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Original Article

An RP-HPLC Method for the Concurrent Measurement of Almitrine and Raubasine in Tablet and Bulk Forms: Development and Validation

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ABSTRACT

Background: This study work introduces a straightforward, sensitive, specific, and efficient stability-indicating HPLC approach designed for the simultaneous measurement of Almitrine and Raubasine in both pre-formulation and drug formulation contexts. **Materials and Methods:** The suggested RP-HPLC method was established using a Zorbax Eclipse XDB-C_{18} column (250x4.6 mm, 5 µm) at ambient temperature, employing an isocratic mobile phase of orthophosphoric acid (0.1%) in water and acetonitrile (40:60) at a flow rate of 1 mL/min. **Results:** The eluted analytes were detected at a wavelength of 248 nm. In accordance with ICH recommendations, the validity and stability of the medications under various environmental circumstances were assessed. The established method exhibited linearity within the concentration ranges of 1-12 µg/mL for Almitrine and 3-36 µg/mL for Raubasine respectively. The retention times for Raubasine and Almitrine were determined to be 2.839 and 2.089 respectively. The accuracy achieved by the proposed approach ranged from 99.01 to 100.7. The Relative Standard Deviation (RSD) for the precision research results was determined to be less than 1%. **Conclusion:** The established RP-HPLC method exhibited exceptional precision, specificity, sensitivity, and stability indication. The proposed analytical approach offered a sensitive and specific test for the measurement of Almitrine and Raubasine. Consequently, the method can be employed in the quality control department for routine examination of Almitrine and Raubasine estimate.

Keywords: Almitrine, Isocratic elution, Raubasine, RP-HPLC, Simultaneous.

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INTRODUCTION

Raubasine (RBS), also known as Ajmaline [-[(19α)-16, 17 didehydro-19-methyl-oxayohimban-16-carboxylic acid methyl esters] is an alkaloid derived from the plant *Rauwolfia serpentina*. Renowned for its antiarrhythmic properties, it is widely used to manage cardiac rhythm disorders, including ventricular tachycardia and atrial fibrillation (National Centre for Biotechnology Information, 2024). Its primary mechanism of action involves inhibiting cardiac sodium channels, crucial for generating and propagating action potentials in heart muscle cells thereby regulating heart rhythms (Synapse, June 2024). Almitrine (ALM) is a well-established drug known for enhancing Hypoxic Pulmonary Vasoconstriction (HPV) through a calcium-mediated vasoconstrictor effect specific to pulmonary arteries. It has been successfully utilized in hypoxemic patients with Acute Respiratory Distress Syndrome (ARDS), demonstrating its efficacy in improving oxygenation (Aronson, 2016). The combination of RBS and ALM has proven to be a promising therapy for age-related cerebral disorders and post-stroke rehabilitation. Clinical studies conducted across Europe consistently demonstrate its effectiveness in improving cognitive impairments, neurosensory vascular issues, and related conditions. Notable trials highlight significant improvements in memory, concentration, and mental alertness, particularly in vascular-related cases, with superior outcomes compared to placebo treatments. Its additional benefits in managing visual and vestibular disorders further reinforce its therapeutic potential. With a well-tolerated profile and established dosing regimen, this combination represents a valuable option for enhancing cognitive and vascular health in aging populations (Bentué-Ferrer, 1998). An extensive literature review indicates that while several analytical methods have been reported for the individual estimation of RBS (Rao *et al*., 1981; Ylinen*et al*., 1990; Auriola *et al*., 1990; Cieri 1995; Tikhomiroff and Jolicoeur 2002; Stoeckigt *et al*., 2002; Srivastav *et al*., 2006) and ALM (Kaiser *et al*., 1987; Geary and Stavchansky 1992; Luijten *et al*., 1998; Voisin 1981) a limited number of methods

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are available for their simultaneous estimation in combination (El-Sayed 2009a; El-Sayed 2009b; El-Sayed 2009c; El-Sayed 2011; Wang *et al*., 1997). The development of novel analytical methods is crucial for advancing research. In this context, the author has undertaken the development of a new RP-HPLC method for the simultaneous estimation of these drugs. This effort aims to deepen our understanding of their combination, potentially uncovering new therapeutic applications and broadening their role in medical practice. The chemical structures of RBS and ALM are depicted (Figure 1).

MATERIALS AND METHODS

Chemicals and solvents

All chemicals were of analytical reagent grade. Deionized water, obtained from a Milli-Q system, was used for all experiments. ALM and RBS were procured from Sigma-Aldrich Chemicals, Mumbai, India. Acetonitrile and orthophosphoric acid were purchased from Ranbaxy Fine Chemical Limited, New Delhi, India.

Apparatus and Chromatographic Conditions

The HPLC analysis was performed on a 2996 HPLC system. Separation was carried out using a Zorbax Eclipse XDB-C18 column (250×4.6 mm, 5 µm). The column temperature was maintained at 40ºC, and the mobile phase flow rate was set at 1 mL/min under isocratic conditions, using a mixture of 0.1% orthophosphoric acid in water and acetonitrile in a 40:60 ratio (v/v). The injection volume was 20 µL, and detection was achieved at 248 nm using a UV detector. The method yielded retention times of approximately 2.839 min for ALM and 2.089 min for RBS, with a total run time optimized accordingly. For each injection, a run time of 10 min was allowed in order to ensure full detection of both drugs and their clearance from the system before the next injection.

Generation of stock and working standard solutions

Distinct standard stock solutions for ALM and RBS were created by dissolving 30 mg of ALM and 10 mg of RBS in a diluent of acetonitrile and water in a 1:1 ratio. Each solution was prepared to a final volume of 100 mL in a volumetric flask. 5 mL of the prepared stock solutions were precisely measured and diluted to 50 mL using the identical diluent, yielding working standard solutions with concentrations of 30 µg/mL for ALM and 10 µg/mL for RBS.

Preparation of samples

Twenty Truxil tablets were precisely weighed, pulverized into a fine powder form, and thoroughly homogenized with a mill and pestle. A 600 mg aliquot of the powdered sample was weighed and placed into a 100 mL volumetric flask. Following the addition of 50 mL of the dilution solution the resulting mixture was subjected to sonication for 30 min in order to guarantee full dissolution. The solution was subsequently diluted to the mark with the identical solvent and strained through a 0.45 µm membrane filter. Afterwards, 5.0 mL of the filtered sample was aliquoted into a 50 mL volumetric flask and diluted to the mark. An injection quantity equal to 20 µL of the formulated sample solution was utilized for HPLC analysis.

Method Validation

Specificity

To prove the absence of interference with the elution of TXL in standard specimens or pharmaceutical formulations, the specificity and selectivity of the method were investigated by setting up the chromatographic conditions as described and following the system to equilibrate introducing of blank samples (diluent).

Precision

Intra- and inter-day assay variation were used to assess the method's precision (Table 2). The former involved analyzing standard and sample solutions of ALM and RBS in six replicates on the same day, while the latter involved analyzing same solutions in six repetitions on three separate days.

Accuracy

The sample solution was mixed with a known amount of a constant-concentration standard drug solution (10% of Tuxil standard solution) and accuracy recovery trials were conducted at 80%, 100%, and 120. Each concentration was injected into the column for 3 times and percent recovered was calculated.

Linearity

Calibration curves were constructed in order to assess the method's linearity. For this, standard ALM and RBS solutions in a range of concentrations (10%-120%) were employed. The column was equilibrated with the mobile phase for at least 30 min prior to the injection of the solutions. The calibration curves were created by plotting the chromatograms' peak regions against the concentrations. Regression analysis was used to determine the calibration formula and correlation coefficients for the seven standard concentrations.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

This approach was used to analyze a set of dilutions at levels of 20%, 10%, 5%, 2%, 1%, 0.5%, 0.2%, 0.1%, 0.05%, 0.02%, 0.01%, and 0.005% to determine the Limits of Detection (LOD) and Quantification (LOQ) for MGB and SLD. Signal-to-noise ratios were recorded to evaluate sensitivity. The concentration at which the signal-to-noise ratio was roughly 3:1 was determined to be the LOD, and the concentration at which the LOQ was roughly

10:1, guaranteeing a Relative Standard Deviation (%RSD) of 10% or lower (*n*=3).

System Suitability

Prior to conducting the primary analysis, the system suitability was assessed using metrics such as tailing factor, relative standard deviation of peak area, retention, theoretical plate number of the column (for column efficiency), and retention time.

Robustness

By examining standard solutions under typical operating settings, the method's robustness was assessed. Minor adjustments were made to the method's parameters, such as altering the flow rate and using different columns.

RESULTS

The suitable mobile phase used determines whether successful isolation and resolution in HPLC analysis of substances like ALM and RBS is possible. By use of a trial-and-error approach, the process was tuned so that the pH was changed to stabilize and improve the retention of ionized chemicals. In a non-polar environment, the mobile phase consisted in Acetonitrile (ACN) in water and 0.1% Orthophosphoric Acid using a 40:60 ratio. From one Zorbax Eclipse XDB-C18 column, ALM and RBS were separated using an optimal wavelength of 248 nm. The %recovery was calculated to lie within 99.01-100.7 using correlation values of 0.9996 and 0.9998 for ALM at 1 μg/mL and 12 μg/mL respectively. An HPLC test turned up RBS at -98.6% and ALM at 99.3%.

DISCUSSION

Optimization of chromatographic conditions

Choosing the right mobile phase is essential to getting adequate separation and resolution when using HPLC to examine substances like ALM and RBS. Using a trial-and-error approach, the process was optimized to produce a chromatogram with appropriate system suitability parameters and good resolution. Since RBS is a polar chemical, the pH needs to be changed to produce an atmosphere that stabilizes and improves the retention of ionizable substances. To improve the elution of non-polar substances like ALM, the mobile phase's polarity must be decreased concurrently. To meet the specified conditions, a mixture of 0.1% Orthophosphoric Acid in water that operates as the aqueous phase (polar) and Acetonitrile (ACN), which functions as the organic phase (non-polar), is designated as the mobile phase. The 40:60 ratio of Water to ACN establishes a somewhat non-polar environment, conducive to the separation of RBS and ALM. In order to separate ALM and RBS under the given HPLC circumstances, the Zorbax Eclipse XDB-C18 column offers the required selectivity and compatibility, reducing peak tailing and enhancing resolution. The wavelength was chosen using the UV spectrum's absorption maxima for the two medications. The ideal wavelength for each of the active components was 248 nm.

Method Validation

Specificity

Since the blank had no interfering peak during the ALM and RBS retention time, there was no interference with the ALM and RBS peaks. The chromatographic peak clarity seen in the chromatography images (Figure 2) can be used to determine the method's specificity.

Precision

The results of the tests, including the mean, standard variation, relative standard deviation, and relative percentage error, are shown in Table 1. A low standard deviation provides support for the precision.

Accuracy

The % recovery was calculated and was found to be in the range of 99.01-100.7. The results for accuracy indicate that the % recovery

Table 1: Precision metrics.

	RBS	ALM
Standard concentration (μg mL^{-1}).	90	400
Mean of measured concentration (μg mL^{-1})±SD.	90.58±0.13	402.90±0.06
RSD (%).	0.14	0.02
Percentage relative error.	0.64	0.72

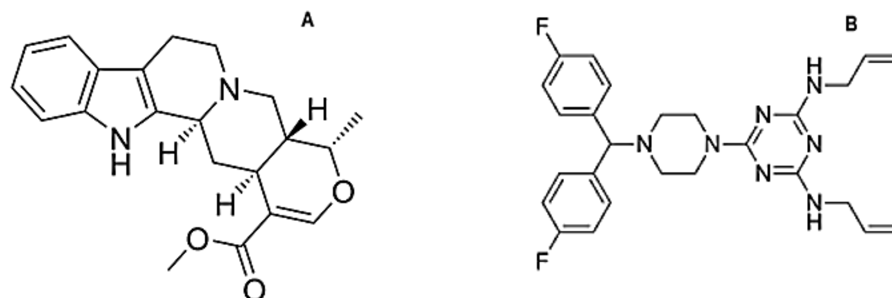

Figure 1: Chemical configuration of A) RBS B) ALM.

values are within the range which indicates that the method is accurate as it meets the necessary criteria. The results are shown in Table 2.

Linearity

Figure 3 showed a plot of the data with the regression line's slope and y-intercept, a correlation value of 0.9996 and 0.9998 for ALM and RBS, respectively, and linearity ranges of 3 µg/mL to 36 µg/mL for ALM and 1 µg/mL to 12 µg/mL for RBS.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

This method produced LOD levels of 0.2% indicating notable sensitivity. For ALM and RBS, the LOQ value was at 0.60%.

System Suitability

The outcome for these settings is displayed in Table 3. For analysis, the column efficiency was significantly higher, i.e. ≥2000. Additionally, the tailing factor was within the range of ≥1.2. Additionally, the computed relative standard deviation for the peak area and retention duration (mean of six replicates) likewise fell within the acceptable range. The suggested approach will be appropriate for routine analysis based on all of these factors.

Robustness

As shown in the Table 4 the percentage of recovery fell between 97.0% and 103.0%, and the RSD for both active components was less than 3.0%. This indicates robustness, since changes in the mobile phase flow speed and the use of different columns had little effect.

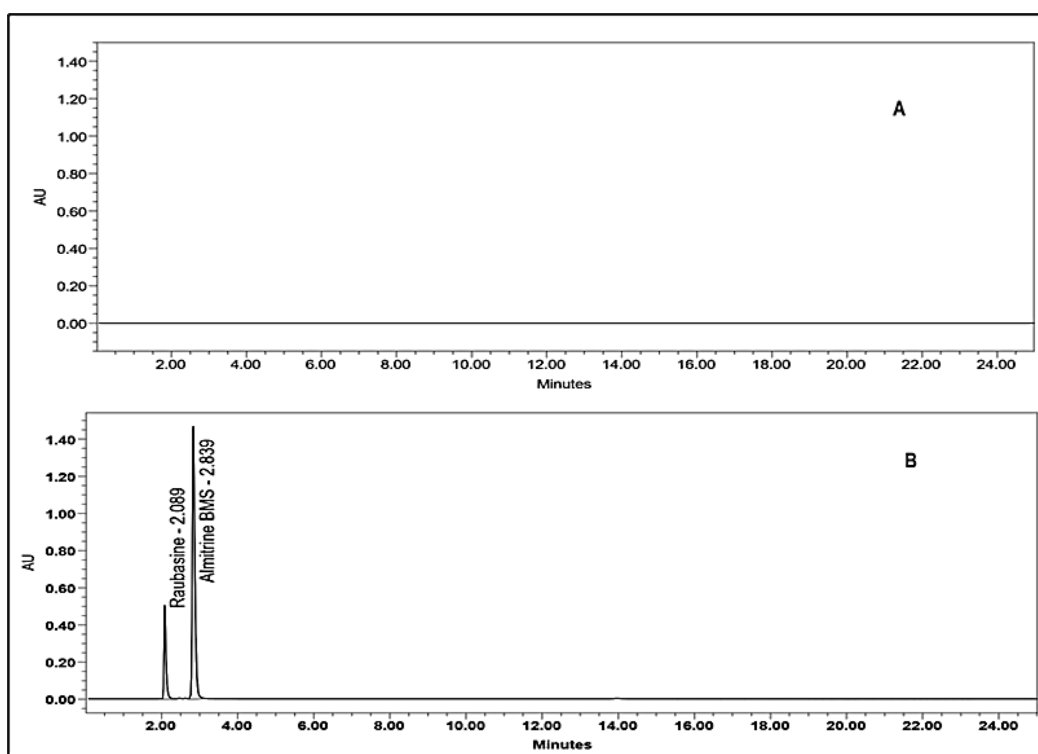

Figure 2: Recorded chromatograms for: (A) Blank (B) Standard Solution.

Table 2: Analytical accuracy testing data for ALM and RBS.

Accuracy level	Initial Concentration (µg/mL)	Spiked Concentration (µg/mL)	Concentration recovered (µg/mL)	% Recovery	% RSD
ALM					
80	24	3	26.69±0.006	98.84	0.023
100	30	3	32.87±0.005	99.92	0.016
120	36	3	38.81±0.065	99.51	0.169
RBS					
80	8	1	8.853±0.010	98.36	0.113
100	10	1	10.89±0.013	98.97	0.123
120	12	1	12.950±0.008	99.61	0.060

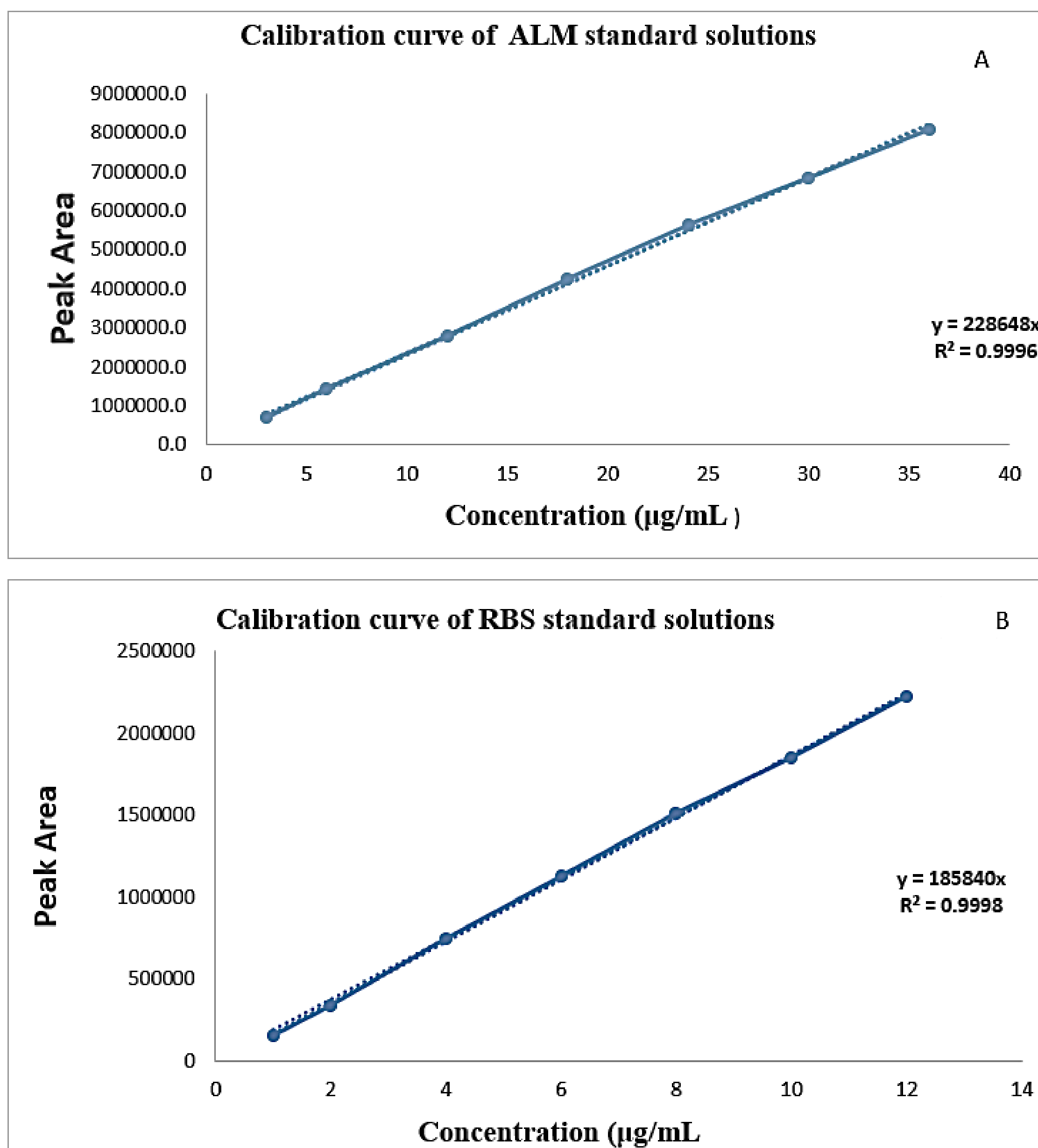

Figure 3: Linearity Validation Through HPLC Calibration Curve for: A) ALM and B) RBS.

Table 3: An overview of research on system appropriateness.

System suitability parameters	ALM	%RSD*	RBS	%RSD*
Peak Area (mV)	6784097±998.1	0.1	1824032±1824.8	0.1
Retention Time (min)	2.839±0.005	0.16	2.089±0.003	0.140
Theoretical plates	8565.96	0.0052	8736.77	0.013
Tailing Factor	1.35	0.0012	1.43	0.0031
Resolution	6.63			

*n=6 determinations.

Table 4: Findings from Robustness Assessments.

	ALM	RBS
Change in column		
Assay	99.4%	99.49%
%RSD	0.1	0.3
Flow increase (1.1 mL/min)		
Assay	99.24%	99.5%
%RSD	0.2	0.1
Flow decrease (0.9 mL/min)		
Assay	99.11%	99.31%
%RSD	0.2	0.1

Mean*n=3 Determinations.

Table 5: HPLC assay results for tablet formulations.

	Labeled amount	Measured amount	% Purity	SD	%RSD
ALM	30	29.8	99.3	0.242	0.81
RBS	10	9.68	98.6	0.045	0.46

Applications of the developed analytical method in the assay of formulations

The proposed method was further applied to the analysis of pharmaceutical dosage forms. Three batches of the tablets were selected for evaluation, and six replicates from each batch were analyzed using the developed HPLC method. The results, summarized in Table 5, demonstrated excellent agreement with the labeled content of ALM and RBS as claimed by the manufacturer. This confirms the method's reliability and suitability for routine quality control of ALM and RBS in their dosage form.

CONCLUSION

In conclusion, a simultaneous HPLC method was successfully developed, optimized, and validated for the effective separation and detection of ALM and RBS in their combined dosage form. The method exhibited high selectivity, precision, accuracy, and robustness, aligning with the requirements outlined in ICH guidelines. Its simplicity and reliability make it well-suited for routine quality control of dosage forms. The isocratic elution technique employed in the method capitalizes on the distinct polarities of ALM and RBS, ensuring efficient separation. By strategically adjusting the composition of the mobile phase over time, the differential interactions of each compound with the stationary phase were utilized to achieve precise and effective separation. This approach underscores the method's utility in the quality assessment of pharmaceutical formulations.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ABBREVIATIONS

ALM: Almitrine; **RBS:** Raubasine; **RP-HPLC:** Reverse Phase High-Performance Liquid Chromatography; **HPV:** Hypoxic Pulmonary Vasoconstriction; **ARDS:** Acute Respiratory Distress Syndrome; **ICH:** International Council for Harmonisation; **ACN:** Acetonitrile; **SD:** Standard Deviation; **RSD:** Relative Standard Deviation; **LOQ:** Limit of Quantification; **LOD:** Limit of Detection; **UV:** Ultraviolet; **TXL:** Truxil; **%RSD:** Percentage Relative Standard Deviation; **%LOD:** Percentage Limit of Detection; **%LOQ:** Percentage Limit of Quantification; **nm:** Nanometer; **µg/mL:** Microgram per Milliliter; **mL:** Milliliter; **µm:** Micrometer; **°C:** Degrees Celsius; **HPLC:** High-Performance Liquid Chromatography; **NCBI:** National Centre for Biotechnology Information; **GIET:** Godavari Institute of Engineering and Technology; **SD:** Standard Deviation; **%:** Percentage.

SUMMARY

HPLC analysis of ALM and RBS requires the right mobile phase. The pH was adjusted by trial-and-error to stabilize and improve ionized chemical retention. The mobile phase was 40:60 Acetonitrile (ACN) in water and 0.1% orthophosphoric acid. At optimum 248 nm, the Zorbax Eclipse XDB-C18 column separated ALM and RBS. The Zorbax Eclipse XDB-C18 column reduced peak tailing and improved resolution with selectivity and compatibility. The linearity ranges for ALM and RBS are 3 to 36 µg/mL and 1 to 12 µg/mL, respectively, with correlation values of 0.9996 and 0.9998. This approach yielded 0.2% LOD and 0.60%

LOQ for ALM and RBS. The recovery rate for ALM and RBS was 99.01-100.7.

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