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VALIDATED RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LOSARTAN AND AMLODIPINE IN BULK AND TABLET DOSAGE FORM

Akula Srilekha[1], K. Usha

[1]Department Of Pharmaceutical Analysis, Princeton College Of Pharmacy, Pocharam, Hyderabad, Telangana 500088.

Abstract:

A simple, precise, and validated Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method was developed for the simultaneous estimation of Losartan and Amlodipine in bulk and tablet dosage forms. The chromatographic analysis was carried out using a Develosil C18 column (4.6 mm × 250 mm, 5 µm) maintained at ambient temperature. The mobile phase comprised Acetonitrile and Acetate buffer (pH 4.3) in the ratio of 35:65% v/v, delivered at a flow rate of 1 mL/min. Detection was performed at a wavelength of 238 nm, with an injection volume of 20 µL and a run time of 6 minutes.

The method produced well-resolved peaks for both Losartan and Amlodipine with consistent retention times and no interference from excipients. It was validated in accordance with ICH Q2(R1) guidelines, demonstrating excellent linearity, precision, accuracy, specificity, and robustness. The results confirm that this RP-HPLC method is suitable for the routine quality control of Losartan and Amlodipine in combined pharmaceutical dosage forms.

Keywords: *RP-HPLC, Losartan, Amlodipine, Develosil C18 column, linearity, accuracy.*

Corresponding author:

Akula Srilekha,

Department of Pharmaceutical Analysis, Princeton college of Pharmacy, Pocharam, Hyderabad

Mobile:

Email Id: *princeton.pharmacy@gmail.com*

QR CODE

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INTRODUCTION:

Chromatography is a widely used laboratory technique for separating and analyzing components of a mixture based on their differential interactions with a stationary phase and a mobile phase. It is crucial in many fields such as chemistry, biochemistry, pharmaceuticals, and environmental science for purifying, identifying, and quantifying compounds.

The term "chromatography" was coined by the Russian scientist Mikhail Tsvet in 1903, when he developed the technique to separate plant pigments. The process involves a sample being passed through a medium (stationary phase), where the different components of the sample are separated based on their varying affinities for the stationary phase and their solubility in the mobile phase.

Types of Chromatography:

1. **Paper Chromatography**: A simple technique where a liquid sample is separated on a paper strip, typically used for small-scale analysis.
2. **Thin-Layer Chromatography (TLC):** A more advanced technique that uses a thin layer of adsorbent material (like silica gel) on a plate to separate compounds.
3. **Gas Chromatography (GC):** Involves a gaseous mobile phase and is used primarily for separating volatile compounds.
4. **Liquid Chromatography (LC):** Uses a liquid mobile phase to separate components and is often employed in high-performance liquid chromatography (HPLC).
5. **High-Performance Liquid Chromatography (HPLC):** A more sophisticated form of liquid chromatography that offers higher resolution and is commonly used for the analysis of pharmaceuticals, biochemical substances, and environmental samples.
6. **Ion-Exchange Chromatography**: Separates ions and polar molecules based on their charge and affinity for the stationary phase.
7. **Size-Exclusion Chromatography (SEC):** Separates components based on their size, with larger molecules eluting faster than smaller ones.

Principle of Chromatography:

The separation in chromatography occurs due to differences in the distribution of sample components between the stationary phase (often a solid or liquid) and the mobile phase (gas or liquid). The components of the sample travel at different rates, allowing them to be isolated from each other. The rate at which a compound moves through the system depends on its chemical affinity for the stationary phase, its size, and its solubility in the mobile phase.

Applications of Chromatography:

- Purification of compounds: Isolating pure substances from mixtures.
- Identification and quantification: Analyzing complex mixtures for identification and concentration of components.
- Pharmaceutical analysis: Monitoring drug formulations, detecting impurities, and ensuring quality control.
- Environmental analysis: Detecting pollutants in air, water, and soil.

High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid. High performance liquid chromatography (HPLC) is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product.[1] The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. [2] The sample compound with the greater affinity to the stationary layer will travel slower and for a shorter distance in comparison to compounds with less affinity which travel faster and for a longer distance. [3] The High-Performance Liquid Chromatography is more versatile than gas chromatography since

(a) it is not limited to volatile and thermally stable samples, and

(b) the choice of mobile and stationary phases is wider. [4] HPLC has numerous advantages like

- Simultaneous Analysis
- High Resolution
- High Sensitivity
- Good repeatability
- Small sample size
- Moderate analysis condition.
- Easy to fractionate the sample and purify. [5]

Classification of HPLC can be done as:

- preparative HPLC and analytical HPLC (based on scale of operation)
- affinity chromatography, adsorption chromatography, size exclusion chromatography, ion exchange chromatography, chiral phase

chromatography (based on principle of separation)
- gradient separation and isocratic separation, (based on elution technique)
- normal phase chromatography and reverse phase chromatography (based on modes of operation).[6]

A. Normal phase chromatography:

In normal phase chromatography, mobile phase is non-polar and stationary phase is polar. Hence, the station phase retains the polar analyte. An increase in polarity of solute molecules increases the adsorption capacity leading to an increased elution time. Chemically modified silica (cyanopropyl, aminopropyl and diol) is used as a stationary phase in this chromatography. [7] For example. A typical column has an internal diameter of around 4.6 mm, and a length in the range of 150 to 250 mm. Polar compounds in the mixture that are passed through the column will stick longer to the polar silica than the non-polar compounds. Therefore, the non-polar ones will pass more quickly through the column.[8]

B. RP-HPLC (Reversed phase HPLC):

RP-HPLC has a non-polar stationary phase and polar or moderately polar mobile phase. RP-HPLC is based on the principle of hydrophobic interaction [9].In a mixture of components those analytes which are relatively less polar will be retained by the non-polar stationary phase longer than those which are relatively more polar. Therefore the most polar component will elute first. [10]

METHOD DEVELOPMENT ON HPLC

A step involved in method development of HPLC is as follows:

1. Understanding the Physicochemical properties of drug molecule.
2. Selection of chromatographic conditions.
3. Developing the approach of analysis.
4. Sample preparations
5. Method optimization
6. Method validation

1. Understanding the Physicochemical properties of drug molecule.

Physicochemical properties of a drug molecule play an important role in method development. For Method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. The solubility of molecules can be explained on the basis of the polarity of molecules. Polar, e.g. water, and nonpolar, e.g. benzene, solvents do not mix. In general, like dissolves like i.e., materials with similar polarity are soluble in each other. The selection of mobile phase or diluents is based on the solubility of analyte. The analyte must be soluble in diluents and must not react with any of its component. pH and pKa plays an important role in HPLC method development. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion.

pH = - log10[H3O+]

Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times. [11-12]

1.Selection of chromatographic conditions:

Selection of column:

Selection of the stationary phase/column is the first and the most important step in method development. The development of a rugged and reproducible method is impossible without the availability of a stable, high-performance column. To avoid problems from irreproducible sample retention during method development, it is important that columns be stable and reproducible. A C8 or C18 column made from specially purified, less acidic silica and designed specifically for the separation of basic compounds is generally suitable for all samples and is strongly recommended. [13] Column dimensions, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favored in most of the present HPLC columns due to several physical characteristics. [14]

Buffer Selection

Choice of buffer is governed by the pH that is desired. The typical pH range for reversed phase on silica-based packing is pH 2 to 8. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value <2 units of the desired mobile phase pH.

General consideration for buffer selection:

1. Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
2. Some salt buffers are hygroscopic and this may lead to changes in the chromatography like increased tailing of basic compounds and possibly selectivity differences.
3. Ammonium salts are generally more soluble in organic/water mobile phases·
4. Trifluoroacetic acid can degrade with time. It is volatile and absorbs at low UV wavelengths.

5. Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier at all. The growth accumulates on column inlets and can damage chromatographic performance.
6. At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
7. Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 - 48 hrs. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.
8. After buffers are prepared, they should be filtered through a 0.2-µm filter.
9. Mobile phases should be degassed. [15]

10. EXPERIMENTAL METHODS

11. INSTRUMENTS USED

12. Table-: Instruments used

S.No	Instruments and Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

13. CHEMICALS USED:

14. Table-: Chemicals used

S.No	Chemical	Names
1	Losartan	Provided by Sura Pharma labs
2	Amlodipine	Provided by Sura Pharma labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

15. HPLC METHOD DEVELOPMENT:

16. TRAILS

17. Preparation of standard solution:

Accurately weigh and transfer 10 mg of Losartan and Amlodipine working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicated to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.2ml of Losartan and 0.6ml of Amlodipine from the above stock solutions

into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:
Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:
Initially the mobile phase tried was Methanol: Water and ACN: Water with varying proportions. Finally, the mobile phase was optimized to Acetonitrile and Acetate buffer (pH-4.3) in proportion 35:65 v/v respectively.

Optimization of Column:
The method was performed with various C18 columns like Symmetry, X terra and ODS column. Develosil C18 (4.6mm×250mm) 5µm particle size Column was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used	:	Waters Alliance 2695 HPLC with PDA Detector 996 model.
Temperature	:	Ambient
Column	:	Develosil C18 (4.6mm×250mm) 5µm particle size Column
Mobile phase	:	Acetonitrile and Acetate buffer (pH-4.3) (35:65% v/v)
Flow rate	:	1ml/min
Wavelength	:	238nm
Injection volume	:	20µl
Run time	:	6minutes

VALIDATION

PREPARATION OF MOBILE PHASE:

Preparation of mobile phase:
Accurately measured 350ml of Acetonitrile (35%) of and 650ml of Acetate buffer (65%) were mixed and degassed in a digital ultra-sonicated for 20 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent.

VALIDATION PARAMETERS

SYSTEM SUITABILITY
Accurately weigh and transfer 10 mg of Losartan and Amlodipine working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicated to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette out 0.2ml of Losartan and 0.6ml of Amlodipine from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

SPECIFICITY STUDY OF DRUG:

Preparation of Standard Solution:
Accurately weigh and transfer 10 mg of Losartan and Amlodipine working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicated to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette out 0.2ml of Losartan and 0.6ml of Amlodipine from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution:
Take average weight of Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Losartan and Amlodipine sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicated to dissolve it completely and make volume up to the mark with the same solvent. Filter the sample solution by using injection filter which contains 0.45µ pore size.

Further pipette out 0.2ml of Losartan and 0.6ml of Amlodipine Sample solution from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:
Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

8. RESULTS & DISCUSSION:

Trial 5 Optimized Chromatogram (Standard)

Mobile phase ratio : Acetonitrile and Acetate buffer (pH-4.3) (35:65% v/v)
Column : Develosil C18 (4.6mm×250mm) 5µm particle size Column
Column temperature : Ambient
Wavelength : 238nm
Flow rate : 1ml/min
Injection volume : 20µl
Run time : 6minutes

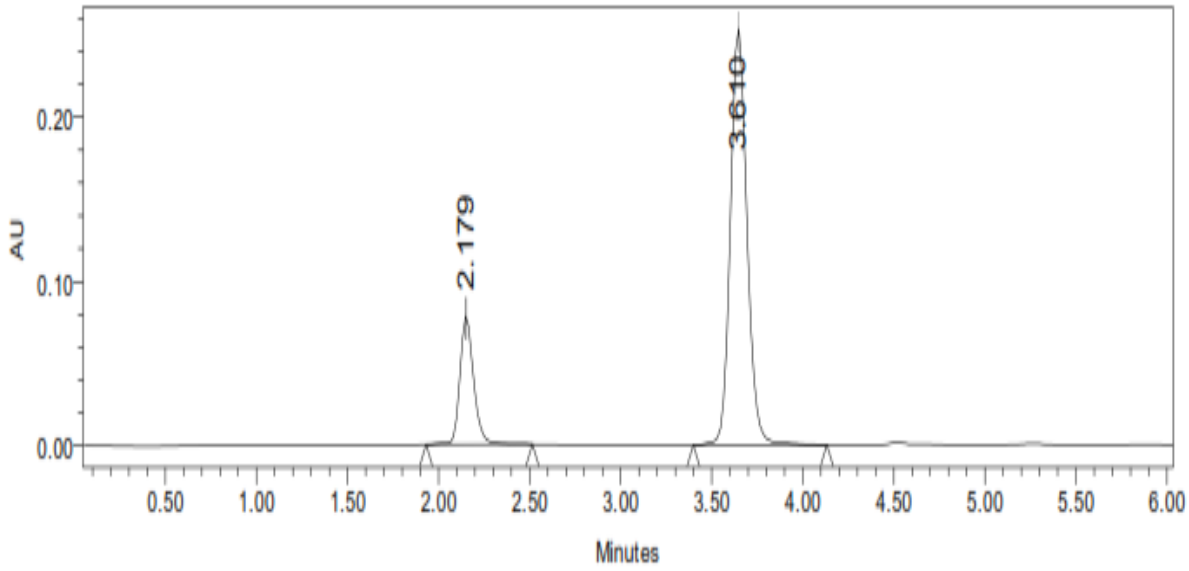


Figure-: Optimized Chromatogram (Standard)

Table-: Optimized Chromatogram (Standard)

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Losartan	2.179	513567	78659	1.2	4536	
2	Amlodipine	3.610	1625892	265321	1.1	7985	9.8

System Suitability:

Table-: Results of system suitability for Losartan

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Losartan	2.152	513652	78542	4698	1.2
2	Losartan	2.157	513524	78654	4785	1.2
3	Losartan	2.141	513425	78541	4682	1.2
4	Losartan	2.133	513647	78454	4854	1.2
5	Losartan	2.166	514824	78655	4872	1.2
Mean			513814.4			
Std. Dev.			572.2004			
% RSD			0.111363			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

Table-: Results of system suitability for Amlodipine

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Amlodipine	3.674	1635285	265421	7985	1.1	10.1
2	Amlodipine	3.631	1635241	265484	7898	1.1	10.1
3	Amlodipine	3.625	1652547	253498	7954	1.1	10.1
4	Amlodipine	3.692	1658458	265241	7965	1.1	10.1
5	Amlodipine	3.629	1652894	265348	7985	1.1	10.1
Mean			1646885				
Std. Dev.			10865.58				
% RSD			0.659766				

Assay (Standard):

Table-: Peak results for assay standard of Losartan

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Losartan	2.152	513538	78074	1.2	4562	1
2	Losartan	2.198	513975	79001	1.2	4620	2
3	Losartan	2.179	513283	78048	1.2	4652	3

Table-: Peak results for assay standard of Amlodipine

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Amlodipine	3.646	1625632	265325	1.1	7949	1
2	Amlodipine	3.604	1635458	265423	1.1	7919	2
3	Amlodipine	3.610	1635241	265874	1.1	7926	3

Assay (Sample):

Table-: Peak results for Assay sample of Losartan

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection	% of Assay
1	Losartan	2.152	513265	78548	1.2	4582	1	100.1
2	Losartan	2.150	513254	78547	1.2	4658	2	100.1
3	Losartan	2.187	513876	78498	1.2	4597	3	99.9

Table-: Peak results for Assay sample of Amlodipine

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection	% of Assay
1	Amlodipine	3.646	1625284	78569	1.1	7985	1	100.0
2	Amlodipine	3.651	1624613	78547	1.1	7898	2	100.7
3	Amlodipine	3.601	1625874	78462	1.1	7854	3	100.6

LINEARITY

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY OF LOSARTAN:

Concentration µg/ml	Average Peak Area
10	245899
15	365687
20	481526
25	589854
30	705882

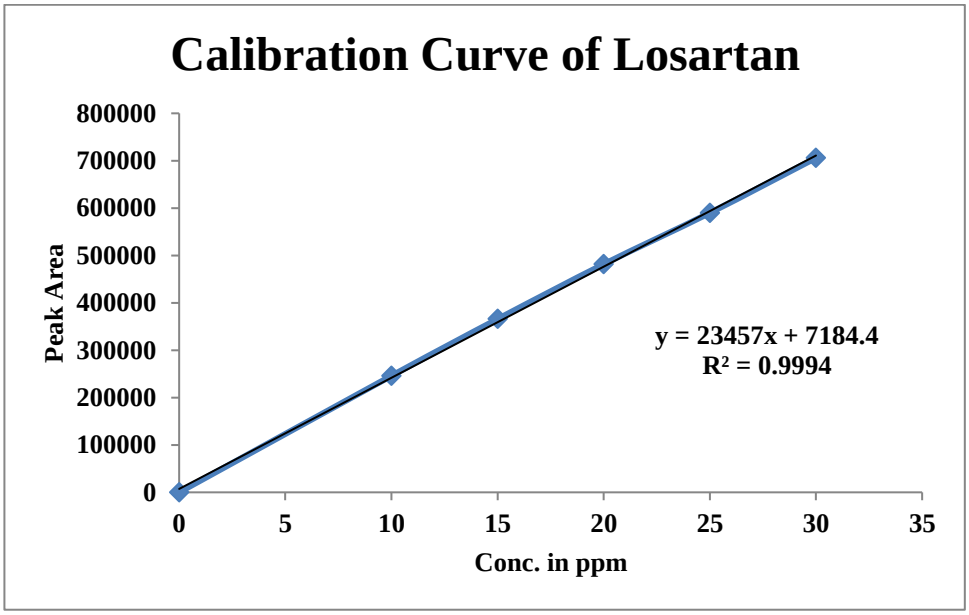


Fig-: Calibration Graph of Losartan

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY OF AMLODIPINE:

Concentration µg/ml	Average Peak Area
30	863094
45	1249397
60	1678592
75	2050412
90	2468444

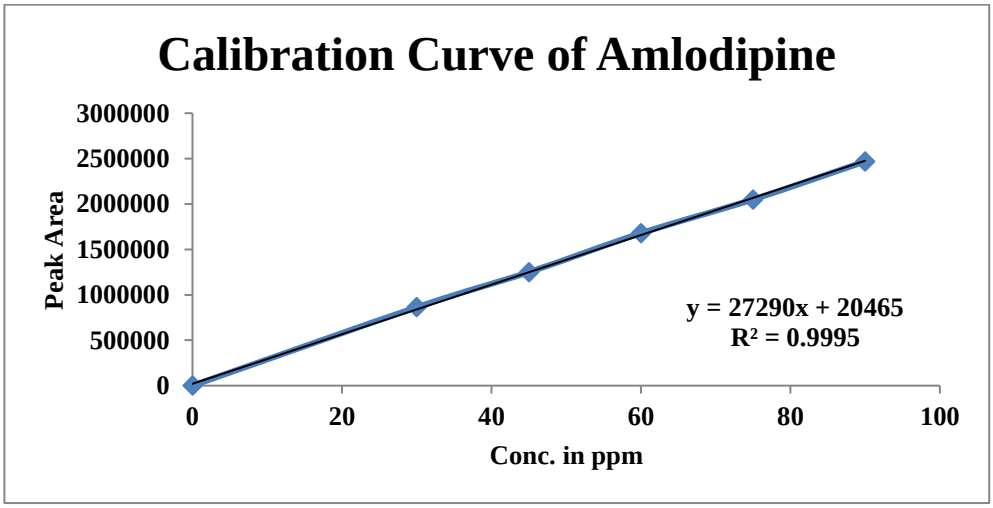


Fig-: Calibration Curve of Amlodipine

REPEATABILITY

Table-: Results of repeatability for Losartan:

S. No	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Losartan	2.157	513568	78546	1.2	4528
2	Losartan	2.159	513685	78541	1.2	4572
3	Losartan	2.186	513659	79852	1.2	4598
4	Losartan	2.160	513254	78498	1.3	4529
5	Losartan	2.170	513647	77898	1.2	4572
Mean			513562.6			
Std.dev			177.9475			
%RSD			0.03465			

Acceptance criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table-: Results of repeatability for Amlodipine:

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Amlodipine	3.603	1635625	265325	1.1	7985
2	Amlodipine	3.608	1658744	264588	1.1	7859
3	Amlodipine	3.600	1652985	265985	1.2	7845
4	Amlodipine	3.696	1645898	264898	1.1	7969
5	Amlodipine	3.629	1652364	268489	1.1	7846
Mean			1649123			
Std.dev			8811.631			
%RSD			0.534322			

Intermediate precision:

Table-: Results of Intermediate precision for Losartan

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Losartan	2.198	514658	78698	4658	1.2
2	Losartan	2.196	514354	78599	4598	1.2
3	Losartan	2.160	513985	79854	4652	1.2
4	Losartan	2.160	514875	79879	4561	1.2
5	Losartan	2.160	514658	79865	4659	1.2
6	Losartan	2.186	516452	79854	4589	1.2
Mean			514830.3			
Std. Dev.			852.3705			
% RSD			0.165563			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2.

Table-: Results of Intermediate precision for Amlodipine

S.No	Peak Name	Rt	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	Resolution
1	Amlodipine	3.623	1645875	266589	7985	1.1	10.1
2	Amlodipine	3.611	1658554	265898	8001	1.1	10.1
3	Amlodipine	3.696	1649854	265415	7985	1.1	10.1
4	Amlodipine	3.696	1659842	265154	7956	1.1	10.1
5	Amlodipine	3.696	1645985	266598	7985	1.1	10.1
6	Amlodipine	3.642	1659852	265341	8002	1.1	10.1
Mean			1653327				
Std. Dev.			6838.733				
% RSD			0.413635				

ACCURACY

Table-: The accuracy results for Losartan

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	245954	10	10.179	101.79%	101.36%
100%	483747	20	20.316	101.58%	
150%	715961	30	30.	100.72%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

Table-: The accuracy results for Amlodipine

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	842287	30	30.114	100.38%	100.26%
100%	1659744	60	60.068	100.113%	
150%	2483885	90	90.268	100.297%	

ROBUSTNESS

LOSARTAN

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	513567	2.179	4536	1.2
Less Flow rate of 0.9 mL/min	523652	2.210	4462.3	0.9
More Flow rate of 1.1 mL/min	502146	2.184	4325.1	1.0
Less organic phase	521574	2.200	4632.4	0.9
More Organic phase	502416	2.172	4190.8	0.8

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

AMLODIPINE

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	1625892	3.610	4536	1.1
Less Flow rate of 0.9 mL/min	1758455	4.498	4426.4	0.9
More Flow rate of 1.1 mL/min	1742514	3.505	4421.5	0.8
Less organic phase	1726451	4.504	4355.1	0.9
More organic phase	1725466	3.512	4426.6	0.9

CONCLUSION:

A validated Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method was developed for the simultaneous estimation of Losartan and Amlodipine in both bulk drugs and tablet dosage forms. The goal was to establish a simple, accurate, and reproducible analytical method that meets ICH validation guidelines and can be applied for routine quality control analysis.
Chromatographic separation was performed using a Develosil C18 column (4.6 mm × 250 mm, 5 µm particle size) under ambient temperature conditions. The optimized mobile phase consisted of Acetonitrile and Acetate buffer (pH 4.3) in a 35:65% v/v ratio, pumped at a flow rate of 1 mL/min. Detection was carried out at a wavelength of 238 nm, with an injection volume of 20 µL, and a total run time of 6 minutes. Under the optimized chromatographic conditions, both Losartan and Amlodipine were well separated with sharp, distinct peaks and consistent retention times. The method was validated according to ICH Q2(R1) guidelines, and it showed excellent results for linearity, precision, accuracy, specificity, and robustness, confirming the method's reliability and suitability for pharmaceutical analysis.

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