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Development and Validation of a simple RP-HPLC method for the simultaneous determination of Amlodipine besylate and Glimepiride. Application to in-vitro release study of bilayer tablet

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ABSTRACT

A simple, rapid, and precise RP-HPLC method for simultaneous analysis of Amlodipine besylate and Glimepiride in bulk and its pharmaceutical formulations has been developed and validated. Amlodipine besylate was separated from Glimepiride by using Grace Smart Altima C8 column (25 cm × 4.6 mm, 5-µm) with a mobile phase consisting of acetonitrile: 20mM phosphate buffer (55:45 (v/v), pH 3.5) a flow rate of 1 mL/min and detection wavelength at 230 nm. Amlodipine besylate and Glimepiride were eluted with retention times of 5.47 min and 14.17 min respectively. The method was validated for accuracy, precision, linearity, specificity and sensitivity in accordance with ICH (Q2B) guidelines. The results of all the validation parameters were found to be within the acceptable limits. The calibration plots were linear over the concentration ranges from 70-3000ng/mL for Amlodipine besylate and 100-3000ng/mL for glimepiride. The limit of detection and limit of quantification were found to be 19.4ng/mL and 58.8ng/mL for amlodipine besylate, 25.6ng/mL and 76.2ng/mL for glimepiride respectively for both the drugs. From the results it is suggested that the method is simple, reproducible, accurate and precise. The method was successfully applied for the determination of content and the dissolution profile of the combined bilayer tablet dosage form.

Keywords: Amlodipine, Glimepiride, RP-HPLC, Simultaneous determination, Validation.

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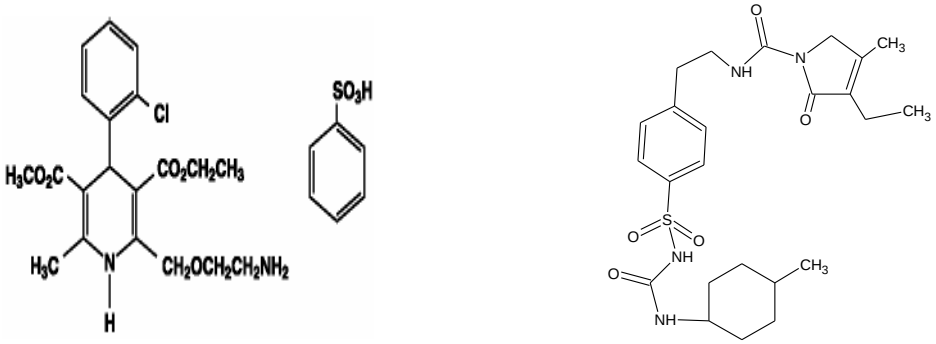
INTRODUCTION

Amlodipine besylate (AMD) is a white crystalline powder, slightly soluble in water and sparingly soluble in ethanol.AMD is (Figure 1a) chemically described as 3-ethyl-5-methyl (±)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate, monobenzenesulphonate (MW 567.1) [1, 2]. AMD inhibits calcium ion influx across cell membranes selectively, with a greater effect on vascular smooth muscle cells than on cardiac muscle cells. Serum calcium concentration is not affected by AMD. AMD is a peripheral arterial vasodilator that acts directly on vascular smooth muscle to cause a reduction in peripheral vascular resistance and reduction in blood pressure [3,4]. Glimepiride (GLM) is a white to yellowish-white, crystalline, odorless to practically odorless powder and is practically insoluble in water. Chemically it is (Figure 1b) 1-[[ρ-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido) ethyl] phenyl] sulfonyl]-3-(trans-4-methylcyclohexyl) urea. GLM acts by lowering blood glucose appears to be dependent on stimulating the release of insulin from functioning pancreatic beta cells, and increasing sensitivity of peripheral tissues to insulin. GLM is likely binds to ATP-sensitive potassium channel receptors on the pancreatic cell surface, reducing potassium conductance and causing depolarization of the membrane. Membrane depolarization stimulates calcium ion influx through voltage-sensitive calcium channels. This increase in intracellular calcium ion concentration induces the secretion of insulin [5-7].



(a) (b)

Figure. 1. Chemical structures of drugs used in this method. (a) Amlodipine besylate and (b) Glimepiride

The literature survey revealed that no HPLC methods were reported for the simultaneous determination of AMD and GLM till date. Methods are available for the quantification of AMD individually and with other combinations other than GLM [8-10]. Methods are available for the quantification of GLM individually and with other combinations other than AMD [11-16]. The objective of the present investigation was to develop a simple and novel method for the

simultaneous determination AMD and GLM by employing high pressure liquid chromatography method. The scope of this method was to determine the drug content and in-vitro release of each drug from the newly formulated combined bilayer tablet dosage form. The analytical method employed for the quantitative determination of drug in formulation plays a significant role in the evaluation and interpretation of drug release from the formulation. Therefore, a complete validation of analytical method was performed accordance to ICH guidelines[17] to yield reliable results that could be satisfactorily interpreted.

MATERIALS AND METHODS

Instrumentation

The instruments employed in this study were follows; HPLC-1260 infinity series with auto-sampler, Agilent Technologies, Dissolution apparatus- Labindia, Mumbai, India. Analytical balance- Afcoset, Mumbai, India, pH meter- Systronics, Ahmadabad, India.

Standards and chemicals

AMD and GLM were gift samples obtained from AurobindoPharma (Hyderabad, India). HPLC grade water from(SD fine chemicals),Acetonitrile of HPLC grade were purchased from Merck Ltd. (Mumbai, India), and o-phosphoric acid, Sodium dihydrogen phosphate of A.R. From S. D. Fine chemicals Pvt.Ltd Mumbai, India.

Stock and working solution preparation

***Preparation of standard stock solution*:**

10mg of AMD and GLM was weighed and transferred into a 10mL volumetric flask dissolved and the volume was made up with methanol. A standard solution of AMD and GLM was prepared by suitable dilution of the stock solution with the mobile phase. The sample was prepared by spiking the required volume of working stock solution (from the stock solution, 100µg/mL) into 10 mL volumetric flasks and volume made up of with the mobile phase for linearity and validation of method.

***Preparation of a buffer*:**

1.36g of potassium dihydrogen orthophosphate was weighed and dissolved in 500ml of HPLC grade water and the pH was adjusted to 3.5 by using orthophosphoric acid.

Sample preparation:

The sample from the dosage form prepared by transferring the 1mL of solution of extracted drugs from the dosage forms with methanol, into the 10 ml volumetric flask and volume made up of with mobile phase.

Method validation

The validation parameters like linearity, sensitivity, accuracy, precision, recovery and stability of drugs, was done according to the ICH guidelines[17]. Selectivity is studied by comparing the chromatograms obtained from the blank sample with the chromatogram obtained from a standard drug mixture. Calibration curves are prepared by assaying standard samples contains two drugs, ranging from 50-3000 ng/mL. The linearity of the method was determined by plotting the peak area (*y*) of the drug versus the nominal concentration (*x*) of drug, respectively. The calibration curves are constructed by least squares linear regression.

Intra- and inter-day accuracy and precision of this method were determined at three different concentration levels on 3 different days, and on each day, three replicates were analyzed with independently prepared calibration curves. The accuracy and precision are expressed as percentage accuracy and relative standard deviation (R.S.D., %) respectively and calculated by using equations (1) and (2).

$$\% \text{ Accuracy} = \frac{\text{Mean observed concentration}}{\text{Nominal concentration}} \text{ X } 100 \quad ----\text{Eq (1)}$$

$$\% \text{ RSD} = \frac{\text{Standad deviation}}{\text{Mean}} \text{ X } 100 ----- \text{Eq (2)}$$

The limit of detection (LOD) and limit of quantification (LOQ) are defined as the lowest concentration giving a signal-to-noise ratio of at least 3-fold and 10-fold, respectively. The LOD and LOQ of this method were verified based on the standard deviation of response and slope by using the equations (3) and (4).

$$\text{LOD} = \frac{3.3\ \sigma}{\text{Slope}} \quad ----- \text{Eq. (3)}$$

$$\text{LOQ} = \frac{10\ \sigma}{\text{Slope}} \quad ----- \text{Eq. (4)}$$

Where σ =standard deviation of intercept from calibration curve

Slope = Average slope of the calibration curve

The stability of the drug solution was determined for the short-term by keeping at room temperature (25°C) for 24h. Auto sampler stability was determined by storing the samples for 24 h in the auto sampler. Each sample injected three times into HPLC and concentrations obtained were compared with the nominal values of the QC samples. The stress studies were carried out by taking 100mg of each drug into 100mL volumetric flask and added 1ml of 0.1N hydrochloric acid for acid hydrolysis, 0.1N sodium hydroxide for alkali hydrolysis and 10% hydrogen

peroxide for oxidation[18], then samples were kept in water bath at 60°C for 1h and volume made up with mobile phase, to get suitable concentrations and injected into the HPLC.

Analysis of dosage form:

20 Tablets were weighed, and finely powdered. From this an accurately weighed sample of powdered tablets equivalent to 4mg of AMD and 2mg of GLM [equivalent to one tablet] was extracted with methanol in a 100ml volumetric flask using ultra sonicator. This solution was filtered through Whatmann No.1 filter paper. The solution obtained was diluted with the mobile phase to obtain a concentration in the range of linearity previously determined. All determinations were carried out in six replicates. The amount of drug recovered was calculated from the linearity graph. The dissolution was carried out for 8h, first 2h in acidic media pH 1.2 and then in pH 6.8 buffers at 100 rpm and samples were collected at different time interval and made up of a suitable dilution with the mobile phase and injected into the HPLC system. The concentration or release of each drug was calculated from the respective linearity graph.

RESULTS AND DISCUSSION

Method optimization:

AMD and GLM are hydrophobic, almost insoluble in aqueous solutions and are freely soluble in methanol; the reverse-phase chromatography was adopted. Hydrophobic C8 a stationary phase column was tried. During the method development, top priority was given for the complete separation of AMD, GLM. The chromatographic method was optimized by changing various parameters, such as the pH of the mobile phase, organic modifier and buffer used in the mobile phase and composition of the mobile phase. Buffers like Ammonium acetate, phosphate buffer in various strengths were tried along with methanol and acetonitrile as organic solvent. A mixture of acetonitrile and phosphate buffer (pH 3.5) (in the proportions of 40:60, 45:55, 50:50, 65:45, 60:40, 65:35, 80:20, 90:10, 70:30 (v/v), were tested as a mobile phase with Grace Smart C-8 column. The mobile phase composition of 45:55 v/v buffers: ACN was shown good resolution, retention time with minimal tailing factor in acceptable range. The method was optimized with the mobile phase composition of acetonitrile and phosphate buffer 55:45 (v/v).

Buffer molarity of 10, 20 and 50 mM was tested. There were no significant changes in the chromatographic response and peak shape with change in buffer molarity. A buffer molarity of 20 mM was selected for further analysis.

After several trials, the method was optimized as a mixture of 20mM potassium dihydrogen phosphate buffer (pH 3.5) and Acetonitrile (45:55 v/v), at a flow rate of 1mL/min, at 230nm for

run time of 16 min. These chromatographic conditions achieved satisfactory resolution, retention time and tailing for both drugs of AMD and GLM. The (Figure 2) shows that chromatogram of AMD and GLM and these are well separated from each other.

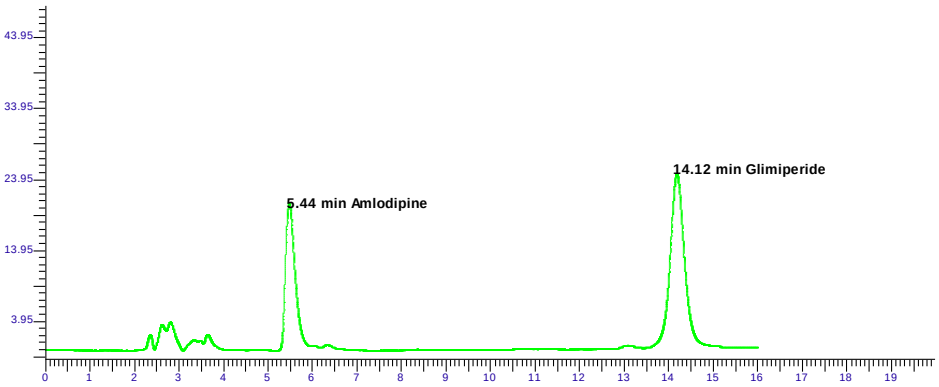


Figure 2 Standard chromatogram of AMD and GLM.

System suitability:

To check the system suitability, working stock standard of individual drugs were injected into HPLC to determine the individual retention times of drugs. Then working standard mixture solution was injected five times and a relative standard deviation (RSD) for five consecutive injections ≤2, resolution between two adjacent peaks ≥2 and tailing factor ≤2 acceptable values[19]were considered. Resolution (R), relative standard deviation from five replicates injections of a working standard mixture solution, tailing factor (T), retention time and peak area of individual drugs are presented in(Table 1). System suitability test confirmed that the chromatographic system was adequate for the analysis planned to be done.

Table.1. System suitability parameters of Amlodipine besylate (AMD) and Glimepiride (GLM)

Parameters	**Results**				**Required limits**
	AMD		**GLM**		
	Mean±SD	%RSD	Mean±SD	%RSD	
Retention time in minutes (R_t)	5.46±0.015	0.279	14.16±0.025	0.177	RSD≤2
Theoretical plates (N)	3347±39	1.16	8397±55	0.654	N>2000
Tailing Factor (T)	1.27±0.007	0.524	1.04±0.008	0.776	T≤2
Resolution(R_s)	7.74±0.056	0.730	18.53±0.056	0.302	R_s>2

Values are expressed in Mean± SD

METHOD VALIDATION

Selectivity:

The selectivity of the present method was established by checking the blank sample and observed the chromatogram. There was no interference found at retention times of AMD and

TEL in the blanks, indicates the selectivity of the method. The carryover effect of the present method was established by using six injections of blank and an upper limit of quantification (ULOQ) of AMD and GLM. These samples were analysed alternately to check any carryover in the blank sample. In this study there were no such effects observed.

Linearity:

The linearity of this method is evaluated by linear regression analysis, which is calculated by the least square method and the drug is linear in the concentration range of 70-3000 ng/mL for AMD and 100-3000ng/mL for GLM. Calibration standards are prepared by spiking required volume of working standard (100µg/mL) solution into a different 10 ml volumetric flasks and volume made up with methanol to yield concentrations of 70, 100, 200, 500, 1000, 2000 and 3000ng/mL of AMD and GLM. The resultant peak area of each drug was measured. Calibration curve is plotted between peak areas of drug against concentration of the drug. The (Figure 3) shows the linearity graph regression coefficient (r^2) including the slope and y-intercept.

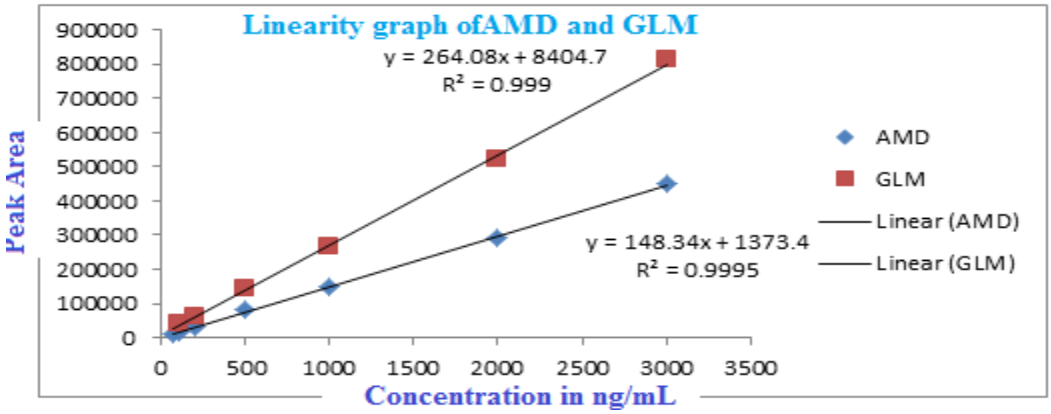


Figure 3. Linearity graph of AMD and GLM.

Sensitivity:

The limit of detection (LOD) and limit of quantification (LOQ) are defined as the lowest concentration giving a signal-to-noise ratio of at least 3-fold and 10-fold, respectively. The LOD and LOQ of this method were verified based on the standard deviation of response and slope found to be 19.42 ng/mL, 58.87ng/mL for AMD and 25.16 ng/mL, 76.25ng/mL.

Intra-day and Inter-day Precision and Accuracy*:*

The intra- and inter-day precision and accuracy of this method is determined by analysing replicates of QC samples at three concentrations on 3 different days. The coefficients of variation for the intra- and inter-day precision were <2.3%. The intra- and inter-day accuracies are 95-10-106.90% of AMD and 95.57-102.32% for GLM. The low levels of coefficients of variation (Table.2), indicate the method is accurate and precise.

Table.2. Intra and Inter-day accuracy, precision of AMD and GLM

Concentration (ng/mL)	Inter-day (n=6)				Intra-day (n-9)			
	AMD		GLM		AMD		GLM	
	Accuracy	RSD (%)	Accuracy	RSD (%)	Accuracy	RSD (%)	Accuracy	RSD (%)
1000	104.98±1.69	1.61	97.55±1.15	1.18	95.10±1.04	1.09	96.87±1.40	1.45
1500	99.13±2.95	2.97	100.82±1.68	1.67	106.94±1.09	1.02	95.90±0.47	0.49
2000	105.42±0.62	0.59	102.32±1.13	1.11	98.46±0.85	0.86	98.13±0.83	0.84

Values expressed Mean±SD

Robustness and Ruggedness:

Robustness of the method was done by changing slight variation in the parameters like mobile phase composition, flow rate and wavelength. Present method did not show any significant change when the critical parameters were modified. The tailing factor for both the drugs was always less than 2.0 and the components were well separated under all the changes carried out. Considering the modifications in the system suitability parameters and the specificity of the method, as well as carrying the experiment at room temperature, shows that the method conditions were robust. Ruggedness is studied along with precision and accuracy of batches where the effect of the column and analyst change are observed. The observed value for analyst variation and results obtained for precision and accuracy are within the acceptance criteria (i.e. there are no changes in the retention time, recovery and precision of the drug) according to ICH[17].

Stability studies:

The stability of the drug was studied at different conditions for quality control (QC) of samples. The samples were analyzed and compared with freshly analyzed QC samples, no difference were found in accuracy and precision. The **(**Table.3) represents the stability data of AMD and GLM at different conditions. In the forced degradation studies there was no degradation product was identified. The chromatograms of stress conditions are shown in (Figure. 4).

Table.3. Auto sampler and short-term stability of AMD and GLM (n=3)

Conc. (ng/mL)	Auto-sampler stability				Short-term stability			
	AMD		GLM		AMD		GLM	
	Accuracy	RSD (%)	Accuracy	RSD (%)	Accuracy	RSD (%)	Accuracy	RSD (%)
1000	105.23±1.18	1.12	98.40±1.30	1.32	105.71±1.31	1.24	106.88±1.53	1.43
1500	100.17±1.52	.52	101.78±1.66	1.63	99.96±1.43	1.43	100.62±0.98	0.99
2000	109.24±1.08	0.99	106.23±1.30	1.23	107.66±1.43	1.33	107.27±1.71	1.59

Values expressed Mean±SD

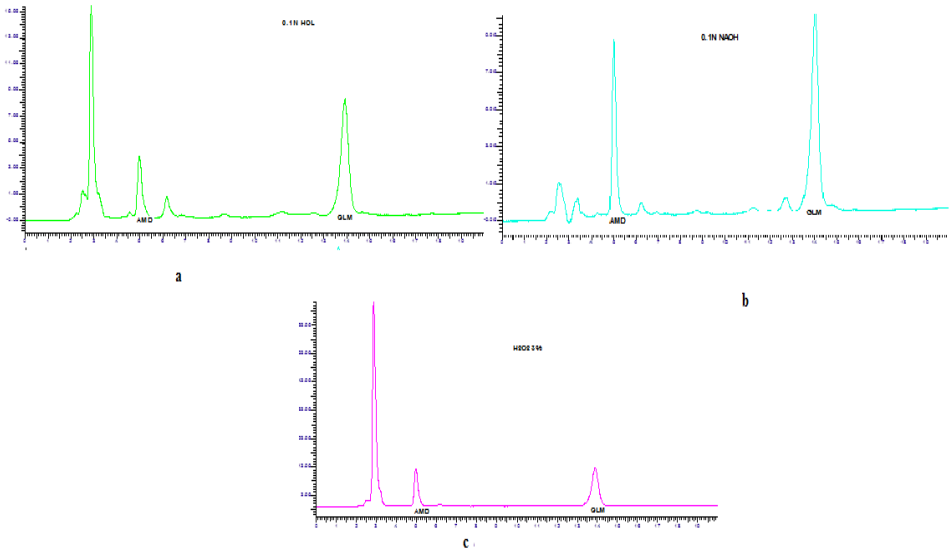


Figure 4. Chromatograms of AMD and GLM under stress conditions (a) in 0.1N hydrochloric acid (b) in 0.1N sodium hydroxide (c) 3% hydrogen peroxide

Application of method for assay and dissolution of dosage form:

The amount of each drug in the formulation has been determined and the percentage recovery was found to be 90.16- 105.88% for F2-F6 of AMD and F2-F5 for GLM, the data were represented in (Table.4).The assay of F1 formulation was found to be less than 80% for both drugs. The dissolution profiles of F2-F5 formulations have been studied for 8 h and F6 formulation for 2h to check the release of AMD. The dissolution was not performed for F1 formulation as it does not meet the requirement of content of drugs for assay. The (Figure 5) shows the dissolution profiles of formulations. Release profile of GLM was extended to 8h and more than 85% of AMD was released within 30 min for F3-F6 and it was extended to 1 h for F2 formulation to release 85% of AMD.

Table.4. Assay of formulation (n=6) of Amlodipine besylate and Glimepiride:

Labelled amount(mg)		**Formulations**	**Calculated amount (mg)**		**Assay (%)**	
AMD	GLM		AMD	GLM	AMD	GLM
4	2	F1	2.5	1.3	64.98±1.49	69.59±12.33
		F2	3.7	1.8	94.68±4.85	92.37±1.70
		F3	4.08	1.9	102.12±19.43	95.27±5.83
	4	F4	3.88	2.1	97.15±19.28	105.8±7.60
		F5	3.6	3.6	90.10±0.36	90.36±2.35
		F6	3.69	1.4	92.32±0.21	35.95±2.73

Values are expressed in Mean ±SD

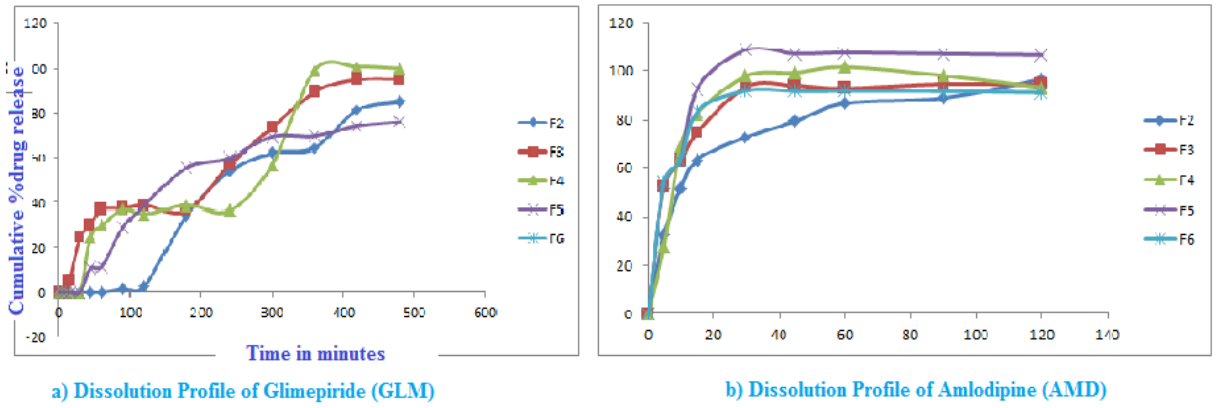


Figure 5. Dissolution profile of AMD and GLM from the bi-layered tablet

CONCLUSION:

The developed method possess good selectivity, specificity, there is no interference found in the blanks at retention times of AMD and GLM and good correlation between the peak area and concentration of the drug under prescribed conditions and also the recoveries are found to be >90.1% for F2-F6 of AMD and F2-F5 of GLM. The observation of % RSD less than 5 for both intra- and inter-day measurements also indicates a high degree of precision. A linearity range of 70-3000 ng/mL for AMD and GLM; this linearity range covers all the strengths of AMD and GLM. This can be applicable for the simultaneous determination of this combination (AMD and GLM) for further investigation.

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