e-ISSN: 0975-9506, p-ISSN: 2961-6093

Available online on www.ijpqa.com doi: 10.25258/ijpqa.16.6.8

International Journal of Pharmaceutical Quality Assurance 2025; 16(6); 38-49

Original Research Article

Green Assessment of Analytical Procedure for the Determination of Anti-Hypertensive Drugs by HPLC

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Received: 25-03-2025 / Revised: 23-04-2025 / Accepted: 26-05-2025
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Conflict of interest: Nil

Abstract:

Background: The objective of the proposed study was to develop and validate a stability-indicating greener chromatographic method for the simultaneous analysis of anti-hypertensives drugs for amlodipine besylate (AML) and folic acid (FOL) in synthetic mixtures , assessing its environmental impact using various greenness evaluation tools — an emerging advancement in the field of analytical chemistry. AML, a calcium channel blocker used for hypertension management, is currently under investigation in Phase III clinical trials (CN111481554A) in combination with FOL. This combination has demonstrated potential for enhanced blood pressure reduction, reduced cardiovascular and cerebrovascular risk, and improved protection of vital organs, including the heart, brain, and kidneys.

Methods: Chromatographic separation was achieved using a Shimadzu P-series C18 column (250 mm × 4.6 mm, 5 µm particle size). The mobile phase, consisting of methanol, acetonitrile, and buffer (adjusted to pH 3.5 using orthophosphoric acid) in a 30:50:20 (v/v/v) ratio, was delivered at a flow rate of 1.0 mL/min. Detection was carried out at a wavelength of 235 nm using a PDA detector. The method validation was performed according to ICH Q2 (R2) guidelines. Forced degradation studies were conducted under acidic (0.1N HCl), alkaline (0.1N NaOH), oxidative (3% H_2O_2), thermal (60°C for 3 hours), and photolytic (UV light exposure) conditions.

Results and Discussion: Retention times were recorded as 2.011 minutes for AML and 4.019 minutes for FOL. The method exhibited linear calibration curves over the concentration ranges of 50–300 µg/mL for AML and 8–48 µg/mL for FOL, with correlation coefficients (R^2) of 0.9967 and 0.9975, respectively. Sensitivity was demonstrated through low detection and quantification limits. Accuracy and precision were within acceptable limits, and the results were reproducible. Forced degradation studies indicated significant degradation under acidic, alkaline, and oxidative conditions, while thermal and photolytic conditions induced comparatively minor degradation. This validated method is suitable for stability and simultaneous quantification studies of AML and FOL in pharmaceutical formulations. Assessment of method was carried out using the two different tools. The developed method is anticipated to be eco-friendly, alternative to developed method in regard to safe solvent, less toxic and less run time.

Conclusion: The stability-indicating method is reliable and ideal for routine analysis and stability studies in quality control lab user greener solvent.

Keywords: Amlodipine besylate (AML), Folic acid (FOL), Stability Indicating High-Performance Liquid Chromatography, Forced degradation study, Method validation.

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Introduction

Amlodipine is a long-acting calcium channel blocker (CCB) that belongs to the dihydropyridine class. Its chemical structure consists of a 1,4-dihydropyridine ring with various substitutions, which confer its pharmacological properties. The molecular formula of amlodipine is $C_{20}H_{25}ClN_2O_5$, and its IUPAC name is 3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate. Amlodipine's biological activity is primarily attributed to its ability to inhibit L-type calcium channels in vascular smooth muscle and cardiac muscle, result-

ing in vasodilation and reduced myocardial oxygen demand [1].

Its prolonged half-life allows for once-daily dosing, making it highly effective for controlling blood pressure and preventing angina episodes [2].AML was approved on the date of 6/14/1996 under the brand name of Norvasc manufactured by Pfizer Central Research under application number of 6/14/1996 by the U.S. Food and Drug Administration.

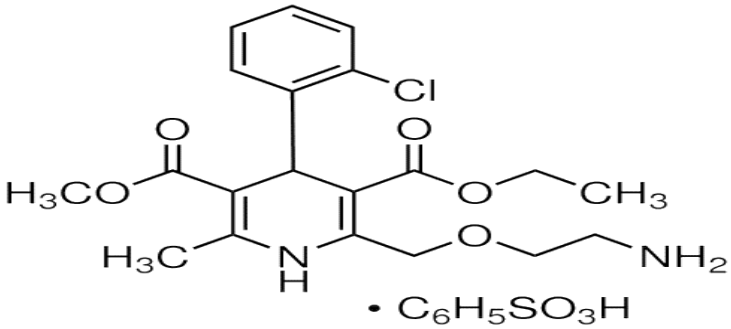

Figure 1: Structure of amlodipine besylate

Folic acid, also known as pteroylmonoglutamic acid, is a water-soluble B-vitamin (vitamin B9) essential for DNA synthesis, amino acid metabolism, and red blood cell production.

Chemically, it contains a pteridine ring and has the molecular formula $C_{19}H_{19}N_7O_6$ [4]. One of its critical physiological roles includes reducing homocysteine levels, which has been associated with a decreased risk of neural tube defects (NTDs) during fetal development [5].

Folic acid is widely used in clinical practice, not only for its established role in preventing NTDs but also for off-label indications such as improving cognitive function and reducing cardiovascular risk factors [6], approved U.S. Food and Drug Administration (FDA) in 1943.

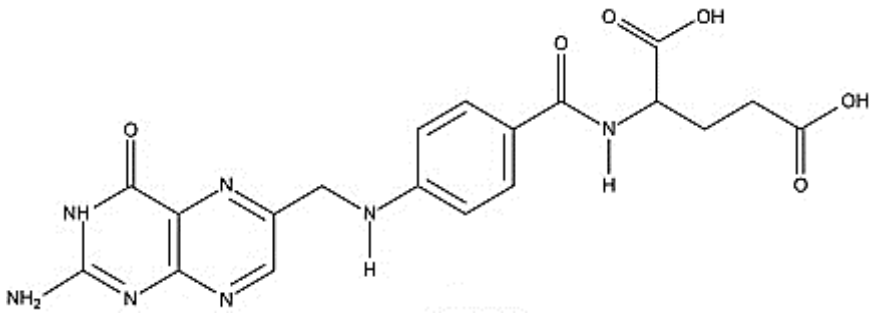

Figure 2: Structure of folic acid

The combination of amlodipine and folic acid offers potential benefits for patients with hypertension and cardiovascular disease by improving vascular function, reducing homocysteine levels, and lowering stroke risk. Further studies are needed to establish optimal dosing and patient selection criteria for maximizing therapeutic benefits.[6-11]

Existing literature reveals that UV spectroscopy, HPLC, and HPTLC have been primarily used to analyze AML [12-25] and FOL[26–33] individually in bulk and pharmaceutical dosage forms. However, to date, no method has been reported for the simultaneous analysis of these two compounds. To address this, a new, simple, precise, and stability-indicating RP-HPLC method was developed and validated for the simultaneous quantification of AML and FOL. A simulated mixture was used to emulate the formulation under study, as the combination is still undergoing phase III clinical trials (CN111481554A) and commercial formulations are not yet available. This combination therapy, consisting of 5 mg of AML and 0.4 mg of FOL, aims has stable and smooth function of lowering blood pressure, more effectively reducing risk of cardiovascular and cerebrovascular diseases, and better protecting effect on heart, brain and kidney.

The proposed method facilitates accurate quantification and reliable stability assessment, which are essential for quality control applications. Stress testing was performed in accordance with ICH

guidelines [34] to evaluate the stability of the drug components under various stress conditions, further supporting the method's robustness for routine use to have a stable and smooth function of lowering blood pressure, more effectively reducing risk of cardiovascular and cerebrovascular diseases, and better protecting the heart.

Green chemistry refers to the method designed to minimize or eliminate the use and production of hazardous materials, including solvents, reagents, and by products that pose risks to human health or the environment. Green chemistry focus on designing for every stage of the chemical life cycle, minimizing the inherent hazards of chemical processes and products by optimizing their intrinsic properties, and establishing a logical framework of guidelines for sustainable chemical design. These principles ensure that chemical processes are developed with environmental and human safety in mind, promoting sustainability and reducing harmful impacts.

Several metrics have been developed to assess the environmental impact of analytical techniques. While some are specific to particular methods, others are widely applicable across various analytical procedures. The Complex Green Analytical Procedure Index (GAPI) and Analytical GREEness (AGREE) were used to evaluate the novel HPLC technique. A simple and effective tool for interpreting such assessments is the Complex Green Analytical Procedure Index (Complex GAPI), which utilizes pictograms for easy visualization. Complex GAPI evaluates various stages of the analytical process, including sampling, sample preparation, solvent and reagent consumption, instrumentation, and waste generation. Notably, it covers a wider range of criteria compared to other green metrics like complex GAPI. Complex GAPI assigns one of three color codes — green**,** yellow or red — to indicate the environmental impact of each step. To assess the environmental friendliness of the proposed method relative to existing techniques, two approaches were used: the AGREE & GAPI pictogram analysis.

Materials and Methods

Chemicals and Reagents: The AML standard was procured from Zydus Pharma Ltd., Ahmedabad, Gujarat, India, and the FOL standard from Sun Pharma Ltd., Vadodara, and Gujarat, India. Chromatographic-grade solvents (water, methanol, and acetonitrile) and analytical-grade chemicals (triethylamine, sodium hydroxide, potassium dihydrogen phosphate, hydrogen peroxide, ammonium acetate, and hydrochloric acid) were procured from Ranchem Ltd., Mumbai, India.

Instrumentation: A Shimadzu P-series integrated HPLC system (Model LC-20AD) equipped with a quaternary gradient unit, LC-20AD solvent delivery module, DGU-20AR degassing unit, CTO-10ASVP column oven, SPD-M40 photodiode array (PDA) detector, and SIL-20AC programmable auto-sampler was utilized for chromatographic analysis. The system was managed and controlled using LAB SOLUTION software. A Shimpack ODS C18 column (250 mm × 4.6 mm, 5 µm particle size) was used for the chromatographic separation, ensuring effective resolution and reproducibility.

Preparation of the Buffer Solution: Prepare 250 mL of HPLC-grade water and adjust its pH to 3.5 using 0.1% orthophosphoric acid. Stir well to achieve uniformity, then sonicate the solution to promote dissolution and consistency. Finally, filter it through a 0.45 µm membrane to eliminate particulates and impurities.

Preparation of Mobile Phase: The mobile phase comprised a buffer (pH 3.5, adjusted with orthophosphoric acid), methanol, and acetonitrile in a 30:10:20 (v/v/v) ratio.

Preparation of Standard Solution: 50 mg AML and 8 mg FOL were weighed and added to a 100 ml flask. After sonication in 50 ml methanol until dissolved, the solution was diluted to 100 ml with diluent, yielding Stock solutions of 500 µg/ml of AML and 80 µg/ml FOL. 5 mL of each stock was then diluted to 10 ml, resulting in final concentrations of 500 µg/ml AML and 80 µg/ml FOL.

Preparation of Linearity Solutions: Precise aliquots (1–6 mL) of the stock solutions were transferred into 10 mL volumetric flasks and diluted to volume with the mobile phase, yielding final concentrations of 50–300 µg/mL for AML and 8–24 µg/mL for FOL. Retention times and peak areas were recorded to generate six calibration curves. A calibration plot of peak area versus analyte concentration was constructed, and linear regression analysis was performed to assess the correlation.

Analysis of synthetic mixture: Synthetic mixture was prepared by mixing amlodipine besylate (AML) (50.0 mg), folic acid (FOL) (8 mg) with starch (140.0 mg), Hydroxy propyle methayl cellulose E5 (30.0 mg), Polly vinayl pyrrolidone (20.0mg) magnesium stearate (2.5 mg) and talc (1.0mg). Weigh accurately a powder equivalent to 5 mg of amlodipine besylate (0.8 mg folic acid) & transfer in 10 ml volumetric flask dissolved in 5.0 ml with Methanol and sonicated for 5 min then diluted with methanol to reach the desired solution strength (500 and 80 µg/ml), Filter with 0.45 micron syringe filter. Further diluted 2 ml of above solution to 10 ml volumetric flask and volume was make up to the mark with diluent. Concentration of amlodipine besylate (AML) was 100 µg/ml and for folic acid (FOL) 16 µg/mL.

Analytical Method Validation: Analytical validation is the systematic method of verifying that a

chosen analytical technique can yield precise, reliable, and repeatable results appropriate for the intended use.

Linearity: The method was evaluated within the concentration ranges of 50–300 µg/ml for AML and 8-48 µg/ml for FOL. Calibration solutions were constructed by transferring 1– 6 ml aliquots of stock solutions into 10 ml flasks and adjusting volumes with the Mobile phase. A calibration curve of Peak area versus concentration was plotted, and linearity was assessed based on slope, intercept, and correlation coefficient, yielding good linearity with an R^2 value near 0.9967 and 0.9975 with regression line equation for AML & FOL.

Precision: Result should be expressed as Relative Standard Deviation or Co-efficient of variance.

Repeatability: Peak areas were measured following six injections of a standard solution containing 150 µg/ml of AML and 24 µg/ml of FOL. The RSD was determined to assess the method's precision.

Inter-day Precision: A standard solution containing AML at concentrations of 50,150, and 300 µg/ml and FOL at concentrations of 8, 24 and 48 µg/ml was injected three times within the same day. The RSD was determined from the recorded peak areas to assess the intraday precision of the method.

Intra-day precision: A standard solution containing AML at concentrations of 50, 150 and 300 µg/ml and FOL at concentrations of 8, 24 and 48 µg/ml was injected over three different days. Peak areas were measured, and the RSD was calculated to assess intraday precision.

Accuracy: Accuracy of analysis was determined by calculating recovery of drug addiction method at three different concentration level (50%, 100% & 150%). Standard AML (50,100,150 µg/ml) & Standard FOL (8, 16, 24 µg/ml) was spiked to pre-quantified target sample of 100 µg/ml of AML & 16 µg/ml of FOL. The % drug recovery was calculated at each level.

Limit of Detection & Limit of Quantitation: The LOD was estimated using 3 calibration curves to assess linearity, with the formula LOD = 3.3 × (SD/Slope), where Slope is the mean slope of the curves and SD is the standard deviation of the Y-intercept. The LOQ was determined using the formula LOQ = 10 × (SD/Slope).

Robustness: A solution of 150 µg/ml AML and 24 µg/ml FOL was analyzed under deliberate variations to assess the robustness of the method. The variables tested included mobile phase ratio, wavelength (235 ± 3 nm), and flow rate (1.0 ± 0.1 mL/min). The method's reliability was determined by calculating the RSD of the results.

Forced Degradation Study of AML and FOL:

Acid hydrolysis: A stock solution of AML (1500 µg/mL) and FOL (240 µg/mL) was mixed with 5 mL of 0.1N HCl in a 10 mL flask, refluxed at 50°C for 1 hour, then neutralized with 5 mL of 0.1N NaOH and diluted to volume with methanol. The solution was further diluted with the mobile phase to obtain final concentrations of 150 µg/mL AML and 24 µg/mL FOL.

Alkaline Hydrolysis: A stock solution of AML (1500 µg/mL) and FOL (240 µg/mL) was mixed with 5 mL of 0.1N NaoH in a 10 mL flask, refluxed at 50°C for 1 hour, then neutralized with 5 mL of 0.1N Hcl and diluted to volume with methanol. The solution was further diluted with the mobile phase to obtain final concentrations of 150 µg/mL AML and 24 µg/mL FOL.

Oxidative Stress: A stock solution of AML (1500 µg/mL) and FOL (240 µg/mL) was mixed with 5 mL of 3% Hydrogen peroxide in a 10 mL flask, place the flask at room temperature for 1 hour, The solution was further diluted with the mobile phase to obtain final concentrations of 150 µg/mL AML and 24 µg/mL FOL.

Thermal Degradation: Thermal degradation was conducted by placing 100 mg of amlodipine besylate (AML) and 16 mg of folic acid (FOL) in a Petri dish and subjecting them to a hot air oven at 60°C for 2 hours.

Photo degradation: Photolytic degradation was performed by spreading 100 mg of AML and 16 mg of FOL as a thin layer on a Petri plate and exposing them to UV light in a chamber for 24 hours.

The thermally and photolytically stressed samples were transferred to a 100 mL volumetric flask containing methanol as a diluent, sonicated for 10 minutes, and diluted to volume to achieve a concentration of 1000 µg/mL AML and 160 µg/mL FOL.

A1.5 mL aliquot of this stock solution was further diluted to 10 mL with methanol and filtered through a 0.45 µm syringe filter before analysis. The final concentration of AML and FOL (150 µg/mL and 24 µg/mL, respectively) was injected into the HPLC system for analysis.

Results and Discussion

The accuracy, precision, efficacy, and specificity of the proposed approach for the simultaneous quantification of AML and FOL were demonstrated. At a flow rate of 1 mL/min, the optimized mobile phase (Buffer pH 3.5, Methanol, and Acetonitrile 20:30:50 % v/v/v) demonstrated excellent repeatability and reproducibility.

UV detection at 235 nm was employed for quantification, with retention times for AML and FOL being 2.011 and 4.019 minutes, respectively the results are presented comprehensively in Table 1.

The method was evaluated based on the ICH guidelines. (Acceptance criteria- Theoretical plate: >2000, Resolution: >1.5, Tailing factor: <2, RSD: <2, Specificity**:** No Interference)

Table 1: Regression analysis of Calibration curve

Parameter	**AML**	**FOL**
Detection Wavelength	235 nm	
Linearity range	50-300 µg/ml	8-48 µg/ml
Regression equation	y = 1591.7x - 7891.8	y = 3982.8x + 4587.7
Regression coefficient (r^2)	0.9967	0.9975
Mean of slope	1585	637
Standard deviation of intercept	2219.75	473.04
Limit of Detection	4.622	2.44
Limit of Quantitation	14.007	7.42
Retention time	2.051 min ± 0.02	4.055 min ± 0.039
Resolution	0.00	6.007 ± 0.067
Theoretical plate	6111 ± 70.22	8460 ± 99.68
Tailing factor	0.82 ± 0.01	0.92 0.01
		0.93

Selection of Diluent: According to solubility, AML and FOL were soluble in methanol. Methanol was therefore chosen as the diluent.

Selection of Wavelength: AML and FOL solutions were individually prepared in methanol at a conc of 10 µg/ml. Each solution was scanned using a UV-Vis spectrophotometer over the 200–400 nm wavelength range. Based on the overlay spectra of AML and FOL, the analytical wavelength of 235 nm was selected, as both compounds exhibited significant absorbance at this wavelength.

Optimization of mobile phase: AML and FOL displayed well-resolved peaks with retention periods of 2.822 and 6.012 minutes, respectively, using a mobile phase of buffer (pH 3.5, adjusted with orthophosphoric acid), methanol, and acetonitrile (20:30:50 v/v/v), under isocratic conditions (Figure 2).

This synthetic was chosen for chromatographic development, with a constant flow rate of 1 ml/min.

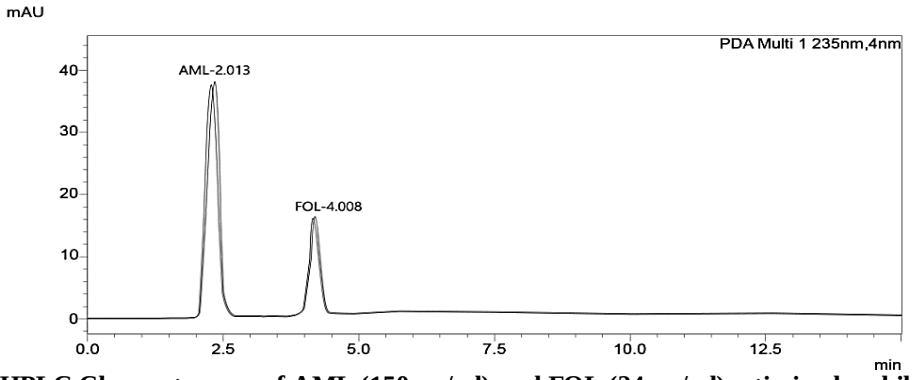

Figure 3: HPLC Chromatogram of AML (150 µg/ml) and FOL (24 µg/ml)optimized mobile phase in standard drugs and synthetic mixture.

Linearity: The linear regression examination of the calibration data revealed a strong linear correlation between peak areas and concentrations for AML and FOL within their respective concentration ranges of 50-300 µg/ml and 8-48 µg/ml.

The correlation coefficients (r^2) for AML and FOL were found to be 0.9967 and 0.9975, respectively. These results indicate that, within the specified concentration ranges, the analytical technique demonstrates near-compliance for AML and acceptable linearity for FOL.

Precision: The precision method was quantified by performing six replicate measurements of the level of each analyte. The RSD values were determined to be 0.46% for AML and 0.55% for FOL. Intra-day and inter-day variations were further assessed to evaluate precision. RSD values for inter-day precision, derived from six replicate studies conducted over multiple days, ranged from 1.42% to 1.80% for AML and from 1.00% to 1.60% for FOL. intra-day precision, assessed from six replicates analyzed on the same day, produced RSD values for AML ranging from 0.60% to 1.20% and for FOL ranging from 0.60% to 1.21%. These results validate the Accuracy and Robustness of the analytical technique within the specified concentration ranges.

Accuracy: The standard spiking method was employed to quantify the analyte concentrations in spiked solutions to assess the accuracy of the method. Accuracy was assessed through % recoveries, which ranged from 98.22% to 100.4% for AML and from 98.41% to 101.29% for FOL and the results are provided in Table 2. These findings indicate that the method demonstrates optimal accuracy and reliability within the established concentration ranges.

Table 2: Recovery data for AML and FOL

Level	Conc. of Synthetic mixture (µg/ml)		Amount of Std. added (µg/ml)		Total amount (µg/ml)		Total amount Recovered (µg/ml) Mean ± SD		% Recovery	
	AML	FOL	AML	FOL	AML	FOL	AML	FOL	AML	FOL
0	100	16	0	0	100	16	100.42±0.52	16.1±0.11	100.42	100.63
50	100	16	50	12.5	150	24	150.51±2.09	24.31±0.24	100.34	101.29
100	100	16	100	25	200	32	196.43±2.33	31.49±0.32	98.22	98.41
150	100	16	150	37.5	250	40	245.71±2.75	39.98±0.46	98.28	99.95

LOD & LOQ: Calculations of the signal-to-noise ratio were used to quantify the LOD & LOQ. The experimentally determined LOD values were 4.62 µg/ml for AML and 2.44 µg/ml for FOL. Similarly, the LOQ values for AML and FOL were observed to be 14.07 µg/ml and 7.42 µg/ml, respectively.

Robustness: The reliability of the proposed analytical method was quantifying by making small, deliberate modifications to the optimized method parameters.

The effects of these modifications on the method's performance were evaluated.

The method under the investigated conditions is confirmed by the minimal variability observed in the analytical results (Table 3).

Table: 3 Robustness data of AML and FOL (n=3)

Parameters	Normal condition	Drug	Change in condition	Area (Mean ± SD) (n=3)	% RSD
Detection wavelength	235 nm	AML (150 µg/ml)	232 nm	222865.9 ± 2101.04	0.94
			238 nm	222266.75 ± 2874.29	1.29
		FOL (24 µg/ml)	232 nm	105293.79 ± 940.83	0.89
			238 nm	101789.07 ± 761.46	0.75
Flow rate	1.0 ml/min.	AML (150 µg/ml)	0.9 ml/min.	219549.38 ± 2343.36	1.07
			1.1 ml/min.	221313.23 ± 2032.45	0.92
		FOL (24 µg/ml)	0.9 ml/min.	103799.11 ± 1286.94	1.24
			1.1 ml/min.	102552.67 ± 1267.52	1.24
Mobile Phase composition	Methanol, Acetonitrile, Buffer (adjust pH 3.5 with ortho phosphoric acid), 30:50:20 v/v/v	AML (150 µg/ml)	35:45:20 v/v/v	222217.39 ± 2199.28	0.99
			35:50:15 v/v/v	219631.1 ± 3170.93	1.44
		FOL (24 µg/ml)	35:45:20 v/v/v	104099.44 ± 875.8	0.84
			35:50:15 v/v/v	104012.61 ± 1313.26	1.26
pH of Mobile phase	pH 3.5	AML (150 µg/ml)	3.8	223013.07 ± 2125.45	0.95
			3.2	222634.66 ± 3443.96	1.55
		FOL (24 µg/ml)	3.8	104167.54 ± 769.45	0.74
			3.2	104080.71 ± 1408.84	1.35

Analysis of synthetic mixture: The assay % was quantified through the injection of the sample solution into the chromatographic system in three replicates. The mean assay percentages were calculated as 101.51% for AML and 102.04% for FOL. The representative chromatogram is illustrated in Figure 6, with results detailed in Table 4.

Table 4: Assay of AML and FOL

Drug	Conc. (µg/ml)	Amount found (µg/ml)	Amount found Mean ± SD (n=3)	% Assay Mean ± SD (n=3)
Amlodipine Besylate (AML)	100	101.50	101.51 ± 1.44	101.51 ± 1.44
		100.07		
		102.95		

Folic Acid (FOL)	16	16.24 16.42 16.31	16.33 ± 0.09	102.04 ± 0.54

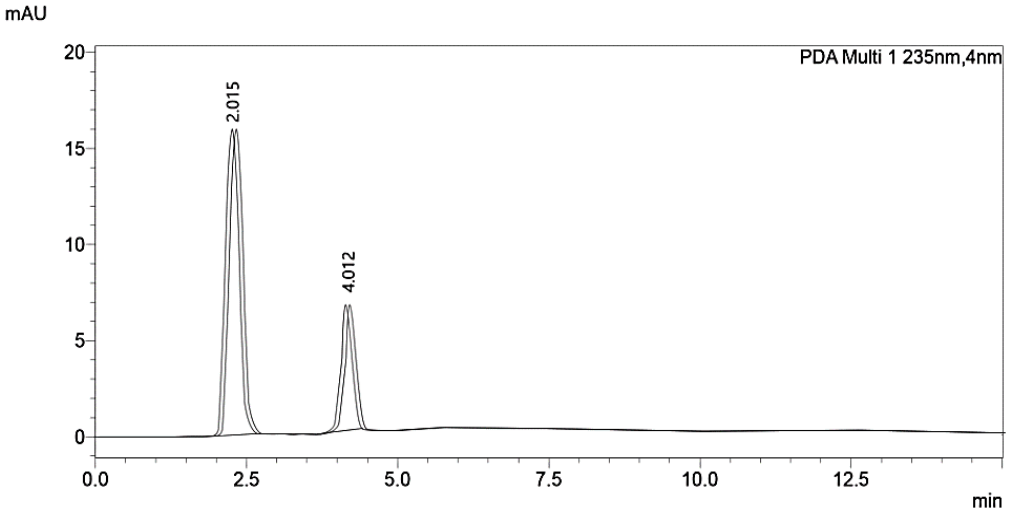

Figure 6: HPLC chromatogram of AML (100 µg/ml) and Folic Acid (16 µg/ml) in bulk and synthetic mixture.

Forced Degradation Study: A forced degradation analysis was conducted to determine the intrinsic stability of the drugs. In this study, individual APIs and their mixture were subjected to various stress conditions. The working solutions of both drugs were utilized for the forced degradation experiments. The applied stress conditions included oxidation, photostability, thermal stability, base hydrolysis, and acid hydrolysis.

The representative chromatogram is depicted in Figure (7-11) with the results summarized in Table 5.

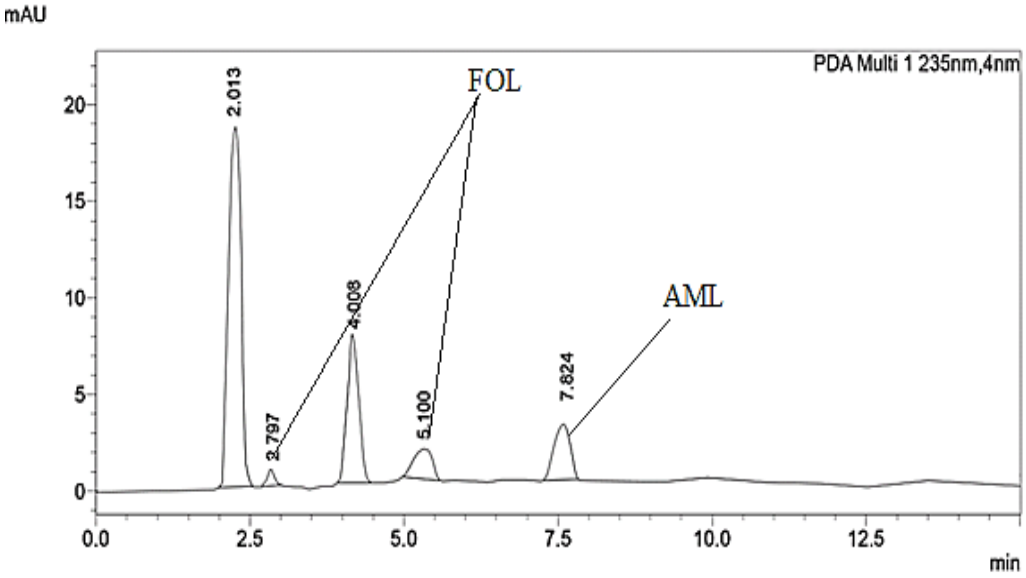

Figure 4: Chromatogram of acid stressed of AML and FOL mixtures

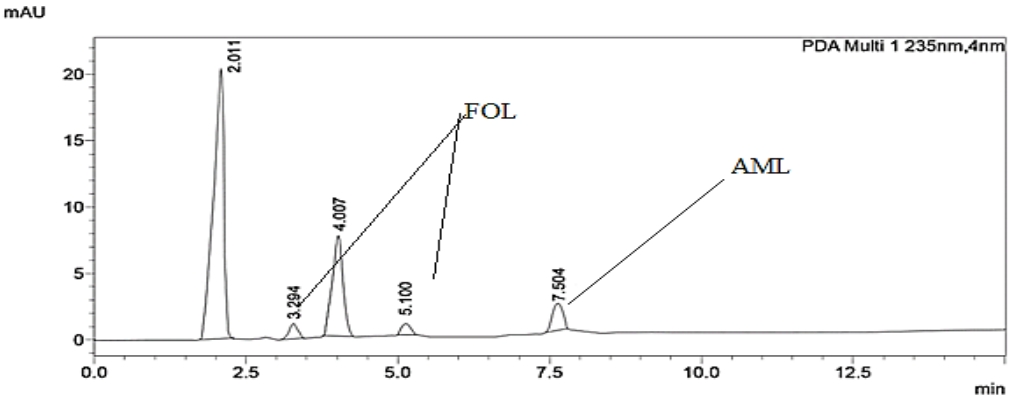

Figure 5: Chromatogram of Base stressed of AML and FOL mixtures.

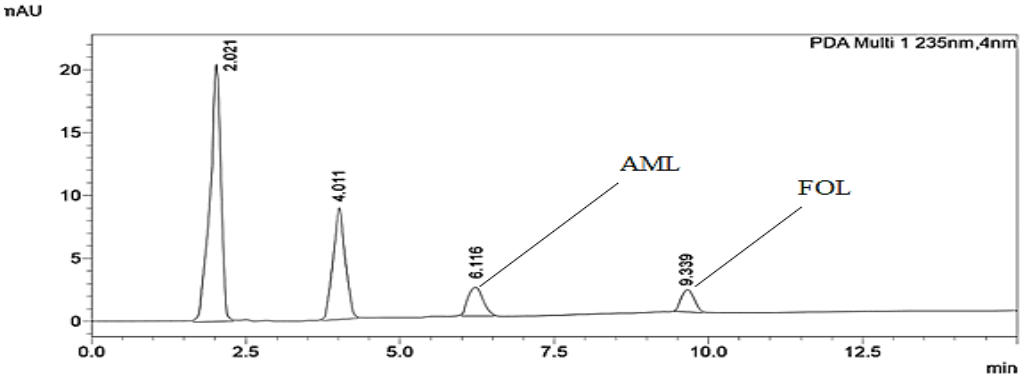

Figure 6: Chromatogram of Hydrogen peroxide stressed of AML and FOL mixtures

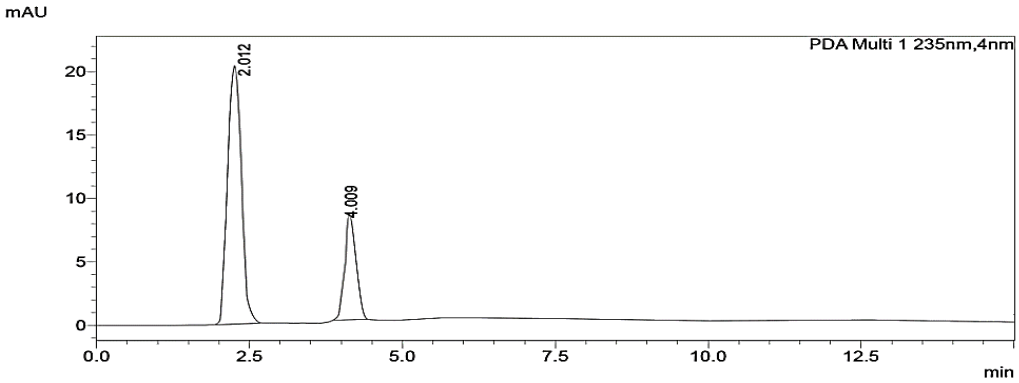

Figure 7: Chromatogram of thermal stressed of AML and FOL mixtures

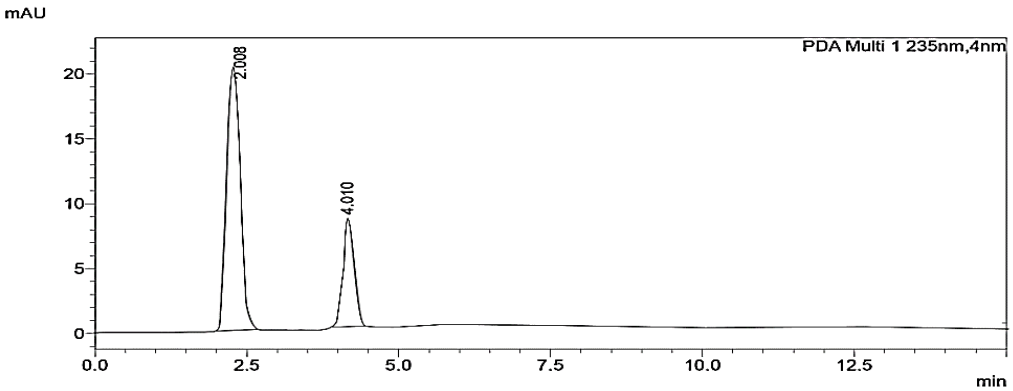

Figure 8: Chromatogram of photolytic stressed AML and FOL mixtures.

Table 5: Summary of forced degradation studies:

Stress Condition	Amlodipine Besylate (150 µg/ml)				Folic Acid (24 µg/ml)			
	Area	% Drug Recovered	% Drug degrade	Degradant Retention time (min)	Area	% Drug Recovered	% Drug degraded	Degradant Retention time (min)
Acid hydrolysis (0.1N HCl) Duration = 1 hr at 50^0C	203467.20	88.53	11.47	7.826	87974	87.24	12.76	2.798 and 5.105
Base hydrolysis (0.1N NaOH) Duration = 2 hr at 60^0C	207345.78	90.15	9.85	7.504	91100	90.51	9.49	3.294 and 5.100
Hydrogen peroxide (3% H_2O_2) Duration = 1 hr at 60^0C	209023.35	90.85	9.15	9.434	86095	85.27	14.73	6.169
Thermal Degradation (3 hr at 60^0 C)	216480.14	93.98	6.02	-	95064	94.65	5.35	-
Light Degradation (under UV light 254nm wavelength for 6 hrs)	214844.72	93.29	6.71	-	96075	95.71	4.29	-

Evaluation of Greenness by AGREE SOFTWARE: The AGREE tool is utilized to assess the environmental sustainability of the existing methodology. AGREE is an analytical tool that may evaluate the environmental impact of analytical methods, regardless of the specific technique utilized. This tool was constructed utilizing the 12 key green analytical chemistry factors, which encompass sample preparation, derivatization, automation, simplicity, solvent toxicity, number of samples tested, number of components analyzed in a single test, energy consumption per analysis, and analysis time.

The outcome is transformed into a scale ranging from 0 to 1. The greenness character of the present HPLC technique was assessed using this instrument. The results indicated that the devised approach is environmentally sustainable, achieving a score of 0.72 depicted in Fig no -13. Valuation of Greenness by AGREE SOFTWARE

The AGREE tool is utilized to assess the environmental sustainability of the existing methodology. AGREE is an analytical tool that may evaluate the environmental impact of analytical methods, regardless of the specific technique utilized. This tool was constructed utilizing the 12 key green analytical chemistry factors, which encompass sample preparation, derivatization, automation, simplicity, solvent toxicity, number of samples tested, number of components analyzed in a single test, energy consumption per analysis, and analysis time. The outcome is transformed into a scale ranging from 0 to 1. The greenness character of the present HPLC technique was assessed using this instrument. The results indicated that the devised approach is environmentally sustainable, achieving a score of 0.72 depicted in Fig no -13.

Assessment of Greenness for Developed Method:

Complex GAPI: The Complex GAPI is made up of five pentagrams that are used to measure and quantify the environmental impact of every step of the developed process using a different colour code: green, yellow and red which indicate a low, medium and high environmental impact. Greener colour shades indicate high environmental safety and less risk to analysts. For the developed Method, its shows nine green shaded pentagrams, two with yellow shaded and three with Red as show in pictogram. This indicates that proposed method is very much eco-friendly and analyst safety as given in Figure 9.

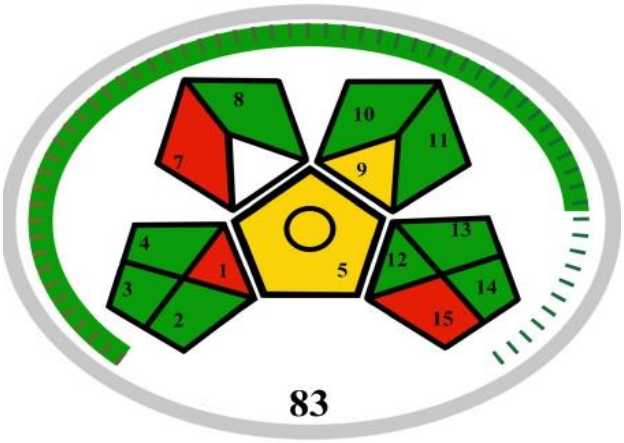

Figure 9: Complex GAPI (Green Analytical Procedure Index).

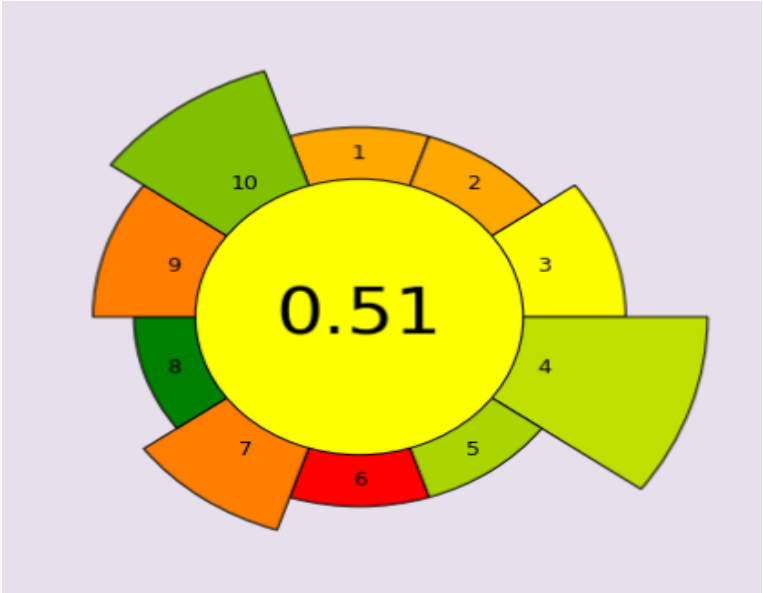

Figure 10: AGREEprep

The AGREEprep is utilized to assess the environmental sustainability of the existing methodology. AGREEprep is an analytical tool that may evaluate the environmental impact of analytical methods, regardless of the specific technique utilized. This tool was constructed utilizing the 1 key green analytical chemistry factors, which encompass sample preparation, derivatization, automation, simplicity, solvent toxicity, number of samples tested, number of components analyzed in a single test, energy consumption per analysis, and analysis time. The outcome is transformed into a scale ranging from 0 to 1. The greenness character of the present HPLC technique was assessed instrument. The results indicated that devised approach is environmentally sustainable, achieving a score of 0.51 depicted in Fig: 10

Evaluation of Greenness by AGREE SOFTWARE and GAPI tool is utilized to assess the environmental sustainability of the existing methodology. AGREE is an analytical tool that may evaluate the environmental impact of analytical methods, regardless of the specific technique utilized. This tool was constructed utilizing the 12 key green analytical chemistry factors, which encompass sample preparation, derivatization, automation, simplicity, solvent toxicity, number of samples tested, number of components analyzed in a single test, energy consumption per analysis, and analysis time.

The outcome is transformed into a scale ranging from 0 to 1. The greenness character of the present HPLC technique was assessed using this instrument. The results indicated that, devised approach is environmentally sustainable, achieving a score of 0.72 depicted in Fig: 10.

Conclusion

To our knowledge, no analytical method has been previously reported for the simultaneous quantification of the above combination. The developed stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method

demonstrates effective separation and accurate quantification of both analytes within synthetic mixtures. Moreover, it offers critical insights into their stability profiles under various ICH-recommended stress conditions as per the [Q2 (R2)] guidelines. The analytes were subjected to forced degradation studies, confirming the method's robustness and ability to resolve degradation products. Method validation was performed following ICH [Q2 (R2)] guidelines, with all validation parameters falling within the prescribed acceptance limits. Therefore, the established method is deemed suitable for routine quality control and stability testing of AML & FOL in pharmaceutical formulations. According to the AGREE prep and Complex GAPI greenness, the suggested technique can reduce the environmental effect of other organic mobile phase solvents, such as acetonitrile, and improve safety for analysts doing regular analysis in quality control.

Acknowledgement: We appreciate the contribution of every member involved in completing this article.

Data Availability: The data supporting the finding of this study are available from corresponding author upon reasonable request.

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