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Analytical Methods Development Ai\D Validation F'or Simultaneous Estimation of ' F'olic Acid and Amlodipine Besylate in Synthetic Mixture

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ABSTRACT
Folic Acid is a Vitamin B supplement that may be used to prevent and treat folate deficiency that can causes megaloblastic anemia. Amlodipine Besylate is in a class of medication called calcium channel blockers. It can help to prevent heart disease, heart attacks and strokes. The action of Amlodipine and Folic acid preparation on hypertension and cardiovascular in renal hypertensive patients with hyperhomocysteinemia. The present study aims to develop and validate simple, precise, and reproducible analytical methods for their simultaneous estimation in a synthetic mixture. Two UV spectrophotometric methods, namely First Order Derivative and Simultaneous Equation methods, along with a RP-HPLC method, were developed and validated as per ICH Q2 (R2) guideline. For First Order Derivative Method, UV detection was performed at 250 nm was selected as the zero-crossing point of Folic Acid and 314 nm was selected as the zero-crossing point of Amlodipine Besylate. For Simultaneous Equation Method, UV detection was performed at 284 nm and 236 nm of Folic Acid and 236 nm and 284 nm of Amlodipine Besylate because Folic Acid and Amlodipine Besylate give absorbance on both wavelengths 236 nm and 284 nm. In RP-HPLC method, mobile phase Phosphate Buffer (pH 3.2 adjusted with 10% ortho phosphoric acid): Methanol: Acetonitrile (50:25:25 %v/v/v) at 265 nm was chosen for high-resolution peaks. Retention time was found to be 2.4 min and 5 min for Folic Acid and Amlodipine Besylate, respectively. All methods exhibited excellent linearity within the concentration range of 0.8-4.0 µg/ml for Folic acid and 5-25 µg/ml for Amlodipine Besylate, with correlation coefficients greater than 0.997. Accuracy was confirmed by recovery studies (99.5%-99.9%), and precision showed %RSD below 2%. Low LOD and LOQ values indicated high sensitivity. Assay results were within acceptable limits (99.5%-99.9%). The developed methods were found to be accurate, precise, sensitive, and reproducible with no interference from excipients. These methods can be effectively applied for routine quality control analysis of synthetic mixture.

1. INTRODUCTION:

The **WHO** recent epidemiological interest focuses on H-type hypertension- the coexistence of high blood pressure and high homocysteine (HHcy). The epidemiology of combining folic acid (FA) and amlodipine primarily focuses on the management of **H-type hypertension**- a condition characterized by high blood pressure coupled with elevated plasma homocysteine (tHcy) levels. This combination is significant because both factors synergistically increase the risk of cardiovascular and cerebrovascular diseases, particularly stroke. The combination of Folic Acid and Amlodipine Besylate

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was studied under clinical trial phase 4 [1]. And it was proved that this combination safe and effective to Homocysteine. A combination of amlodipine and folic acid can be more effective than amlodipine alone in lowering blood pressure and plasma homocysteine levels, especially in patients with high blood pressure and high homocysteine levels who are intolerant to ACE inhibitors. Studies suggested that combining amlodipine with folic acid can lead to a greater reduction in both blood pressure and homocysteine levels compared to using amlodipine alone [2]. the combination of amlodipine and folic acid showed promise in managing high blood pressure and reducing homocysteine levels, potentially leading to better cardiovascular outcomes [3].

Folic Acid (2S)-[4-(2-amino-4-hydroroxypteridin-6-yl) methylamino] glutamic acid [4]. Amlodipine Besylate 3-O-ethyl-5-O-methyl2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-di-hydropyridine-3,5-dicarboxylate benzenesulfonate [5-6]. Despite the availability of analytical methods[7] for the individual estimation of these drugs, no validated analytical method has yet been reported for their simultaneous estimation. Therefore, the present study aims to develop and validate simple, accurate, and reproducible UV spectrophotometric and RP-HPLC methods for the simultaneous estimation of Folic Acid and Amlodipine Besylate in a synthetic mixture. A literature survey reveals that several analytical methods have been reported for the estimation of Folic Acid and Amlodipine Besylate either individually or in combination with other active pharmaceutical ingredients. Literature survey reveals stability indicating UV spectrophotometric [8-9], RP-HPLC [10-12], stability indicating HPLC [13-15], Liquid Chromatography-Tandem Mass Spectrometric Method for Determination of Folic Acid in Human Plasma [16], Thin Layer Chromatography Densitometric Method [17], UPLC after Solid Phase Extraction [18], HPLC-UV [19, 20] method for the estimation of Folic Acid alone and in combinations. Several analytical methods have been reported for the quantitative determination Amlodipine Besylate such as UV spectrophotometry [21], RP-HPLC and UV-spectrophotometric methods [22] and HPTLC-UV for Amlodipine Besylate and Azilsartan mixture in Human plasma [23], and Ultra-Performance Liquid Chromatography Method for Simultaneous Quantification of Hydrochlorothiazide, Amlodipine besylate and Valsartan in Market Fixed Dose Combination [24]. However, no analytical method has been reported for the simultaneous estimation of Folic Acid and Amlodipine Besylate in a synthetic mixture. Hence, the present study aims to develop and validate simple, economical, precise, and robust Two UV spectrophotometric (Vierodt's and first-order derivative) and RP-HPLC methods for the simultaneous estimation of Folic Acid and Amlodipine, in accordance with ICH Q2 (R2) guideline [25] within all Validation Parameters.

2. Experimental Materials and Instrumentation:

2.1 Chemicals and Reagents:

Folic acid was obtained as a gift sample from Strava Healthcare Pvt. Ltd., Ahmedabad, India. Amlodipine besylate was kindly provided as a gift sample by SG Healthcare Pvt. Ltd., Ahmedabad, India. HPLC grade solvents, including methanol, acetonitrile, and water, were procured from Finar Chemicals Pvt. Ltd., India. Ortho phosphoric acid and methanol (analytical reagent grade) were obtained from Astron Chemical India. All chemicals and reagents used in the study were of analytical or HPLC grade and were used as received without further purification.

2.2 instrumentation:

The spectrophotometric measurements were performed using a UV-Visible spectrophotometer (Shimadzu-1900, UV Probe 2.7 version software) with a spectral bandwidth of 1 nm was employed for all spectroscopic measurements, using a pair of 1.0 cm matched quartz cells over the range of 200-400 nm. For chromatographic information acquisition and analysis, High-Performance Liquid Chromatography system Systronic RP-HPLC (SYS-LC-138) with UV Detector was utilized together. The pH of the buffer solution was observed utilizing the Chemi Line pH meter. The Scale-Tec analytical balance was utilized to weigh the samples. The HPLC mobile phase was subjected to sonication using an Sonicator- Digital Pro^{+}, PS-10A, (Broleo). Chromatographic separation was achieved using a Kromstar C_{18} column (250 × 4.6 mm, 5 µm).

2.3 Analytical Conditions:

In accordance with ICH Q2 (R2) [25] requirements, the analytical conditions for a simultaneous technique for the measurement of Folic Acid and Amlodipine Besylate in UV and HPLC were optimized and validated. For UV Spectroscopy Methanol was used as a Solvent. Detection wavelength (λmax) of FOL and AMLO were 284 nm and 236 nm, respectively. The first-order derivative UV spectra were derived from the zero-order spectra using methanol as the solvent. Quantitative analysis was performed at the zero-crossing point (ZCP) of Folic Acid at 250 nm for the estimation of Amlodipine Besylate, and at the ZCP of Amlodipine Besylate at 314 nm for the estimation of Folic Acid. For Simultaneous equation as Vierordt's method the absorption maxima (λmax) were established at 284 nm for Folic Acid and 236 nm for Amlodipine Besylate. This procedure applies the Simultaneous

Equation technique based on Vierodt's principle, where the precise concentration of each drug within the sample is calculated according to the mathematical expressions. For RP-HPLC, Kromstar C_{18} (250 mm × 4.6 mm, 5 µm) was used in the procedure. The mobile phase consisted of Phosphate Buffer (pH 3.2 adjusted with 10% ortho phosphoric acid): Methanol: Acetonitrile (50:25:25 %v/v/v) 265 nm wavelength was selected for RP-HPLC, with 1 mL/min flow rate.

2.4 Preparation of Solutions:

2.4.1 Preparation of Stock Solution:

Accurately weighed 10 mg of Folic Acid and 10 mg of Amlodipine Besylate were individually transferred into separate 100 mL volumetric flasks and dissolved in methanol. The solutions were sonicated to ensure complete dissolution, and the volume was made up to the mark with methanol to obtain standard stock solutions having a concentration of Folic Acid 100 µg/mL of and 100 µg/mL of Amlodipine Besylate, respectively.

2.4.2 Preparation standard solution

Accurately 2 ml of [the mixture solution of Folic Acid (8 µg/ml) and 2 ml Amlodipine Besylate (50 µg/ml)] was pipetted out into 10 ml volumetric flask and the volume was adjusted up to the mark with Methanol. Final concentration of Folic Acid was 1.6 µg/ml and Amlodipine Besylate 10 µg/ml.

2.4.3 Preparation of standard working solution

The concentration ranges of 0.8-4.0 µg/mL of Folic Acid and 5-25 µg/mL of Amlodipine Besylate formed, from each stock solution, Folic Acid (100 µg/ml) (0.08, 0.16, 0.24, 0.32 and 4.0 ml) and Amlodipine Besylate (100 µg/ml) (0.5, 1, 1.5, 2 and 2.5 ml) were pipetted out in ten different 10 ml volumetric flasks and made up to mark with Methanol to obtained] 0.8, 1.6, 2.4, 3.2 and 4.0 µg/ml of and 5, 10, 15, 20 and 25 µg/ml respectively. Under the optimized spectrophotometric conditions, the samples were analyzed using a 1 cm quartz cuvette in the UV spectrophotometer. Similarly, the optimized chromatographic conditions, 20 µL of each standard working solution were injected into RP-HPLC system by Hamilton syringe and analyzed.

2.4.5 Preparation of 10% Orthophosphoric acid"

10% orthophosphoric acid was prepared by diluting 1.0 ml of concentrated ortho phosphoric acid in 10 ml HPLC grade water.

2.4.5 Preparation of 10mM Phosphate Buffer"

Accurately weighed 0.272 gm potassium dihydrogen phosphate (KH_2PO_4) was transferred it in 200 ml HPLC grade water and allowed it to dissolve. It was filtered through 0.45 µm membrane filter and sonicated for about 10 min. Buffer pH was adjusted to 3.2 with 10% ortho phosphoric acid.

3. METHODOLOGY:

3.1 ***UV methods*****:**

3.1.1 ***Method I: First Order Derivative UV Method***

Accurately 2 ml of [the mixture solution of Folic Acid (8 µg/ml) and 2 ml Amlodipine Besylate (50 µg/ml)] was pipetted out into 10 ml volumetric flask and the volume was adjusted up to the mark with Methanol. Final concentration of Folic Acid was 1.6 µg/ml and Amlodipine Besylate 10 µg/ml. Each solution was scanned in the range of 200-400 nm. In zero order UV spectra, Folic Acid exhibited an absorption maximum at 284 nm while Amlodipine Besylate showed an absorption maximum at 236 nm in Figure 1. Folic Acid and Amlodipine Besylate standard stock solutions were prepared in Methanol at concentrations of 100 µg/mL and 100 µg/mL, respectively. A small amount of each stock solution was taken and placed into 10 mL volumetric flasks. Methanol was used to adjust the volumes to the mark, resulting in final concentrations of FOL ranging from 0.8 to 4.0 µg/mL and AMLO ranging from 5 to 25 µg/mL. All zero-order absorption UV spectra were converted to first-order derivative UV spectra. Calibration functions were established by plotting first-order derivative absorbance against corresponding concentrations for each analyte. Appropriate volume, 0.16 mL of FOL and 1.0 ml AMLO standard stock solution was transferred to two separate 10 mL volumetric flasks and the volume was adjusted to mark with methanol to get concentration 1.6 and 10 µg/mL, respectively. The solutions were scanned separately in the UV-region i.e., 400-200 nm. The zero-order UV absorption spectra of FOL and AMLO in Methanol shown in Figure 1. The zero-order spectrum was processed to obtain first-derivative spectrum. The two first derivative spectra were overlaid which showed that FOL showed zero crossing at 314 nm, while AMLO showed zero crossing at 250 nm which showed in Figure 2. The determinations were made at 314 nm for Folic acid (ZCP of Amlodipine Besylate) and 250 nm for Amlodipine Besylate (ZCP of Folic acid).

3.1.2 Method II: Simultaneous equation as Vierordt's method

Solutions of Folic acid (1.6 µg/ml) and of Amlodipine Besylate (10 µg/ml) prepared in methanol were subjected to a spectral scan from 200 to 400 nm at a medium speed, utilizing pure methanol as the reagent blank. For the analytical determination, the absorption maxima (λmax) were established at 284 nm for Folic acid and 236 nm for Amlodipine Besylate. This procedure applies the Simultaneous Equation technique based on Vierodt's principle, where the precise concentration

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of each drug within the sample is calculated according to the following mathematical expressions.

Standard Stock solutions of Folic acid and Amlodipine Besylate in the concentration range 0.8-4.0 µg/mL and 5-25 µg/ml were made in the methanol and absorbance of these solutions was measured at 284 nm and 236 nm. Calibration curves were plotted to confirm the Beer's law and the absorptivity values calculated at the respective wavelengths for both the drugs. Two simultaneous equations as below were formed using these absorptivity values A (1%, 1 cm).

At λ1 A1=ax1bCx+ay1bCy…….(1)
At λ2A2= ax2bCx+ay2 bCy…… (2)
For measurements in 1 cm cells b=1,
Rearrange eq. (2),
Cy= A2− ax2Cx/ ay2
Substituting for Cy in eq (1) and rearranging
Cx = A2ay1−A1 ay2/ax2 ay1−ax1 ay2……..(3)
Cy = A1ax2−A2 ax1/ax2 ay1−ax1 ay2……..(4)

Where Cx and Cy are the concentration of Folic acid and Amlodipine Besylate, respectively, A1 and A2 are absorbance at 284 nm and 236 nm, respectively, ax1 and ax2 are absorptivity of at Folic acid 284 nm and 236nm, respectively; ay1 and ay2 are absorptivity of at Amlodipine Besylate 236 nm and 284 nm, respectively. By solving the two simultaneous equations, the concentrations of Folic acid and Amlodipine Besylate in sample solutions were obtained.

3.2 Method III: Reverse Phase High Performance Liquid Chromatography Method

For RP-HPLC, the analysis was carried out using an isocratic elution technique using a mobile phase comprised of different mobile phases such as at Phosphate Buffer (pH 3.2 adjusted with 10% ortho phosphoric acid): Methanol: Acetonitrile (50:25:25 %v/v/v) at a flow rate of 1 mL/min found better separation of both the drug peaks. Prior to usage, the solvents were filtered through a 0.45 µm filter and sonicated for 30 min. The stationary phase was a Kromstar C_{18} (250 mm × 4.6 mm, 5 µm), and the eluent was observed by a U.V Detector from 200 to 400 nm, alongside chromatograms extracted at 265 nm (figure 1). The calibration curves were prepared by measuring the peak areas of FOL and AMLO and plotted their values against the pertinent concentrations. In accordance, the equations for linear regression were calculated.

3.3 Method Validation

The analytical procedures employed in this study were validated in accordance with the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), ICH Q2 (R2): *Validation of Analytical Procedures* [25]. Validation parameters evaluated included specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), and robustness.

3.3.1 Specificity

Specificity denotes the capacity of an analytical procedure to accurately and distinctly quantify the target analyte despite the potential interference of co-existing substances. Within a complex sample, these extraneous components commonly encompass synthesis impurities, degradation products, or various matrix constituents that could otherwise confound the measurement.

3.3.2 Linearity and Range: (n=6)

The linearity of Folic acid and Amlodipine Besylate was found to be in the range of 0.8-4.0 µg/mL and 5-25 µg/mL, respectively. Plot the calibration curve of peak area vs. concentration (µg/mL). Linearity of both the drugs were checked in term of slope, intercept and correlation coefficient.

3.3.3 Precision

The Intraday and Interday precisions also referred to as repeatability and intermediate accuracy, respectively were used to assess the precision of Methods I and II. The experiment was conducted on the same day and for the next three days for both Intraday and Interday precision, analysing freshly made solutions at concentrations of 0.8, 1.6, and 2.4 µg/mL of FOL and 5, 10, and 15 µg/mL of AMLO. To assess intermediate precision, the mean absorbance (UV) and peak area (HPLC) were recorded for each set of experiments. For repeatability, 16 µg/mL of FOL and 10 µg/mL of AMLO were used. The results were represented as a percentage Relative Standard Deviation (RSD), with a value of less than two considered acceptable. This meticulous approach ensures a comprehensive evaluation of the precision of the analytical methods, providing confidence in the reliability and consistency of the results obtained for the concentrations of FOL &AMLO in the tested solutions.

3.3.4 Limit of Detection (LOD)

Limit of detection can be calculated using following equation as per ICH guidelines.

$$\mathrm{LOD} = 3.3 * \frac{\sigma}{S}$$

Where, σ = standard deviation of the calibration curve

S = slope of the calibration curve

3.3.5 Limit of Quantification (LOQ)

Limit of quantification can be calculated using following equation using the standard deviation of the Y-intercept (σ) and the mean slope (S) of the

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calibration curve according to ICH Q2 (R2) guideline.

$$\text{LOQ} = 10 * \frac{\sigma}{S}$$

3.3.6 Accuracy (Recovery study) (n=3)

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50 %, 100 %, 150 % and the values were measured for Folic acid (1.6 µg/mL) and Amlodipine Besylate (10 µg/mL). This performance was done in triplicate. The accuracy of the method was determined by calculating recovery of Folic acid and Amlodipine Besylate by the standard addition method.

3.3.7 Assay as analysis of Synthetic Mixture

The synthetic mixture of Folic Acid and Amlodipine Besylate were prepared in the dose ratio of 0.8:5. A synthetic mixture equivalent to 100 mg was prepared by accurately weighing Folic acid (1.6 mg) and Amlodipine Besylate (10 mg). Microcrystal Cellulose (MCC) (3.5 mg), Lactose (27.7 mg), Magnesium Stearate (6 mg), Talc (4 mg), and Croscarmellose Sodium (3 mg) were used as excipients. All the components were transferred into a mortar and blended thoroughly using a pestle to obtain a homogeneous synthetic mixture. This mixture was transferred in 100 ml volumetric flask and allowed to sonicate and made up to mark with Methanol. This solution was filtered through Whatmann filter paper. The filtrate was diluted to the mark with Methanol. The mixture contains 16 µg/mL of Folic Acid and 100 µg/mL of Amlodipine Besylate.

3.3.7.1 Preparation of sample solution

Accurately 1 ml of the above [mixture solution of Folic Acid (16 µg/ml) and Amlodipine Besylate. (100 µg/ml)] was pipetted out into 10 ml volumetric flask and the volume was adjusted up to the mark with Methanol. Final concentration of was Folic Acid 1.6 µg/ml and Amlodipine Besylate 10 µg/ml. Then analysed using the previously described UV-spectrophotometric and chromatographic conditions. The concentrations of FOL and AMLO were calculated using a regression equation.

3.3.8 Robustness

The robustness of analytical methods becomes evaluated to decide their ability to face up to minor variations in approach situations. For the HPLC technique, samples have been subjected to evaluation below changed situations, which include adjustments inside the flow rate (± 0.1 mL/min), detection wavelength (± 2 nm), and natural content material (± 2 %) inside the mobile segment. The resulting results on machine suitability parameters have been intently monitored. In the times of Methods I and II, distinct analysts conducted sample analyses to evaluate the robustness of the strategies.

3.3.9 System Suitability Tests

A system suitability test is an integral part of liquid chromatography. They are used to verify that resolution and reproducibility of chromatography system are adequate for the analysis to be done. The test includes the Resolution, Column efficiency, Tailing factor and Theoretical plates.

4. RESULTS AND DISCUSSION:

4.1 UV Methods

The first-order derivative UV spectrophotometric method provides distinct advantages over conventional zero-order UV techniques by enhancing spectral resolution and reducing baseline drift, thereby improving selectivity and accuracy. Measurement at zero-crossing wavelengths allows selective quantification of analytes in the presence of overlapping spectra without prior separation. In comparison with higher-order derivative methods, the first-order derivative approach offers a better signal-to-noise ratio, resulting in improved precision and reproducibility. Additionally, the method is simple, rapid, cost-effective, and requires minimal sample preparation, making it well suited for routine quality-control analysis of multicomponent pharmaceutical formulations.

4.1.1 Selection of wavelength for Folic Acid and Amlodipine Besylate

The remarkable absorbance Folic Acid of exhibited an absorption maximum at 284 nm, while Amlodipine Besylate showed an absorption maximum at 236 nm [figure 1]

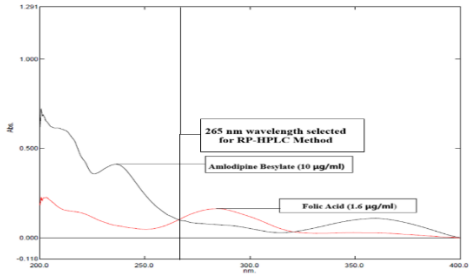

Figure 1: Overlain UV Spectra of Folic acid (1.6 µg/ml) and Amlodipine Besylate (10 µg/ml) in Methanol (Zero Order)

4.1.2 First order derivative UV Method Development

The FOL and AMLO overlapping absorption throughout the 200 - 400 nm range is shown by these spectra, which makes it more difficult to quantify the pharmaceuticals using traditional UV spectrophotometry without accounting for the overlap. The sum of the absorbances of the two

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compounds may be used to calculate the overall absorbance of a solution containing a combination of both at a certain wavelength. In situations where the levels of the two medicinal drugs overlap, the method entails figuring out the quantity of each drug using their zero-order spectra. The first derivative corresponding to each absorption spectrum of each drug was recorded, using $\Delta\lambda = 2$ nm and scaling factor 4. The amplitude values were measured at 314nm (λ1) (ZCP of AMLO) for Folic Acid and 250 (λ2) (ZCP of FOL) for Amlodipine Besylate showed in Figure 2. To determine the wavelength for measurement, Folic Acid (1.6 µg/ml) and Amlodipine Besylate (10 µg/ml) solutions were scanned between 200-400 nm.

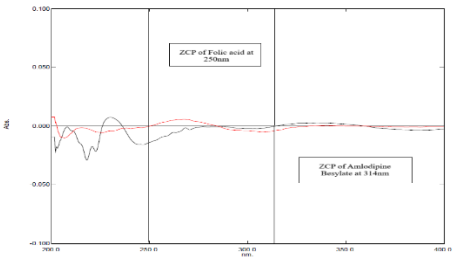

Figure 2: Overlain UV Spectra of Folic Acid (1.6 µg/ml) and Amlodipine Besylate (10 µg/ml) in Methanol (First order)

4.1.2 Simultaneous equation (Vierordt's) Method

For the simultaneous equation method, standard solutions of Folic Acid (1.6 µg/mL) and Amlodipine Besylate (10 µg/mL) in methanol were subjected to spectral scanning between 200 and 400 nm at medium speed, with methanol employed as the blank solution. For the analytical determination, the absorption maxima (λmax) were established at 284 nm for Folic Acid and 236 nm for Amlodipine Besylate (Figure 1). This procedure applies the Simultaneous Equation technique based on Vierodt's principle, where the precise concentration of each drug within the sample is calculated.

For multi-component UV analysis, Vierordt's method is named after the German scientist Karl Vierordt. UV Spectra Folic Acid (0.8-4.0 µg/mL) and Amlodipine Besylate (5-25 µg/mL) over the linearity and range had been showed in Figure 3(A) and 3(B) respectively.

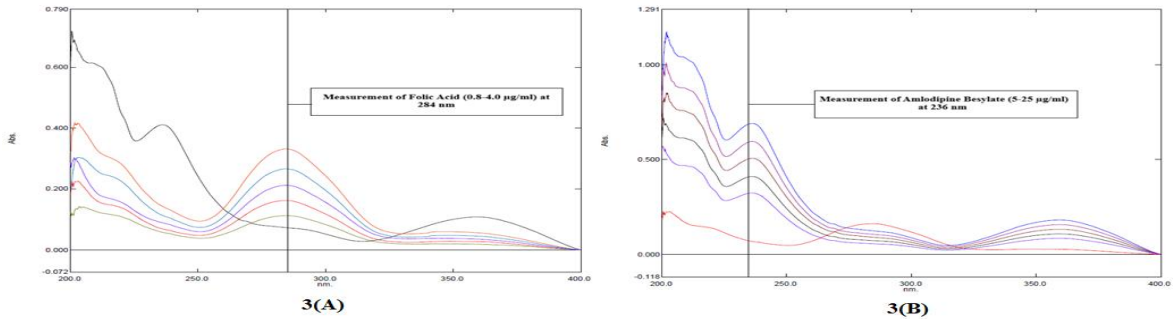

Figure 3: Overlain UV Spectra of 3(A) Folic Acid (0.8-4.0) at 284 nm and 3(B) Amlodipine Besylate (5-25 µg/ml) at 236 nm

4.2 RP-HPLC Method

Reverse-phase high-performance liquid chromatography (RP-HPLC) was selected for the analysis due to its high resolution, sensitivity, and reproducibility in the separation and quantification of compounds with varying polarity. The technique offers excellent peak symmetry, shorter analysis time, and superior compatibility with aqueous and organic mobile phases, making it particularly suitable for routine quality control and analytical applications. In addition, RP-HPLC provides high method robustness and ease of method optimization, while requiring minimal sample preparation. Owing to these advantages and its wide regulatory acceptance, RP-HPLC is extensively employed in pharmaceutical and analytical research, making it an appropriate and reliable choice for the present study. C_{18} column was selected because it is least polar compare to C_4 and C_8 columns. C_{18} column allows eluting polar compounds more quickly compare to non-polar compounds.

4.2.1 Selection detection wavelength

The sensitivity of RP-HPLC method that uses UV detection depends upon proper selection of detection wavelength. At 265 nm both drugs give good peak height and shape. So,265 nm was selected for simultaneous estimation Folic Acid and Amlodipine Besylate of in synthetic mixture. Overlain UV spectra of Folic Acid (1.6 µg/ml) and Amlodipine Besylate (10 µg/ml) in Methanol showed in figure 1.

4.2.2 Chromatography

The mobile phase Phosphate Buffer (pH 3.2 adjusted with 10% ortho phosphoric acid): Methanol: Acetonitrile (50:25:25 % v/v/v) was selected because it was found to ideally resolve the peaks with retention time 2.4 min and 5.0 min for Folic Acid and Amlodipine Besylate, respectively. Kromstar C_{18} (250×4.6 mm, 5 µm) column was used

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for separation of Folic Acid and Amlodipine Besylate with flow rate of 1.0 ml/min. Figure 4 showed RP-HPLC Chromatogram of Folic Acid (1.6 µg/ml) and Amlodipine Besylate (10 µg/ml).

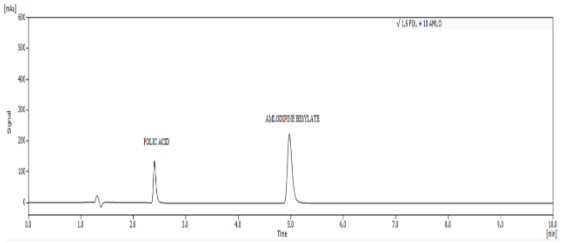


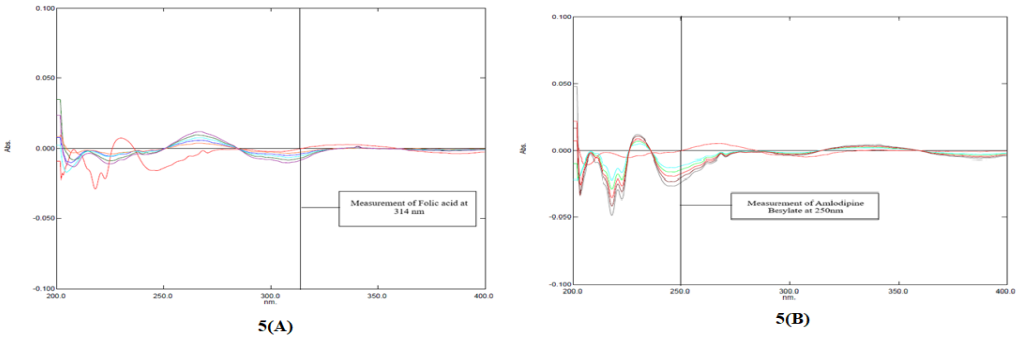
Figure 4: RP-HPLC Chromatogram of Folic Acid (1.6 µg/ml) and Amlodipine Besylate (10 µg/ml) in Phosphate Buffer (pH 3.2 adjusted with 10% ortho phosphoric acid): Methanol: Acetonitrile (50:25:25 % v/v/v) at 265 nm {Run time: 10 min, Flow rate: 1 ml/min}

4.3 VALIDATION OF THE PROPOSED METHODS

4.3.1 Validation Parameters of the UV Method

4.3.1.1 Linearity and range

For FOL and AMLO, the absorbances ranged from 0.8-4.0 µg/mL at 314 nm and 5-25 µg/mL at 250 nm showed in Figure 5(A) and 5(B), respectively.



5(A) 5(B)

Figure 5: Overlain UV Spectra of 5(A)Folic acid (0.8-4.0 µg/ml) at 314 nm and 5(B)Amlodipine Besylate (5-25 µg/ml) at 250 nm

A linear relationship was found and calibration curve was plotted for concentration vs. absorbance. For FOL, the calibration curve equation, y = 0.0013x + 0.002while for AMLO, it was y = 0.0007x + 0.0077. Results showed that the correlation coefficient (R^2) was between 1.0 and 0.997 (Table 1).

For simultaneous Equation Method UV Spectra of Folic Acid (0.8-4.0 µg/ml) and Amlodipine Besylate (5-25 µg/ml) over the linearity and range had been showed in Figure 3(A) and 3(B), respectively. For UV, of Folic Acid exhibited a linear response in the concentration range of 0.8-4.0 µg/mL at 284 nm and 236 nm. The correlation coefficients (r^2) were found to be 0.9979 and 0.9995 at, 284 nm and 236 nm respectively, indicating excellent linearity. The mean absorbance values (n = 6) showed low standard deviation with %RSD values below 1.5%, demonstrating good precision and Amlodipine Besylate repeatability showed linearity over the concentration range of 5-25 µg/mL at, 284 nm and 236 nm with correlation coefficients (r^2) of 0.9987 and 0.9997, respectively. The Linearity Data are Summarized in Table 2.

Table 1: Linearity data of FOL and AMLO by UV (first order) and RP-HPLC

Parameters	UV Spectrophotometry		RP-HPLC	
	FOL at 314 nm	AMLO at 250 nm	FOL at 265 nm	AMLO at 265 nm
Linearity Range	0.8-4.0 µg/ml	5-25 µg/ml	0.8-4.0 µg/ml	5-25 µg/ml
Correlation Coefficient	1	0.997	0.9991	0.9992
LOD	0.09	0.97	0.04	0.38
LOQ	0.29	2.94	0.11	1.14

Table 2: Linearity data of FOL and AMLO for Simultaneous equation UV method

Sr. No.	Parameters	Folic Acid		Amlodipine Besylate	
1	Wavelength (nm)	284 nm	236 nm	236 nm	284 nm
2	Beer's Law Limit (µg/ml)	0.8-4.0 µg/ml	5-25 µg/ml	0.8-4.0 µg/ml	5-25 µg/ml
3	Correlation Coefficient (r^2)	0.9995	0.9979	0.9997	0.9987
4	LOD (µg/ml)	0.05	0.10	0.46	0.86
5	LOQ (µg/ml)	0.16	0.31	1.39	2.61

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4.3.1.2 Calculation for Simultaneous Equation Method for Folic Acid and Amlodipine Besylate in Synthetic Mixture.

Folic Acid (1.6 µg /ml) and Amlodipine Besylate (10 µg/ml) in Methanol, both the solutions were scanned over range of 200-400 nm against Methanol as blank, using medium scan speed. The sampling wavelength for analysis includes, Absorption maxima (λmax) of Folic Acid was 284 nm, Absorption maxima (λmax) Amlodipine Besylate of was 236 nm. The method employs Simultaneous Equation as per Vierodt's method and the concentrations of drugs in sample solution were determined by using the following formula:

For Folic Acid (X),

$$Cx = \frac{A2\ ay1 - A1\ ay2}{a\ x2\ aY1 - a\ x1ay2}$$

For Amlodipine Besylate (Y),

$$Cy = \frac{A1ax2 - A2\ a\ x1}{ax2\ aY1 - a\ x1ay2}$$

Where, ax_1 Where ax_1 and ax_2 represented the absorptivity of Folic Acid at 284 nm and 236 nm, respectively; ay_1 and ay_2 denoted the absorptivity of Amlodipine Besylate at 236 nm and 284 nm, respectively; and A_1 and A_2 corresponded to the absorbance of the sample measured at 284 nm and 236 nm, respectively

$$Cx = \frac{A2\ ay1 - A1\ ay2}{a\ x2\ aY1 - a\ x1ay2}$$

Cx = (0.072) (0.038) – (0.163) (0.006) / (0.042) (0.038) – (0.097) (0.006)
=(0.0027) – (0.0009) / (0.0015) - (0.0005)
= (0.0018) / (0.0011)
=1.6 µg/mL

Where, ax_1= Absorptivity of Folic Acid at 284 nm (λ_1): 0.097
ax_2= Absorptivity of Folic Acid at 236 nm (λ_2): 0.042
ay_1= Absorptivity of Amlodipine Besylate at 236 nm (λ_1): 0.038
ay_2= Absorptivity of Amlodipine Besylate at 284 nm (λ_2): 0.006
A_1= Absorbance of Folic Acid at 284 nm, (λ_1): 0.163
A_2= Absorbance of Folic Acid at 236 nm, (λ_2): 0.072

$$\mathbf{Cy = \frac{A1ax2 - A2\ a\ x1}{ax2\ aY1 - a\ x1ay2}}$$

C y = (0.410)(0.042) - (0.072)(0.097) / (0.042)(0.038) – (0.097)(0.006)
= 0.0172– 0.0069 / 0.0015 – 0.0005
= 0.0103 / 0.0011
= 9.3 µg/mL

4.3.1.3Precision

Methodological precision was evaluated through intraday, inter-day, and repeatability assessments using triplicate analyses of Folic Acid (0.8, 1.6, and 2.4 µg/mL) and Amlodipine Besylate (5,10 and 15 µg/ml) across three consecutive days and within a single diurnal period. Absorbance values were recorded for these concentrations to establish intermediate precision, while repeatability was specifically determined using concentrations of 1.6 µg/ml for Folic Acid and 10 µg/ml for Amlodipine Besylate. The outcomes, expressed as Relative Standard Deviation (% RSD) for each precision parameters were less than two.

4.3.1.3 LOD and LOQ:

The limits of detection (LOD) and quantification (LOQ) are calculated using the standard deviation responses and slopes obtained from the calibration curves of each drug at their specific wavelengths. The results of LOD and LOQ were displayed in Table 1 & 2.

4.3.1.4 Accuracy

The accuracy of the technique recuperation accomplished by means of standard addition approach. To pre-analyzed pattern acknowledged quantity of general FOL and AMLO spiked in extraordinary concentrations. The restoration was executed in three stages 50 %, 100 % and 150 % of FOL and AMLO. Accuracy was carried out by the Recovery Studies (standard addition method). The results were stipulated in triplicate and the accuracy indicated by % recovery. For first order UV spectrophotometric method, The % Recovery was obtained in range of 99.62%-99.75% for Folic Acid and 99.85%-90.96%for Amlodipine Besylate, were showed in Table 3. And For the Simultaneous Equation method, The % Recovery was obtained in range of 99.66%-99.80% Folic Acid and 99.91%-99.97%for Amlodipine Besylate, were showed in Table 3.

Table 3: Recovery study data for UV (First order) and RP-HPLC Method

UV method						
Name of Drug	**% Level of recovery**	**Test Amount (µg/ml)**	**Amount of drug taken (µg/ml)**	**Total Std Amt (µg/ml)**	**Total amount Recovered (µg/ml)**	**% Mean Recovery ± SD(n=3)**
Folic Acid	50	1.6	0.8	2.4	2.392	99.66±0.01311
	100	1.6	1.6	3.2	3.193	99.78±0.02955
	150	1.6	2.4	4.0	3.992	99.80±0.00755
Amlodipine Besylate	50	10	5	15	14.990	99.93±0.01000

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	100	10	10	20	19.983	99.91±0.02858
	150	10	15	25	24.993	99.97±0.10066
RP-HPLC Method						
Folic Acid	50	1.6	0.8	2.4	2.389	99.54±0.7412
	100	1.6	1.6	3.2	3.191	99.71±0.6358
	150	1.6	2.4	4.0	3.995	99.87±0.9253
Amlodipine Besylate	50	10	5	15	14.988	99.92±0.9426
	100	10	10	20	19.99	99.95±0.7561
	150	10	15	25	24.995	99.98±0.4357

Table 4: Recovery study for simultaneous equation UV method

Name of Drug	% Level of recovery	Test Amount (µg/ml)	Amount of drug taken (µg/ml)	Total Std. Amt (µg/ml)	Total amount Recovered (µg/ml)	% Mean Recovery ± SD(n=3)
Folic Acid	50	1.6	0.8	2.4	2.392	99.66±0.01311
	100	1.6	1.6	3.2	3.193	99.78±0.02955
	150	1.6	2.4	4.0	3.992	99.80±0.00755
Amlodipine Besylate	50	10	5	15	14.990	99.93±0.01000
	100	10	10	20	19.983	99.91±0.02858
	150	10	15	25	24.993	99.97±0.10066

4.3.1.5 Assay of synthetic mixture

From assay, Final concentration of was Folic Acid 1.6 µg/mL and Amlodipine Besylate 10 µg/mL were run into UV and RP-HPLC the Percentage assay of were found to be 99.07 % and 99.64 %, respectively for UV (first order) For Simultaneous equation method The percentage Assay of Folic Acid and Amlodipine Besylate were found to be 99.60% & 99.35% Respectively. Its results showed in Table 5.

Table 5: Analysis of synthetic mixture for UV and RP-HPLC Method

UV- Method (First order)				
Name of Drug	**Amount in synthetic mixture (µg/ml)**	**Mean Amount found (µg/ml)**	**% Assay ± SD (n=3)**	**%RSD**
Folic Acid	1.6	1.597	99.81±0.1890	0.19
Amlodipine Besylate	10	9.993	99.93±0.057	0.05
UV- Method (Simultaneous equation method)				
Folic Acid	1.6	1.598	99.87±0.00058	0.03
Amlodipine Besylate	10	9.998	99.98±0.02042	0.20
RP-HPLC Method				
Folic Acid	1.6	1.593	99.56±0.5375	1.11
Amlodipine Besylate	10	9.995	99.95±0.7134	1.07

4.3.2 Validation Parameters of the RP-HPLC Method

4.3.2.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. It was proved by comparing the chromatogram of mobile phase, test preparation solution to show that there was no interference of mobile phase and excipients peaks with peak of Folic Acid and Amlodipine Besylate

4.3.2.2 Linearity

The RP-HPLC chromatogram of Folic Acid (0.8-4.0 µg/mL) and Folic Acid (5-25 µg/mL) at 265 nm showed in figure 1. The Peak Area was found. Linearity was calculated using calibration curves were plotted between concentrations and peak areas. The regression equation of calibration curve was generated y = 953.87x + 144.99 for FOL and y = 135.73x + 646.03for AMLO. The correlation coefficient (R^2) values were observed to be 0.9991 and 0.9992. (Table 1).

4.3.2.3 Precision

Concentrations of 0.8, 1.6 and 2.4 µg/mL for FOL and 5, 10 and 15 µg/mL for AMLO were selected for precision studies. On the same day, the peak area of the prepared solutions was measured at three different time intervals at the selected wavelength. Similarly, on the first, second, and third days, the peak area of the same solutions was measured to evaluate inter-day precision. Each solution was prepared and analyzed in triplicate.

4.3.2.4 Accuracy

The accuracy of the technique recuperation was decided change into accomplished by means of standard addition approach. To pre-analyzed pattern acknowledged quantity of general FOL and AMLO spiked in extraordinary concentrations. The restoration was executed in three stages 50 %, 100 % and 150 % of fashionable FOL and AMLO. The results were studied in triplicate and the accuracy changed into indicated by% recovery (Table 3). Accuracy was carried out by the Recovery Studies.

For HPLC, The % Recovery was obtained in range of 99.54%-99.87%for Folic Acid and 99.92%-99.98% for Amlodipine Besylate were showed in Table 3. The mean percentage recovery values for both drugs were found to be within the ICH-accepted range of 98-102%, with low standard deviation. These results confirmed the accuracy, trueness, and reliability of the RP-HPLC method and indicated that excipients present in the synthetic mixture did not interfere with the estimation of either drug.

4.3.2.5 LOD and LOQ

LOD Values were found to be 0.04 and 0.38 µg/mL for and Folic Acid and for Amlodipine Besylate respectively. LOQ Values were found to be 0.11 and 1.146 µg/mL, respectively for Folic Acid and Amlodipine Besylate. These results showed in Table 1.

4.3.2.6 Assay

From assay, Final concentration of was Folic Acid 1.6 µg/mL and Amlodipine Besylate 10 µg/mL were injected into HPLC System and The Percentage assay of Folic Acid and Amlodipine Besylate were found to be 99.56 % and 99.56 %, respectively. Results showed in Table 5.

4.3.2.7 Robustness

Chromatographic analysis was used to analyze the effects of changes in analysts, and the results showed that there was no statistically significant difference in the% RSD of technique II. Additionally, small changes were performed to assess the robustness of the created HPLC procedures. The approaches robustness was demonstrated by the% RSD, which remained constant despite minor variations in flow rate, run time, and detection. It was determined that the created approaches were essential.

5. CONCLUSION:

The present study successfully demonstrates the development and validation of UV spectrophotometric and RP-HPLC methods for the simultaneous estimation of Folic Acid and Amlodipine Besylate in a synthetic mixture. Both methods were validated in accordance with the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 (R2) and showed excellent linearity, accuracy, precision, sensitivity, and reproducibility. Both UV methods **Simultaneous Equation** and **First Order Derivative** method and RP-HPLC method were exhibited **excellent linearity, precision, and accuracy**, and were successfully validated according to **ICH Q2 (R2)** guideline. There was **no interference** from excipients, confirming the specificity of the methods. These methods can be reliably applied for **routine quality control** of combined Folic Acid and Amlodipine Besylate **in synthetic mixture.** Furthermore, the proposed methods can be extended for the analysis of marketed formulations and stability studies, highlighting their potential applicability in regulatory and industrial quality-control settings.

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CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

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