Original Research Article

Analytical Method Development and Validation For Simultaneous Estimation of Amlodipine Besylate and Indapamide by using UV VIS Spectrophotometer and RPHPLC in bulk and dosage form

Abstract:

In HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried to separate active ingredients.The objective of this study was to develop a rapid and sensitive RP-HPLC method for the analysis of Amlodipine Besylate and Indapamide in bulk drug and pharmaceutical dosage form by using the most commonly employed C-18 column with UV-detection.The system with Acetonitrile: Acetate buffer pH-5 (40: 60 v/v) and 1.2 mL / min flow rate was selected as mobile phase.The samples collected are Amlodipine Besylate (Gift sample procured from Matrix Laboratories Ltd., Hyderabad.) and Indapamide (Gift sample procured from Yarrow Chem Laboratories Ltd.).This method has been found to be better than previously reported methods, due to its wider range of linearity, use of readily available mobile phase, lack of extraction procedures. Hence above method can be used in quality control for routine analysis of finished products of Amlodipine Besylate and Indapamide simultaneously without any interference.

Keywords:

Amlodipine Besylate and Indapamide, UV VIS Spectrophotometer, RPHPLC, Mobile phase, Stationary Phase, C-18 Column, Retention time, Retention factor, Regression coefficient.

Introduction:

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products.

Basic criteria for new method development of drug analysis:

- 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.
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

Analytical chemistry is a science deals with the identification, characterization and estimation of the components of a sample. The primary interest of an analytical chemist is to develop experimental methods of measurement to obtain information about the qualitative and quantitative tests for given composition of a sample. Analytical chemistry involves a multi sided approach to obtain information of every individual chemical species present in any sample. A knowledge of analytical chemistry helps to develop methods, select an appropriate instruments, and strategies to obtain information on the composition and nature of sample.

Materials

Drug Sample:

- Amlodipine Besylate (Gift sample procured from Matrix Laboratories Ltd., Hyderabad.)
- Indapamide (Gift sample procured from Yarrow Chem Laboratories Ltd.).

Chemicals Required:

For UV-VIS Spectrophotometry

- Methanol AR grade (S. D. Fine-chem limited, Mumbai).
- Distilled water

For HPLC

- Methanol (Merck specialties private limited, Mumbai).
- Double distilled water (Merck specialties private limited, Mumbai)

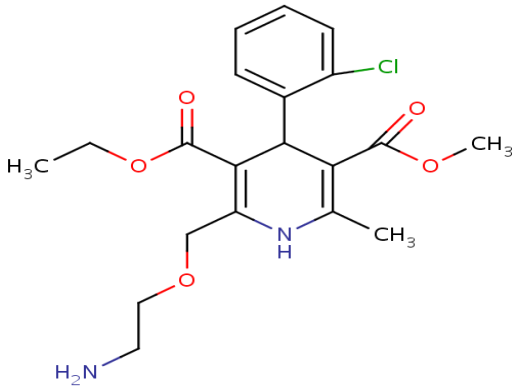
4.2 Instruments

1. Axis Ag N 204-PO digital balance.
2. Elico LI 120 pH meter.
3. 1.5LH Ultrasonic bath sonicator.
4. Elico SL 218 double beam UV-Vis spectrophotometer,
   With Wide Range Photodiode detection and fixed 10 mm path holders for reference and sample. (Instrument-1)
5. Elico SL 210 double beam UV-Vis spectrophotometer,
   With Wide Range Photodiode detection and fixed 10 mm path holders for reference and sample. (Instrument-2)
6. Agilent 1120 compact LC system.
   Agilent 1120 Compact LC Includes isocratic pump, manual injector, variable wavelength detector, Ezchrome Elite Compact software, LMD software, startup column.

Drug Profile of Amlodipine Besylate:

Amlodipine Besylate is a dihydropyridine calcium-channel blocker.

1. **Structure :**



2. **Chemical Name** **:** 3-Ethyl 5-methyl 2-(2-aminoethoxymethyl)4-(2-chlorophenyl)-1,4-dihydro-6-methylpyridine-3,5 dicarboxylate mono benzene sulphonate

3. **Synonyms** **:**Amlodipine Benzene sulfonate

4. **Molecular Formula** **:**$C_{20}H_{25}ClN_2O_5$

5. **Molecular Weight** **:**567.1

6. **Melting Point** **:**178-179 °C

7. **Description** **:**A white or almost white powder.

8. **Solubility** **:** Slightly soluble in water, isopropyl alcohol; freely soluble in methyl alcohol

9. **pKa** **:**9.45

10. **Category** **:** It is a dihydropyridine calcium channel blocker
For treatment of Hypertension and Angina pectoris

11. **Dosage Forms** **:** Tablets , Capsules

12. **Brand Names** **:** Amlodac , Amlopres

13. **Mechanism of Action:** Amlodipine decreases arterial smooth muscle contractility and subsequent vasoconstriction by inhibiting the influx of calcium ions through L-type calcium channels.

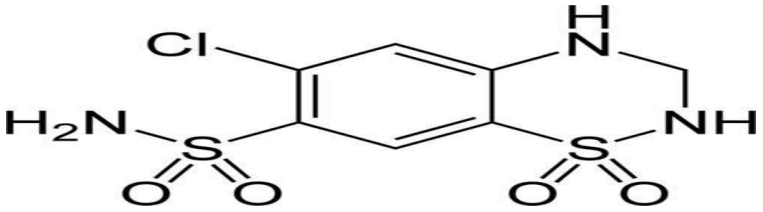
14. Pharmacodynamics/ Kinetics

Absorption	**:**	well absorption
Distribution	**:**	Protein binding 97.6%
Metabolism	**:**	Extensively metabolized in the liver
Bioavailability	**:**	60-65%
Half-life Elimination:		35-50 hrs
Excretion	**:**	Primarily urine

15. Use **:** Treatment of Hypertension and Angina Pectoris

4.4. Drug Profile of Indapamide

1. Structure **:**



2. **Chemical Name** **:** 3-(aminosulfonyl)-4- chloro-N-(2,3-dihydro-2- methyl-1H-indol-1-yl).

3. **Molecular Formula** **:** $C_{16}\ H_{16}ClN_3O_3S$

4. **Molecular Weight** **:** 365.8

5. **Melting Point** **:**160 -162°C

6. **Description** **:** White solid

7. **Solubility** **:** Soluble in methanol, Slightly soluble in Acetone, insoluble in Water.

8. **Category** **:** For treatment of Hypertension**.**

9. **pKa** **:** 8.8

10. **Dosage Forms** **:** Tablets.

11. **Brand Names** **:** Idatix ,Natrlix

12. **Mechanism of Action:** Indapamide blocks the slow component of delayed rectifier potassium current (IKs) without altering the rapid component (IKr) or the inward rectifier current. Specifically it blocks or antagonizes the action the proteins KCNQ1 and KCNE1. Indapamide is also thought to stimulate the synthesis of the vasodilatory hypotensive prostaglandin PGE2.

13. Pharmacodynamics/ Kinetics

Absorption : C_{max} is approximately 115-260 ng/ml. The T_{max} is 2h.

Distribution : 71% to 79% is protein bound

Metabolism : Extensively metabolized

Elimination : The $t_{1/2}$ is 26h. More than 70% is excreted in urine and 23% is excreted in GI tract probably including the biliary route

14. Use **:** Treatment of high blood pressure and kidney problems

5.1. Estimation of Indapamideby UV-Spectrophotometric Method

A. Method Development

1. Solvent Selection:

In order to select suitable solvent for determination of Indapamide, various solvents were selected for the solubility studies and it was found that Indapamide was freely soluble in the following solvents; Methanol, Ethanol…etc., and slightly soluble in water. In the present investigation, Methanol was used as primary solvent and distilled water was used as secondary solvent.

2. Preparation of Standard Stock Solution:

Standard stock solution was prepared by dissolving accurately weighed 25mg of Indapamide in Methanol and the volume was made up to 25 ml with distilled water in a 25 ml volumetric flask (Stock solution-I, 1000 µg/ ml). 1.0 ml of stock solution-I was diluted to 10 ml with solvent (Stock solution-II, 10 µg/ ml).

3. Determination of λ max:

The solution (10µg/ml) was scanned in the UV region of 200-400 nm, which shows maximum absorbance at 280 nm and the absorbance curve was given in **figure 7.**

4. Study of Beer-Lambert's law:

Aliquots of standard solution of ranging from 10-50µg/ ml were transferred into a series of 10 ml volumetric flasks. The volume in each flask was made up to 10 ml with solvent and the absorbance were measured at 280 nm against solvent blank and the absorbance values were shown in **table 1**. The obtained absorbance values are plotted against the concentration of Indapamide to get the calibration curve and were represented as **figure 8**. The concentration of the unknown sample was determined from the calibration graph.

Optical Parameters of the Calibration curve:

The regression equation and correlation coefficient were determined and were given in **table 2.**

B. Application of Proposed Method for Analysis of Tablet Formulation

For analysis of commercial formulation, 20 tablets of Indapamide were weighed, powdered in glass mortar and the powder equivalent to 25mg of Indapamide was weighed accurately and transferred into a 25ml standard volumetric flask. The contents were dissolved in solvent and sonicated for few minutes. This solution was filtered through (0.45µ) Whatmann filter paper no. 41. 1.0 ml of the filtrate was diluted to 10 ml with solvent to get the solution of 100 µg/ ml. An appropriate aliquot of 3.0ml of test solution was diluted to 10mlto produce the concentration 30µg/ml. The absorbance of the solution was recorded at 280 nm and the concentration of the Indapamide was determined by linear regression equation. Results were shown in **table 3.**

C. Validation of Spectrophotometric Method:

The following parameters were determined to validate the developed analytical method as per ICH guidelines (ICH Q2B, 1996).

1. Accuracy:

Accuracy is the closeness of the test results obtained by the method to the true value. To assess the accuracy of the proposed method, recovery experiment was performed at three different levels i.e. 50%, 100% and 150%. To the pre analyzed sample solution a known amount of standard drug solution was added at three different levels and absorbance were recorded. The recovery values were summarized in **table 4.**

2. 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. It provides an indication of random error results and was expressed as coefficient of variation (CV).

Intra and Inter-day Precision:

The precision of the proposed method was ascertained by actual determination of six replicates of fixed concentration (50 µg/ml) of the drug within the Beer's range and variation of results within the same day (intra-day), variation of results between days (inter-day) was analyzed and was shown in **table 5.**

3. Linearity:

This is the method ability to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range.

The linearity of the method was demonstrated over the concentration range of 10-50 µg/ml of the target concentration. Aliquots of 10, 20, 30, 40 and 50 µg/ ml are prepared from Stock solution (1000µg/ml); Calibration curve was plotted and presented in **figure 8.**

4. Ruggedness:

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures etc.

The solution of 50µg/mL was prepared and analyzed with change in the analytical conditions like different Instruments (Elico SL 218 and Elico SL 210) and different analysts (Analyst-1 and Analyst-2). The results were given in **table 6.**

5. LOD and LOQ:

The LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S (Where, σ is the standard deviation of y intercepts obtained from the replicate measurements (n=6) and S is mean of the slopes of the calibration curves) and were given in **table 2.**

6. Molar Extinction Coefficient (lit. mol^{-1} cm^{-1}):

It can be calculated as,

Molar extinction coefficient = $\frac{A}{C} X L$ **Equation 20**

Where,

A= Absorbance of drug,

C= Concentration of drug in gm/100mL,

L= Path length

The results were given in **table 2.**

7. Sandell's Sensitivity (µg/cm^2/0.001 absorbance units):

It can be calculated as,

Sandell's sensitivity = $\frac{C}{A} X\ 0.001$ **Equation 21**

Where,

C= Concentration of drug,

A= Absorbance of drug.

The results were given in **table 2.**

5.2 Estimation of Amlodipine Besylate by UV-Spectrophotometric Method

A. Method Development

1. Solvent Selection:

In order to select suitable solvent for determination of Amlodipine Besylate, various solvents were selected for the solubility studies and it was found that Amlodipine Besylate was freely soluble in the following solvents: Methanol, Ethanol, acetone, acetonitrile…etc., and slightly is soluble in water. In the present investigation Methanol was used as primary solvent and distilled water was used as secondary solvent.

2. Preparation of Standard Stock Solution:

Standard stock solution was prepared by dissolving accurately weighed 25mg of Amlodipine Besylate in Methanol and the volume was made up to 25 ml with distilled water in a

25ml volumetric flask (Stock solution-I, 1000 µg/ mL). 1.0ml of stock solution-I was diluted to 10 ml with distilled water (Stock solution-II, 10 µg/ mL).

3. Determination of λ max:

The solution (10µg/ml) was scanned in the UV region of 200-400 nm, which shows maximum absorbance at 360 nm and the absorbance curve was given in***Figure 9***

4. Study of Beer-Lambert's law:

Aliquots of standard solution of Amlodipine besylate ranging from 10-50µg/ml were transferred into a series of 10 ml volumetric flasks. The volume in each flask was made up to 10 ml with solvent and the absorbance were measured at 360 nm against solvent blank and the absorbance values were shown in **table 7**. The obtained absorbance values are plotted against the concentration of Amlodipine Besylate to get the calibration curve and were shown in **figure 10**. The concentration of the unknown sample was determined from the calibration graph.

Optical Parameters of the Calibration curve:

The regression equation and correlation coefficient were determined and were given in **table 8.**

B. Application of Proposed Method for Analysis of Tablet Formulation

For analysis of commercial formulation, 20 tablets of Amlodipine Besylate(amlo-10mg) were weighed, powdered in glass mortar and the powder equivalent to 25 mg of Amlodipine Besylate was weighed accurately and transferred into a 25ml standard volumetric flask. The contents were dissolved in solvent and sonicated for few minutes. This solution was filtered through (0.45µ) Whatmann filter paper no. 41. 1 ml of the filtrate was diluted to 10 ml with solvent to get the solution of 100 µg/ ml. An appropriate aliquot of 3.0 ml of test solution was diluted to 10 ml to get a concentration of 30 µg/ ml. The absorbance of the solution was recorded at 360nm and the concentration of the Amlodipine Besylate was determined by linear regression equation. Results were shown in **table 9.**

C. Validation of Spectrophotometric Method

The following parameters were determined to validate the developed analytical method as per ICH guidelines (ICH Q2B, 1996).

1. Accuracy:

Accuracy is the closeness of the test results obtained by the method to the true value. To assess the accuracy of the proposed method, recovery experiment was performed at three different levels i.e. 50%, 100% and 150%. To the pre analyzed sample solution a known amount of standard drug solution was added at three different levels and absorbance were recorded. The recovery values were summarized in **table 10.**

2. 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. It provides an indication of random error results and was expressed as coefficient of variation (CV).

Intra and Inter-day Precision:

The precision of the proposed method was as certained by actual determination of six replicates of fixed concentration (50µg/ml) of the drug within the Beer's range and variation of results within the same day (intra-day), variation of results between days (inter-day) was analyzed and was shown in **table 11.**

3. Linearity:

This is the method ability to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range.

The linearity of the method was demonstrated over the concentration range of 10-50 µg/ ml of the target concentration. Aliquots of 10, 20, 30, 40 and 50 µg/ ml are prepared from Stock solution(1000µg/ml); Calibration curve was plotted and presented in **figure 10.**

4. Ruggedness:

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures etc.

The solution of 50µg/mL was prepared and analyzed with change in the analytical conditions like different instruments (Elico SL 218 and Elico SL 210) and different analysts (Analyst-1 and Analyst-2). The results were given in **table 12.**

5. LOD and LOQ:

The LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S (Where, σ is the standard deviation of y intercepts obtained from the replicate measurements (n=6) and S is mean of the slopes of the calibration curves) and were given in **table 8.**

6. Molar Extinction Coefficient (lit. mol^{-1} cm^{-1}):

It can be calculated as,

Molar extinction coefficient $= \frac{A}{C} XL$ **Equation 20**

Where,

A= Absorbance of drug,

C= Concentration of drug in gm/100mL,

L= Path length

The results were given in **table 8.**

7. Sandell's Sensitivity (µg/cm^2/0.001 absorbance units):

It can be calculated as,

Sandell's sensitivity $= \frac{C}{A} X\ 0.001$ **Equation 21**

Where,

C= Concentration of drug,

A= Absorbance of drug.

The results were given in **table 8.**

5.3. Simultaneous Estimation of Amlodipine Besylate and Indapamideby UV-Spectrophotometric Method

Simultaneous Equation Method *(Vierodt's method)*

If sample contains two absorbing substances (x and y) and each of which absorbs at the λmax of the other, then it may be possible to determine both the drugs by the technique of simultaneous equation. The information required is:

λ_1: Wavelength maxima for drug x

λ_2: Wavelength maxima for drug y

ax_1 and ax_2: Absorptivity of X at λ_1 and λ_2

ay_1 and ay_2: Absorptivity of Y at λ_1 and λ_2

A_1: Absorbance of sample at λ_1

A_2: Absorbance of sample at λ_2

Let Cx and Cy be the concentration of X and Y respectively in the diluted sample: Two equations are constructed based upon the fact that at λ1 and λ2, the absorbance of the mixture is the sum of the individual absorbances of X and Y.

At λ_1

$$\mathbf{A_1 = ax_1bCx + ay_1bCy} \qquad \textbf{Equation 22}$$

At λ_2

$$\mathbf{A_2 = ax_2bCx + ay_2bCy} \qquad \textbf{Equation 23}$$

For measurements in 1 cm cells, b = 1

Concentrations of X and Y can be determined by the equations

$$\mathbf{Cx} = \frac{A2ay1 - A1ay2}{ax2ay1 - ax1ay2} \qquad \textbf{Equation 24}$$

$$Cy = \frac{A1ax2 - A2ax1}{ax2ay1 - ax1ay2} \qquad \textbf{Equation 25}$$

A. Selection of Sampling Wavelength for Analysis and Preparation of Standard

1. Solvent Used

Distilled water

2. Preparation of Standard Stock Solution

25 mg each of standard Amlodipine Besylate and Indapamidewere weighed accurately and transferred in to two separate 25mL flasks, dissolved in 10mL of solvent and made up to the mark with distilled water to obtain a final concentration of 1000 µg/mL of each Amlodipine Besylate and Indapamide(standard stock solutions A1 and A2 respectively). From the above stock solution 'A1' and 'A2' 1 mL aliquots were pipetted in to two separate volumetric flasks and dissolved in 5mL of solvent and made up to the mark with distilled water to obtain a final concentration of 100µg/mL. (Standard stock solutions 'B1' and 'B2' respectively).

3. Selection of Analytical Wavelengths

Appropriate dilution of the standard stock solutions 'A1' and 'A2' were scanned separately in the entire ultraviolet range. The λmax of each standard was selected in such a way that at each absorption maxima the difference in absorption of the two components should be as

large as possible. The two wavelengths were 360nm and 280nm for Amlodipine Besylate and Indapamide respectively. At 360nm Amlodipine Besylate has higher absorbance than Indapamide and at 280nm Indapamide has higher absorbance than Amlodipine Besylate which were shown in **figure 11.**

4. Selection of Analytical Concentration Range and Construction of Calibration Graph

Amlodipine Besylate: Appropriate aliquots ranging from 0.1 mL to 0.5mL (1mL=100 µg/mL) was pipetted out in to a series of 10mL volumetric flasks. The volume was made up to the mark with distilled water to obtain a concentration range, ranging from 10-50µg/mL (10, 20, 30, 40, 50 µg/mL). Absorbance of the above solutions was

measured at 360 nm and a calibration curve of absorbance against concentration was plotted.

Indapamide: Appropriate aliquots ranging from 0.1 mL to 0.5mL (1mL=100 µg/mL) was pipetted out in to a series of 10mL volumetric flasks. The volume was made up to the mark with distilled water to obtain a concentration range, ranging from 10-50µg/mL (10, 20, 30, 40, 50 µg/mL). Absorbance of the above solutions was measured at 280 nm and a calibration curve of absorbance against concentration was plotted.

Both drugs follow Beer lambert's law in the concentration range of 10-50 µg/mL. Regression equation was established and the correlation coefficient was determined. The results were given in **table 13 and 14**and calibration curves of both the drugs were shown in **figure 12.**

B. Analysis of Tablet Formulation

Twenty tablets of Amlodipine Besylate and Indapamide combination dosage forms (Amlodac-D) were weighed and their average weight was determined. The tablets were crushed in to fine powder. From the tablet triturate a tablet mass equivalent to 12.5mg of Amlodipine Besylate or 25 mg of Indapamide was transferred in to a 25mL volumetric flask , dissolved in a small quantity of methanol by sonication for 10min and finally the volume was made up to the mark with methanol. The resultant solution was filtered through a Whatmann filter paper no. 41

and used as sample stock solution 'A' (500µg/mL Amlodipine Besylate and 1000µg/mL Indapamide).

From the above stock solution 1mL aliquot was transferred in to a 10 mL volumetric flask, dissolved in a small quantity of distilled water and the volume was made up to the mark with distilled water to obtain a final concentration of 50µg/mL Amlodipine Besylate and 100µg/mL Indapamide. This solution was used as the sample stock solution 'B'.

1.0mL of the sample stock solution 'B' was transferred in to a 10 mL volumetric flask, dissolved in a small quantity of distilled water and the volume was made up to the mark with distilled water. The absorbance of the resultant solution was measured at the two absorption maxima 360nm and 280nm. This absorbance was noted as A_1 and A_2 respectively and amount of the drugs present was calculated using simultaneous equation method and the results were given in **table 15.**

C. Method Validation

The following parameters were determined to validate the developed analytical method as per ICH guidelines (ICH Q2B, 1996).

1. Accuracy:

Accuracy is the closeness of the test results obtained by the method to the true value. To study the accuracy, 20 tablets were weighed and powdered and analysis of the same was carried out. Recovery studies were carried out by addition of known amount of the known amount of Amlodipine Besylate and Indapamide to the sample at three different concentration levels i.e. 50%, 100% and 150% (Standard addition method). The results were given in **table 16**

2. 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. It

provides an indication of random error results and was expressed as relative standard deviation (coefficient of variation)

Procedure for the Determination of Intra-day Precision

In intraday precision six replicate sample matrices separately containing 50µg/mL of Amlodipine Besylate and Indapamide were analysed at different time intervals on the same day at 360nm and 280 nm respectively. The variation of the results within the same day was analysed and statistically validated.

Procedure for the Determination of Inter-day Precision

In inter-day precision six replicate sample matrices separately containing 50µg/mL of Amlodipine Besylate and Indapamide were analysed on different days at 360nm and 280 nm respectively. The variation of the results was analysed and statistically validated, which were given in **table 17 and 18.**

3. Linearity and Range:

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in the sample with in a given range. Appropriate aliquots ranging from 0.1mL to 0.5mL were pipetted out separately from the 'standard stock solution B1 and B2' out in to a series of 10mL volumetric flasks. The volume was made up to the mark with distilled water to obtain a concentration range, ranging from 10-50µg/mL (10, 20, 30, 40, 50µg/mL). Absorbance of the above solutions was measured at 360nm and 280 nm respectively.

A calibration curve of concentration vs. absorbance was established and shown in **figure 12.** Both drugs follow Beer's lamberts law in the concentration range of 10-50µg/mL. Regression equation was established and the correlation coefficient was determined. The optical and regression parameters were given in **table 14**.

4. Ruggedness:

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures etc.

The solution of 50µg/mL of Amlodipine Besylate and Indapamide was prepared separately and analyzed with change in the analytical conditions like different instruments (Elico SL 218 and Elico SL 210) and different analysts (Analyst-1 and Analyst-2) and the results were given in **table 19 and 20.**

5. LOD and LOQ:

The LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S (Where, σ is the standard deviation of y intercepts obtained from the replicate measurements (n=3) and S is mean of the slopes of the calibration curves) and were given in **table 14.**

5. Determination of Molar Extinction Coefficient:

The absorbance of all the concentrations of Amlodipine Besylate(10-50µg/mL) and Indapamide (10-50µg/mL) were determined at the absorption maximum 360nm and 280nm respectively. The molar extinction coefficient was determined using the formula

$$\text{Molar extinction coefficient} = A/C \times L \qquad \textbf{Equation 20}$$

Where,

A= Absorbance of drug,

C= Concentration of drug in gm/100mL,

L= Path length

The results were given in **table 14.**

6. Sandell's Sensitivity (µg/cm^2/0.001 absorbance units):

It can be calculated as,

Sandell's sensitivity = $C/A \times 0.001$ **Equation 21**

Where,

C = Concentration of drug,

A = Absorbance of drug.

The results were given in **table 14.**

5.4. Simultaneous Estimation of Amlodipine Besylate and Indapamide by RP-HPLC Method

A. Selection of Sampling Wavelength for Analysis and Preparation of Standard Calibration Curves.

1. Selection of Mobile Phase

The standard solutions containing Amlodipine Besylate and Indapamidewere injected into the HPLC system and run in different solvent systems. By studying literature survey, different mobile phases in different proportions and different pH were tried in order to find the best conditions for the separation.

It was found that acetonitrile and acetate buffer of pH- 5 gives satisfactory results as compared to other mobile phases. This mobile phase system was tried with different proportions and using different flow rates. Finally, the optimal composition of the mobile phase was determined to be Acetonitrile : Acetate Buffer pH-5(40:60v/v)

2. Preparation of Mobile Phase

Mobile phase was prepared by mixing Acetontrile and Acetate Buffer pH-5 in the ratio of 40:60 and was initially filtered through 0.45µm millipore membrane filter and sonicated for 15 min before use.

Preparation of Acetate Buffer pH-5:

Dissolve 13.6g of sodium acetate in 200ml of water and add 6.0ml of glacial acetic acid to adjust the pH and the volume is made upto 1000ml.

3. Preparation of Standard Stock Solution

The separate stock solutions of AMB and IND were prepared by accurately weighing 25mg each into a separate 25 ml volumetric flasks A and B and made up to the volume with mobile phase to get 1000µg/ml respectively. From the above standard stock solutions 1.0ml from

volumetric flask A and 1.0 ml from volumetric flask B was transferred to a 10 ml volumetric flask and made up to the volume with

same mobile phase to get 100µg/ml and 100µg/ml of AMB and IND respectively (Working stock solution).

4. Selection of Analytical Wavelength

By appropriate dilution of each standard stock solution with mobile phase, various concentrations of Amlodipine Besylate and Indapamide were prepared separately. Each solution was scanned using double beam UV visible spectrophotometer between the range of 200 nm to 400 nm and their spectra was overlaid. From the overlain spectra shown in **figure 13** of Amlodipine Besylate and Indapamide, 268 nm was selected as analytical wavelength for Multicomponent analysis using HPLC method.

5. Optimized Chromatographic Conditions

Mobile phase consisting of Acetonitrile : Acetate Buffer pH-5 (40:60 v/v) was used in isocratic mode. The mobile phase was initially filtered through 0.45µm millipore membrane filter and sonicated for 15 min before use. The flow rate was maintained at 1 ml/min and the injection volume was 20µL. UV detection was performed at 268 nm and the separation was achieved at ambient temperature.

6. Selection of Analytical Concentration Range and Construction of Calibration Curve for Amlodipine Besylate and s

Appropriate aliquots ranging from 0.5ml to 2.5ml were pipetted out from the working stock solution (100 µg/ml of Amlodipine Besylate) and 1.0ml to 5.0ml (from 100µg/ml of Indapamide) in to a series of 10 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 5-25 µg/ml of Amlodipine Besylate 10-50µg/ml of Indapamide Chromatograms representing linearity was shown in **figure 16 to 20.** Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 20 µL of each concentration of the drug were injected into the HPLC system three times separately and their chromatograms were recorded under the same chromatographic conditions as described above.

Peak areas were recorded for all the peaks and a standard calibration curve of area against concentration was plotted as concentration of the drug Vs peak area (**figure 21 and 22**). The results were shown in **table 22**. Both the drugs follow the Beer's Lambert's law in the concentration range of 5-25µg/ml of Amlodipine Besylate and 10-50µg/ml of. Indapamide

The linearity of calibration curves and adherence of the system to Beer's Lambert's law was validated by high value of correlation coefficient and less than 2% percent relative standard deviation (%RSD) for the intercept value which were shown in **table 22.**

B. Analysis of Tablet Formulation.

The tablets were initially powdered and an amount equivalent to 12.5mg of Amlodipine Besylate and 25 mg of Indapamide was accurately weighed into a 25 ml volumetric flask, mixed with 20ml of mobile phase. The solution was made up to the volume with mobile phase and sonicated for 5 minutes. The solution was then filtered through 0.45µm millipore membrane filter. Final stock containing 10µg/ml and 5µg/ml of Indapamide and Amlodipine Besylate respectively was prepared by subsequent dilution with the same mobile phase. 20µL of sample solution was injected into chromatographic system and the peak responses were measured. The solution was injected three times into the column. The amount present in each tablet was calculated by comparing the areas of test with that of the standard. A typical chromatogram of test solution containing 10 µg/ml of Indapamide and 5µg/ml of Amlodipine Besylate was shown in **figure 23**. The results were shown in **table 23**.

C. Method Validation

The method was validated according to ICH Q2 B guidelines for validation of analytical procedures in order to determine system suitability, linearity, sensitivity, precision, accuracy and robustness for the analytes (ICH Q2B, 1996).

1. System Suitability:

The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from six replicate injections for Amlodipine Besylate and Indapamide retention times and peak areas.

System suitability was carried out by injecting 100% concentration (sample having 50µg/ml of Indapamide and 25µg/ml of Amlodipine Besylate) into the HPLC system. This was repeated for six times under similar condition. The tailing factor (T) and no. of theoretical plates (N) obtained were shown in **figure 24** and the results were given in **table 24 and 25.**

2. Accuracy:

To confirm the accuracy of the proposed method, recovery experiments were performed by standard addition technique. In this method a known quantity of pure drug was added at three different levels i.e. 50 %, 100% and 150% to pre-analyzed sample solutions and calculated the recovery of Amlodipine Besylate and Indapamide for each concentration. Chromatograms showing different levels of recovery were shown in **figure 25 to 27**. The results of recovery studies by proposed method were validated by statistical evaluation and were given in **table 26.**

3. Linearity and Range:

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. Linearity of the method was determined by means of calibration curve using different concentration of the drugs. Linearity was evaluated by visual inspection of a calibration curve shown in **figure 21 and 22.** The linearity of the method was determined in concentration range of 5-25µg/ml for Amlodipine Besylate and 10-50µg/ml for Indapamide. Each solution was injected in triplicate. Chromatograms representing linearity were shown in **figure 16 to 20.** The slope, intercept was reported as required by ICH which were given in **table 21 and 22.**

4. Precision:

The precision of an analytical method was studied by performing intraday and inter day precision.

Intraday Precision

Variation of results within the same day was analyzed. Intraday precision was determined by analyzing a set of six combined standard solutions of Amlodipine Besylate (25µg/ml) and Indapamide (50µg/ml) in linearity range as 100% concentration at three different time intervals on same day. Chromatogram representing intraday precision was shown in **figure 28** and the results were given in **table 27.**

5. Specificity and Selectivity:

The specificity of the RP-HPLC method was determined by complete separation of Amlodipine Besylate and Indapamide with parameters like retention time (Rt), resolution (Rs) and tailing factor (T_f). Here tailing factor for peaks of Amlodipine Besylate and Indapamide was less than 2% and resolution was also more than 1%. The average retention time and standard deviation for Amlodipine Besylate and Indapamide were found to be satisfactory for six determinations of sample solution containing 25µg/ml of Amlodipine Besylate and 50µg/ml of Indapamide respectively. The peaks obtained for Amlodipine Besylate and Indapamide were sharp and have clear baseline separation as none of the excipients interfered with the analytes of interest. The chromatogram to represent specificity was shown in **figure 29** and the results were given in **table 28.**

6. Robustness:

The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters like different column temperature, different analytical wavelength, different flow rate. The solution containing 25µg/ml of Amlodipine Besylate and 50µg/ml of Indapamide was injected into sample injector of HPLC three times under different parameters like deliberate variations in flow rate (±0.2mL/min) and detection wavelength (± 2 nm).

Chromatogram representing robustness was shown in **figure 30 and 31**for change in flow rate and the results were given in **table 29.** Chromatogram representing robustness was shown in **figure 32 and 33** for change in detection wavelength and the results were given in **table 30**.

7. Ruggedness:

The evaluation of ruggedness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters like different instruments, analysts, laboratories, reagents, days etc. The solution containing 25 µg/ml of Amlodipine Besylate and 50 µg/ml of Indapamide was injected into HPLC three times under different parameters like different analysts. Chromatogram representing ruggedness was shown in **figure 34 and 35** for change in analysts and the results were given in **table 31.**

8. LOD and LOQ:

The LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S (Where, σ is the standard deviation of the responses and S is mean of the slopes of the calibration curves). The results were given in **table 22.**

6.1. Estimation of Indapamide by UV-Spectrophotometric Method

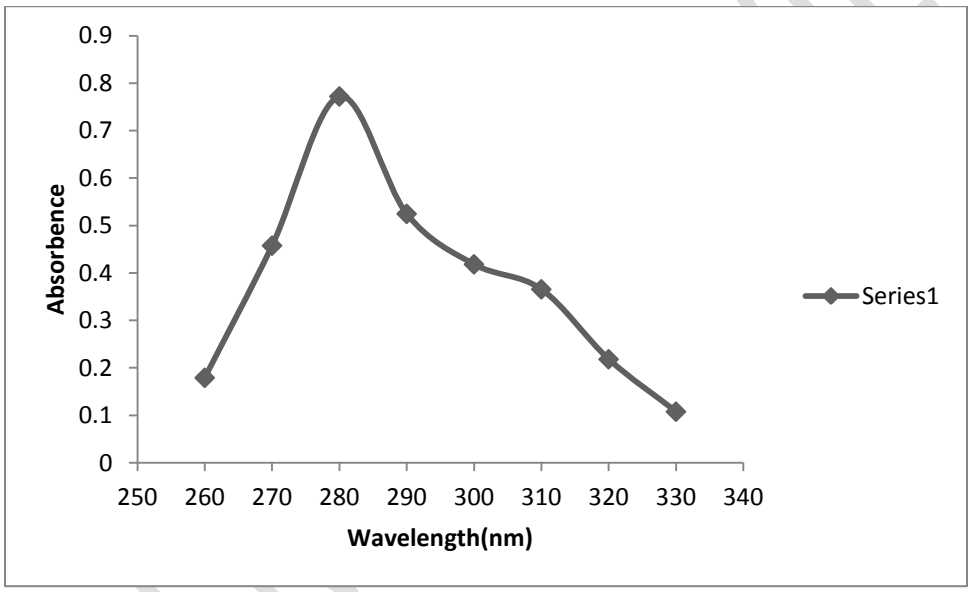


Figure 7: Absorption Curve of Indapamide

Table 1: Linearity Data of Indapamide at 280nm

S.NO	Concentration of Indapamide (µg/ml)	Absorbance at 280nm
1	10	0.1039
2	20	0.2345

3	30	0.3441
4	40	0.4543
5	50	0.5787

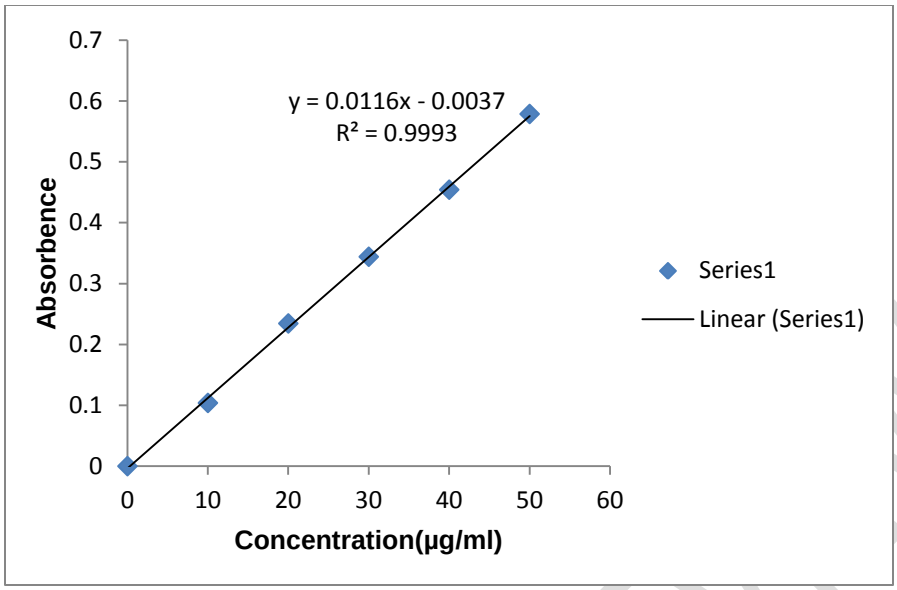


Figure 8: Calibration Curve of Indapamide

Table 2: Optical and Regression Parameters of the Calibration Curve Obtained by UV Spectrophotometric Method

Parameter	Indapamide
Linearity Range (µg/mL)	10-50
Molar Extinction Coefficient (lit.mol^{-1} cm^{-1})	117.25
Sandell's Sensitivity (µgcm^{-2}/ 0.001 abs units)	0.085
Regression Equation (Y*)	Y=0.116x-0.0037
Slope (m)	0.116
Intercept (c)	0.0037
Regression coefficient (r^2)	0.999
LOD (µg/mL)	1.452
LOQ (µg/mL)	4.40

*Y=mX+C where X is the concentration of Indapamide in µg/mL and Y is the absorbance at the respective λmax

Table 3: Assay of Indapamide Tablet Formulation

Estimation of content of Indapamide			
Dosage form	Labeled claim (mg)	Amount estimated (mg)	% Purity
Indapamide	1.5mg	1.47	98.4% w/w

Table 4: Accuracy Data of Indapamide

S. No	Level of Recovery	Amount Added (µg/ml)		Total Amount Recovered (µg/ml)	% Recovery (w/w)
		Test	Stnd		
1	50%	5	5	9.92	99.2
2	100%	5	15	20.12	100.6
3	150%	5	25	29.98	99.9
Mean Recovery = 99.2-100.6%					

Table 5: Precision Data of Indapamide

S. No	Conc. (µg/ml)	Absorbance							
		Intraday Precision					Interday Precision		
		0 hrs	2 hrs	4 hrs	6 hrs	8 hrs	Day 1	Day 2	Day 3
1	50	0.578	0.581	0.581	0.585	0.579	0.581	0.589	0.581
2	50	0.568	0.578	0.579	0.583	0.587	0.586	0.587	0.592
3	50	0.577	0.580	0.580	0.576	0.585	0.582	0.586	0.590
4	50	0.576	0.577	0.589	0.585	0.583	0.589	0.590	0.589
5	50	0.571	0.578	0.580	0.582	0.582	0.590	0.582	0.590
6	50	0.561	0.576	0.581	0.579	0.585	0.581	0.585	0.581
Mean		0.571	0.578	0.581	0.581	0.583	0.584	0.586	0.587
SD		0.0065	0.0018	0.0036	0.0035	0.0028	0.0040	0.0028	0.0048
%RSD		1.1	0.31	0.61	0.60	0.48	0.68	0.47	0.81

Table 6: Ruggedness Data of Indapamide

S. No	Conditions	Conc. (µg/mL)	Absorbance	Mean	SD	%RSD
1	Analyst – 1	50	0.5779	0.5791	0.0019	0.328
2		50	0.5781			
3		50	0.5814			
4	Analyst-2	50	0.5789	0.5786	0.0016	0.276
5		50	0.5801			
6		50	0.5769			
7	Instrument-1	50	0.5799	0.5797	0.005	0.862
8		50	0.5791			
9		50	0.5801			
10	Instrument-2	50	0.5808	0.5812	0.0051	0.877
11		50	0.5811			
12		50	0.5818			

6.2. Estimation of Amlodipine Besylate by UV-Spectrophotometric Method

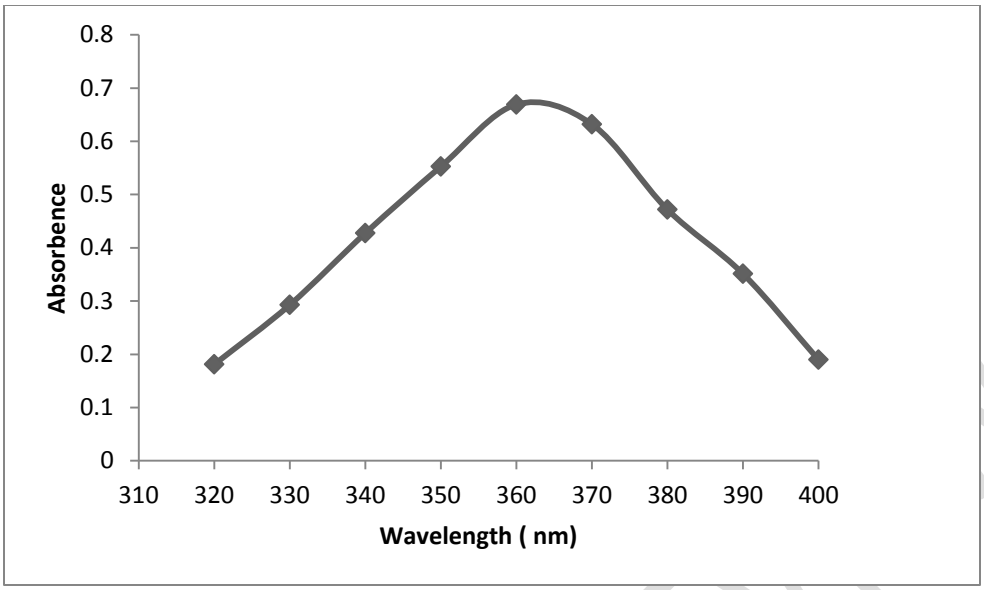


Figure 9: Absorption Curve of Amlodipine Besylate

Table 7: Linearity Data at 360nm for Amlodipine Besylate

S. No.	Concentration of Amlodipine Besylate (µg/mL)	Absorbance at 360 nm
1	0	0
2	10	0.1330
3	20	0.2451
4	30	0.3580
5	40	0.4810
6	50	0.6100

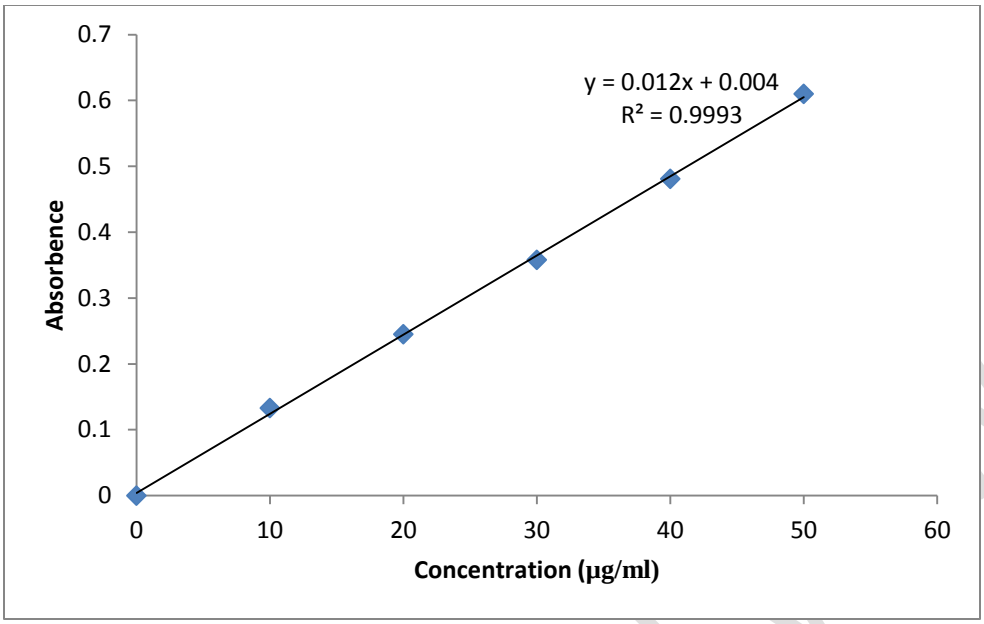


Figure 10: Calibration Curve of Amlodipine Besylate

Table 8: Optical and Regression Parameters of the Calibration Curve Obtained by UV Spectrophotometric Method

Parameter	Amlodipine Besylate
Linearity Range (µg/mL)	10-50
Molar Extinction Coefficient (lit.mol^{-1} cm^{-1})	122.5
Sandell's Sensitivity (µgcm^{-2}/ 0.001 abs units)	0.081
Regression Equation (Y*)	Y=0.012x + 0.004
Slope (m)	0.012

Intercept (c)	0.004
Regression Coefficient (r^2)	0.9993
LOD (µg/mL)	0.36
LOQ (µg/mL)	1.10

*Y=mX+C where X is the concentration of Amlodipine Besylate in µg/mL and Y is the absorbance at the respective λmax

Table 9: Assay of Amlodipine Besylate Tablet Formulation (Amlodac)

Estimation of content of Amlodipine Besylate			
Dosage form	Labeled claim (mg)	Amount estimated (mg)	% Purity
Amlodipine Besylate	5mg	4.91	98.2% w/w

Table 10: Accuracy Data of Amlodipine Besylate

S. No	**Level of Recovery**	**Amount Added (µg/ml)**		**Total Amount Recovered (µg/ml)**	**% Recovery (w/w)**
		Test	**Stnd**		

1	50%	5	5	9.96	99.6
2	100%	5	15	20.19	100.9
3	150%	5	25	30.43	101.4
Mean Recovery = 99.6-101.4%					

Table 11: Precision Data of Amlodipine Besylate

S. NO	Conc. (µg/ml)	Absorbance							
		Intraday Precision					Interday Precision		
		0 hrs	2 hrs	4 hrs	6 hrs	8 hrs	Day 1	Day 2	Day 3
1	50	0.614	0.620	0.621	0.609	0.609	0.611	0.621	0.629
2	50	0.615	0.619	0.628	0.610	0.610	0.601	0.628	0.630
3	50	0.628	0.622	0.619	0.615	0.615	0.609	0.630	0.625
4	50	0.617	0.628	0.625	0.613	0.617	0.601	0.625	0.633
5	50	0.615	0.628	0.628	0.620	0.619	0.607	0.627	0.626
6	50	0.628	0.628	0.619	0.619	0.615	0.612	0.622	0.629
Mean		0.619	0.624	0.623	0.614	0.614	0.606	0.625	0.628
SD		0.0066	0.0043	0.0042	0.0045	0.0039	0.0048	0.0035	0.0028
%RSD		1.06	0.68	0.67	0.73	0.63	0.80	0.56	0.44

Table 12: Ruggedness Data of Amlodipine Besylate

S. No	Conditions	Conc. (µg/mL)	Absorbance	Mean	SD	%RSD
1	Analyst - 1	50	0.6190	0.6160	0.00260	0.422
2		50	0.6141			
3		50	0.6150			
4	Analyst-2	50	0.6170	0.6153	0.00152	0.247
5		50	0.6140			
6		50	0.6150			
7	Instrument-1	50	0.6091	0.6078	0.00615	1.010
8		50	0.6011			
9		50	0.6132			
10	Instrument-2	50	0.6131	0.6163	0.00281	0.454
11		50	0.6177			
12		50	0.6182			

6.3. Simultaneous Estimation of Amlodipine Besylate and Indapamideby UV-Spectrophotometric Method

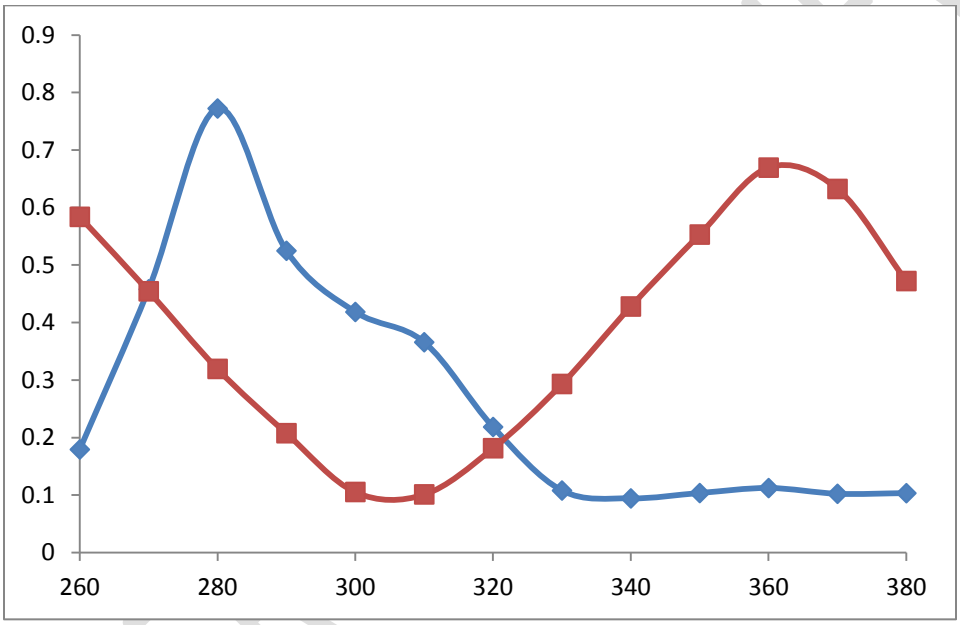


Figure 11: Absorption Curve of Indapamideand Amlodipine Besylate

Table 13: Linearity Data at 280 nm for Indapamide and 360nm for Amlodipine Besylate

S. No	Concentration	Absorbance	
		Indapamide	Amlodipine Besylate
1	0	0	0

2	10	0.1311	0.1081
3	20	0.2360	0.2260
4	30	0.3631	0.3300
5	40	0.4720	0.4451
6	50	0.600	0.5602

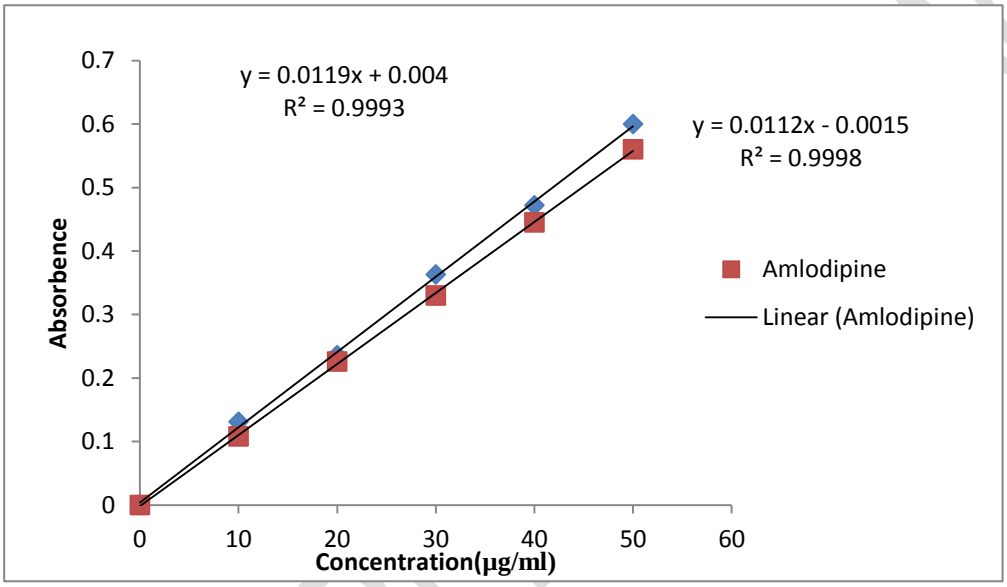


Figure 12: Calibration Curve of Indapamideand Amlodipine Besylate

Table 14: Optical and Regression Parameters of the Calibration Curve Obtained by UV Spectrophotometric Method.

Parameter	Amlodipine Besylate	Indapamide
Linearity Range (µg/mL)	10-50	10-50
Λmax	360	280
Molar Extinction Coefficient (lit.mol^{-1} cm^{-1})	113	118
Sandell's Sensitivity (µgcm^{-2}/ 0.001 abs units)	0.088	0.084
Regression Equation (Y*)	Y=0.0112x-0.0015	Y=0.0119x+0.004

Slope (m)	0.0112	0.0119
Intercept (c)	0.0015	0.004
Regression Coefficient (r^2)	0.9998	0.9993
LOD (µg/mL)	0.39	1.43
LOQ (µg/mL)	1.12	4.40

*Y=mX+C where X is the concentration of drug in µg/mL and Y is the absorbance at the respective λmax

Table 15: Assay of Amlodipine Besylate and Indapamide in Tablet Formulation

S. No	Amount Present in (mg/tab)		Amount Obtained in (mg/tab)		Label Claim %w/w	
	AMB	IND	AMB	IND	AMB	IND
1	5	1.5	4.96	1.49	99.2	99.8

Table 16: Determination of Accuracy for Amlodipine Besylate and Indapamide

Recovery level	Amount of Standard drug added (µg/ml)		Amount of test added(µg/ml)		Total Amount Recovered (µg/ml)		% Recovery w/w	
	AMB	IND	AMB	IND	AMB	IND	AMB	IND
50	5	10	5	10	9.90	19.9	99.07	99.5

100	15	30	5	10	20.24	40.05	101.2	100.5
150	25	50	5	10	30.5	60.9	100.4	101.5

Table 17: Precision Data of Amlodipine Besylate

S. NO	Conc. (µg/ml)	Absorbance							
		Intraday Precision					Inter day Precision		
		0 hrs	2 hrs	4 hrs	6 hrs	8 hrs	Day 1	Day 2	Day 3
1	50	0.571	0.572	0.571	0.579	0.580	0.588	0.581	0.592
2	50	0.570	0.577	0.570	0.582	0.583	0.586	0.589	0.587
3	50	0.579	0.571	0.569	0.583	0.586	0.580	0.590	0.581
4	50	0.574	0.572	0.564	0.576	0.579	0.579	0.593	0.582
5	50	0.573	0.576	0.574	0.580	0.580	0.580	0.592	0.586
6	50	0.577	0.571	0.576	0.579	0.588	0.581	0.593	0.581
Mean		0.574	0.573	0.570	0.579	0.582	0.582	0.589	0.584

SD	0.00346	0.00263	0.0041	0.0024	0.0036	0.0037	0.0045	0.0043
%RSD	0.6	0.45	0.73	0.41	0.61	0.63	0.76	0.73

Table 18: Precision Data of Indapamide

S. NO	Conc. (µg/ml)	Absorbance							
		Intraday Precision					Interday Precision		
		0 hrs	2 hrs	4 hrs	6 hrs	8 hrs	Day 1	Day 2	Day 3
1	50	0.707	0.708	0.714	0.718	0.719	0.721	0.729	0.731
2	50	0.701	0.705	0.714	0.714	0.715	0.723	0.725	0.728
3	50	0.710	0.706	0.707	0.716	0.709	0.728	0.719	0.729
4	50	0.712	0.714	0.709	0.720	0.721	0.723	0.728	0.733
5	50	0.708	0.711	0.704	0.719	0.720	0.718	0.726	0.728
6	50	0.710	0.712	0.704	0.720	0.719	0.719	0.719	0.723
Mean		0.708	0.709	0.708	0.717	0.717	0.722	0.724	0.728
SD		0.0038	0.0035	0.0045	0.0024	0.0044	0.0035	0.0043	0.0033
%RSD		0.5	0.5	0.64	0.33	0.61	0.48	0.59	0.45

Table 19: Ruggedness Data of Amlodipine Besylate

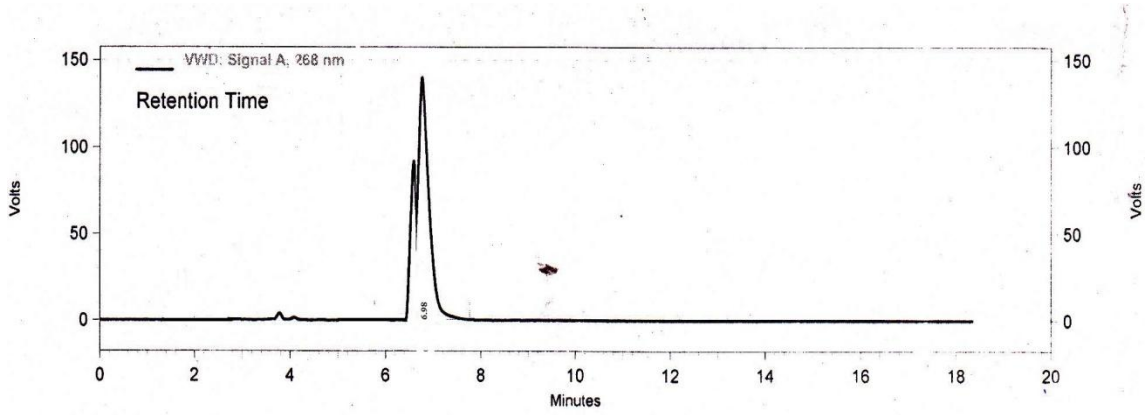
S. No	Conditions	Conc. (µg/mL)	Absorbance	Mean	SD	%RSD
1	Analyst - 1	50	0.571	0.571	0.00152	0.26
2		50	0.570			
3		50	0.573			
4	Analyst-2	50	0.574	0.574	0.001	0.17
5		50	0.573			
6		50	0.575			
7	Instrument-1	50	0.577	0.576	0.0057	0.11
8		50	0.576			
9		50	0.577			
10	Instrument-2	50	0.571	0.571	0.0057	0.10
11		50	0.572			
12		50	0.571			

Table 20: Ruggedness Data of Indapamide

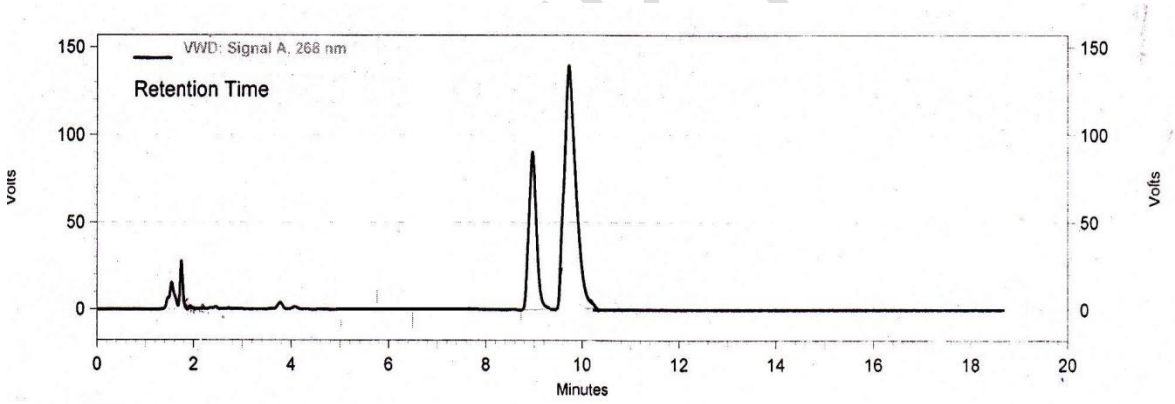
S. No	Conditions	Conc. (µg/mL)	Absorbance	Mean	SD	%RSD
1	Analyst - 1	50	0.707	0.706	0.00264	0.37
2		50	0.708			
3		50	0.703			
4	Analyst-2	50	0.701	0.702	0.00115	0.16
5		50	0.703			
6		50	0.703			
7	Instrument-1	50	0.708	0.706	0.00152	0.21
8		50	0.706			
9		50	0.705			
10	Instrument-2	50	0.708	0.706	0.00288	0.4
11		50	0.708			

12		50	0.703			

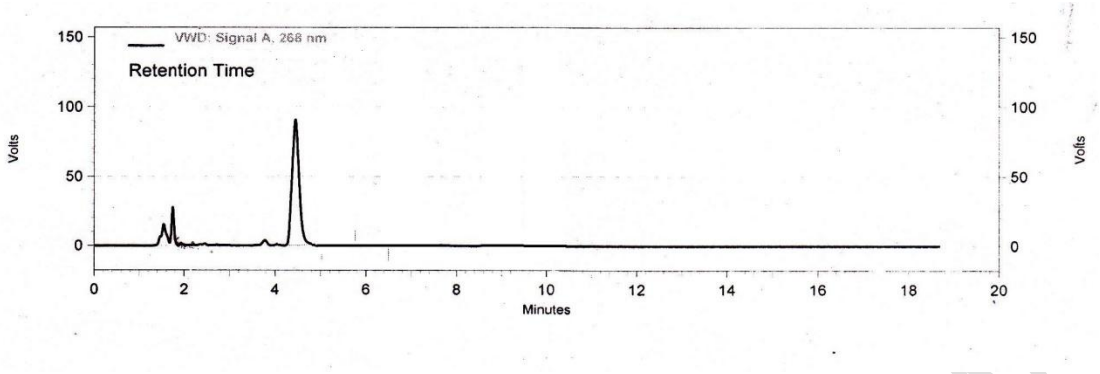
Chromatographic Trials:



Chromatographic Trial-I



Chromatographic Trial-II



Chromatographic Trial-III

Trials	Mobile phase Composition	Flow Rate ml/min	Inference
1	Methanol : Water (70:30)	1.0	Splitting of peaks
2	Methanol:ACN:Acetate Buffer pH-4.6 (60:20:20)	1.0	Less resolution with tailing of peaks
3	Methanol: Phosphate Buffer pH- 6.8 (70:30)	1.0	Single drug is detected

Optimized Chromatographic Conditions

Mobile phase	Acetonitrile:Acetate Buffer pH-5 (40:60)
Mode of Operation	Isocratic

Column	Kromasil 100-5C_{18} column [250mm x 4.6mm].
Column temperature	Ambient
Flow rate	1.2 ml/min
Detection wavelength	268nm
Injection volume	20 µl
Run time	20 minutes

6.4. Simultaneous Estimation of Amlodipine Besylate and Indapamideby RP-HPLC Method

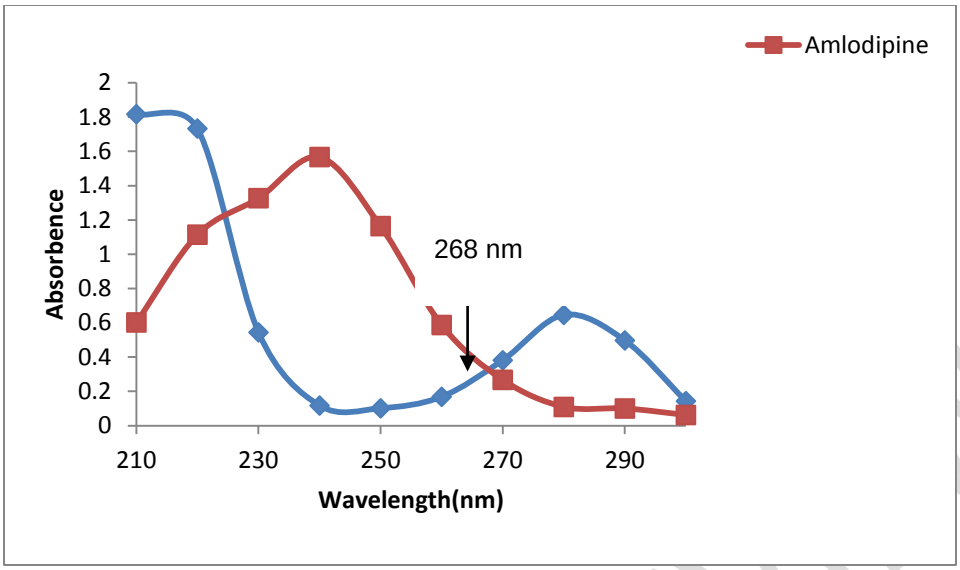


Figure 13: Overlain Spectra of* Indapamide *and Amlodipine Besylate

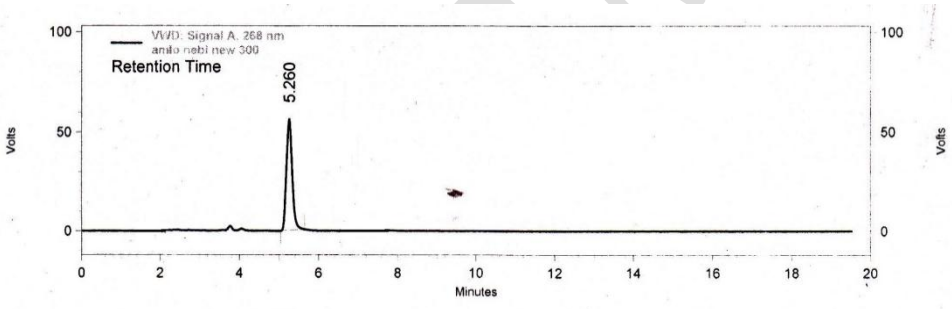


Figure 14: RP-HPLC Chromatogram of Amlodipine Besylate

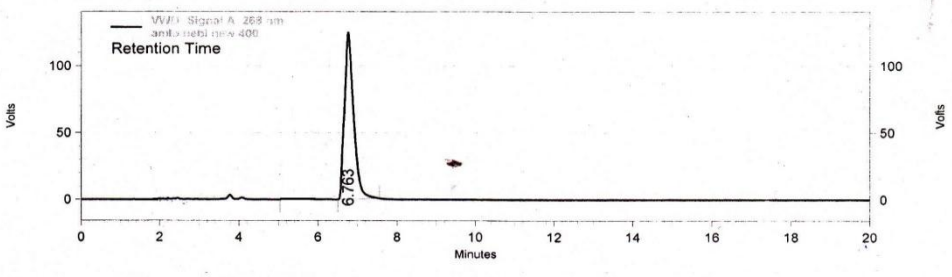


***Figure 15: RP-HPLC Chromatogram of* Indapamide**

Linearity:

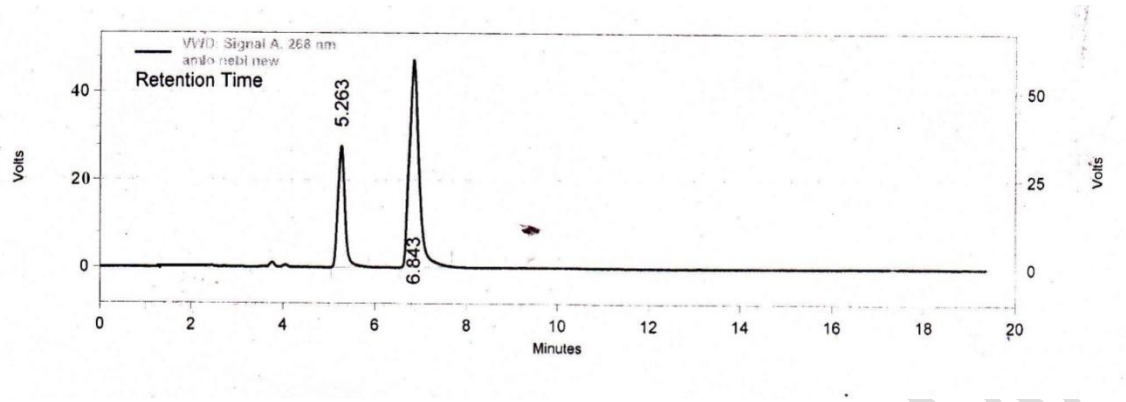


Figure 16: RP-HPLC Chromatogram of Linearity-1

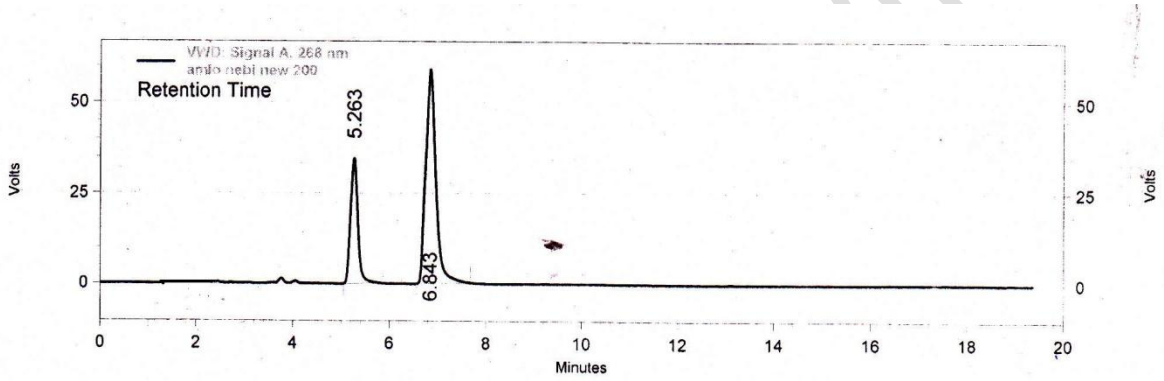


Figure 17: RP-HPLC Chromatogram of Linearity-2

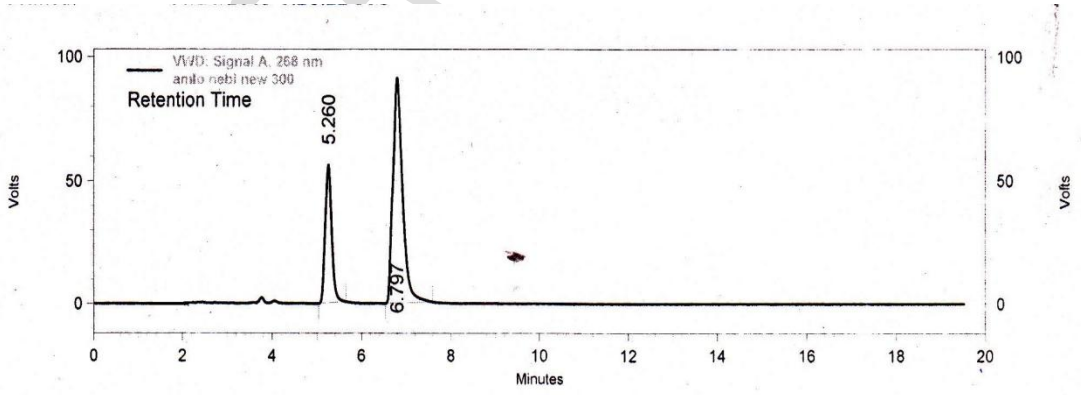


Figure 18: RP-HPLC Chromatogram of Linearity-3

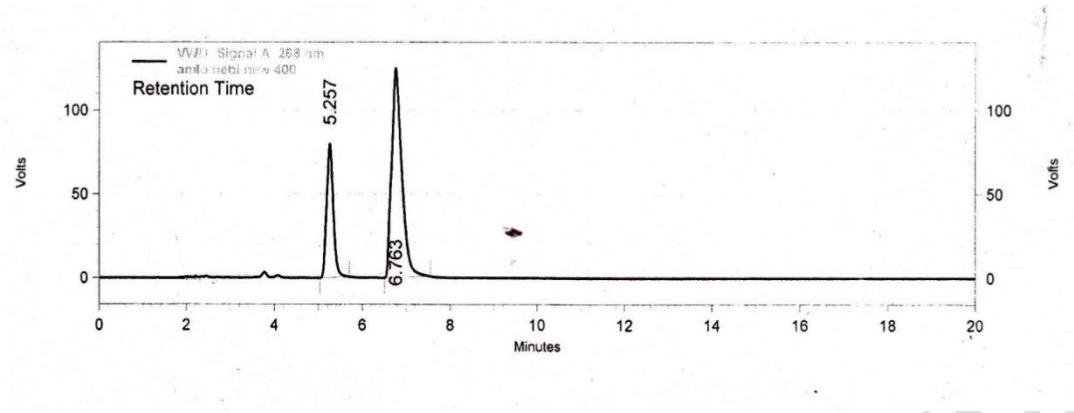


Figure 19: RP-HPLC Chromatogram of Linearity-4

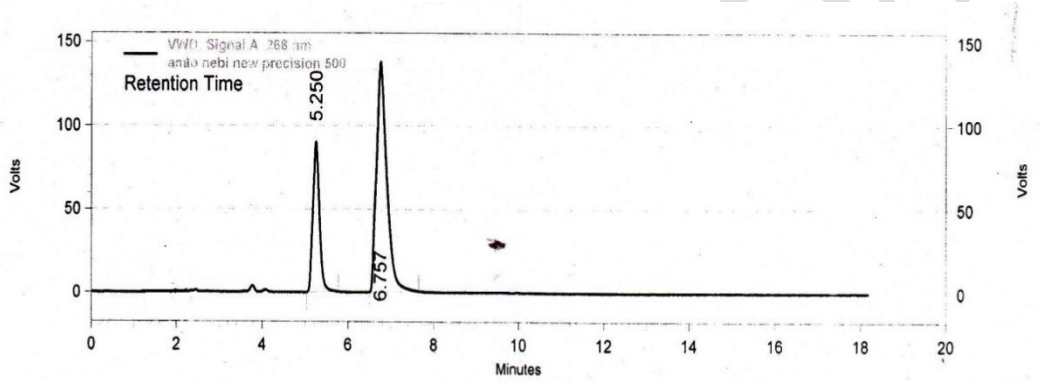


Figure 20: RP-HPLC Chromatogram of Linearity-5

S. No	Amlodipine Besylate			Indapamide		
	Conc. (µg/mL)	R_t(min)	Peak Area	Conc. (µg/mL)	R_t (min)	Peak Area
0	0	0	0	0	0	0
1	5	5.2	3294662	10	6.8	7935414
2	10	5.2	6134216	20	6.8	14519997
3	15	5.2	9512573	30	6.7	22950353
4	20	5.2	12678890	40	6.7	30745235
5	25	5.2	15537733	50	6.7	37670976

Table 21: Linearity Data of Amlodipine Besylate and* Indapamide *at 268 nm by RP−HPLC Method

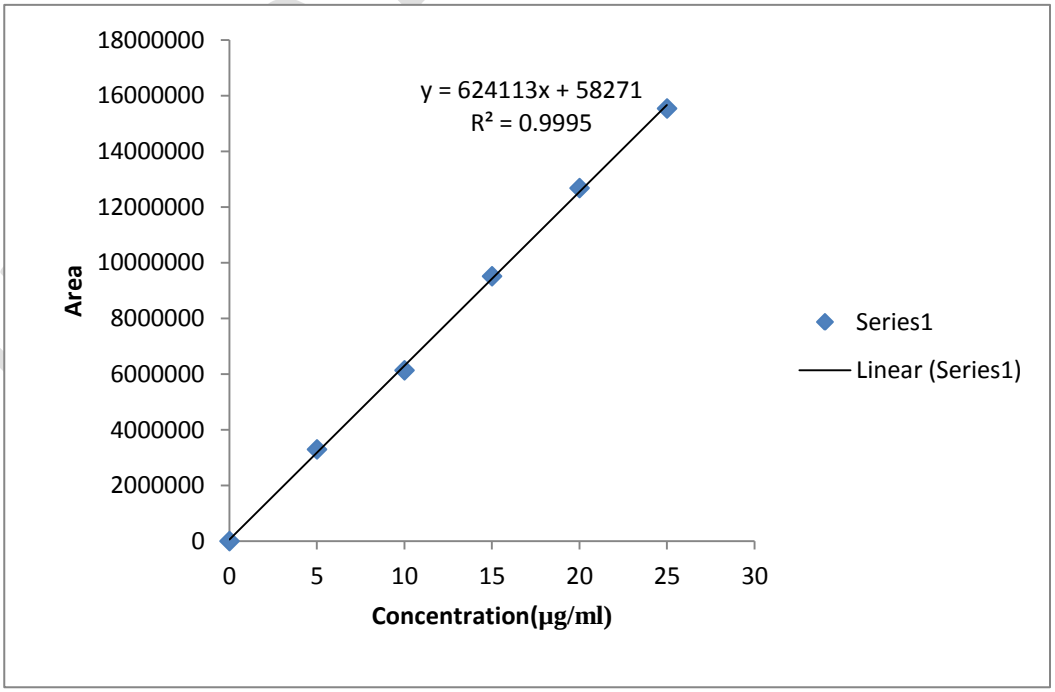


Figure 21: Calibration Curve of Amlodipine Besylate at 268 nm by RP−HPLC Method

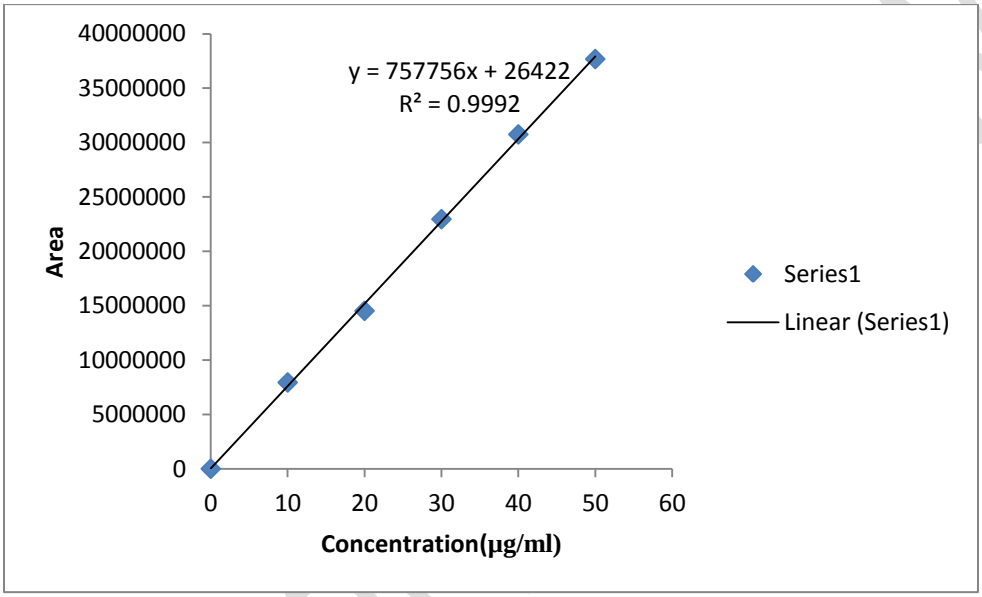


Figure 22: Calibration Curve of **Indapamide** *at 268 nm by RP−HPLC Method*

Table 22: Statistical Data of Amlodipine Besylte and **Indapamide** *at 268 nm by RP−HPLC Method*

Parameter	Amlodipine Besylate	Indapamide
Linearity Range (µg/mL)	5-25	10-50
Regression Equation	Y=624113x + 58271	Y=757756x + 26422
Slope (m)	624113	757756
Intercept (c)	58271	26422

Regression Coefficient (r^2)	0.9995	0.9992
Limit of Detection (µg/mL)	0.36	1.43
Limit of Quantitation (µg/mL)	1.10	4.41

ASSAY:

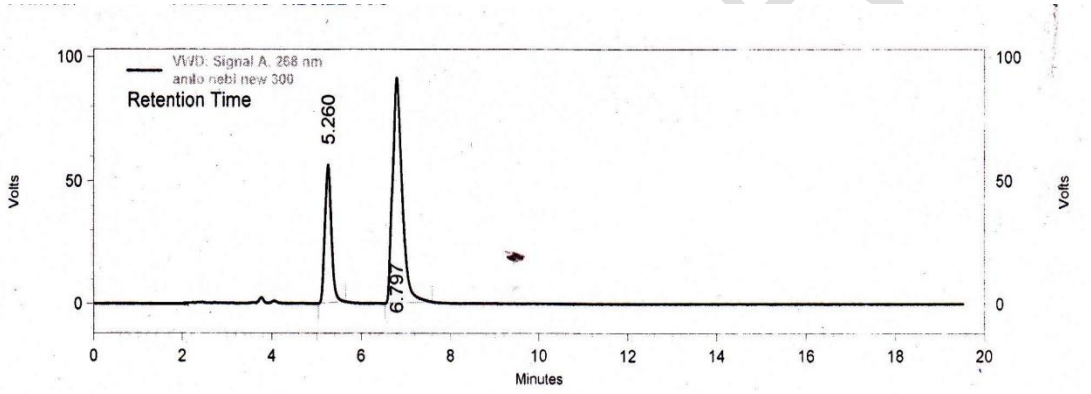


Figure 23: RP-HPLC Chromatogram of Test Formulation

Table 23: **Assay of Amlodipine Besylate and Indapamide in Tablet Formulation**

S. No	Amount Present in (mg/tab)		Amount Obtained in (mg/tab)		Label Claim %w/w	
	AMB	IND	AMB	IND	AMB	IND
1	5	1.5	4.99	1.49	99.8	99.2

System Suitability

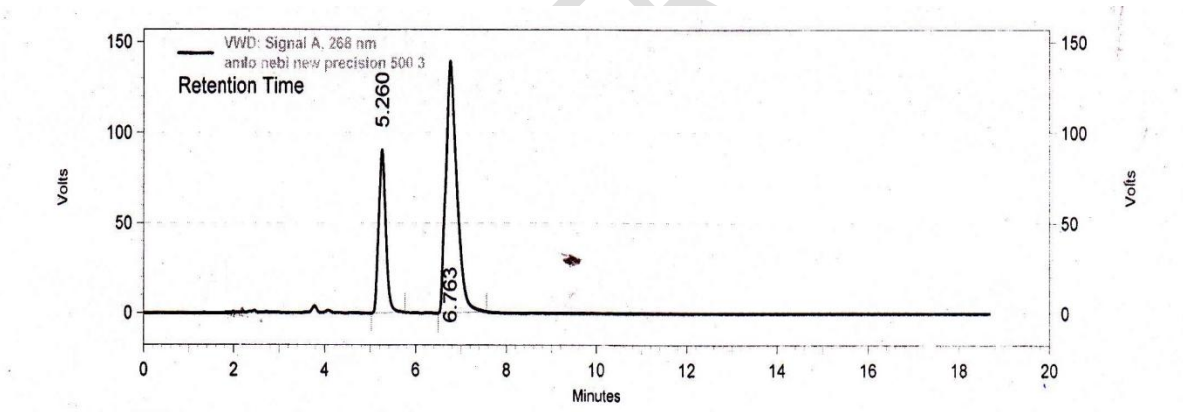


Figure 24: RP-HPLC Chromatogram to Show System Suitability Parameters

Table 24: System Suitability Data of Amlodipine Besylate and Indapamide

S. No	Amlodipine Besylate			*Indapamide*		
	Conc. (µg/mL)	**R_t (min)**	**Peak Area**	**Conc. (µg/mL)**	**R_t (min)**	**Peak Area**
1	25	5.253	16194461	50	6.763	38670976
2	25	5.260	16098038	50	6.763	38896370
3	25	5.260	16418135	50	6.763	38318024
4	25	5.267	16071855	50	6.763	38701120
5	25	5.317	16084531	50	6.833	38554476
6	25	5.260	16135461	50	6.763	38456783
Mean		5.26	16167080		6.77	38599624
SD		0.023	130711		0.028	202578
% RSD		0.44	0.80		0.42	0.52

Table 25: Summary of System Suitability Parameters

Parameters	**Amlodipine Besylate**	***Indapamide***
Retention Time (min)	5.26	6.76
Resolution (R_s)	1.5	
Tailing Factor (T)	1.12	1.03
Theoretical Plates (N)	11230	10463

Accuracy:

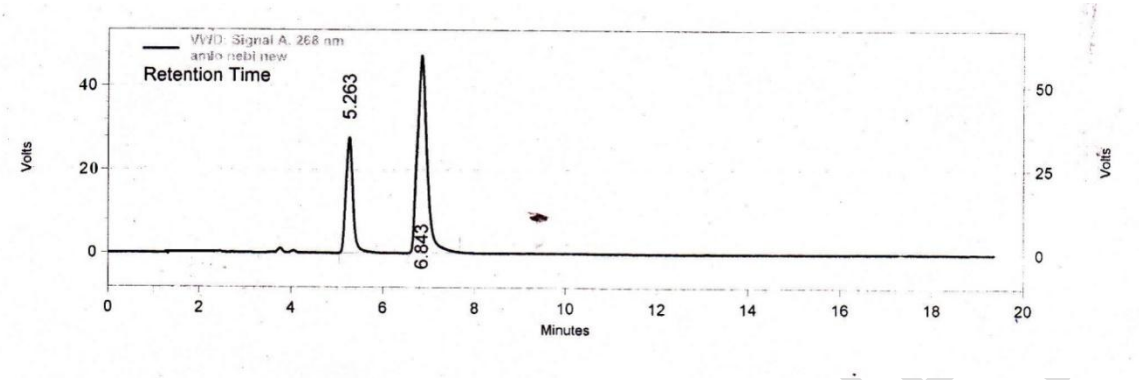


Figure 25: RP-HPLC Chromatogram of 50% Recovery Level

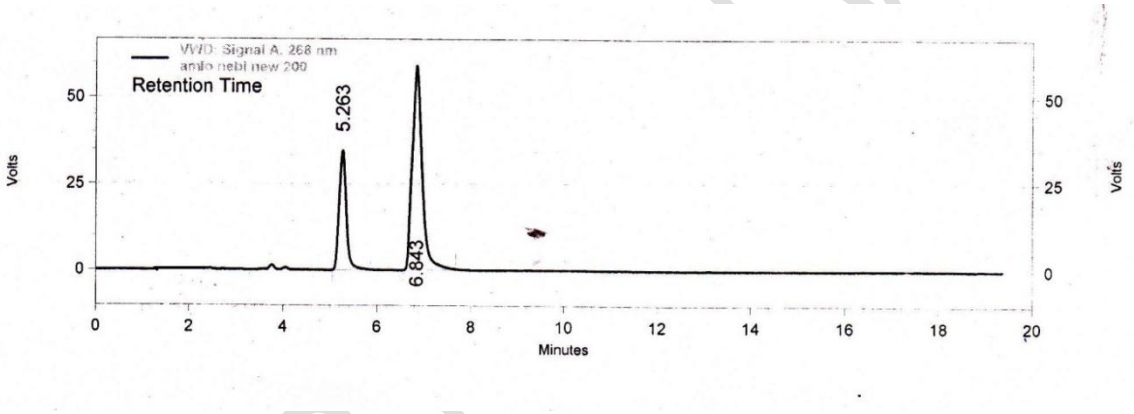


Figure 26: RP-HPLC Chromatogram of 100% Recovery Level

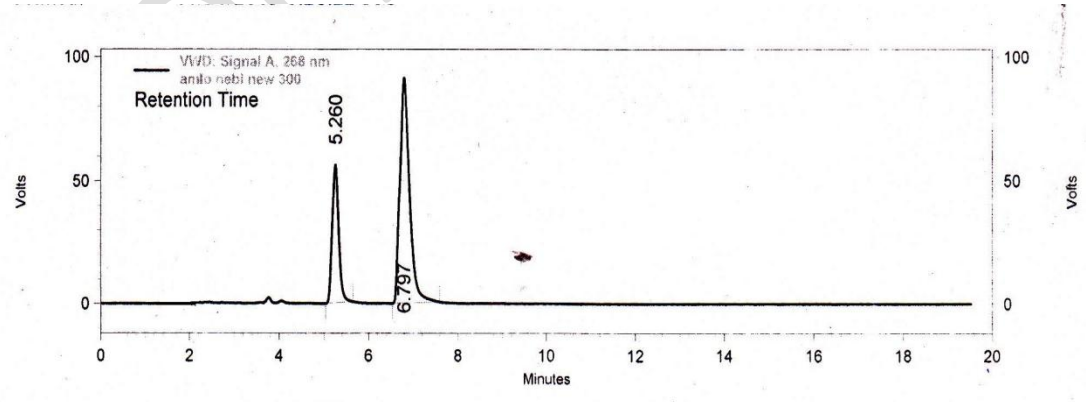


Figure 27: RP-HPLC Chromatogram of 150% Recovery Level

Table 26: Determination of Accuracy for Amlodipine Besylate and Indapamide by RP-HPLC Method

Recovery level	Amount of Standard drug added (µg/ml)		Amount of test added(µg/ml)		Total Amount Recovered (µg/ml)		% Recovery w/w	
	AMB	IND	AMB	IND	AMB	IND	AMB	IND
50%	2.5	5	2.5	5	4.95	9.92	99.0	99.2
100%	7.5	15	2.5	5	10.12	20.02	101.2	100.1
150%	12.5	25	2.5	5	15.23	30.3	101.5	101.0

Precision:

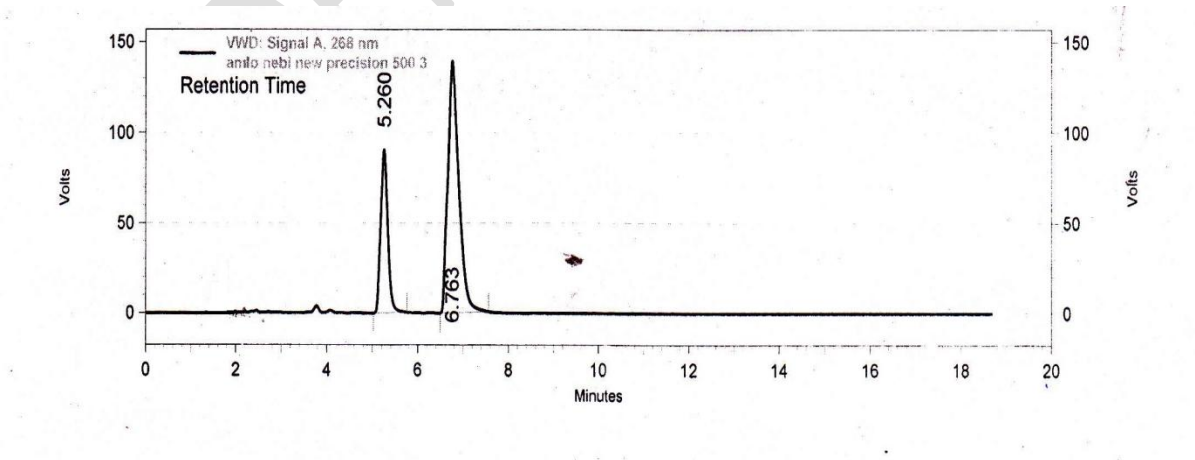


Figure 28: RP-HPLC Chromatogram to Show Intraday Precision of Amlodipine Besylate and Indapamide

Table 27: Determination of Precision for Amlodipine Besylate and Indapamideby RP-HPLC

S. No	Amlodipine Besylate (25µg/mL)	*Indapamide* (50µg/mL)
	Peak Areas	
	Intraday	Intra day
1	16098038	38896370
2	16148135	38318024
3	16135461	38554476
4	16071855	38701120
5	16146983	39318724
6	16108432	38963452
Mean	16118150	38792027
SD	30568	348441
% RSD	0.18	0.89

Specificity:

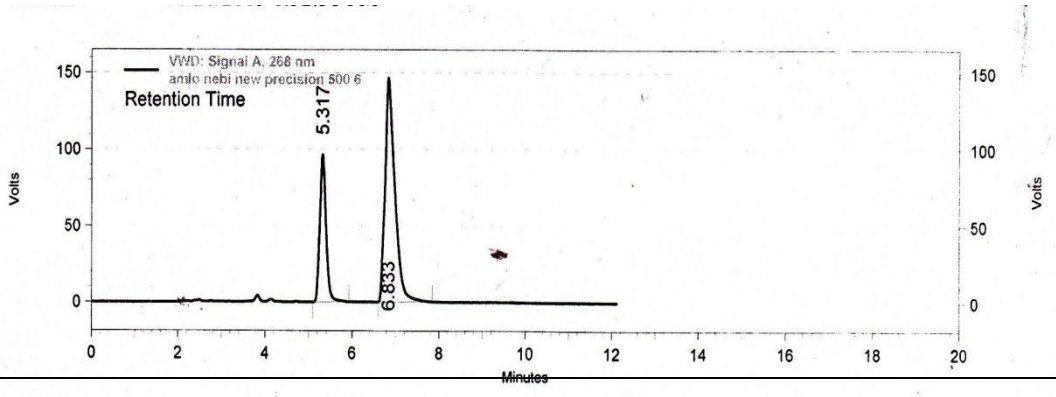


Figure 29: RP-HPLC Chromatogram to Show Specificity of Sample Solution

Table 28: Specificity Parameters of Amlodipine Besylate and Indapamide by RP-HPLC

Parameters	**Amlodipine Besylate**	***Indapamide***
Retention Time (min)	5.31	6.83
Resolution (R_s)	1.5	
Tailing Factor (T)	1.12	1.05
Theoretical Plates (N)	11234	10357

Robustness:

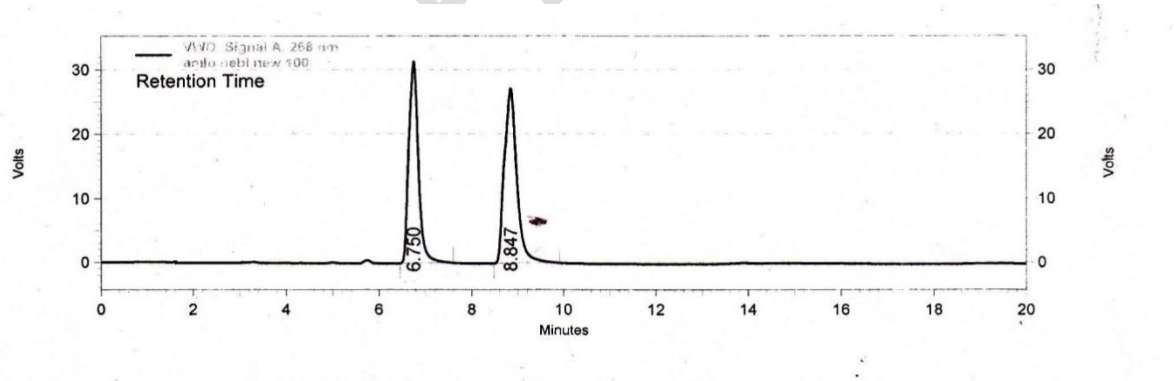


Figure 30: RP-HPLC Chromatogram of Robustness Study for Amlodipine Besylate and Indapamideat Flow rate 1.0 mL/min

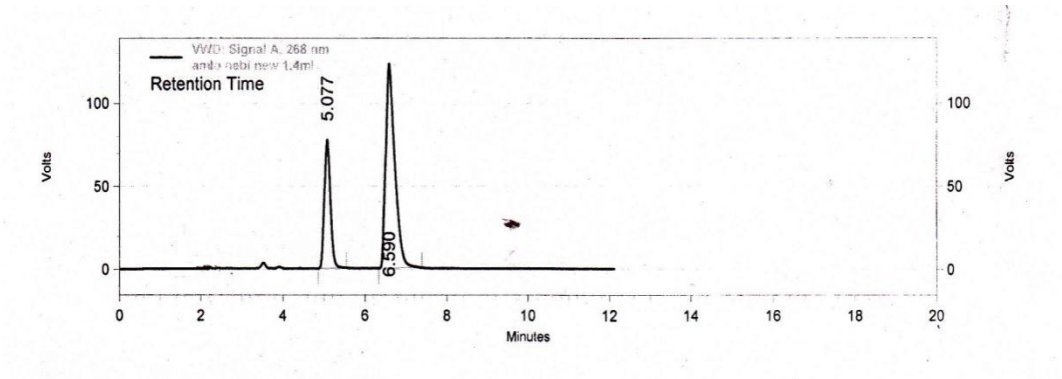


Figure 31: RP-HPLC Chromatogram of Robustness Study for Amlodipine Besylate and Indapamide at Flow rate 1.4 mL/min

Table 29: Robustness Data with Change in Flow Rate

S. No	Flow Rate (±0.2mL)	Amlodipine Besylate		*Indapamide*	
		R_t (min)	**Peak Area**	**R_t (min)**	**Peak Area**
1	1.0	6.75	16041855	8.84	37901120
2	1.0	6.72	16085451	8.81	38975391
3	1.0	6.75	16183467	8.83	38113462
Mean		6.74	16103591	8.82	38329991
SD		0.0173	72527.8	0.0152	568927.1
% RSD		0.21	0.4	0.17	1.4
1	1.4	5.07	16102361	6.59	37667892
2	1.4	5.07	16035432	6.51	37745217
3	1.4	5.06	16096532	6.55	38235685
Mean		5.06	16078108	6.55	37882931
SD		0.00577	37073.5	0.04	307930
% RSD		0.11	0.23	0.61	0.81

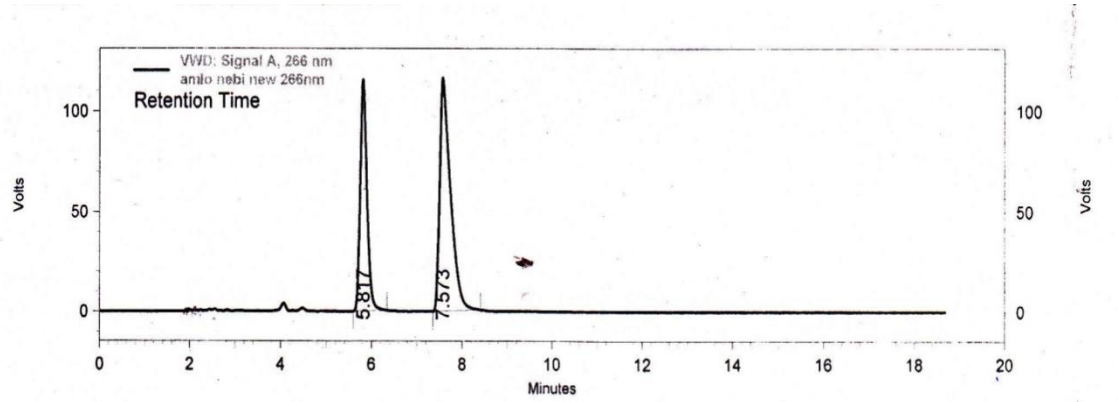


Figure 32: RP-HPLC Chromatogram of Robustness Study for Amlodipine Besylate and Indapamide at Detection Wavelength 266nm

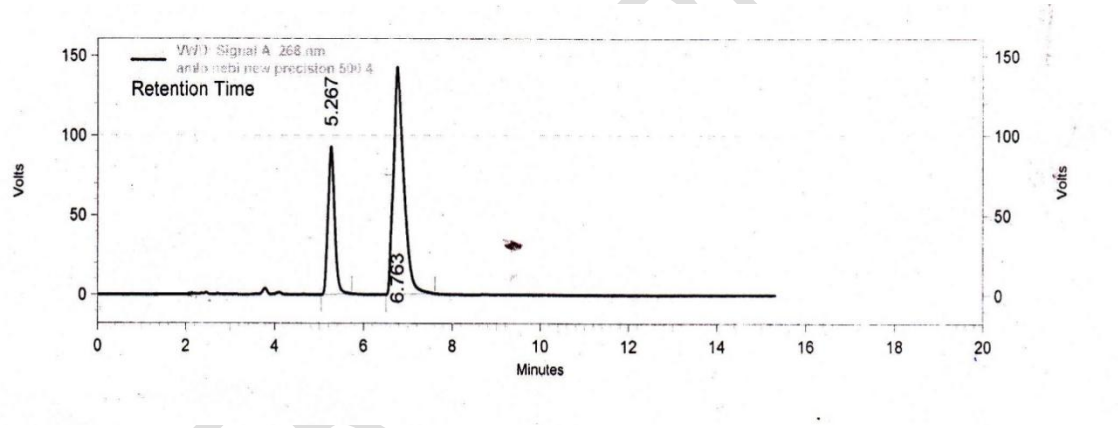


Figure 33: RP-HPLC Chromatogram of Robustness Study for Amlodipine Besylate and Indapamide at Detection Wavelength 270nm

Table 30: Robustness Data with Change in Detection Wavelength

S. No	Detection wavelength (±0.2nm)	Amlodipine Besylate		*Indapamide*	
		R_t (min)	Peak Area	R_t (min)	Peak Area
1	266	5.81	20111207	7.51	32374028
2	266	5.85	20323156	7.53	33065421

3	266	5.82	20574539	7.57	32687435
Mean		5.82	20336300	7.53	32708961
SD		0.0208	231945	0.0305	346198
% RSD		0.35	1.14	0.40	1.05
1	270	5.26	16198038	6.76	39286370
2	270	5.21	16206739	6.77	39669832
3	270	5.21	16176893	6.72	39915673
Mean		5.22	16193890	6.75	39623958
SD		0.0288	15349	0.0264	317149
% RSD		0.55	0.14	0.39	0.80

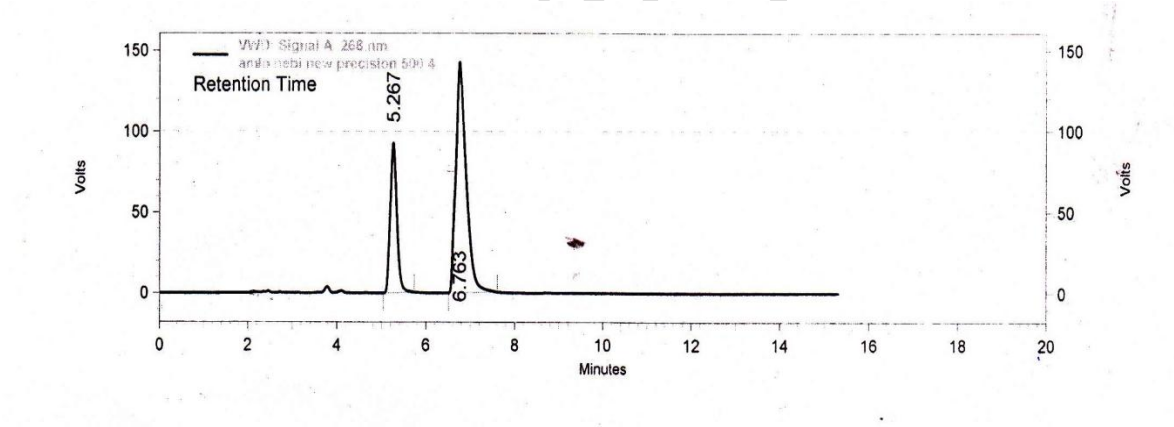


Figure 34: RP-HPLC Chromatogram of Ruggedness Study for Amlodipine Besylate and Indapamideby Analyst-I

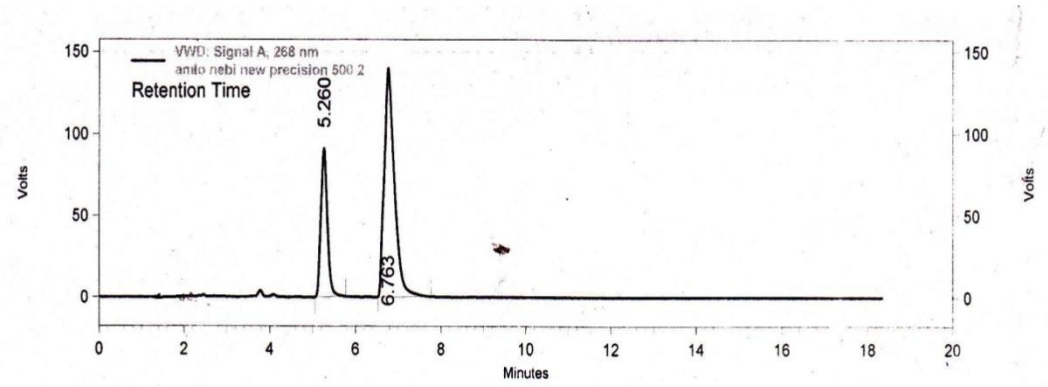


Figure 35: RP-HPLC Chromatogram of Ruggedness Study for Amlodipine Besylate and Indapamide by Analyst-2

Table 31: Ruggedness Data of Amlodipine Besylate and Indapamide

S. No.	Condition	Amlodipine Besylate		*Indapamide*	
		Rt	**Peak Area**	**Rt**	**Peak Area**
1	**Analyst-1**	5.26	16098038	6.76	38896370
2		5.31	16124589	6.76	38796853
3		5.31	16135461	6.83	38554476

Mean		5.29	16119362	6.78	38749233
SD		0.0288	19251	0.040	175851
%RSD		0.54	0.11	0.6	0.45
4	**Analyst-2**	5.31	16148761	6.83	38914560
5		5.26	16125896	6.76	39058621
6		5.26	16114986	6.59	38954368
Mean		5.27	16129881	6.72	38975849
SD		0.0288	17236	0.123	74394
%RSD		0.54	0.10	1.8	0.19

7.1. Estimation of *Indapamide* by UV-Spectrophotometric Method

The objectives of the proposed work was to develop some new and sensitive analytical methods for the determination of Indapamide and to validate the methods according to USP and ICH guidelines and applying the same for its estimation in pharmaceutical formulations.

The absorption spectra were recorded in the wavelength region of 200-400 nm which was shown in **figure 7** and the λmax was found to be at 280nm. Beer's law range was confirmed by the linearity of the calibration curve of Indapamide, which was shown in **figure 8.**Indapamide showed linearity in the concentration range of 10-50 µg/ml.

The optical characteristics such as absorption maxima, Beer's law limits, molar absorptivity, slope (b), intercept (C), correlation coefficient (r^2) obtained from different concentrations, percent relative standard deviation, LOD and LOQ values were given in **table 2**with satisfactory results**.** The quantitative estimation was carried out on formulation. The percentage purity of the marketed formulation was found to be 98.4%w/w. The results were given in **table 3.**

The accuracy of the methods was confirmed by the recovery studies, by adding known amount of the pure drug to the formulation and the analytical data were given in **table 4.**

The percentage recovery was found to be between 99.2 – 100.6%w/w, shows that the method was free from the interference of excipients used in the formulation.

The % RSD values were less than 2 indicating the precision of the methodology and low standard error values indicates the accuracy of the method. The statistical data's were given in **table 5.**

Results obtained for the proposed methods confirm the suitability of these methods for pharmaceutical dosage forms. The other active ingredients and excipients usually present in the pharmaceutical dosage forms did not interfere in the estimation, when commercial dosage forms were analyzed by these methods.

The results obtained in Ruggedness test (%RSD ≤ 2) expresses the reproducibility of the method. The ruggedness results were shown in **table 6.**

The developed UV Spectrophotometric method was found to be rapid, simple, precise, accurate and economic for routine estimation of Indapamide in commercial dosage forms.

7.2. Estimation of Amlodipine Besylate by UV-Spectrophotometric Method

The objectives of the proposed work was to develop some new and sensitive analytical methods for the determination of Amlodipine Besylate and to validate the methods according to USP and ICH guidelines and applying the same for its estimation in pharmaceutical formulations.

The absorption spectra were recorded in the wavelength region of 200-400 nm which was shown in **figure 9**and the λmax was found to be at 360 nm. Beer's law range was confirmed by the linearity of the calibration curve of Amlodipine Besylate, which was represented in **figure 10.** Amlodipine Besylate showed linearity in the concentration range of 10-50 µg/mL.

The optical characteristics such as absorption maxima, Beer's law limits, molar absorptivity, slope (b), intercept (C), correlation coefficient (r2) obtained from different concentrations, percent relative standard deviation, LOD and LOQ values were shown in **table 8** with satisfactory results.

The quantitative estimation was carried out on formulation. The percentage purity of the marketed formulation was found to be 98.2%w/w. The results were given in **table 9**.

The accuracy of the methods was confirmed by the recovery studies, by adding known amount of the pure drug to the formulation and the analytical data are presented in **table 10**.

The percentage recovery was found to be between 99.6 – 101.4%w/w, shows that the method was free from the interference of excipients used in the formulation.

The % RSD values are less than 2 indicating the precision of the methodology. The statistical data's were given in **table 11.** Results obtained for the proposed methods confirm the suitability of these methods for pharmaceutical dosage forms. The other active ingredients and excipients usually present in the pharmaceutical dosage forms did not interfere in the estimation, when commercial dosage forms were analyzed by these methods.

The results obtained in Ruggedness test (%RSD ≤ 2) expresses the reproducibility of the method. The ruggedness results were shown in **table 12.**

The developed UV Spectrophotometric method was found to be rapid, simple, precise, accurate and economic for routine estimation of Amlodipine Besylate in commercial dosage forms.

7.3. Simultaneous Estimation of Amlodipine Besylate and Indapamide by UV-Spectrophotometric Method

The objective of the proposed work was to develop UV simultaneous method for the determination of Amlodipine Besylate and Indapamide, and to validate the methods according to ICH guidelines and applying the same for its estimation in marketed formulations.

This is a very simple method and requires knowledge of molar absorptivity of the components which should be determined very accurately. It only requires measurement of absorbance at 280 nm and 360 nm which were selected as the maximum absorbance, the absorption curve was shown in **figure 11** and few simple calculations, which can be done manually. The percent label claim in given tablet formulation was found to be 99.2% w/w for Amlodipine Besylate and 99.80% w/w for Indapamide, which were found to be satisfactory and the results were given in **table 15**.

The method was validated as per ICH norms. Accurate results were obtained by utilizing the proposed methods for the quantitation of Amlodipine Besylate and Indapamide and a good agreement with the results obtained by the reported methods was found. For UV spectrophotometric method, linearity was obtained in the concentration range of 10-50µg/mL for both the drugs and the results were given in **table 13** with calibration curves shown in **figure 12**

High % recovery greater than 98% (99.07-100.4% w/w for Amlodipine Besylate and 99.5-101.5% w/w for Indapamide) showed that the method is free from the interference of excipients used in the formulation. The results were given in **table 16**. The value of standard deviation and % RSD were found to be < 2%, indicated the high precision of the method. The results were given in **table 17and 18.**

Detection limit and quantitation limit were determined from the standard deviation of y-intercepts of six calibration curves and average slope of the same. LOD and LOQ of Amlodipine Besylate were found to be 0.39µg/mL and 1.12µg/mL at 360nm. LOD and LOQ of Indapamide were found to be 1.43 µg/mL and 4.40µg/mL at 280nm. The reliability of the proposed method was evaluated and the results were shown in **table 14.**

The results obtained in Ruggedness test (%RSD < 2) expresses the reproducibility of the method. The ruggedness results were shown in **table 19 and 20.**

From the validation results the developed UV Spectrophotometric method was found to be rapid, simple, precise, accurate and economic for routine estimation of Amlodipine and Indapamide in commercial dosage forms.

7.4. Simultaneous Estimation of Amlodipine Besylate and Indapamide by RP-HPLC Method

In HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried to separate active ingredients.

The objective of this study was to develop a rapid and sensitive RP-HPLC method for the analysis of Amlodipine Besylate and Indapamide in bulk drug and pharmaceutical dosage form by using the most commonly employed C-18 column with UV-detection.

Initially, various mobile phase compositions were tried to elute the drug. Mobile phase ratio and flow rate were selected based on peak parameters (height, capacity, theoretical plates, tailing or symmetry factor), run time and resolution. The system with Acetonitrile: Acetate buffer pH-5 (40: 60 v/v) and 1.2 mL / min flow rate was selected.

The optimum wavelength selected was 268 nm from the overlain spectra obtained which was shown in **figure 13** at which better detector response for the drug was obtained. The retention time for Amlodipine Besylate and Indapamidewas found to be 5.2 min and 6.7 min respectively which were shown in **figure 14 and15.** The linearity was observed in concentration range of 5-25 µg/ mL and 10-50 µg/ mL for Amlodipine Besylate and Indapamide respectively. Calibration curves of the respective drugs were shown in **figure 21 and 22.**Summary of linearity results were given in **table 22.**

System suitability was assessed by injecting 5 replicate injections of 100% test concentration. Number of theoretical plates was more than 2000 for both the drugs and tailing factor was less than 1.5 for both Amlodipine Besylate and Indapamide was reported. A Resolution of greater than 1 was observed. The

relative retention times of six replicate injections and system suitability parameters were given in **table 24 and 25.**

The low % RSD values (≤ 2) indicated that the method was precise and accurate. The mean recoveries were found in the range of 99.0 – 101.5% w/w for Amlodipine Besylate and 99.2-101.0%w/w for Indapamide.

Specificity of the chromatographic method was tested by injecting sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Amlodipine Besylate and

Indapamide at 5.2min and 6.7min respectively without any interference. Thus the developed method was specific to Amlodipine Besylate and Indapamide and the parameters were given in **table 28.**

The robustness of an analytical method was determined by analysis of aliquots from homogenous lots by differing physical parameters such as change in flow rate to 1.2 ± 0.2 mL and changing detection wavelength 268nm ± 2nm. The obtained values were given in **table 29 and 30.** These values with low % RSD (<2) indicated that the method was quite robust.

Ruggedness of the proposed method was determined by analysis of aliquots from homogeneous slot by different analysts, using similar operational and environmental conditions; the % RSD reported was found to be less than 2 and these values were listed in **table 31**.

The proposed method was validated in accordance with ICH parameters and was applied for analysis of the same in marketed formulations. The content of each component in the formulation was estimated by comparing the peak area of the test sample with that of the peak area of the standard and the results were given in **table 23**which were found to be 99.8% w/w for Amlodipine Besylate and 99.2% w/w for Indapamide respectively.High % recovery and low % RSD suggested that the method can be applicable for the routine analysis of commercial formulations.

Hence, the developed HPLC method can be adopted for the routine analysis of Amlodipine Besylate and Indapamide in pharmaceutical formulations.

Drug combinations are commonly used clinically and analyst is required to develop suitable methods of their analysis. For routine analytical purposes it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with good accuracy and precision. The commonly used tests of pharmaceutical analysis generally entail compendia testing method development, setting specifications, and method validation.

Analytical testing is one of the more interesting ways for scientists to take part in quality process by providing actual data on the identity, content and purity of the drug products. New methods are now being developed with a great deal of consideration to worldwide

harmonization. As a result, new products can be assured to have comparable quality and can be brought to international markets faster.

A liquid chromatographic technique coupled with spectrophotometric analysis is a versatile tool that can generate extensive analytical data that is highly useful in the routine drug analysis. For routine analytical purposes it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with good accuracy and precision.

In the present work, an attempt was made to provide a newer, simple, accurate and low cost spectrophotometric and HPLC methods for the effective quantitative determination of Amlodipine Besylate and Indapamideas an active pharmaceutical ingredients as well as in pharmaceutical preparations in their single and combined dosage forms, without the interferences of other constituent in combined formulations. Hence it is planned to develop both HPLC and Spectrophotometric methods.

The results were summarized as follows

8.1. Estimation of Amlodipine Besylate and Indapamide by UV-Spectrophotometric Method

Table 32: Summarized Results of UV- Spectrophotometric Methods

Parameter	**Results**	
	Amlodipine Besylate	**Indapamide**

λ max	360	280
Beer's Law Range (µg/mL)	10-50	10-50
Regression Equation	Y=0.012x + 0.004	Y=0.116x-0.0037
Correlation Coefficient (r^2)	0.9993	0.9991
Molar Extinction Coefficient ($lit.mol^{-1}cm^{-1}$)	122.5	117.25
Sandell's Sensitivity (µg. cm^{-2}/0.001 abs units)	0.081	0.085
% Recovery (w/w)	99.6-101.4%	99.2-100.6%
LOD (µg/mL)	0.36	1.45
LOQ (µg/mL)	1.10	4.40
Assay (% Purity) w/w	98.2%	98.4
Precision (%RSD)		
Intraday Precision	0.63 – 1.06	0.4 - 1.1
Inter day Precision	0.80	0.68
Ruggedness (%RSD)		
Analyst 1	0.42	0.32
Analyst 2	0.24	0.27
Instrument 1	1.01	0.86
Instrument 2	0.40	0.87

8.2. Simultaneous Estimation of Amlodipine Besylate and Indapamide by UV-Spectrophotometric Method

Table 33: Summarized Results of Simultaneous UV-Spectrophotometric Method

Parameter	Results	
	Amlodipine Besylate	**Indapamide**
λ max	360	280
Beer's Law Range (µg/mL)	10-50	10-50
Regression Equation	Y=0.0112x-0.0015	Y=0.0119x+0.004
Correlation Coefficient (r^2)	0.9998	0.9993
Molar Extinction Coefficient ($lit.mol^{-1}cm^{-1}$)	113	118
Sandell's Sensitivity (µg. cm^{-2}/0.001 abs units)	0.088	0.084
% Recovery	99.07-101.2%	99.5-101.5%
LOD (µg/mL)	0.39	1.43
LOQ (µg/mL)	1.12	4.40
Assay (% Purity) w/w	99.2%	99.8%
Precision (%RSD)		
Intraday Precision	0.41-0.73	0.33-0.64
Inter day Precision	0.63	0.48
Ruggedness (%RSD)		
Analyst 1	0.26	0.37
Analyst 2	0.17	0.16
Instrument 1	0.11	0.21
Instrument 2	0.10	0.4

8.3. Simultaneous Estimation of Amlodipine Besylate and Indapamideby RP-HPLC Method

Table 34: Summarized Results of RP-HPLC Method

Parameter	Results	
	Amlodipine Besylate	**Indapamide**
Detection Wavelength	268	
R_t (min)	5.2	6.7
Beer's Law Range (µg/mL)	5-25	10-50
Regression Equation	Y=624113x + 58271	Y=757756x + 26422
Correlation Coefficient (r^2)	0.9995	0.9992
% Recovery (w/w)	99.0-101.5%	99.2-101.5%
LOD (µg/mL)	0.36	1.43
LOQ (µg/mL)	1.10	4.41
Assay (% purity) w/w	99.8%	99.2%
Precision		
Intraday Precision	0.18	0.89
Robustness		
Flow Rate 1.0 mL/min	0.4	1.4
Flow Rate 1.4 mL/min	0.23	0.81
Detection Wavelength 266 nm	1.14	1.05
Detection Wavelength 270 nm	0.14	0.80
Ruggedness		
Analyst 1	0.11	0.45
Analyst 2	0.10	0.19

8.4. Conclusions

Development of methods to achieve the final goal of ensuring the quantity of drug substances and drug products is not a trivial undertaking. The capabilities of the methods developed were complementary to each other. Hence they can be regarded as simple, specific and sensitive methods for the estimation of Amlodipine Besylate and Indapamide in single and combined pharmaceutical dosage forms. The developed UV Spectrophotometric methods and RP-HPLC methods were validated according to ICH guidelines and were found to be applicable for the routine analysis of Amlodipine Besylate and Indapamide in their single and combined dosage forms.

The proposed UV methods were simple, sensitive and reliable with good precision and accuracy. This method was specific while estimating the commercial formulations without interference of excipients and other additives. Hence, this method can be used for the estimation of Amlodipine Besylate and Indapamidein bulk samples and their pharmaceutical formulations individually and in combination by simultaneous equation method.

This method has been found to be better than previously reported methods, due to its wider range of linearity, use of readily available mobile phase, lack of extraction procedures. Hence above method can be used in quality control for routine analysis of finished products of Amlodipine Besylate and Indapamide simultaneously without any interference.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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