732	101.4		
3	1403.50	0.4366	0.3762	101.8		
4	1402.52	0.4319	0.3792	100.5		
5	1401.90	0.4329	0.3781	100.9		
6	1400.50	0.4346	0.3774	100.3		

Intermediate precision

Intermediate precision test was determined between different analysts. The value of percentage RSD was below 2.0%, showed Intermediate precision of developed analytical method. The results are presented in Table 8.16

TABLE 8.16: Intermediate precision data for Rosuvastatin calcium and Hydrochlorthiazide

Sr. No	% Assay intermediate precision					
	Rosuvastatin calcium (10 µg/ml)					
	Absorbance			% Assay	% Mean	% RSD
	wt (mg)	243 nm	270 nm			
1	1401.18	0.4344	0.3768	98.9	99.2	0.7
2	1402.80	0.4341	0.3732	99.4		
3	1401.29	0.4366	0.3762	98.3		
4	1400.12	0.4319	0.3792	100.1		
5	1400.80	0.4329	0.3781	98.7		
6	1403.50	0.4346	0.3774	99.9		
Hydrochlorthiazide (12.5 µg/ml)						
Sr. No	Absorbance			% Assay	% Mean	% RSD
	wt (mg)	243 nm	270 nm			
1	1401.18	0.4344	0.3768	100.9	100.7	0.9
2	1402.80	0.4341	0.3732	101.7		
3	1401.29	0.4366	0.3762	100.4		
4	1400.12	0.4319	0.3792	100.1		
5	1400.80	0.4329	0.3781	99.2		
6	1403.50	0.4346	0.3774	101.8		

Accuracy

The difference between theoretical added sample amount to the placebo and practically achieved sample amount from placebo (after UV analysis) is called accuracy of analytical method. Accuracy was determined at three different level 80%, 100% and 120% of the target concentration in triplicate. The results are presented in Table 8.17 and Table 8.18.

TABLE 8.17: Accuracy data of Rosuvastatin calcium for UV analysis

Level	Placebo (mg)	Absorbance		Amount of drug added (µg/ml)	Amount of drug recovered (µg/ml)	Recovery ± SD (%) (N=3)	Mean ± SD (%)	% RSD
		243 nm	270 nm					
80%	1312.56	0.3475	0.301	8.05	8.01	99.5±0.1	99.8±1.6	1.6
	1312.42	0.3471	0.3051	8.12	7.99	98.4±0.1		
	1312.60	0.3478	0.3056	8.14	8.26	101.5±0.2		
100%	1312.31	0.4344	0.3768	10.15	10.01	98.6±0.1	99.3±0.8	0.8
	1312.25	0.4342	0.3764	10.18	10.09	99.1±0.2		
	1312.46	0.434	0.3761	10.2	10.22	100.2±0.2		
120%	1312.85	0.5212	0.4521	12.1	12.26	101.3±0.1	100.6±1.2	1.2
	1312.66	0.5209	0.4518	12.08	12.23	101.2±0.2		
	1312.75	0.5216	0.4524	12.25	12.15	99.2±0.1		

TABLE 8.18: Accuracy data of Hydrochlorthiazide for UV analysis.

Level	Placebo (mg)	Absorbance		Amount of Drug added (µg/ml)	Amount of Drug recovered (µg/ml)	Recovery ± SD (%) (N=3)	Mean ± SD (%)	% RSD
		243 nm	270 nm					
80%	1312.56	0.3475	0.301	10.05	10.14	100.9±0.1	101.0±0.4	0.4
	1312.42	0.3471	0.3051	10.09	10.16	100.7±0.2		
	1312.60	0.3478	0.3056	10.07	10.21	101.4±0.3		
100%	1312.31	0.4344	0.3768	12.52	12.41	99.1±0.1	100.3±1.0	1.0
	1312.25	0.4342	0.3764	12.48	12.68	100.8±0.2		
	1312.46	0.434	0.3761	12.58	12.65	100.6±0.2		
120%	1312.85	0.5212	0.4521	15.01	14.99	99.9±0.2	100.9±0.9	0.9
	1312.66	0.5209	0.4518	15.09	15.3	101.4±0.3		
	1312.75	0.5216	0.4524	15.22	15.43	101.4±0.2		

Solution stability

The standard and sample solutions were found stable up to 24 hours at room temperature. The results are presented in Table 8.19 and Table 8.20.

TABLE 8.19: Standard solution stability UV data of Rosuvastatin calcium and Hydrochlorthiazide

Time (hr)	**Absorbance**		**% Difference**	
	243 nm	**270 nm**	**Rosuvastatin calcium**	**Hydrochlorthiazide**
0	0.4349	0.3761	==	==
8	0.4341	0.3759	0.44	0.39
24	0.434	0.3739	0.48	0.42
% Mean RSD			0.5	0.4

TABLE 8.20: Sample solution stability UV data of Rosuvastatin calcium and Hydrochlorthiazide

Time (hr)	**Absorbance**		**% Difference**	
	243 nm	**270 nm**	**Rosuvastatin calcium**	**Hydrochlorthiazide**
0	0.4347	0.3764	==	==
8	0.4340	0.3761	0.18	0.17
24	0.4338	0.3729	0.4	0.44
% Mean RSD			0.3	0.3

LOD (LIMIT OF DETECTION AND LOQ (LIMIT OF QUANTITATION)

The results of LOD and LOQ are mentioned in Table 8.21.

TABLE 8.21: LOD and LOQ Data for sample solution

Parameters	**Rosuvastatin calcium**	**Hydrochlorthiazide**
LOD	1.0000 µg/ml	1.2500 µg/ml
LOQ	3.3000 µg/ml	4.1250 µg/ml

TABLE 8.22: Summary of validation parameters of UV method for simultaneous estimation of Rosuvastatin calcium and Hydrochlorthiazide

Parameters of validation	**Acceptance criteria**	**Rosuvastatin calcium**		**Hydrochlorthiazide**	
Range of Linearity	Follows Beer Lambert's law	5 – 15 µg/ml		6.25-18.75 µg/ml	
Correlation Coefficient	R > 0.990 or 0.995	243 nm	270 nm	243 nm	270 nm
		0.9998	0.9994	0.9998	0.9998
Regression coefficient	$R^2 > 0.990$ or 0.995	0.9995	0.9988	0.9975	0.9996
Regression equation	-	Y= 0.0297x+ 0.0394	Y= 0.0167x + 0.038	Y=0.0121x+0.0211	Y=0.0244x-0.0056
LOD	-	1.0000 µg/ml		1.2500 µg/ml	
LOQ	-	3.3000 µg/ml		4.1250 µg/ml	
Repeatability	RSD < 2%	0.30%		0.60 %	
Intermediate precision	RSD < 2%	0.70%		0.90 %	
Accuracy	Recovery 98- 102%	98.1% to 101.4%		99.1 % to 101.3 %	
Specificity	No interference of blank, Placebo	No interference of blank, Placebo		No interference of blank, Placebo	
Solution Stability	> 12 hour	Stable for 24 hr %RSD = 0.3%		Stable for 24 hr %RSD = 0.3%	

TABLE 8.23: Marketed formulation assay comparison for Rosuvastatin calcium and Hydrochlorthiazide

Assay by HPLC						
Brand Name	**Label claim (mg)**		**Amount Found**		**% Assay ± % CV**	
	Rosuvastatin calcium	**Hydrochlorthiazide**	**Rosuvastatin calcium**	**Hydrochlorthiazide**	**Rosuvastatin calcium (N=3)**	**Hydrochlorthiazide (N=3)**
Zyrova H	10	12.5	10.12	12.48	101.12 ± 0.14	99.84 ± 0.27
Rozustat H	10	12.5	9.98	12.42	99.8 ± 0.27	99.36 ± 0.47
Assay by UV						
Brand Name	**Label claim (mg)**		**Amount Found**		**% Assay ± % CV**	
	Rosuvastatin calcium	**Hydrochlorthiazide**	**Rosuvastatin calcium**	**Hydrochlorthiazide**	**Rosuvastatin calcium (N=3)**	**Hydrochlorthiazide (N=3)**
Zyrova H	10	12.5	10.07	12.49	100.07 ± 0.19	99.92 ± 0.47
Rozustat H	10	12.5	10.03	12.56	100.03 ± 0.17	100.48 ± 0.52

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CHAPTER-9

RP-HPLC and HPTLC method for Metoprolol succinate and Chlorthalidone

9.1 RP-HPLC method for Metoprolol succinate and Chlorthalidone [1-4]

TABLE 9.1: Drug & marketed formulation details of Metoprolol succinate and Chlorthalidone

Drug	Company	
Metoprolol succinate	Cadila healthcare ltd, Ankleshwar, Gujarat	
Chlorthalidone	Glenmark pharmaceuticals ltd, Ankleshwar Gujarat	
Drug name	**Marketed formulation-I**	**Marketed formulation-II**
Metoprolol succinate 50 mg + Chlorthalidone 12.5 mg	Vinicor D tablets IPCA laboratories ltd Kandivali (West), Mumbai, Maharashtra	Promet D tablets Grownbury pharma pvt. ltd Krishna nagar, New Delhi

Experimental work

Chromatographic condition

Column	: Inerstil ODS 3 (4.6 X 100 mm), 5µm.
Detector	: 254 nm
Injection volume	: 20 µl
Flow Rate	: 1.0 mL min^{-1}
Temperature	: 30º C
Run Time	: 14 minutes
Mobile Phase	: Diammonium hydrogen phosphate buffer (at pH 5.5): methanol (70:30, v/v)
Diluent	: Methanol as diluent I and mobile phase as diluent II

Buffer preparation

Accurately weighed 1.3 g diammonium hydrogen phosphate was dissolved in to 1000 mL milli-q water and 10 ml triethylamine was added to this buffer solution, than pH was adjusted to 5.5 with diluted ortho-phosphoric acid solution.

Preparation of standard solution

Approximately 100 mg of Metoprolol succinate and 25 mg of Chlorthalidone reference standards were accurately weighed and transferred to a 200 ml volumetric flask. The weighed sample was dissolved in methanol and sonicated for 10 minutes. The weighed sample was made up to volume with methanol to produce a standard stock solution. 10 ml volume of aliquot from the standard stock solution was appropriately diluted to 25 ml with mobile phase obtain final standard concentration of Metoprolol succinate (200 μg/ml) and Chlorthalidone (50 μg/ml), respectively.

Preparation of test solution

Twenty tablets were weighed accurately and finely powdered. Tablet powder equivalent to 250 mg of Metoprolol succinate and 62.5 mg of Chlorthalidone (equivalent to 5 tablets powder) was transferred to a 500 ml volumetric flask. 200 ml of methanol was added to above flask and flask was sonicated for 30 minutes. The solution was made up to mark with methanol. 10 ml volume of aliquot was transferred to 25 ml volumetric flask and volume was made up to mark with diluent II to obtain final standard concentration of Metoprolol succinate (200 μg/ml) and Chlorthalidone (50 μg/ml). The test solution was filtered through 0.45µ (PVDF Millipore Filter) and analyzed by using HPLC. The detection wavelength of 254 nm was chosen in order to achieve a good sensitivity for quantitative determination of Metoprolol succinate and Chlorthalidone in solid dosage form. The retention time for Metoprolol succinate was 6.9 min and Chlorthalidone was 9.9 min. The isocratic program throughout HPLC method was adopted to analyze both components in a single run as shown in Figure 9.1

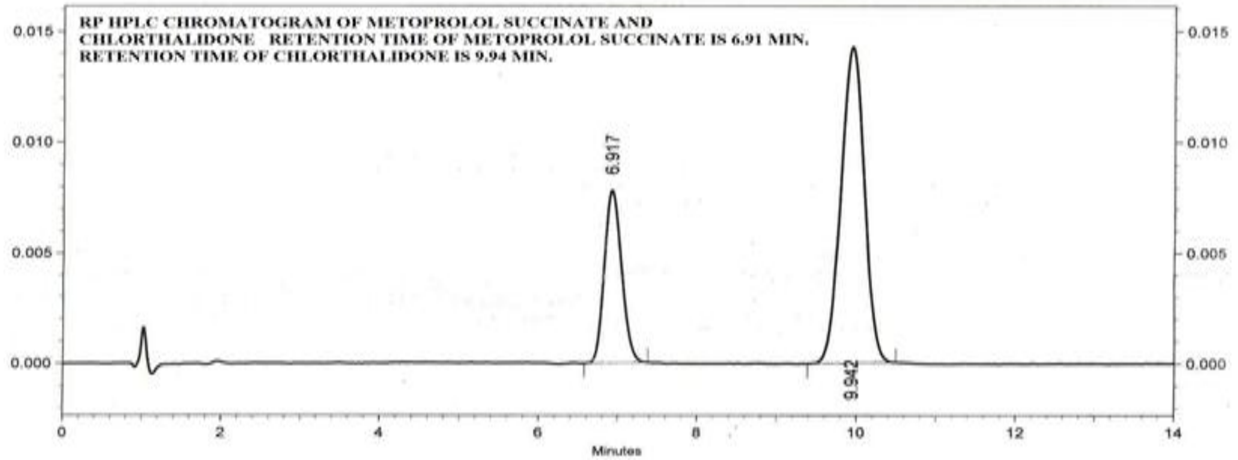

FIGURE 9.1: HPLC chromatogram of Metoprolol succinate and Chlorthalidone.

Method validation [5-9]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1). The developed method validated with respect to parameters such as system suitability, solution stability, specificity, linearity, repeatability, accuracy, intermediate precision, robustness, limit of detection and limit of quantitation.

System suitability and system precision

System suitability test were performed to check repeatability of system for analysis performed. The results for system suitability parameters were found satisfactory. The results of system suitability and system precision are presented in Table 9.2.

TABLE 9.2: System suitability parameters of RP-HPLC analysis for Metoprolol succinate and Chlorthalidone

Drug	Retention time ± SD (min) (N=5)	Theoretical plates ± SD (N=5)	Asymmetry ± SD (N=5)	% RSD (N=5)	Resolution ± SD (N=5)
Metoprolol succinate	6.9 ± 0.0039	4536 ± 235	1.22±0.10	0.2	-
Chlorthalidone	9.9 ± 0.0058	5221±142	1.03±0.11	0.1	6.65 ±0.04

Solution stability

Standard and sample solutions were kept at room temperature (25°C) for 24 hours and solution stability data after 0 hours, 8 hours and 24 hours were calculated. The change in % RSD was calculated. Standard solution and sample solution of concentration of Metoprolol succinate (200 µg/ml) and Chlorthalidone (50 µg/ml) were taken to test solution stability. It was found that change in % RSD for standard and sample solution was not more than 2%. The results of solution stability are summarized in Table 9.3. The standard and sample solutions were found stable up to 24 hours at room temperature.

TABLE 9.3: RP-HPLC data of standard and sample solution stability for Metoprolol succinate and Chlorthalidone

Standard solution stability				
Time (hr)	**Area**		**% Difference**	
	Metoprolol succinate	**Chlorthalidone**	**Metoprolol succinate**	**Chlorthalidone**
0	119403	292364		
8	119564	293462	0.07	0.27
24	119652	295412	0.11	0.53
% Mean RSD			0.09	0.4
Sample solution stability				
Time (hr)	**Area**		**% Difference**	
	Metoprolol succinate	**Chlorthalidone**	**Metoprolol succinate**	**Chlorthalidone**
0	119542	294526	-	-
8	119856	295846	0.12	0.32
24	119954	296535	0.19	0.35
% Mean RSD			0.16	0.34

Specificity

Specificity of analytical method is ability to measure analyte accurately and specifically in presence of component that may be expected to be present in the sample matrix. Chromatograms of standard, sample, blank and placebo were overlaid for specificity check in sample. Peak purity of standard and sample were obtained using photodiode array detector. The results of peak purity showed peak purity index greater than 0.99. The results of specificity of Metoprolol succinate and Chlorthalidone are summarized in Table 9.4.

TABLE 9.4: RP-HPLC data of specificity study of Metoprolol succinate and Chlorthalidone

Sample	3 point purity	
	Metoprolol succinate	Chlorthalidone
Standard Solution	0.9999	0.9999
Test Solution	0.9999	0.9998

Peak purity

Peak purity curve of Metoprolol Succinate and Chlorthalidone for standard and sample is shown in Figure 9.2 & 9.3.

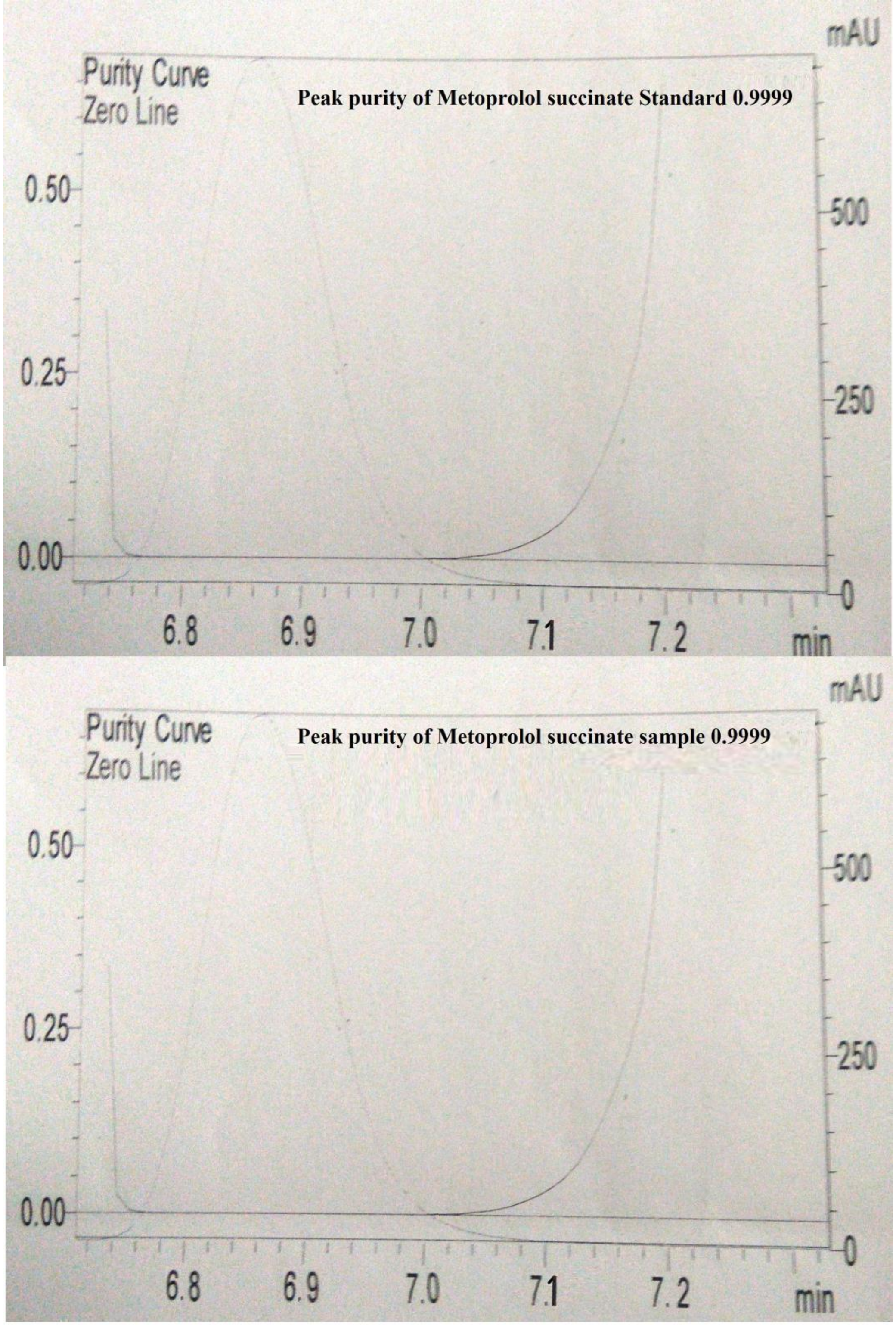

FIGURE 9.2: **Peak purity curve of Metoprolol succinate**

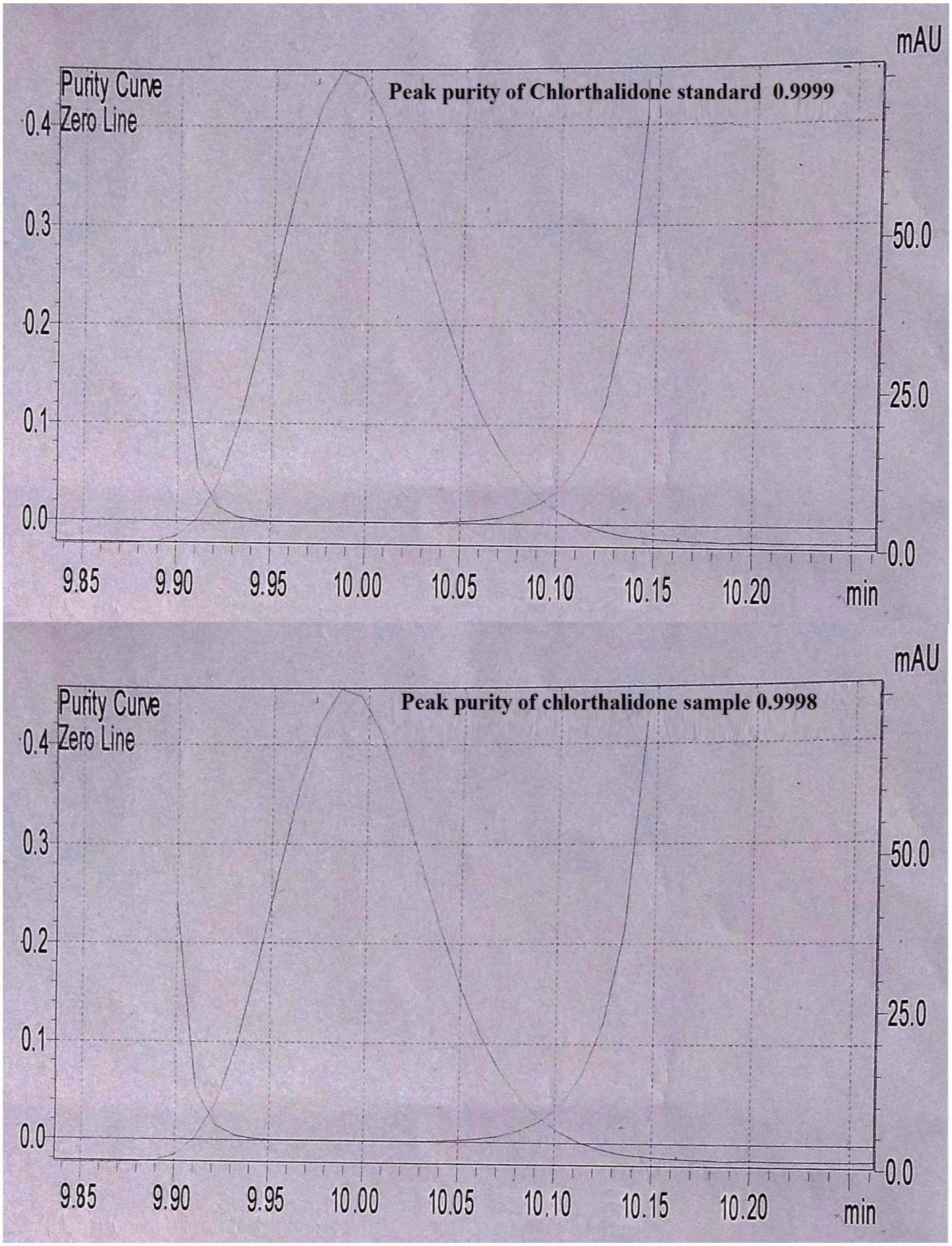

FIGURE 9.3: **Peak purity curve of Chlorthalidone**

Linearity

Appropriate aliquots of Metoprolol succinate and Chlorthalidone standard stock solutions were taken in different 25 ml volumetric flasks. The volume was made upto mark with mobile phase to yield solutions in final concentration range of 50-300 µg mL^{-1} for Metoprolol succinate and 12.5-75 µg mL^{-1} for Chlorthalidone. The solutions were analyzed by using HPLC. Calibration curve for both the drugs are shown in Figure 9.4 and Figure 9.5. The results of linearity are presented in Table 9.5.

TABLE 9.5: RP-HPLC linearity data of Metoprolol succinate and Chlorthalidone

Linearity range	Stock solution of linearity	Diluted to volume ml	Final conc Metoprolol (µg/ml)	Metoprolol area +SD (N=3)
25%	2.50ml	25 ml	50	29851±523
50%	5.00ml	25 ml	100	59702±952
75%	7.50ml	25 ml	150	89552±1051
100%	10.0 ml	25 ml	200	119403±1085
125%	12.5 ml	25 ml	250	149254±1520
150%	15.0 ml	25 ml	300	181105±1652
Linearity range	**Stock solution of linearity**	**Diluted to volume ml**	**Final conc Chlorthalidone (µg/ml)**	**Chlorthalidone area +SD (N=3)**
25%	2.50ml	25 ml	12.5	73091±213
50%	5.00ml	25 ml	25	146182±410
75%	7.50ml	25 ml	37.5	219892±642
100%	10.0 ml	25 ml	50	292364±865
125%	12.5 ml	25 ml	62.5	365621±985
150%	15.0 ml	25 ml	75	445221±1012

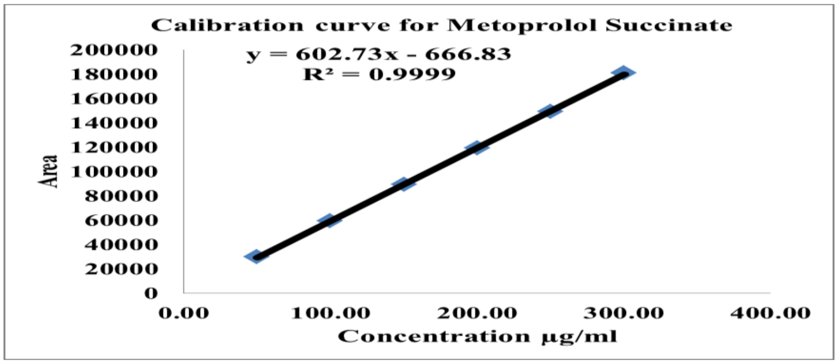

FIGURE 9.4: Calibration curve of Metoprolol succinate for RP-HPLC analysis

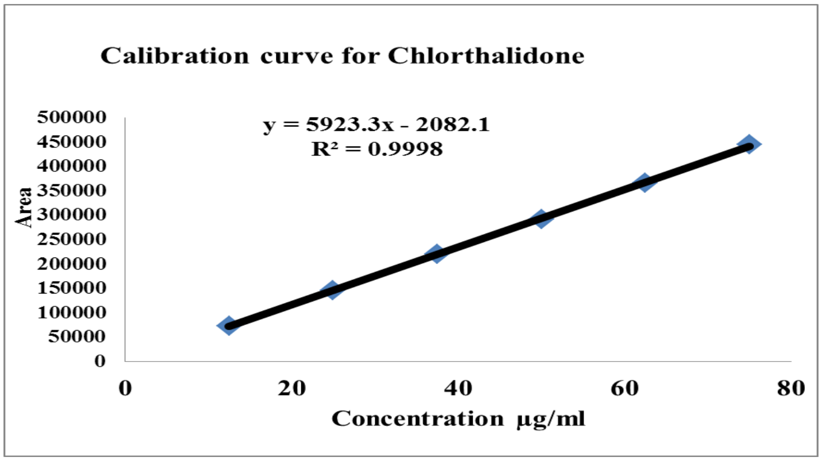

FIGURE 9.5: Calibration curve of Chlorthalidone for RP-HPLC analysis

Repeatability

The method repeatability was done by preparing six different sample preparations by one analyst. Repeatability is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances. Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions like same reagents, equipments, settings and laboratory over a short interval of time. It is normally expected that at least six replicates should be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n value. The RSD values are important for showing degree of variation expected when the analytical procedure is repeated several time in a standard situation. Repeatability should be below 2% for bulk drugs and below 2% for assay in finished product. The results are presented in Table 9.6. The results obtained were within 2% RSD.

Intermediate precision

Intermediate precision test was determined between different analyst, instrument and column. The value of percentage RSD was below 2.0%, showed intermediate precision of developed analytical method. The results are presented in Table 9.6.

TABLE 9.6: Repeatability and intermediate precision RP-HPLC data for Metoprolol succinate and Chlorthalidone.

Repeatability									
Inj no	**Sample wt (mg)**	**Metoprolol succinate (200 µg/ml)**				**Chlorthalidone (50 µg/ml)**			
		Area	% Assay	% Mean	% RSD	Area	% Assay	% Mean	% RSD
1	1070.00	119542	100.4	100.4	0.1	294526	101.0	100.6	0.6
2	1071.30	119430	100.3			295412	101.2		
3	1069.40	119545	100.3			290843	99.8		
4	1069.90	119785	100.6			291452	100.0		
5	1069.80	119625	100.5			294562	101.0		
6	1071.50	119648	100.3			294123	100.7		
Intermediate precision									
1	1073.00	119856	100.6	100.4	0.3	295362	101.3	100.3	0.1
2	1071.30	119692	100.5			294512	100.9		
3	1069.40	119841	100.7			295621	101.4		
4	1069.90	119156	100.1			289999	99.5		
5	1069.80	119025	100.0			289654	99.4		
6	1071.50	119245	100.5			290056	99.3		

Accuracy

The agreement between the theoretical added sample amount to the placebo and practically achieved sample amount from placebo has been employed for the determination of accuracy of analytical method. It was achieved at three different level 50%, 100% and 150% of the target concentration in triplicate. The results are presented in Table 9.7 and Table 9.8.

TABLE 9.7: RP-HPLC accuracy data of Metoprolol succinate

Level	Placebo (mg)	Conc µg/ml	Amount of drug added (mg)	Amount of drug recovered (mg)	Area	Recovery (%) ± SD (N=3)	Mean (%) ± SD	% RSD
50%	756.50	100µg/ml	50.00	49.90	59640	99.9±0.2	98.9±0.9	0.9
	756.41		50.50	50.40	59412	98.6±0.1		
	755.69		50.80	50.70	59123	98.2±0.1		
100%	756.89	200µg/ml	100.20	100.00	119052	99.8±0.1	99.9±0.1	0.1
	756.45		100.40	100.20	119264	100.0±0.3		
	756.82		100.60	100.40	119170	99.9±0.2		
150%	756.84	300µg/ml	150.00	149.70	1795462	100.7±0.1	100.6±0.5	0.5
	756.19		150.40	150.10	180743	101.0±0.1		
	755.89		150.90	150.60	178664	100.0±0.2		

TABLE 9.8: RP-HPLC accuracy data of Chlorthalidone

Level	Placebo (mg)	Conc µg/ml	Amount of drug added (mg)	Amount of drug recovered (mg)	Area	Recovery (%) ± SD (N=3)	Mean (%) ± SD	% RSD
50%	756.50	25 µg/ml	12.50	12.48	147952	98.2±0.1	98.5±0.2	0.2
	756.41		12.80	12.77	146562	98.6±0.2		
	755.69		12.60	12.57	146761	98.6±0.1		
100%	756.89	50 µg/ml	25.20	25.15	294541	100.0±0.3	99.2±0.7	0.7

	756.45		25.40	25.35	292632	99.0±0.2		
	756.82		25.80	25.75	291562	98.7±0.1		
150%	756.84	75 µg/ml	37.50	37.43	441709	98.3±0.2	98.7±0.4	0.4
	756.19		37.60	37.52	441825	99.0±0.1		
	755.89		37.40	37.33	440562	98.7±0.2		

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. Robustness of the method was carried out by deliberately made small changes in the flow rate, pH, and organic phase ratio and column oven temperature. Results are presented in Table 9.9

TABLE 9.9: RP-HPLC robustness data for Metoprolol succinate and Chlorthalidone.

Changing factor	Level	Metoprolol succinate (N=6) %RSD	Chlorthalidone (N=6) %RSD
Column temperature	25°C	0.1	0.4
	35°C	0.1	0.4
Flow rate	1.1 ml/min	0.2	0.8
	0.9 ml/min	0.7	0.2
Organic mixture ratio to buffer (Change in organic mixture composition of mobile Phase upto 2%)	Buffer : methanol (72:28, v/v)	0.2	0.4
	Buffer : methanol (68:32, v/v)	0.5	0.2
pH of buffer used in mobile Phase	pH 5.6 Buffer	0.8	0.1
	pH 5.4 Buffer	0.5	0.5
Mean %RSD		0.4	0.4

LOD (Limit of detection and LOQ (Limit of quantitation)

The results of LOD and LOQ are mentioned in Table 9.10. LOD and LOQ of Metoprolol succinate and Chlorthalidone is calculated based on the standard deviation of response (SD) and slope of calibration curve using formula. LOD= σ/S X 3.3 and LOQ= σ/S X 10.

(where σ is Standard deviation of response and S is slope of calibration curve)

TABLE 9.10: **LOD and LOQ of RP-HPLC data of Metoprolol succinate and Chlorthalidone**

Parameters	**Metoprolol succinate**	**Chlorthalidone**
LOD	1.0000 μg/ml	0.2500 μg/ml
LOQ	3.3000 μg/ml	0.8250 μg/ml

TABLE 9.11: Summary of validation parameters of RP-HPLC data of Metoprolol succinate and Chlorthalidone

Parameters of validation	Acceptance criteria	Metoprolol succinate	Chlorthalidone
Linearity	Covers the entire range	50 – 300 μg/ml	12.5-75 μg/ ml
Correlation coefficient	r > 0.995 or 0.990	0.9999	0.9998
Regression coefficient	r^2> 0.995 or 0.990	0.9999	0.9998
LOD	S/N > 2 or 3	1.0000 μg/ml	0.2500 μg/ml
LOQ	S/N > 10	3.3000 μg/ml	0.8250 μg/ml
Repeatability	RSD < 2%	0.1%	0.6%
Intermediate precision	RSD < 2%	0.3%	1.0%
Accuracy	Recovery 98-102%	98.9% to 100.6%	98.5% to 99.2%
Specificity	No interference of blank, placebo	No interference	No interference
Solution stability	> 12 hour	Stable for 24 hr %RSD = 0.19%	Stable for 24 hr %RSD =0.35%
Robustness	RSD NMT 2% in given condition	Complies	Complies
Flow rate (+ & -),		% RSD 0.5% & 0.3%	% RSD 0.8% & 0.2%
Organic ratio(+ & -),		% RSD 0.1% & 0.7%	% RSD 0.2% & 0.4%
Column temperature(+ & -),		% RSD 0.2% & 0.1%	% RSD 0.7% & 0.4 %
Buffer pH (+ & -)		% RSD 0.8% & 0.5%	% RSD 0.5 % & 0.1%

9.2 HPTLC method for Metoprolol succinate and Chlorthalidone [10-14]

Chromatographic condition:

Stationary phase	: 10 cm × 10 cm precoated silica gel 60 F254 plate.
Mobile phase	: Toluene: Ethyl acetate: Methanol: Triethylamine (6: 1: 1: 0.6, v/v/v/v)
Detection Wavelength	: 230nm
Chamber saturation time	: 15 min
Band width	: 6 mm
Distance between spots	: 10 mm
Rate of spotting	: 150 nl/s
Spotting volume	: 1-14 µl
Distance run	: 90 mm
Slit dimension	: 4 x 0.45mm
Evaluation mode	: Peak area
Lamp	: Deuterium
Diluent	: Methanol

Standard solution

Standard solution of Metoprolol succinate 500 µg/ml and Chlorthalidone 125 µg/ml in diluent was prepared.

Sample solution

Accurately 20 intact tablets were weighed and average weight of tablet was calculated. Crush to powder and mix tablets powder equivalent to 5 tablets (1600 mg) (eq to 250 mg Metoprolol succinate and 62.5 mg Chlorthalidone) diluted to 250 with diluent (Stock solution). Take 10 ml solution from the stock solution and diluted to 20 ml with diluent. (Metoprolol succinate 500 µg/ml, Chlorthalidone 125 µg/ml). HPTLC plate and densitogram is shown in Figure 9.6 and Figure 9.7

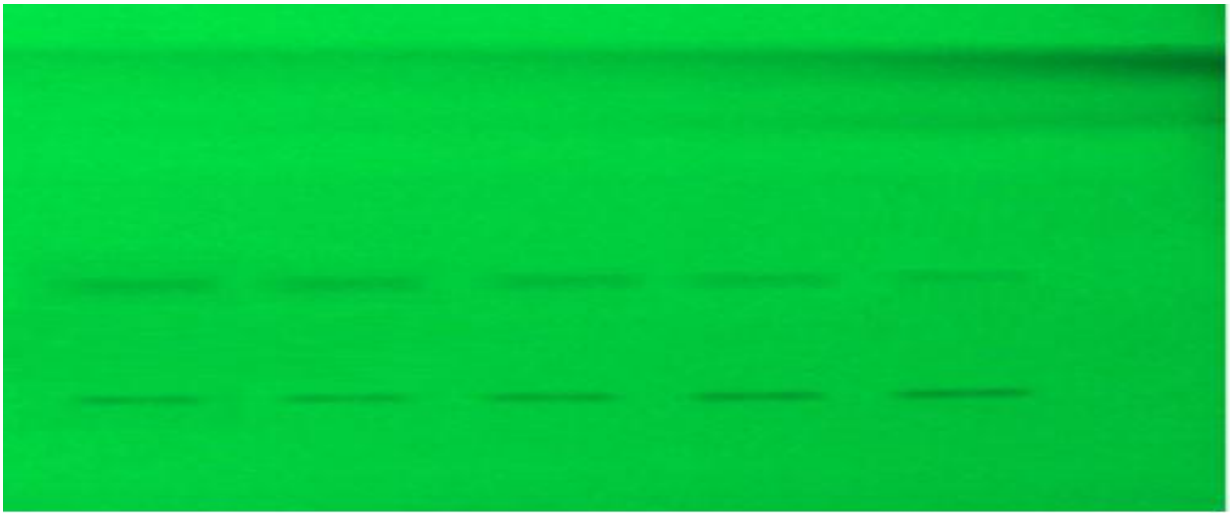

FIGURE 9.6: HPTLC plate for Metoprolol succinate and Chlorthalidone

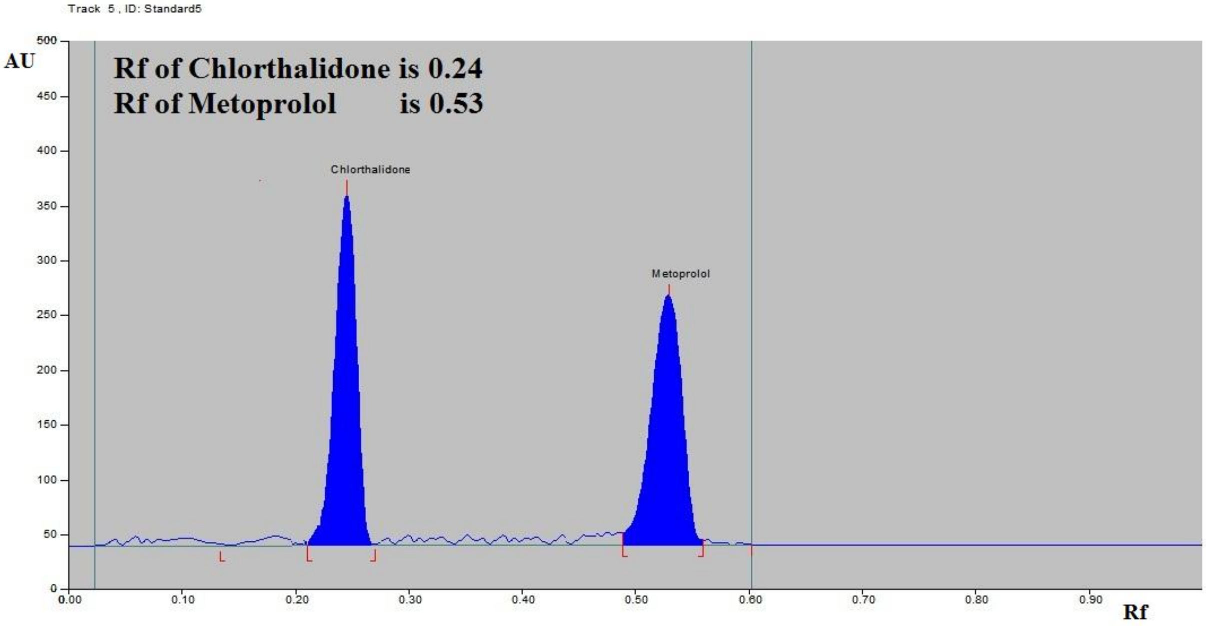

FIGURE 9.7: HPTLC densitogram for Metoprolol succinate and Chlorthalidone

Method validation [15-17]

Validation was carried out with respect to various parameters, as required under ICH guideline Q2 (R1) for parameters linearity, repeatability, accuracy, specificity, intermediate precision, robustness & solution stability.

System suitability and system precision

The results of system suitability and system precision are presented in Table 9.12. The system suitability was performed by injecting 5 replicate of standard solution for Metoprolol succinate and Chlorthalidone.

TABLE 9.12: HPTLC data of system suitability and system precision for Metoprolol succinate and Chlorthalidone

No	Metoprolol succinate (3000 ng/ml)	Chlorthalidone (750 ng/ml)
1	4186.01	5106.75
2	4187.62	5101.14
3	4171.04	5094.63
4	4190.45	5071.14
5	4184.62	5124.63
%RSD	0.16	0.34

Linearity & specificity

Linearity Standard solution of Metoprolol (500 μg/ml) & Chlorthalidone (125 μg/ml) was prepared. From Linearity standard solution 500, 1000, 2000, 3000, 4000, 5000, 6000 and 7000 ng/spot of Metoprolol succinate and 125, 250, 500, 750, 1000, 1250, 1500 and 1750 ng/spot of Chlorthalidone. (100μg/ml = 100 ng/μl) Calibration curves were constructed by plotting peak area versus concentration and regression equations were calculated. Each response was average of three determinations. The results are presented in Table 9.13. The HPTLC plate for Linearity study is shown in Figure 9.8 and overlaid 3D linearity chromatograms is shown in Figure 9.9. Calibration curve for linearity study are shown in Figure 9.10 and Figure 9.11. Diluent, Mobile phase and Placebo interference comparison on HPTLC plate are shown in Figure 9.12. Peak purity spectra are shown in 9.13.

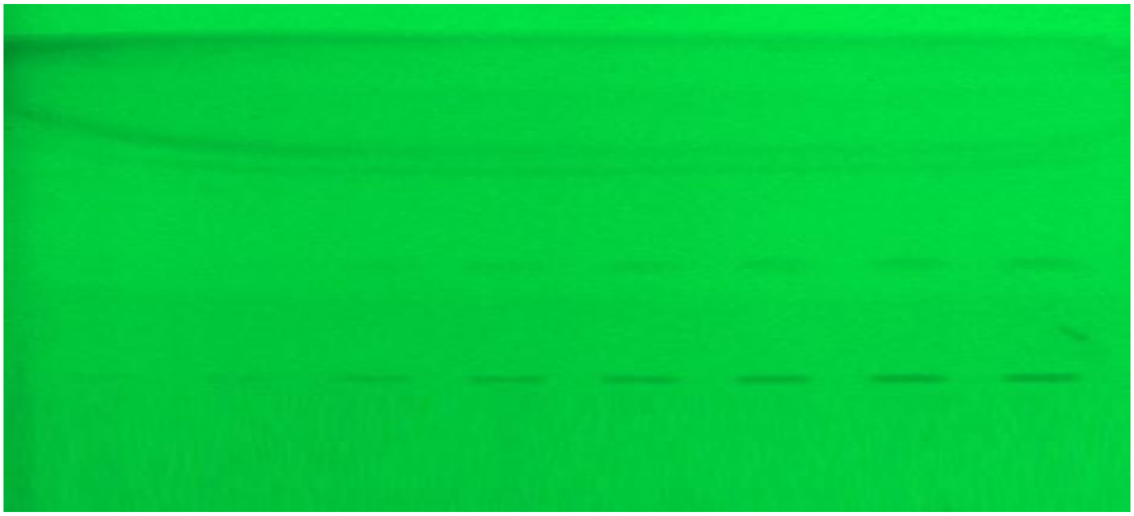

FIGURE 9.8: HPTLC plate for linearity of Metoprolol succinate and Chlorthalidone

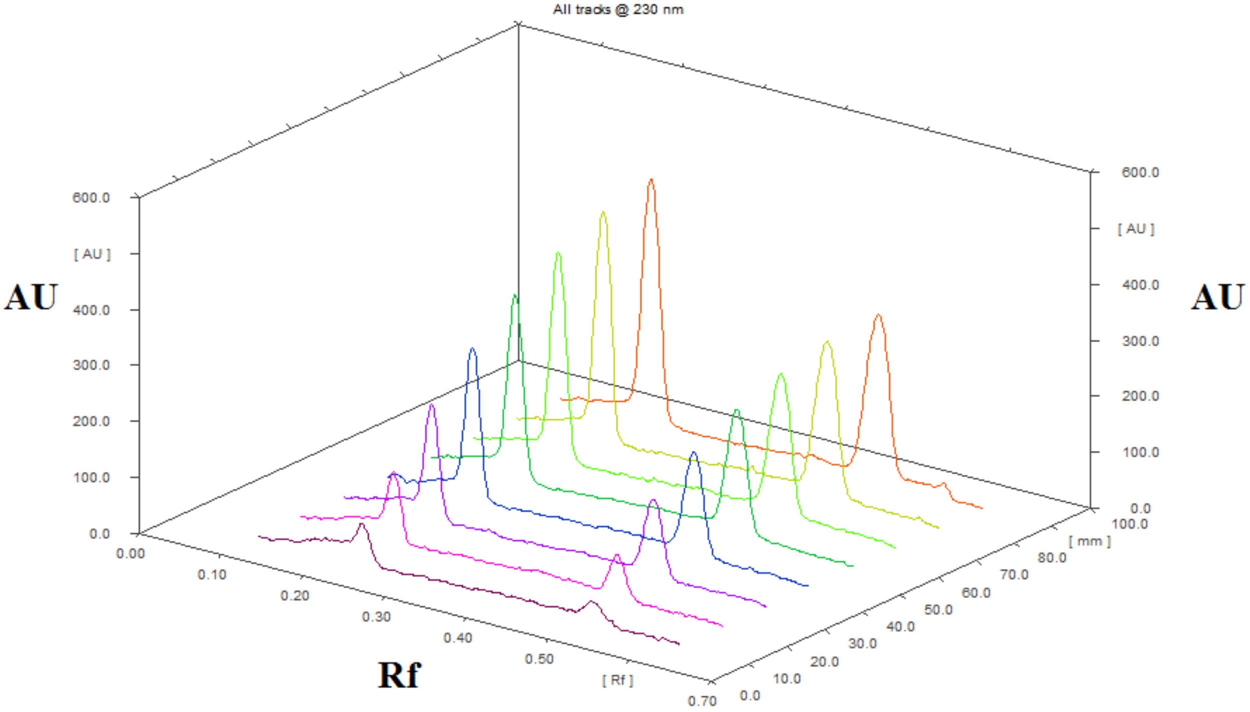

FIGURE 9.9: HPTLC 3D overlay data of linearity for Metoprolol succinate and Chlorthalidone

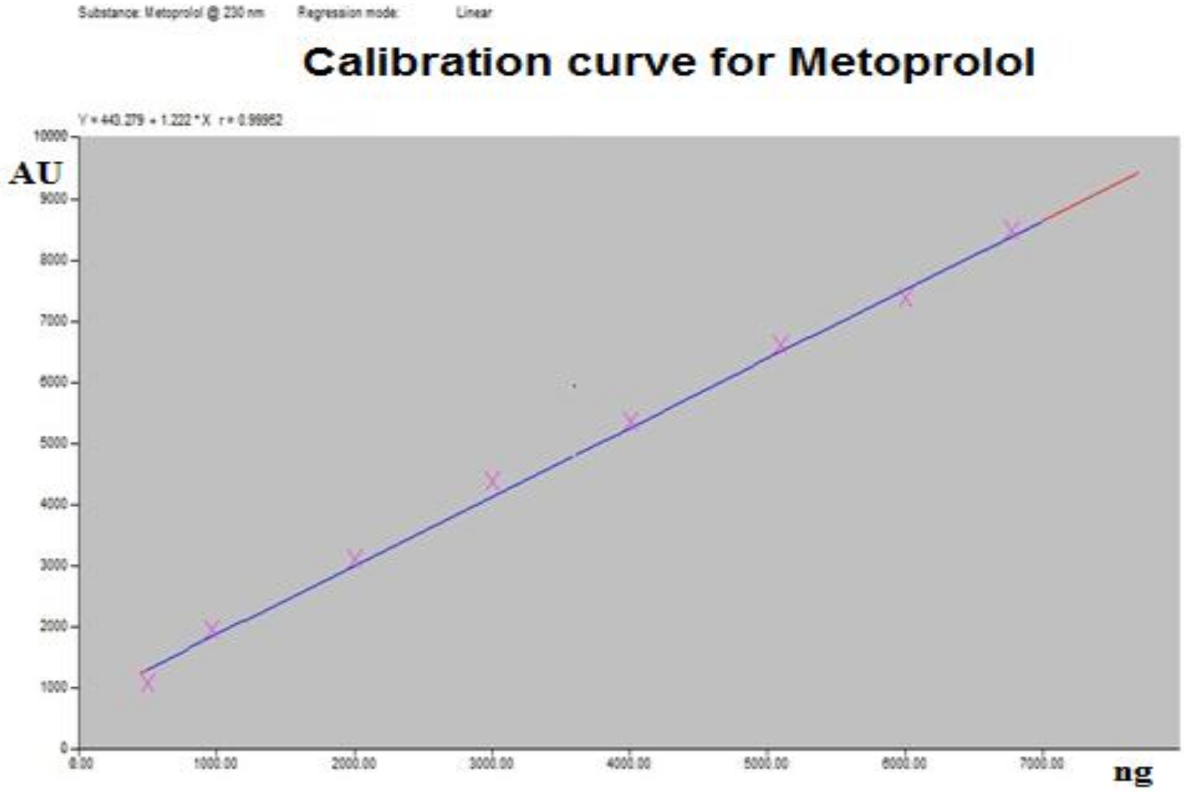

FIGURE 9.10: HPTLC calibration curve for Metoprolol succinate

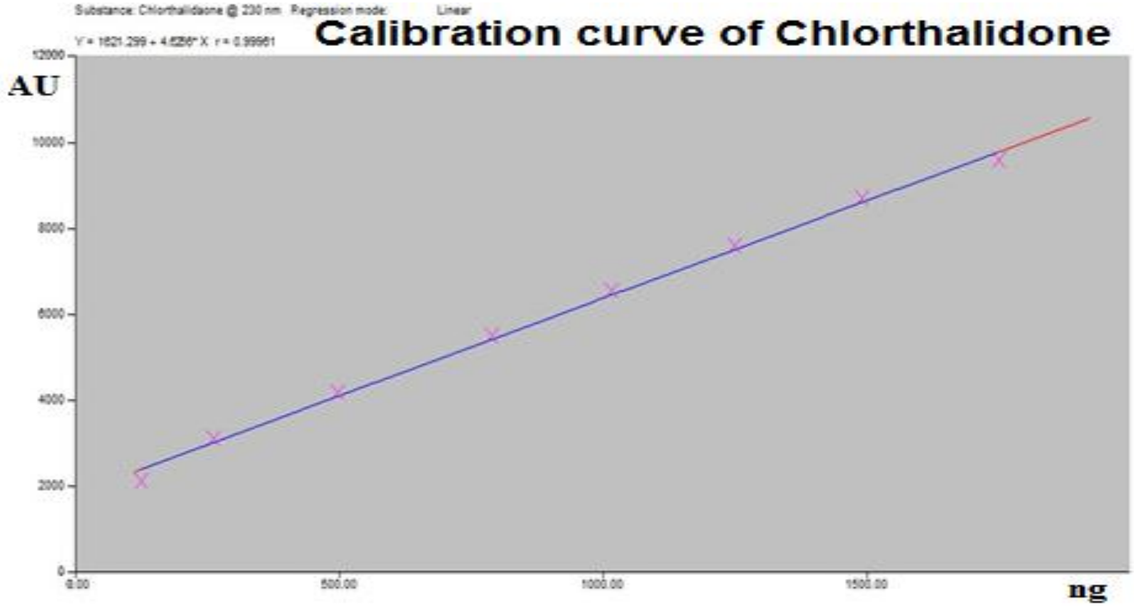

FIGURE 9.11: HPTLC calibration curve for Chlorthalidone

TABLE 9.13: Linearity data for Metoprolol succinate and Chlorthalidone

Injection volume of linearity µl	Metoprolol succinate ng/band	Mean area (N=3) ±SD	%RSD	Chlor-thalidone ng/band	Mean area (N=3) ±SD	%RSD
1	500	938.69 ± 15.20	0.2	125	2109.91 ± 19.15	0.4
2	1000	1708.59 ± 20.23		250	2847.88 ± 22.14	
4	2000	2925.30 ± 22.26		500	3944.64 ± 33.16	
6	3000	4186.01 ± 33.23		750	5106.84 ± 41.94	
8	4000	5363.71± 42.16		1000	6220.41 ± 51.62	
10	5000	6504.24 ± 59.61		1250	7470.64 ± 82.63	
12	6000	7772.24 ± 62.13		1500	8545.41 ± 90.62	
14	7000	8975.35 ± 71.85		1750	9681.29 ± 92.71	

FIGURE 9.12: HPTLC plate for specificity study of Metoprolol succinate and Chlorthalidone

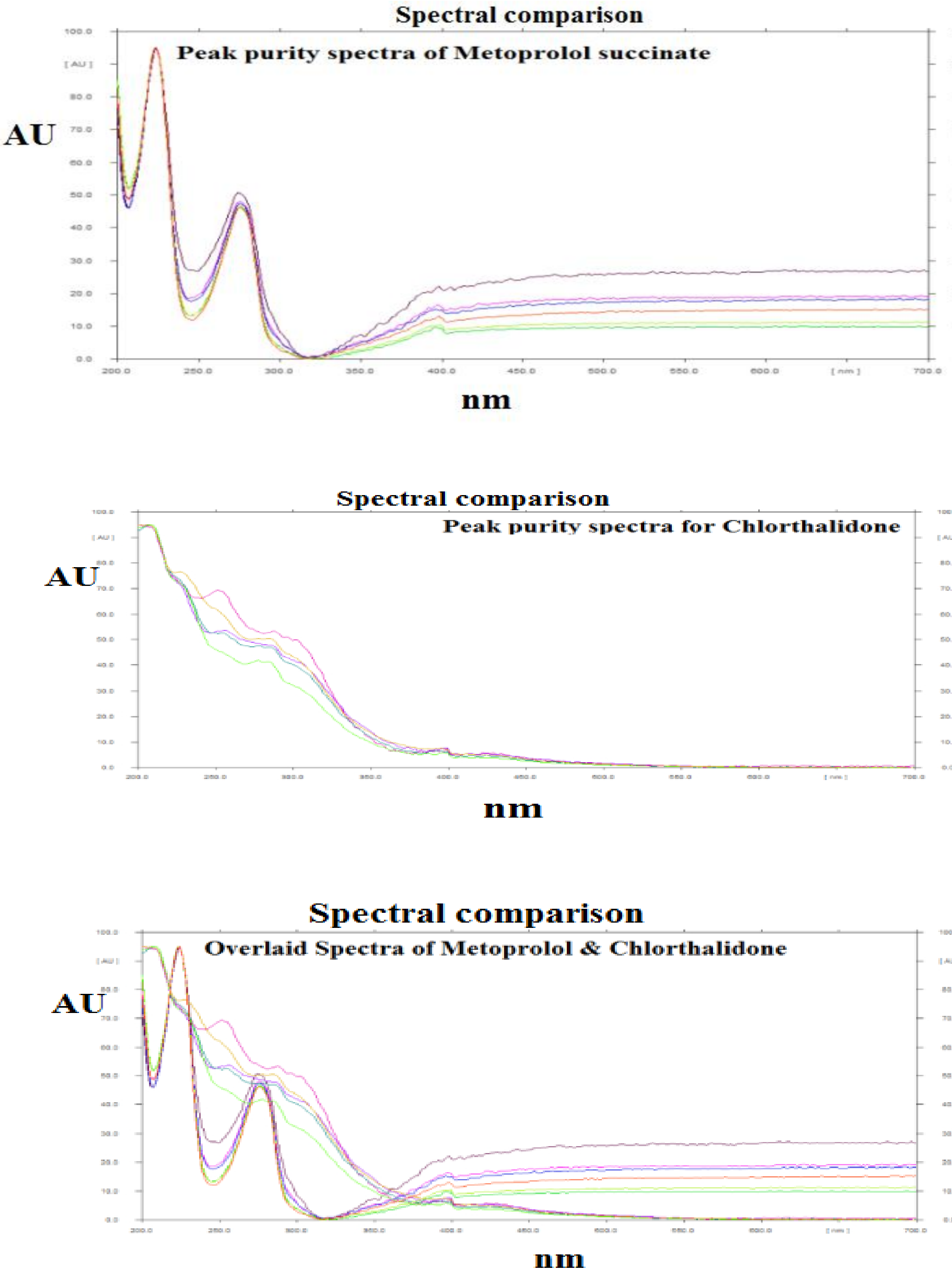

FIGURE 9.13: HPTLC peak purity spectra for Metoprolol succinate and Chlorthalidone

Repeatability & Intermediate precision

The method repeatability & intermediate precision was done as per ICH guidelines. The results were presented in Table 9.14. Overlaid 3D data for Repeatability studies are shown in Figure 9.14. HPTLC plate for repeatability data is shown in Figure 9.15. The results of repeatability and intermediate precision obtained are within 2% RSD.

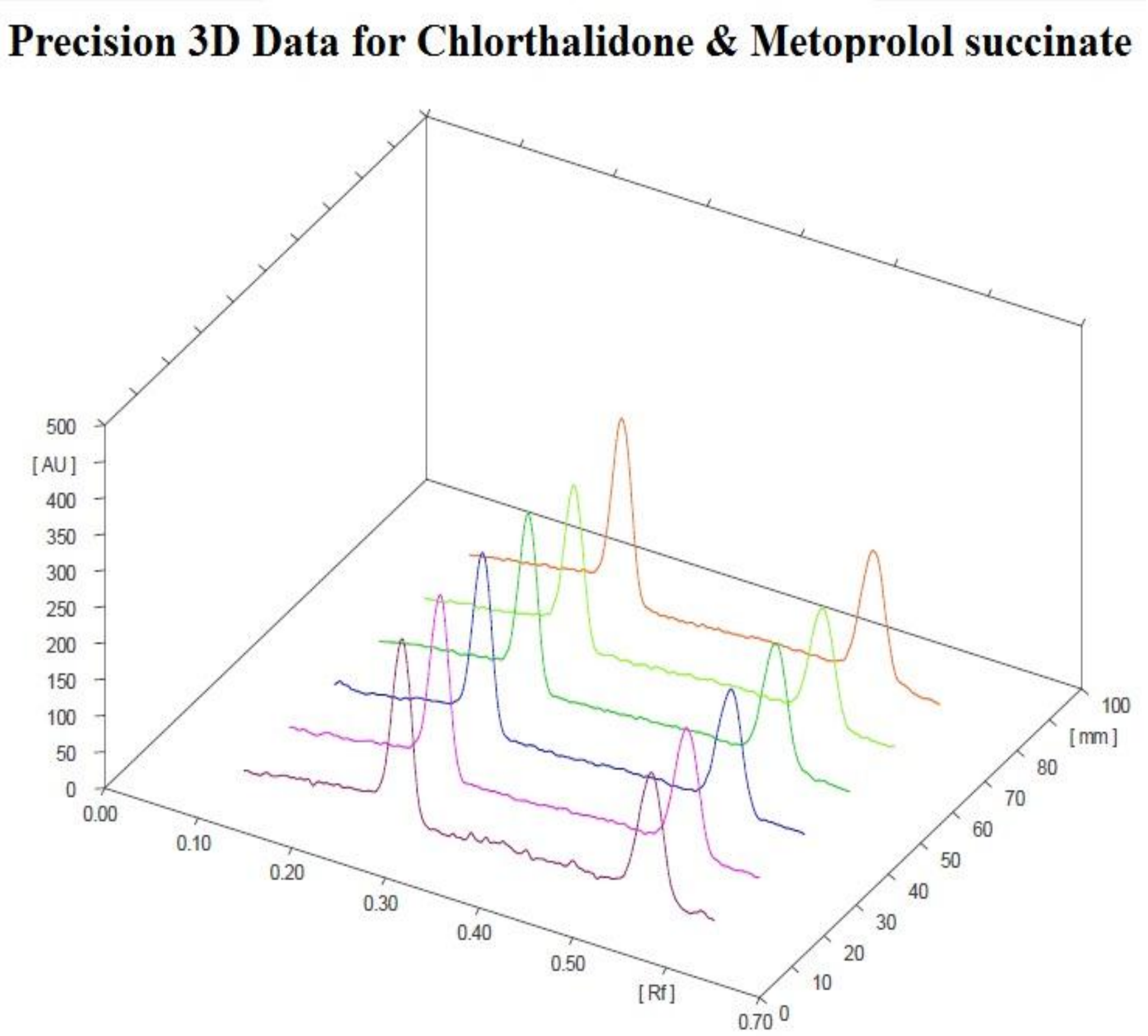

FIGURE 9.14: HPTLC repeatability 3D data for Metoprolol succinate and Chlorthalidone

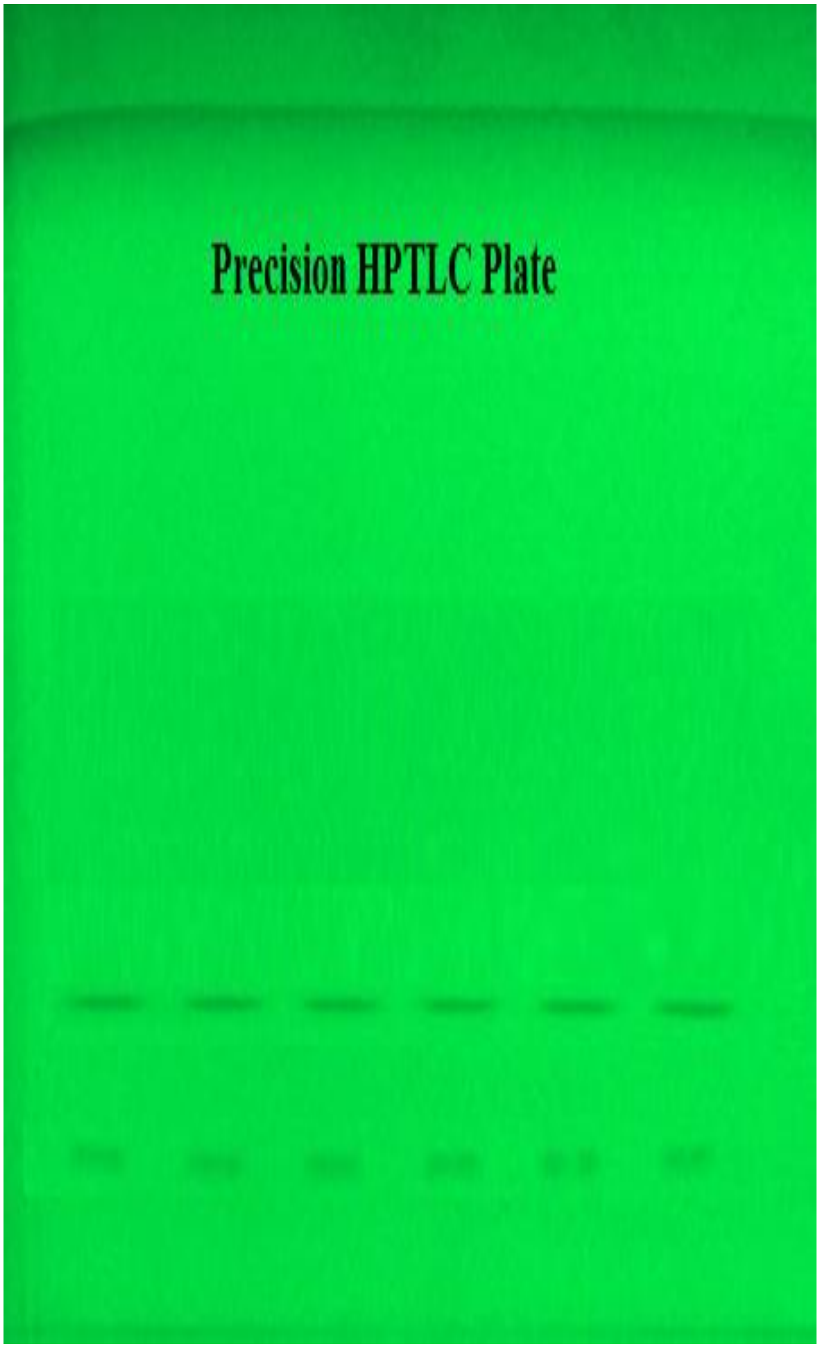

FIGURE 9.15: HPTLC plate for repeatability study of Metoprolol succinate and Chlorthalidone

TABLE 9.14: Results of method repeatability for HPTLC analysis

Sr. No	% Assay repeatability						
	Metoprolol succinate (5µg/ml)						
	Absorbance			**% Assay**	**% Mean**	**SD**	**% RSD**
	Sample wt (mg)	**Conc (ng/ml)**	**Area**				
1	1600.30	750	4190.60	100.8	100.5	0.3593	0.36
2	1601.98	750	4181.62	100.1			
3	1601.96	750	4197.82	100.9			
4	1603.45	750	4194.91	100.4			
5	1606.62	750	4187.67	100.6			
6	1602.46	750	4180.95	99.9			
Chlorthalidone (12.5 µg/ml)							
Sr. No	**Absorbance**			**% Assay**	**% Mean**	**SD**	**% RSD**
	Wt (mg)	**Conc (ng/ml)**	**Area**				
1	1600.30	3000	5108.89	101.4	101.2	0.1825	0.18
2	1601.98	3000	5107.46	101.1			
3	1601.96	3000	5109.46	100.9			
4	1603.45	3000	5101.60	101.1			
5	1606.62	3000	5098.65	101.3			
6	1602.46	3000	5097.14	101.4			

TABLE 9.15: Results of intermediate precision for HPTLC analysis

Sr. No	% Assay intermediate precision					
	Metoprolol succinate (5µg/ml)					
	Absorbance			% Assay	% Mean	% RSD
	Sample wt (mg)	Conc (ng/ml)	Area			
1	1602.40	750	4198.52	100.6	100.5	0.36
2	1605.58	750	4188.96	100.1		
3	1603.16	750	4180.23	99.1		
4	1600.01	750	4195.62	100.8		
5	1609.62	750	4197.26	100.4		
6	1601.24	750	4194.62	101.2		
Chlorthalidone (12.5 µg/ml)						
Sr. No	Absorbance			% Assay	% Mean	% RSD
	wt (mg)	Conc (ng/ml)	Area			
1	1602.40	3000	5104.56	100.1	99.7	0.93
2	1605.58	3000	5101.11	99.7		
3	1603.16	3000	5091.51	98.6		
4	1600.01	3000	5094.63	101.5		
5	1609.62	3000	5098.13	99.3		
6	1601.24	3000	5107.93	99.1		

Accuracy & Solution stability

Accuracy was done by standard addition method. Known amount of standard solutions of Metoprolol succinate (0, 2400, 3000, and 3600 ng/spot) and Chlorthalidone (0, 600, 750 and 900 ng/spot) were added to sample and recovery was performed. Accuracy was determined at three different level 80%, 100% and 120% of the target concentration in triplicate. The results are presented in Table 9.16. The solution stability data are shown in Table 9.17.

TABLE 9.16: HPTLC accuracy data of Metoprolol succinate and Chlorthalidone

Amt. of sample (ng/band)	Levels	Drug added (ng/band)	Drug found (ng/band)	Area mean	Drug recovered mean	% Recovery ± S.D. (N=3)	% RSD
Metoprolol succinate							
3000		-	3000.50	4186.05	3000.80	100.01 ± 0.14	0.1
3000	80 %	2400	5406.60	3345.54	2403.75	99.90 ± 0.34	0.3
3000	100 %	3000	6003.75	4187.19	3004.10	100.02 ± 0.46	0.4
3000	120 %	3600	6605.10	5028.17	3608.16	100.1 ± 0.58	0.6
Chlorthalidone							
750		-	749.80	5106.81	751.75	100.26 ± 0.21	0.2
750	80 %	600	1350.60	4084.79	600.70	99.92 ± 0.54	0.5
750	100 %	750	1502.05	5103.45	751.75	99.93 ± 0.14	0.1
750	120 %	900	1650.35	6139.78	902.25	100.19± 0.29	0.3

TABLE 9.17: HPTLC Solution stability data for Metoprolol succinate and Chlorthalidone

Time (hrs)	Area		Difference (% RSD)	
	Chlorthalidone	Metoprolol succinate	Chlorthalidone	Metoprolol succinate
Standard solution stability				
0	5101.62	4186.17	==	==
24	5090.16	4174.91	0.11	0.13
Sample solution stability				
0	5098.61	4197.93	==	==
24	5081.14	4171.84	0.17	0.31

Robustness

Robustness of the method was carried out by deliberately made small changes in the saturation time and organic solvent of mobile phase. Results are presented in Table 9.17 and Table 9.18.

TABLE 9.18: HPTLC data for robustness of Metoprolol succinate and Chlorthalidone

Metoprolol succinate				**Chlorthalidone**		
No	**System suitability**	**Saturation time -5 min (10min)**	**Saturation time +5 min (20min)**	**System suitability**	**Saturation time -5 min (10min)**	**Saturation time +5 min (20min)**
1	4186.01	4180.01	4206.01	5106.75	5101.52	5129.46
2	4187.62	4177.62	4221.62	5101.14	5074.23	5139.41
3	4171.04	4189.04	4199.04	5094.63	5061.25	5171.46
4	4190.45	4160.45	4239.45	5071.14	5059.62	5181.51
5	4184.62	4164.62	4264.62	5124.63	5139.13	5141.29
%RSD	0.16	0.25	0.56	0.34	0.59	0.39
Overall %RSD	0.3			0.4		

TABLE 9.19: HPTLC data for robustness of Metoprolol succinate and Chlorthalidone

Metoprolol succinate			**Chlorthalidone**	
No	**Organic solvent ratio -10% Toluene (M.P ratio 5.4:1:1:0.6)**	**Organic solvent ratio +10% Toluene (M.P ratio 6.6:1:1:0.6)**	**Organic solvent ratio -10% Toluene (M.P ratio 5.4:1:1:0.6)**	**Organic solvent ratio +10% Toluene (M.P ratio 6.6:1:1:0.6)**
1	4174.01	4195.46	5150.62	5020.72
2	4153.61	4187.5	5181.56	5041.26
3	4141.59	4184.56	5197.41	5074.25
4	4131.52	4171.65	5241.26	5026.29
5	4165.81	4167.56	5162.61	5145.56
%RSD	0.42	0.28	0.68	1.01
Overall %RSD	0.4		0.9	
No	**Wavelength -2 nm (228 nm)**	**Wavelength +2 nm (232 nm)**	**Wavelength -2 nm (228 nm)**	**Wavelength +2 nm (232 nm)**
1	4150.76	4225.62	5111.07	5010.15
2	4152.67	4240.61	5104.98	5015.65
3	4189.12	4245.91	4997.56	5045.91
4	4173.46	4271.62	4991.46	5028.61
5	4191.62	4214.79	4990.61	5031.56
%RSD	0.46	0.51	1.25	0.28
Overall %RSD	0.49		0.8	

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

LOD and LOQ determined as per ICH guidelines. The results of LOD and LOQ are mentioned in Table 9.19.

TABLE 9.20: LOD and LOQ HPTLC data of Metoprolol succinate and Chlorthalidone

Parameters	Metoprolol succinate	Chlorthalidone
Limit of Detection (ng/spot)	100.00 ng/spot	25.00 ng/spot
Limit of Quantitation (ng/spot)	330.00 ng/spot	82.50 ng/spot

Results and discussion

Clarification of variation in strategy from UV to HPTLC for Metoprolol chlorthalidone tablets

An effort was made to develop simultaneous UV method for estimation of metoprolol succinate and chlorthalidone, but due to following drawbacks HPTLC method was developed

- During assay calculation it was found that assay for Metoprolol succinate was obtained 80 % and Chlorthalidone was obtained 120 % due to interference of both drugs at each other's wavelength 276 nm and 225 nm
- Moreover UV method for estimation of Metoprolol succinate and chlorthalidone has already been published so there is no novelty of research for that topic in PhD studies. (RJPT Journal 2011)

Literature review reveals that there is no reported HPTLC method for simultaneous estimation of metoprolol and Chlorthalidone, so the aim was to develop and validate HPTLC method of analysis of Metoprolol succinate and Chlorthalidone. A suitable solvent system for the composition of the mobile phase for development of chromatogram was optimized by testing different solvent mixtures of varying polarity. Various mobile phases were evaluated. Use of hexane, ethylacetate, chloroform and toluene as single component and a short saturation time of 15 min gave slight migration of both drugs. So Methanol: Ethyl acetate (3:7 v/v), Methanol: Ethyl acetate 5:5 v/v, Methanol: Toluene 7:3 v/v, Methanol: Chloroform: TEA (Triethylamine) 6:1:0.1 v/v/v, Methanol: Toluene: TEA (Triethylamine) 3:0.5:0.3, v/v/v were tried. The best results were obtained using Toluene: Ethyl acetate: Methanol: Triethylamine 6:1:1:0.6, v/v/v.

This mobile phase showed a good resolution and a compact spots of Metoprolol succinate and Chlorthalidone. Densitometric scanning of all the tracks at λmax 230 nm showed compound with R_f value 0.24±0.05 identified as Chlorthalidone and 0.53±0.04 identified as Metoprolol succinate. The method was successfully used in the analysis of Metoprolol succinate and Chlorthalidone in tablet dosage form without interference of the formulation excipients.

Significance of 4 components used in mobile phase of HPTLC

Trials were conducted on single, double and triple components mobile phase but satisfactory results of peak shape, spot resolution were not achieved. Toluene is used as non-polar solvent to retain chlorthalidone drug. Ethyl acetate (Semi polar solvent) and methanol (Polar solvent) is used for migration of both spots and proper separation from each other. Triethyl amine is used as peak shape and spot shape modifier for better chromatographic results.

The summary of validation data by HPTLC is shown in Table 9.21.The results of marketed formulation analysis were shown in Table 9.22.

Table 9.21: Summary of HPTLC validation parameters for Metoprolol succinate and Chlorthalidone

Parameter		**Acceptance criteria**	**Metoprolol succinate**	**Chlorthalidone**
Linearity range (ng/spot)		Follows Beer Lambert’s law	500-7000 ng/spot	125-1750 ng/spot
Correlation coefficient (R)		R>0.999 or 0.995	0.9997	0.9998
Regression coefficient (R^2)		R^2 >0.999 or 0.995	0.9995	0.9996
Repeatability (% R.S.D)	Repeatability (Repeatability) (n=6)	%RSD <2%	0.36	0.18
	Intermediate precision (n=6)	%RSD <2%	0.66	0.93
Accuracy (% recovery), (n=3)		98% and 102%	99.9-100.1	99.9-100.2
Limit of detection (ng/spot)		-	100.00	25.00
Limit of quantitation (ng/spot)		-	330.00	82.50
Specificity		No interference from blank, placebo	No interference from blank, placebo	No interference. from blank, placebo
Robustness **Saturation time (+ & -),** **Wavelength (+& -) &** **Organic ratio (+ & -), in mobile phase**		RSD NMT 2% in modified condition	0.56 and 0.25 0.51and 0.46 0.28 and 0.42	0.39 and 0.59 0.28 and 1.25 1.01 and 0.68
Solution stability		> 12 hour	Stable for 24 hr	Stable for 24 hr

TABLE 9.22: Marketed formulation assay comparison for Metoprolol succinate and Chlorthalidone

Assay by HPLC						
	Label claim (mg)		Amount Found		% Assay ± %CV	
Brand Name	Metoprolol succinate	Chlorthalidone	Metoprolol succinate	Chlorthalidone	Metoprolol succinate (N=3)	Chlor-thalidone (N=3)
Vinicor D	50	12.5	50.45	12.52	100.90 ± 0.35	100.16 ± 0.17
Promet D	50	12.5	50.34	12.58	100.08 ± 0.45	100.64 ± 0.47
Assay by HPTLC						
	Label claim (mg)		Amount Found		% Assay ± %CV	
Brand Name	Metoprolol succinate	Chlorthalidone	Metoprolol succinate	Chlorthalidone	Metoprolol succinate (N=3)	Chlor-thalidone (N=3)
Vinicor D	50	12.5	50.25	12.65	100.50 ± 0.55	101.20 ± 0.77
Promet D	50	12.5	50.05	12.48	100.10 ± 0.65	99.80 ± 0.47

9.3 Bibliography and references

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CHAPTER-10

Statistics comparison

10.1 Student t test [1-13]

Student’s test is used for comparison of mean of two samples. t test compares the actual difference between two mean in relation to variation in data obtained from experiment. **Unpaired t-test** compares two different subjects. The **paired t-test** reduces intersubject variability (because it makes comparisons **between** the same subjects).

Unpaired t test is when data collected from two different and independent subjects and size between two samples may or may not be equal. Paired t test are applied when variances between two subjects are equal.

A paired test therefore is a test of null hypothesis that means of two group of subjects that are normally distributed are equal and unpaired t test is test of null hypothesis that two responses which are measured in same unit have a difference with a mean value of zero.

A paired t test is done on subjects that are similar or paired before data is collected and after data is collected. Unpaired t test is done on two independent or different subjects.

A t-test’s statistical significance indicates whether or not the difference between two groups’ averages most likely reflects a “real” difference in the population from which the groups were sampled.

10.2 STATISTICS COMPARISON OF HPLC & UV METHODS FOR OLMESARTAN MEDOXOMIL AND INDAPAMIDE (T-Test: Two-Sample Assuming Equal Variances)

In comparative study, both the methods (i.e. RP-HPLC and UV), for simultaneous estimation of Olmesartan medoxomil and Indapamide were compared for the significant difference between them.

Level of significance: 5%
Degree of freedom: 10
Null hypothesis (h0): There is no significant difference between two analytical methods.
Alternate hypothesis: There is significant difference between two analytical methods.

t-Test: Two-sample assuming equal variances				
Parameters	Olmesartan medoxomil		Indapamide	
	UV	HPLC	UV	HPLC
Mean	99.8	99.9	100.9	101.2
Variance	0.3346	0.0440	0.4896	0.1107
Observations	6	6	6	6
t tabulated	2.228139		2.329139	
t Calculated	0.530740		0.895730	
t tabulated > t calculated	Yes		Yes	
Null Hypothesis	Passes		Passes	
Alternate Hypothesis	Fail		Fail	

Null hypothesis passes; hence there is no significant difference between the two analytical methods employed. Both methods can be used successfully for the simultaneous determination of Olmesartan medoxomil and Indapamide.

10.3 STATISTICS COMPARISON OF HPLC & UV METHODS FOR CHLORTHALIDONE AND OLMESARTAN MEDOXOMIL (T-Test: Two-Sample Assuming Equal Variances)

In comparative study, both the methods (i.e. RP-HPLC and UV), for simultaneous estimation of Olmesartan medoxomil and Chlorthalidone were compared for the significant difference between them.

Level of significance: 5%

Degree of freedom: 10

Null hypothesis (h0): There is no significant difference between two analytical methods.

Alternate hypothesis: There is significant difference between two analytical methods.

t-Test: Two-sample assuming equal variances				
Parameters	Olmesartan medoxomil		Chlorthalidone	
	UV	HPLC	UV	HPLC
Mean	99.5	99.2	99.9	99.4
Variance	0.1786	0.3307	1.1867	0.0640
Observations	6	6	6	6
t tabulated	2.228138		2.228139	
t Calculated	1.258470		1.168163	
t tabulated > t calculated	Yes		Yes	
Null Hypothesis	Passes		Passes	
Alternate Hypothesis	Fail		Fail	

Null hypothesis passes; hence there is no significant difference between the two analytical methods employed. Both methods can be used successfully for the simultaneous determination of Olmesartan medoxomil and Chlorthalidone.

10.4 STATISTICS COMPARISON OF HPLC & UV METHODS FOR NEBIVOLOL HCl AND CHLORTHAIDONE (T-Test: Two-Sample Assuming Equal Variances)

In comparative study, both the methods (i.e. RP-HPLC and UV), for simultaneous estimation of Nebivolol HCl and Chlorthalidone were compared for the significant difference between them.

Level of significance: 5%

Degree of freedom: 10

Null hypothesis: There is no significant difference between two analytical methods.

Alternate hypothesis: There is significant difference between two analytical methods.

t-Test: Two-sample assuming equal variances				
Parameters	Nebivolol HCl		Chlorthalidone	
	UV	HPLC	UV	HPLC
Mean	98.9	99.8	99.7	99.3
Variance	0.8950	0.2817	0.1417	0.1267
Observations	6	6	6	6
t tabulated	2.228138		2.288169	
t calculated	2.182858		1.970276	
t tabulated > t calculated	Yes		Yes	
Null Hypothesis	Passes		Passes	
Alternate Hypothesis	Fail		Fail	

Null hypothesis passes; hence there is no significant difference between the two analytical methods employed. Both methods can be used successfully for the simultaneous determination of Nebivolol HCl and Chlorthalidone.

10.5 STATISTICS COMPARISON OF HPLC & UV METHODS FOR ROSUVASATIN CALCIUM & HYDROCHLORTHIAZIDE (T-Test: Two-Sample assuming equal variances)

In comparative study, both the methods (i.e. RP-HPLC and UV), for simultaneous estimation of Rosuvastatin calcium and Hydrochlorthiazide were compared for the significant difference between them.

Level of significance (α=0.05): 5%

Degree of freedom: 10

Null hypothesis (h0): There is no significant difference between two analytical methods.

Alternate hypothesis: There is significant difference between two analytical methods.

t-Test: Two-sample assuming equal variances				
Parameters	Rosuvastatin calcium		Hydrochlorthiazide	
	UV	HPLC	UV	HPLC
Mean	98.9	99.8	101.0	100.0
Variance	0.8950	0.2817	0.2547	0.8420
Observations	6	6	6	6
t tabulated	2.228138		2.262157	
t Calculated	2.182858		2.177014	
t tabulated > t calculated	Yes		Yes	
Null Hypothesis	Passes		Passes	
Alternate Hypothesis	Fail		Fail	

Null hypothesis passes; hence there is no significant difference between the two analytical methods employed. Both methods can be used successfully for the simultaneous determination of Rosuvastatin calcium and Hydrochlorthiazide.

10.6 STATISTICS COMPARISON OF HPLC & HPTLC METHODS FOR METOPROLOL SUCCINATE AND CHLORTHALIDONE (T-Test: Two-Sample Assuming Equal Variances)

In comparative study, both the methods (i.e. RP-HPLC and HPTLC), for simultaneous estimation of Metoprolol succinate and Chlorthalidone were compared for the significant difference between them.

Level of significance: 5%

Degree of freedom: 10

Null hypothesis (h0): There is no significant difference between two analytical methods.

Alternate hypothesis: There is significant difference between two analytical methods.

t-Test: Two-sample assuming equal variances				
Parameters	Metoprolol Succinate		Chlorthalidone	
	HPTLC	HPLC	HPTLC	HPLC
Mean	100.5	100.4	100.7	100.7
Variance	0.155	0.016	0.0987	0.3378
Observations	6	6	6	6
t tabulated	2.2281388		2.2281388	
t Calculated	0.2961744		0.432626	
t tabulated > t calculated	Yes		Yes	
Null Hypothesis	Passes		Passes	
Alternate Hypothesis	Fail		Fail	

Null hypothesis passes; hence there is no significant difference between the two analytical methods employed. Both methods can be used successfully for the simultaneous determination of Metoprolol succinate and Chlorthalidone.

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CHAPTER-11

Summary and Conclusion

11.1 Summary

- RP-HPLC methods for simultaneous estimation of Olmesartan medoxomil and Indapamide, Olmesartan medoxomil and Chlorthalidone, Nebivolol HCl and Chlorthalidone, Rosuvastatin calcium and Hydrochlorthiazide, Metoprolol succinate and Chlorthalidone in combination drug products was developed.

- UV spectroscopic methods for simultaneous estimation of Olmesartan medoxomil and Indapamide, Olmesartan medoxomil and Chlorthalidone, Nebivolol HCl and Chlorthalidone, Rosuvastatin calcium and Hydrochlorthiazide, Metoprolol succinate and Chlorthalidone in combination drug products was developed.

- HPTLC method for simultaneous estimation of Metoprolol succinate and Chlorthalidone in combination drug product was developed.

- All developed methods to be validated for specificity, linearity, accuracy, repeatability (precision), intermediate precision, limit of detection and limit of quantification, robustness and system suitability.

- Statistical comparison of developed HPLC and UV/HPTLC methods were performed**.**

11.2 Conclusion

Validated RP HPLC method have been developed and successfully applied for simultaneous estimation of Olmesartan medoxomil and Indapamide. Optimization of buffer solution pH, ratio of mobile phase concentration and use of triethylamine in mobile phase as ion pair reagent to improve peak shape was a critical part of method. Optimum resolution & short run time for analysis were key features of method. Acidic pH was kept for buffer solution in mobile phase to retain both peaks in column. Diluent for analysis was kept as water: acetonitrile (50:50 v/v) is easy to prepare (user friendly), compatible with mobile phase (buffer: acetonitrile 60:40 v/v) and improved peak shape for both analytes compared to methanol (100%) and acetonitrile (100%).

Validated RP HPLC method have been developed and successfully applied for simultaneous estimation of Olmesartan medoxomil and Chlorthalidone. Use of sodium dihydrogen phosphate buffer at pH 3.0 and acetonitrile in (48:52) % v/v for mobile phase makes extremely simple method for routine use. Use of mobile phase as diluent for sample improves baseline in HPLC chromatogram and gives proper peak shape of analytes.

Validated RP HPLC method have been developed and successfully applied for simultaneous estimation of Nebivolol hydrochloride and Chlorthalidone. Use of Zorbax eclipse XDB C18 columns (extra Dense Bonding (XDB) of column and double end capping of column makes column perform better. Column has highly, deactivated stationary phase that virtually eliminates undesirable interactions between polar solutes and the silica surface. As a result, superior peak shape, high efficiency and long-term chromatographic reproducibility for drugs estimation. Use of triethylamine in mobile phase as ion pair reagent to improve peak shape was a critical part of method.

Validated RP HPLC method have been developed and successfully applied for simultaneous estimation of Rosuvastatin calcium and Hydrochlorthiazide. Use of ACE C_{18} AR column which has high carbon loading and aromatic group bonded to C18 chain in column for proper peak separation for Rosuvastatin calcium and Hydrochlorthiazide is key feature of developed method. Alkaline pH of buffer solution delayed elution of peaks from column compared to acidic pH of buffer solution.

Validated RP HPLC method have been developed and successfully applied for simultaneous estimation of Metoprolol succinate and Chlorthalidone. Use of methanol in mobile phase as organic solvents to improve peak separation and use of triethylamine in mobile phase as ion pair reagent to improve peak shape was a critical part of method. Use of Inertsil ODS 3 (4.6 x100 mm), 5µ column (smaller length column) shortens run time of method. Use of diammonium hydrogen phosphate buffer at pH 5.5 was critical parameter for proper resolution of both peaks.

Validated UV method have been developed and successfully applied for simultaneous estimation of Olmesartan medoxomil and Indapamide. A predetermined concentration of Indapamide (6 µg/ml) was added to standard and sample solution to obtain concentration of Indapamide in range of UV estimation and to decrease effect of low dose. This predetermined concentration is deducted from final results to get exact concentration of drug. Simultaneous estimation by UV at 240 nm and 256 nm is performed with all results within specification of ICH guidelines.

Validated UV method have been developed and successfully applied for simultaneous estimation of Olmesartan medoxomil and Chlorthalidone. Simultaneous estimation by UV at 220 nm and 254 nm was performed to get all results within specification of ICH guidelines.

Validated UV method have been developed and successfully applied for simultaneous estimation of Nebivolol hydrochloride and Chlorthalidone. Simultaneous estimation by UV at 226 nm and 282 nm was performed to get results within specification of ICH guidelines.

Validated UV method have been developed and successfully applied for simultaneous estimation of Rosuvastatin calcium and Hydrochlorthiazide. Simultaneous estimation by UV at 243 nm and 270 nm was performed to get all results within specification of ICH guidelines.

Validated HPTLC method have been developed and successfully applied for simultaneous estimation of Metoprolol succinate and Chlorthalidone. The developed method is simple, precise, reproducible, specific and accurate. Low cost on solvents and good peak shapes are key features. Detection of both drugs at levels of ng/ml shows sensitivity of method. Proper optimization of mobile phase gives good separation between both peaks. Low chamber saturation time and proper peak shapes suggests efficacy of method. Concentration of triethylamine for proper resolution between peaks is the critical part of the method.

Validated simultaneous RP HPLC method have been developed and successfully applied for estimation of Olmesartan medoxomil and Indapamide, Olmesartan medoxomil and Chlorthalidone, Nebivolol HCl and Chlorthalidone, Rosuvastatin calcium and Hydrochlorthiazide, Metoprolol succinate and Chlorthalidone in their combination drug products on HPLC with PDA detector and isocratic elution. Specificity and selectivity of method was an added asset to method for routine analysis of drug samples in analytical development laboratories and quality control laboratories.

Validated simultaneous UV spectrophotometric methods have been developed and successfully applied for simultaneous estimation of Olmesartan medoxomil and Indapamide, Olmesartan medoxomil and Chlorthalidone, Nebivolol HCl and Chlorthalidone, Rosuvastatin calcium and Hydrochlorthiazide in their combination drug products using UV spectrophotometer. Low interference in UV method gives precise and reproducible results.

Validated HPTLC analytical method has been developed and applied for simultaneous estimation of Metoprolol succinate and Chlorthalidone in their combination drug products. The method can be successfully applied for routine analysis of drug samples in analytical development laboratories and quality control laboratories.

CHAPTER-12

List of Publications

1. Avani Sheth, Chhagan N Patel, Badmanaban Ramlingam, Nehal Shah. Simultaneous estimation of metoprolol succinate and chlorthalidone in pharmaceutical solid dosage form by using a developed and validated reverse phase high performance liquid Chromatographic technique. Scholars' Research Journal, 2012, 2 (1 and 2), 17-21, ISSN No: 2278-8808.
2. Avani Doshi, K. N. Patel, R. Badmanaban, Naman Doshi, Nehal Shah. Simultaneous estimation of Olmesartan medoxomil and Indapamide from bulk and commercial products using a Validated Reverse Phase High Performance Liquid Chromatographic technique. Chronicles of young scientist, 2013, 4(1), 69-73, ISSN No: 2229-5186.
3. Avani Sheth, C N Patel, Nehal Shah. Simultaneous Estimation of Chlorthalidone and Olmesartan Medoxomil from Bulk and Commercial Products using a Validated Reverse Phase High Performance Liquid Chromatographic Technique. Inventi Rapid: Pharm Analysis & Quality Assurance, 2013, (3), 01-05, ISSN No: 0976-3813.
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8. Sheth Avani H, C N Patel, Nehal Shah, Simultaneous Estimation of Metoprolol Succinate and Chlorthalidone in Pharmaceutical Solid Dosage Form by using a Developed and Validated High Performance Thin Layer Chromatographic Technique, Inventi Rapid: Pharm Analysis & Quality Assurance, 2017,3, pp 1-9, ISSN No: 0976-3813.
9. Avani Sheth, C N Patel, Nehal Shah, Ragin shah. Simultaneous estimation of Olmesartan medoxomil and Indapamide by standard addition UV Spectrophotometric method in their combined dosage forms, Indian drugs, ISSN No: 0019-462X.

LIST OF ACCEPTED ARTICLES

10. Avani Sheth, C N Patel, Nehal Shah, Simultaneous UV spectrophotometric method for simultaneous estimation of Rosuvastatin calcium and Hydrochlorthiazide in their combined dosage forms. Indian drugs, (Acceptation date: 31/01/2017).

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Chapter 5

RP-HPLC and UV method for Olmesartan medoxomil and Indapamide

Chapter 6

RP-HPLC and UV method for Chlorthalidone and Olmesartan medoxomil

Chapter 7

RP-HPLC AND UV method for Nebivolol HCl and Chlorthalidone

Chapter 8

RP-HPLC and UV method for Rosuvastatin calcium and Hydrochlorthiazide

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