https://doi.org/10.31925/farmacia.2024.6.11 **ORIGINAL ARTICLE**

DEVELOPMENT AND VALIDATION OF A NOVEL STABILITY-INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS QUANTITATION OF AMLODIPINE, GLIMEPIRIDE AND PERINDOPRIL ARGININE

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Abstract

An accurate, precise, simple and stability-indicating RP-HPLC method was developed and validated for the simultaneous quantitation of perindopril arginine (PA), amlodipine (AML) and glimepiride (GLM) in solution in accordance with the International Conference on Harmonisation (ICH) guidelines. The HPLC analysis was performed using a Supelcosil LC-18 column (25 cm × 4 mm × 5 µm), at a flow rate of 1.0 mL/min, with wavelength of detection at 220.0 nm for PA, AML and GLM. The optimised mobile phase consisted of a mixture of acetonitrile, methanol and phosphate buffer in the ratio of (20:50:30) (v/v/v) which was adjusted to pH 3.5 ± 0.1 with o-phosphoric acid. The proposed method was validated for linearity, system suitability, accuracy, precision (intra- and inter-day) and robustness. The HPLC results displayed good separation and resolution in a total analysis time of 16 minutes where PA, AML and GLM were eluted at retention times of 4.408 min, 5.415 min and 12.189 min, respectively. The optimised method displayed good linearity over the concentration range of 0.25 - 2.00 mg/mL for PA and 0.10 - 0.75 µg/mL for AML and GLM. The recoveries ranged from 98.0 to 102.0% and the RSD% values were found to be less than 2.00 for all drugs. The combination of three drugs was exposed to several forced degradation studies such as acid, base, neutral, thermal, oxidative and photolytic conditions. The forced degradation studies indicated that the optimised method is valid for stability studies.

Rezumat

În acest studiu a fost dezvoltată și validată o metodă cromatografică de înaltă performanță în fază inversă, pentru cuantificarea simultană a perindoprilului (PA), amlodipinei (AML) și glimepiridei (GLM) în soluție, în conformitate cu prevederile ghidului ICH. Analiza HPLC a fost efectuată utilizând o coloană Supelcosil LC-18 (25 cm x 4 mm x 5 um), la un debit de 1,0 mL/min, la lungimea de undă de detecție de 220,0 nm pentru PA, AML și GLM. Faza mobilă optimizată a constat dintr-un amestec de acetonitril, metanol și tampon fosfat în raport 20:50:30 (v/v/v) care a fost ajustat la pH 3,5 ± 0,1 cu acid o-fosforic. Metoda propusă a fost validată pentru liniaritate, adecvarea sistemului, acuratețe, precizie (intra și inter-zi) și robustețe. Rezultatele HPLC au arătat o bună separare și rezoluție într-un timp total de analiză de 16 minute, unde PA, AML și GLM au fost eluate la timpi de retenție de 4,408 min, 5,415 min și, respectiv, 12,189 min. Metoda optimizată a generat o liniaritate bună în intervalul de concentrație de 0,25 - 2,00 mg/mL pentru PA și 0,10 - 0,75 µg/mL pentru AML și GLM. Recuperările au variat între 98,0 și 102,0%, iar valorile RSD% s-au dovedit a fi mai mici de 2,00 pentru toate API. Combinația celor trei substanțe a fost supusă mai multor studii de degradare forțată, cum ar fi condiții acide, bazice, neutre, termice, oxidative și fotolitice.

Keywords: amlodipine, glimepiride, perindopril arginine, HPLC, validation, stability

Introduction

Despite all the breakthroughs in health care practice, hypertension and diabetes prevalence in the adult population is dramatically increasing. According to the world health organization (WHO), more than 400 million people worldwide are diabetic patients, it is estimated this number will be almost doubled by 2045 based on the estimation of the international diabetes federation (IDF) [1, 2]. Moreover, hypertension was

considered the major cause of premature death worldwide. The WHO estimated in 2023 that around 1300 million (1.3 billion) persons worldwide have hypertension and most of them are living in low and middle-income countries [3]. It was no surprise that hypertension and diabetes coexist at a high percentage among the population, in other words, there is a high probability that the same patient has both diseases concurrently. The relationship between insulin resistance and hypertension is documented through several epidemiological studies. Insulin resistance is a predisposing factor for vascular stiffness which can lead to hypertension, and at the same time, hypertension can cause insulin resistance by impairing glucose uptake as it can alter the delivery of insulin and glucose to skeletal muscle cells [4].

Glimepiride (GLM) is a second-generation sulfonylurea and is used to treat type 2 Diabetes mellitus acting by stimulating the insulin secretion from the pancreatic beta cells (insulin secretagogues) [5, 6]. Practically, GLM has a reduced solubility in water and is chemically identified as 1-[[p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]phenyl]sulfonyl]-3-(trans-4-methyl cyclo-hexyl) urea [7]. GLM not only can control the blood sugar level in combination with other antidiabetic agents but also as monotherapy in many cases. Furthermore, some findings support its superiority in comparison to other sulfonylureas such as glibenclamide for patients with cardiovascular complications; by not interfering with the adaptive myocardial protection mechanism of ischemic pre-conditioning [8], and minimal effects on the cardiac and blood flow in comparison to other sulfonylureas [9, 10]. Introducing GLM in the treatment strategies for diabetic patients with hypertension is highly likely due to its relative safety in addition to the recommendations to be used as a second-line or third-line therapy in type 2 diabetes which is favoured by the socioeconomic factors such as the low-cost and the accessibility (availability in most countries) [11].

Amlodipine Besylate (AML) is a potent antihypertensive agent reducing the intracellular calcium ions flow leading to vasodilation and reducing heart contractility to oppose the hypertension mechanism. It is chemically distinguished as a racemic mixture of 3-Ethyl 5-methyl (4RS)-2-[(2-aminoethoxy)-methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulfonate and the (-)-(S)-enantiomer showing superior pharmacological activity [12, 13].

Perindopril arginine (PA) is another antihypertensive agent which is often combined with AML. It works by diminishing the activity of the angiotensin-converting enzyme (ACE) thus interfering with the renin angiotensin aldosterone system (RAAS) the important homeostasis pathway for blood pressure [14, 15].

Fixed dose combinations of PA and AML with different as once-daily tablets are available in the markets under different trade names. It showed superior efficacy and tolerability in comparison to monotherapy use of either one, and hence making it a good candidate to be used for the treatment of hypertension and its complications [16, 17]. So, it was not surprising to find previous analytical methods for quantitation of PA and AML complying with the guidelines and the regulations of International Conference of Harmonization (ICH) [18]: either together [19, 20], or with other antihypertensive agents [21-23]. To the best of our knowledge, there is no single method was developed to determine the concentrations of these three drugs simultaneously, which will be of great value knowing that the treatment for hypertension and diabetes in same patient using the three medications PA, AML and GLM is of great possibility nowadays, especially within the countries with socioeconomical challenges. Several studies have been conducted and many analytical methods have been developed for the simultaneous determination and stability testing of PA, AML and GLM. It revealed that the analytical methods for the individual assessment of the target drugs are available; nonetheless, no reported analytical methods have been described for the assessment of the combination of PA, AML and GLM. Numerous HPLC and spectroscopic methods were reported for the simultaneous estimation of PA, AML and GLM in combination with other drugs in different matrices [19-24]. However, the RP-HPLC method has been commonly used for the determination and stability testing of PA, AML and GLM because of its accuracy, precision, sensitivity and specificity. Thus, it is of great importance to develop and validate an RP-HPLC analytical method for the quantitation of PA, AML and GLM all-in-one combinations. The force degradation studies play a significant part in the development and validation process since they can help to assess the degradation routes and identify the degraded products of drugs, which in return could provide valuable data on the stability of drugs and consequently facilitate the development of pharmaceutical formulations, manufacturing and packing [25].

Sequentially, this process contributes to monitoring the quality of pharmaceutical formulations. It is suggested that stress degradation studies on drugs should be conducted to evaluate the impact of several factors on pharmaceutical formulations such as hydrolysis (acid, base and neutral), thermal (wet and dry), oxidative and photolytic degradation. Consequently, the aim of the current study was to develop and validate a reversed-phase HPLC method for the simultaneous determination of PA, AML and GLM in solutions and to evaluate the forced degradation studies according to the ICH guidelines [18].

Materials and Methods

Chemicals and Reagents

Methanol, Chromosolv. for HPLC ≥ 99.9%, was bought from Honeywell/Riedel-de Haën, (Muskegon, MI, USA).

Acetonitrile, Chromosolv. for HPLC ≥ 99.9%, was purchased from Honeywell/Riedel-de Haën, (Muskegon, MI, USA). Water, for HPLC Plus, was purchased from Carlo Erba (France). Potassium di-hydrogen phosphate was bought from Sigma Aldrich (MO, USA). Ortho-phosphoric acid (85.0%) was purchased from BBC Chemicals (China). Sodium hydroxide (NaOH) was acquired from Xilong Scientific Co. Ltd. (China). Hydrochloric acid (35 - 38%) was purchased from LOBA Chemie (Mumbai, India). Hydrogen peroxide (30%, w/w) was bought from Lab Chem (PA, USA). Amlodipine Besylate (100.0%, w/w) and Glimepiride standards (99.8%, w/w) were donated by JOSWE Medical (Amman, Jordan). Perindopril Arginine standard (99.3%, w/w) was obtained from Tabuk Pharmaceuticals (Riyadh, Saudi Arabia).

Instruments and conditions

The HPLC instrument was manufactured by Hitachi Technologies (Tokyo, Japan). The analysis was performed using a VWR-Hitachi Elite LaChrom system which consists of the following components (solvent delivery pump L-2130, column oven L-2300, sample injection autosampler L-2200, PDA Detector L-2455, an organizer L-2000 system), equipped with a Supelcosil LC-18-column (25 cm × 4 mm × 5 µm, SUPELCO), at a flow rate of 1.0 mL/min, and detection wavelength of 220.0 nm for PA, AML and GLM. The analysis and processing were controlled by EZChrom Elite soft-ware (ver. 3.3.2, Agilent (CA, USA)). The sample injection was conducted using HPLC syringe filters with 0.45 µm pore size. The chromatographic conditions are summarised in Table I.

Table I

The proposed chromatographic conditions

Stationary phase	ODS or C18 (octadecyl silane)
Mobile phase	acetonitrile:MeOH:phosphate buffer (20:50:30)
pH	3.5
Pump mode	isocratic
Detection wavelength	220 nm
Injection volume	15 µL
Flow rate	1.0 mL/min
Column temperature	25ºC
Total run time	16 mins

Development and preparation of mobile phase

More than 36 combinations of the mobile phase preparations were tested. The development included several changes in the following parameters: type of solvent, the composition of the mobile phase, pH of the mobile phase, wavelength of absorption, flow rate and injection volume. The optimum mobile phase used for analysis consisted of a mixture of acetonitrile, methanol and phosphate buffer in the ratio of (20:50:30) (v/v/v) which was adjusted to pH 3.5 ± 0.1 with orthophosphoric acid. The mobile phase was degassed and then was filtered through a 0.45 µm filter. The injection volume was 15 µL.

Preparation of buffer solution

The buffer solution was prepared by dissolving 6.80 g of KH_2PO_4 in 1.00 L of deionised water.

Preparation of standard solutions

The PA standard solution was prepared by transferring 1007.0 mg of the PA standard (equivalent to 1000.0 mg of PA standard) into a volumetric flask (100.0 mL), and about 80.0 mL of methanol was added. Next, the solution was sonicated to dissolve and then it was diluted to the final volume with methanol to prepare a 10.0 mg/mL concentration.

The AML standard solution was prepared by transferring 1000.0 mg of the AML standard (equivalent to 1000.0 mg of AML standard) into a volumetric flask (100.0 mL) and about 80.0 mL of methanol was added. Next, the solution was sonicated to dissolve and then it was diluted to the final volume with methanol to prepare a 10.0 mg/mL concentration.

The GLM standard solution was prepared by transferring 120.2 mg of the GLM standard (equivalent to 120.0 mg of GLM standard) into a volumetric flask (100.0 mL), and about 80.0 mL of methanol was added. Next, the solution was sonicated to dissolve and then it was diluted to the mark with methanol to prepare a 1.2 mg/mL concentration.

Preparation of calibration curves

The calibration curve solutions for the PA, AML and GLM were constructed freshly by diluting the standard stock solution of each drug with methanol in the concentration range of (0.25 - 2.00 mg/mL), (0.10 - 0.75 mg/mL) and (0.10 - 0.75 mg/mL), respectively. Each standard solution was injected in triplicate. A linear relationship was acquired when the drug peak area was plotted against the drug's concentration, and the regression equation was estimated.

Analytical method validation

Several validation factors including limit of detection (LOD), limit of quantitation (LOQ), linearity, system suitability, accuracy, precision, robustness and forced degradation studies were performed according to ICH guidelines. All sample measurements were conducted in triplicates.

System suitability

Fifteen microliters of the three drugs mixture (0.25 mg/mL of PA, 0.10 mg/mL of AML and 0.10 mg/mL of GLM) were injected into HPLC six times under

the optimised and suitable chromatographic conditions, and the analytical method was carried out and validated according to the International Council for Harmonisation guidelines [18]. The peak area, (RSD), retention time, theoretical plates (*N*), resolution, asymmetry factor, retention factor and selectivity factor for each drug were determined. This test was conducted in order to validate and assess the system suitability of the designed method.

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

LOD and LOQ were carried out according to the ICH guidelines. The LOD is the lowest concentration of a certain analyte that can be detected in a solution, but not essentially being quantified, with a signal-to-noise (S/N) ratio of at least 3. The limit of quantitation (LOQ) is known as the lowest concentration of a certain analyte that could be quantified with suitable accuracy and precision, with a signal-to-noise (S/N) ratio of at least 10.

Linearity

The linearity of the designed method was assessed using linear regression analysis by analysing standard mixtures solutions of PA, AML and GLM at six different concentrations, and the linearity of the three drugs was found and evaluated in the concentration range of 0.25 - 2.00 mg/mL for PA, 0.10 - 0.75 mg/mL for AML and GLM. The standard mixture solutions were injected into the HPLC system in triplicates, where the injection volume was (15 µL). The standard calibration curve was plotted between the peak area against concentration. The r-squared correlation coefficient (R^2) and slope were determined.

Accuracy

The accuracy of the designed method was assessed by determining the recovery percentage and relative standard deviation (RSD) of the three drugs (PA, AML and GLM). The accuracy was evaluated by analysing the calibration curve standard solutions and three quality control samples at 80, 100 and 120% levels (low, medium and high). The recoveries of the three added drugs were estimated by correlating the concentration of the pre-analysed drug with the spiked drug's concentration. The concentrations of the three drugs were calculated from the linear regression equations.

Precision

The precision of the proposed HPLC method was determined over three concentration levels (50, 100 and 150%) within the linear range for PA, AML and GLM. The samples were analysed six times (n = 6) in one day and nine times over three sequential days (n = 9), to evaluate the intra-day (repeatability) and inter-day precision (intermediate precision). The precision was expressed as a percentage RSD for the area of each drug (recovery).

Robustness

The robustness evaluation of the proposed method was performed by applying slight variations in several conditions, such as the pH of the mobile phase, wavelength, column temperature and flow rate. The robustness was evaluated for mixtures of the target drugs (PA, AML and GLM) based on the variation in the peak areas (recovery) and their RSD% when compared to the optimised conditions.

Forced degradation studies

The forced degradation studies of PA, AML and GLM were conducted under several conditions such as neutral (unstressed), acid, base, neutral hydrolysis, thermal, oxidative and photolytic degradation. These studies were carried out to illustrate the chemical stability of the drugs. The target drugs mixture was prepared by mixing an appropriate amount of PA (0.75 mg/mL), AML (0.30 mg/mL) and GLM (0.30 mg/mL) in a volumetric flask (10.0 mL) and completed to the mark with methanol. The solutions and samples were analysed using the HPLC in triplicates under the optimised chromatographic conditions.

Acid degradation

The acid degradation was investigated by mixing 1.0 mL of 1.0 M HCl with the drugs mixture in a volumetric flask (10.0 mL) and completed to the mark with methanol. The resulting mixture was then heated in a controlled temperature bath at 60 ± 1°C for a period of 30 minutes.

Base degradation

The base degradation was investigated by mixing 1.0 mL of 1.0 M NaOH with the drugs mixture in a volumetric flask (10.0 mL) and completed to the mark with methanol. The resulting mixture was then heated in a controlled temperature bath at 60 ± 1°C for a period of 30 minutes.

Neutral degradation

The neutral degradation was investigated by applying two different conditions to the original mixture which was prepared by mixing an appropriate amount of PA (0.75 mg/mL), AML (0.30 mg/mL) and GLM (0.30 mg/mL) in a volumetric flask (10.0 mL) and completed to the mark with methanol. The first trial was conducted by storing the mixture, protected from sunlight, at room temperature (25.0ºC) for seven days. The second trial was conducted by storing the mixture, protected from sunlight, in the refrigerator at 2 - 8ºC for seven days.

Thermal degradation

The thermal degradation was performed for both solution and solid states (wet and dry degradation) of the drugs by heating the drugs mixtures at a specific temperature over a specified time period. The solid-state stability experiment was conducted on a sample by heating a suitable amount of the three drugs in a laboratory hot-air oven at 100ºC for 24 hours. Then, the drugs were transferred into a volumetric flask (10.0 mL) and dissolved in methanol. The liquid-state stability experiment was conducted on a sample by heating the drugs mixture solution in a laboratory hot-air oven at 80ºC for 8 hours.

Oxidative degradation.

The oxidative degradation was investigated on the drugs mixture solution using two different concentrations of hydrogen peroxides (H_2O_2) in which the solutions were heated for a certain period of time at a specific temperature. The first mixture was prepared by mixing 1.0 mL of 10% H_2O_2 with the drugs mixture in a volumetric flask (10.0 mL) and completed to the mark with methanol. The second mixture was prepared by mixing 1.0 mL of 30% H_2O_2 with the drugs mixture in a volumetric flask (10.0 mL) and completed to the mark with methanol. Then, each drugs mixture solution was heated in a controlled temperature bath at 60 ± 1°C for a period of 30 minutes.

Photolytic degradation

The photodegradation was conducted on the liquid-state drugs mixture where the solutions were exposed to two different conditions. The first drugs mixture solution was exposed to sunlight for a period of 24 hours. The second drugs mixture solution was exposed to ultra-violet light (UV) at an energy of 200 watt hrs. *per* m^2 and to visible light (Vis) at an illumination of not below than 1.2 million lux hrs for a period of 7 days.

Results and Discussion

The current study indicates the suitability of an RP-HPLC method for the simultaneous determination of PA, AML and GLM in solution. The proposed HPLC method is simple, accurate, fast, robust, sensitive and suitable. It produced a sufficient separation of PA, AML and GLM with good retention times, peak shape, asymmetry factor and resolution. Optimization and development of the mobile phase were conducted where various preparations of mobile phases were tested based on the asymmetric factor, number of theoretical plates, retention time and resolution. Different ratios of aqueous and organic phases were investigated, and the pH was adjusted from 2.0 to 8.0 with o-phosphoric acid and NaOH. After extensive numerous experiments using different combinations, the optimum mobile phase was found to be a mixture of acetonitrile, methanol and phosphate buffer in the ratio of (20:50:30) (v/v/v) which was adjusted to pH 3.5 ± 0.1 with orthophosphoric acid. Furthermore, various wavelengths which range from 205 nm to 245 nm were tested and scanned, taking into consideration the λ_{max} of each drug. Finally, the detection wavelength 220 nm was thoroughly chosen for analysis and quantitation of PA AML and GLM. The flow rate was optimised at 1.0 mL/min. The optimum total run time was 16 minutes with a retention time of 4.408 mins for PA, 5.415 mins for AML and 12.189 mins for GLM. The HPLC-DAD overlay chromatogram of PA, AML and GLM standard mixture calibration curve and the chromatographic analysis results are shown in Figure 1. The designed method was validated according to the following parameters: system suitability, range, linearity, LOD, LOQ, accuracy, precision (intra- and inter-day), robustness and forced degradation studies.

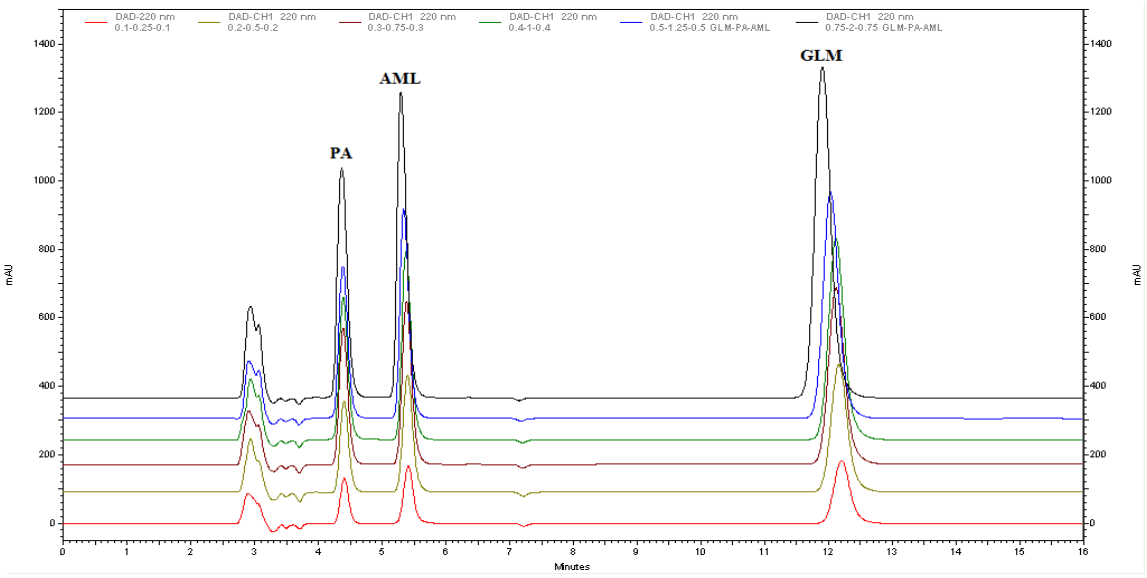

Figure 1.

An overlay chromatogram of the calibration curve for perindopril arginine, amlodipine and glimepiride mixture

Method validation

LOD and LOQ. The LOD values for PA, AML and GLM were found to be 0.0125 mg/mL, 0.010 mg/mL and 0.00625 mg/mL, respectively. While the LOQ values for PA, AML and GLM were found to be 0.075 mg/mL, 0.040 mg/mL and 0.020 mg/mL, respectively.

Linearity. As described earlier, using the optimised condition of the proposed method, the regression analysis was used to evaluate the linearity, relationship between the peak area *versus* concentration, and calculate the correlation coefficients for all drugs. The calibration curve plots of six different concentrations

proved to be linear for PA in the range of 0.25 - 2.00 mg/mL (R^2 = 0.9995), for AML in the range of 0.10 - 0.75 mg/mL (R^2 = 0.9992) and for GLM in the range of 0.10 - 0.75 mg/mL (R^2 = 0.9988). The analysis was conducted and repeated six times to calculate the linear regression equations. The linearity results are summarised in Table II.

Table II

Regression data for calibration curves of PA, AML and GLM

	PA	AML	GLM
Linearity range (mg/mL)	0.25 - 2.00	0.10 - 0.75	0.10 - 0.75
Slope	21908343.08	76178606.79	137748952.7
Intercept	-349088.51	159145.29	130410.81
Correlation coefficient (R^2)	0.9995	0.9992	0.9988
LOD (mg/mL)	0.0125	0.010	0.00625
LOQ (mg/mL)	0.075	0.040	0.020

The system suitability test was applied to the chromatographic results that were obtained using the optimum conditions in order to evaluate several chromatographic parameters such as peak area, RSD, retention time, plates count (*N*), resolution, asymmetry factor, retention factor and selectivity factor. Six injections of the standard mixture solutions of PA, AML and GLM were analysed for this purpose. All of the chromatographic parameters are summarised in Table III.

Table III

The system suitability studies for PA, AML and GLM

	Perindopril Arginine	Amlodipine	Glimepiride
Peak Area	20959005.33	29861316.33	54160604.67
Relative standard deviation (RSD)	4.691%	1.553%	2.735%
Retention time (t_R) ± SD	4.408 ± 0.001	5.415 ± 0.004	12.189 ± 0.016
Theoretical plates (N)	2002.4	2047.9	3048.8
Resolution (Rs)	2.309	---	9.949
Asymmetry factor (AF)	1.079	1.095	1.107
Retention factor (k), Capacity factor	0.520	0.867	3.203
Selectivity factor (α)	1.668	---	3.693

The acceptable limits of the resolution should be ≥ 1.5, column efficiency (plates count) ≥ 2000, asymmetry factor should be ≤ 2 and the RSD% should be ≤ 2. The system suitability chromatographic parameters of the proposed method were found to lie within the acceptance criteria. The system suitability tests confirmed that the proposed method was suitable for the intended analysis and purpose.

The accuracy of the proposed analytical method was evaluated by assessing the recovery of PA, AML and GLM at three quality control samples at 80, 100 and 120% levels (low, medium and high). Every concentration of the quality control samples was analysed in triplicate. The results of recovery studies ranged from 98.632 to 101.928% and the RSD% values were found to be less than 2.00 for all three drugs. The results of percentage recoveries revealed that the data lies within the acceptance range, hence, the proposed method is accurate, and it can be employed for the determination of PA, AML and GLM. The results of the accuracy studies are presented in Table IV.

Table IV

The accuracy studies results (n = 3)

Conc. Level	Amount Added (mg/mL)			Amount Found (mg/mL)			Recovery%			RSD%		
	PA	AML	GLM	PA	AML	GLM	PA	AML	GLM	PA	AML	GLM
80%	0.400	0.180	0.180	0.399	0.182	0.179	99.754	101.022	99.288	0.617	0.927	1.181
100%	0.500	0.200	0.200	0.510	0.197	0.201	101.928	98.632	100.652	0.577	0.073	1.129
120%	0.600	0.220	0.220	0.606	0.218	0.220	100.963	99.166	100.164	0.763	0.772	1.144

The intraday precision (repeatability) was examined by analysing six quality control sample solutions (n = 6) on the same day while the inter-day precision (intermediate) was examined by analysing nine quality control sample solutions (n = 9) over three consecutive days. The assay and RSD% were calculated to evaluate the precision of the peak area of PA, AML and GLM. The intra- and inter-day precision results showed low values of RSD% (RSD% < 2), which suggested that the method is precise. The results of the assay ranged from 98.968 to 101.937% and the RSD% values were found to be less than 2.00 for all three drugs. The results of intra-and inter-day precision are illustrated in Table V.

Table V

The precision studies results

Parameter	Conc. level	Amount Added			Amount Found			Assay%			RSD%		
		PA	AML	GLM	PA	AML	GLM	PA	AML	GLM	PA	AML	GLM
Intra-day precision (n = 6)	50%	0.250	0.100	0.100	0.249	0.102	0.099	99.532	101.968	99.055	1.944	0.866	1.885
	100%	0.500	0.200	0.200	0.507	0.198	0.201	101.363	98.968	100.343	1.081	1.271	0.576
	150%	0.750	0.300	0.300	0.765	0.300	0.297	101.937	100.077	99.046	1.803	0.937	1.254
Inter-day precision (n = 9)	50%	0.250	0.100	0.100	0.251	0.100	0.101	100.231	99.748	100.517	1.915	1.948	1.768
	100%	0.500	0.200	0.200	0.502	0.201	0.202	100.338	100.305	100.752	1.094	1.033	1.019
	150%	0.750	0.300	0.300	0.757	0.300	0.301	100.925	99.875	100.440	1.199	1.285	1.732

The proposed method did not show any considerable change in the recovery percentage when small variations were applied to certain parameters such as pH, wavelength, flow rate and column temperature. The peak area data were acquired and calculated for the recovery and RSD%. The recoveries ranged from 66.215% to 125.208% and the RSD% values were found to be less than 2.00 for all three drugs. In specific cases, the results displayed high recoveries at higher flow rates and longer wavelengths while they displayed smaller recoveries at small flow rates and shorter wavelengths. Rather than that, the recoveries ranged from 98.814% to 102.055%. Due to intentional and slight changes in specific parameters in the method, no significant changes were observed in the peak area and recovery, the proposed method is reasonably robust. The results of robustness studies are illustrated in Table VI.

Forced degradation studies

The forced degradation studies were conducted in several conditions including acid hydrolysis, base hydrolysis, neutral hydrolysis, thermal, oxidative and photolytic degradation using standard mixtures solution of PA, AML and GLM. The solutions were injected in triplicate and the chromatograms of PA, AML and GLM displayed well-separated and resolved peaks of the three drugs. However, in specific stress conditions, the chromatograms displayed the appearance of degradation products or a significant decrease in the peak area. The results of the forced degradation studies are illustrated in Table VII.

Table VI

The robustness results

Parameter	Modification	Recovery%			RSD%		
		PA	AML	GLM	PA	AML	GLM
pH (± 0.2)	3.3	101.900	99.738	100.907	0.152	0.984	0.846
	3.5	100.000	100.000	100.000	0.084	0.046	0.032
	3.7	101.863	102.055	101.581	0.288	0.649	0.506
Flow rate (± 0.2 mL/min)	0.8	123.736	123.769	125.208	1.178	0.657	0.643
	1.0	100.000	100.000	100.000	0.726	0.824	0.871
	1.2	66.215	79.248	90.907	1.744	1.783	1.332
Column Temperature (± 5ºC)	20	99.125	100.055	99.938	0.681	0.966	1.304
	25	100.000	100.000	100.000	0.920	1.502	0.769
	30	99.876	98.814	100.445	0.809	1.919	0.538
Wavelength (± 2 nm)	218	116.026	104.914	94.000	0.212	0.444	0.387
	220	100.000	100.000	100.000	0.098	0.316	0.394
	222	72.202	96.556	112.112	0.837	0.569	0.356

Table VII

The forced degradation studies data

Stress Conditions (n = 3)	PA			AML			GLM		
	Peak Area	Assay %	Deg. %	Peak Area	Assay %	Deg. %	Peak Area	Assay %	Deg. %
Standard (Unstressed)	17744393			25689890			42356600		
Acid Hydrolysis (1 M, 60ºC, 30 mins.)	15489071	87.29	12.71	22708034	88.39	11.61	36885330	87.08	12.92
Base Hydrolysis (1 M, 60ºC, 30 mins.)	16651300	93.84	6.16	22086360	85.97	14.03	40565264	95.77	4.23
Neutral Hydrolysis (25ºC, 7 days)	17042795	96.05	3.95	23675029	92.16	7.84	41557101	98.11	1.89
Neutral Hydrolysis (2 - 8ºC, 7 days)	17563523	98.98	1.02	25251194	98.29	1.71	42288226	99.84	0.16
Thermal (Wet, 80ºC, 8 hrs.)	17063452	96.16	3.84	22829910	88.87	11.13	11475434	27.09	72.91
Thermal (Dry, 100ºC, 24 hrs.)	1792915	10.10	89.90	1792852	6.98	93.02	23241814	54.87	45.13
Oxidative (10% H_2O_2, 60ºC, 30 mins.)	15781857	88.94	11.06	23387356	91.04	8.96	40003044	94.44	5.56
Oxidative (30% H_2O_2, 60ºC, 30 mins.)	15402614	86.80	13.20	22331793	86.93	13.07	38366352	90.58	9.42
Photolytic, UV (≥ 1.2 million lux hours, 200 watt hrs./square, 7 days)	17705755	99.78	0.22	25424349	98.97	1.03	41958446	99.06	0.94
Photolytic, exposed to sun light 24 hrs.)	15096608	85.08	14.92	21867422	85.12	14.88	40002180	94.44	5.56

Acid hydrolysis

The acid degradation studies displayed a decrease in the peak area and height of PA, AML and GLM. The results of acidic degradation of PA, AML and GLM displayed a degradation of 6.16%, 14.03% and 12.92%, respectively. This investigation revealed that the standards mixture solution of the drugs was reasonably stable.

Base hydrolysis

The base degradation studies displayed a small decrease in the peak area and height of PA, AML and GLM. The results of basic degradation of PA, AML and GLM displayed a degradation of approximately 12.71%, 11.61% and 4.23%, respectively. It revealed that the drugs showed good stability compared to that when they were exposed to acid degradation.

Neutral hydrolysis

The neutral hydrolysis displayed high stability and only a slight decrease in the peak area was observed. The chromatograms of the standards mixture solutions, when stored at a temperature of 2 - 8ºC for 7 days, displayed a degradation of 1.02%, 1.71% and 0.16% for PA, AML and GLM, respectively. When the solutions were stored at room temperature (≈ 25ºC) for 7 days, the results displayed a degradation of 3.95%, 7.84% and 1.89% for PA, AML and GLM, respectively.

Thermal degradation

The thermal (liquid state) degradation test displayed good stability for PA and AML while GLM showed the most susceptibility toward thermal degradation. The results indicated a degradation of 3.84%, 11.13% and 72.91% for PA, AML and GLM, respectively. This test resulted in the formation of an extra peak at a retention time of 4.85 minutes which might demonstrate the formation of a degraded product.

The thermal (solid state) degradation test demonstrated poor stability for PA and AML and GLM against thermal stress conditions, and the chromatograms displayed a very significant decrease in the peak area. The results indicated degradation of 89.90%, 93.02% and 45.13% for PA, AML and GLM, respectively. However, no distinct peaks of degraded products were observed.

Oxidative degradation

The oxidative stress conditions were conducted on the drugs mixture solution using 10% and 30% H_2O_2. The drugs displayed a decrease in peak area and reasonable stability against oxidative degradation. When the drugs were treated with 10% H_2O_2, the HPLC results indicated a degradation of 11.06%, 8.96% and 5.56% for PA, AML and GLM, respectively. Furthermore, the drugs were also treated with 30% H_2O_2 and the results revealed degradation of 13.20%, 13.07% and 9.42% for PA, AML and GLM, respectively. This test resulted in the formation of an extra peak of a possible degradation product at a retention time of 3.05 minutes.

Photolytic degradation

The photolytic degradation studies were performed by exposing the drugs mixture solution to UV light (at an illumination of not below than 1.20 million lux hrs) for a period of 7 days. No change in the peak area was observed and the drugs mixture solutions displayed high susceptibility toward photolytic (UV) stress conditions. The results indicated degradation of 0.22%, 1.03% and 0.94% for PA, AML and GLM, respectively. The photolytic degradation was also investigated by exposing the drugs mixture solution to direct sunlight for 24 hrs. The drugs demonstrated good stability against exposure-to-sunlight stress conditions and the results indicated degradation of 14.92%, 14.88% and 5.56% for PA, AML and GLM, respectively.

Conclusions

A simple, accurate, precise and selective RP-HPLC method for the quantitation of PA arginine, AML and GLM was developed and validated in accordance with the ICH guidelines. The chromatograms showed no interference at the retention times of PA, AML and GLM in the blank samples. The data displayed a good correlation between the concentration of each drug with peak area under the developed and optimised conditions. The results of recoveries ranged from 98.0 to 102.0% and the RSD% values were found to be less than 2.00 for all three drugs. Additionally, the stability of PA, AML and GLM was investigated under acid, base, neutral, thermal, oxidative and photolytic conditions. The degraded products were detected under thermal (wet) and oxidative stress conditions. The drugs were found to be highly stable under the stress conditions except for the thermal conditions (wet and dry). Thermal (wet) stress conditions resulted in GLM degradation while thermal (dry) stress conditions resulted in PA, AML and GLM degradation. The proposed stability-indicating method can be employed in routine analysis of pharmaceutical formulations and stability studies of PA, AML and GLM, with a high level of accuracy, intraday and inter-day precision.

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Conflict of interest

The authors declare no conflict of interest.

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