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Development and validation of HPLC method for simultaneous determination of rimantadine marketed product

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ABSTRACT: This study presents the development and validation of a novel high-performance liquid chromatography (HPLC) method for the quantification of rimantadine in pharmaceutical formulations. Rimantadine, an antiviral drug primarily used in the treatment of influenza A, necessitates effective analytical methods for quality control. Our HPLC method employs a reverse-phase column with a mobile phase comprising acetonitrile and 15mM phosphate buffer (pH 3.0) and acetonitrile in the ratio of 65:35 water, under isocratic elution conditions. The detection was carried out using UV spectrophotometry at a wavelength of 270 nm. Method validation was performed in accordance with ICH guidelines, assessing parameters such as specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). The method demonstrated excellent linearity ($r^2 > 0.999$) across a wide concentration range. The precision, expressed as relative standard deviation (RSD), was below 2%, confirming the method's repeatability and reproducibility. Accuracy, tested through recovery studies, ranged from 98% to 102%. The developed method was successfully applied to analyze rimantadine in commercially available rimantadine HCl formulations, with results indicating consistent drug content within the specified limits. This validated HPLC method offers a reliable, efficient, and cost-effective approach for routine quality control analysis of rimantadine in pharmaceutical products.

KEYWORDS: Rimantadine; HPLC; Quantitative; Validation; Antiviral

1. INTRODUCTION

Rimantadine is an adamantane derivative (Figure 1) that has been extensively studied for its pharmacological properties and clinical applications. It has been identified as an effective antiviral agent, particularly in the prophylaxis of influenza A infections [1]. Rimantadine exhibits similar antiviral and neuropharmacological properties as amantadine, its α-methyl derivative, but with fewer adverse effects, especially in elderly patients [2]. Pharmacokinetic studies have shown that rimantadine has a longer plasma half-life and lower urinary excretion than amantadine, indicating its potential for sustained therapeutic effects [3]. Additionally, the pharmacokinetics of rimantadine are predictable, with linear accumulation during repetitive multiple doses [4]. This predictability is advantageous for its clinical use.

Furthermore, rimantadine has been evaluated for its safety and efficacy in various patient populations, including elderly nursing home patients, where it demonstrated a lower rate of central nervous system adverse effects compared to amantadine [5]. However, it is important to note that experience with rimantadine in certain situations, such as controlling influenza outbreaks in nursing homes, is limited compared to amantadine [6]. Nevertheless, rimantadine has been identified as a promising option for the treatment and prevention of influenza A infection, offering desirable features for clinical use [7].

Quantitative methods for the determination of rimantadine have been developed using various analytical techniques[8-10]. Validated a method for the quantitative trace analysis of rimantadine in poultry muscle using column-switch liquid chromatography coupled to tandem mass spectrometry, demonstrating its

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suitability for the analysis of antiviral drugs [11]. Additionally, one study comparing chromatography columns and rapid pretreatment for the simultaneous analysis of rimantadine in chicken muscle using ultra-high-performance liquid chromatography and tandem mass spectrometry was conducted and provided insights into the chemical functionalities and chromatographic separation effectiveness of different columns [12]. Furthermore, presented a method for the simultaneous determination of amantadine and rimantadine in feed by liquid chromatography-Qtrap mass spectrometry with information-dependent acquisition, demonstrating the accuracy and reliability of the developed method [13]. These studies highlight the diverse analytical approaches employed for the quantitative determination of rimantadine, contributing to a comprehensive understanding of its presence in various matrices.

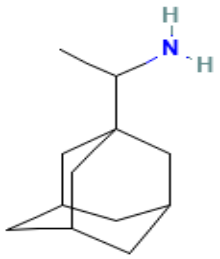


Figure 1. Chemical structure of rimantadine

A validated HPLC method for the determination of rimantadine in biological samples has been reported by, utilizing a monolithic stationary phase and online post-column derivatization for enhanced sensitivity and accuracy [14]. This method offers a reliable approach for the quantitative analysis of rimantadine in human urine samples. Additionally, the study by demonstrated the feasibility of coupling dispersive liquid-liquid microextraction with HPLC for the analysis of rimantadine, providing a sensitive and efficient approach for trace analysis of this compound [15]. These studies contribute to the development of robust analytical methods for the quantitative determination of rimantadine, offering valuable tools for pharmacokinetic studies and clinical applications.

This study aimed to develop and validate a high-performance liquid chromatography (HPLC) method for the quantitative determination of rimantadine hydrochloride in a marketed product. The method was designed to provide accurate and reliable analysis of rimantadine hydrochloride, an antiviral drug used in the treatment of influenza A. The validation studies aimed to assess the specificity, linearity, precision, accuracy, and stability of the developed HPLC method, ensuring its suitability for the quantitative analysis of rimantadine hydrochloride in the marketed product.

2. RESULTS & DISCUSSION

High-performance liquid chromatography (HPLC) is widely used in drug development for the precise and sensitive analysis of pharmaceutical compounds in various matrices. It is extensively applied in bioanalytical studies, pharmacokinetic evaluations, and quality control of drug products. HPLC enables the quantification of drug concentrations in biological fluids, such as plasma and urine, contributing to the assessment of bioavailability, bioequivalence, and pharmacokinetic parameters [16, 17]. Additionally, HPLC methods are essential for the determination of drug content in pharmaceutical formulations, ensuring product quality and consistency [15]. Moreover, HPLC techniques are valuable for the analysis of drug-drug interactions, providing insights into the potential effects of co-administered medications [18]. Furthermore, HPLC has been utilized for the determination of drug levels at potential sites of infection, contributing to the understanding of drug distribution and efficacy [19]. Overall, HPLC plays a pivotal role in drug development, offering versatile and reliable methods for the analysis of pharmaceutical compounds, thereby contributing to the advancement of pharmaceutical research and development. The validation of analytical methods is crucial in drug development to ensure the reliability, accuracy, and robustness of the methods used to analyze pharmaceutical compounds. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines provide a framework for the validation of analytical procedures, encompassing parameters such as specificity, linearity, accuracy, precision, and robustness[20]. Validation studies are essential for demonstrating that the analytical methods are suitable for their intended purpose, whether it is the quantification of active pharmaceutical ingredients in medicinal products, the determination of drug content in pharmaceutical formulations, or the analysis of drug levels in biological fluids for pharmacokinetic evaluations. By adhering to the ICH guidelines, the validation of analytical

methods ensures the generation of reliable data, contributing to the safety, efficacy, and quality of pharmaceutical products throughout the drug development process.

As demonstrated by the linearity of the method, it is suitable for concentrations ranging from 0.1 to 5 mg/ml. The calibration data were obtained by repeating each experiment at all concentrations three times on three separate samples. We plotted the area value observed in HPLC against the concentration and drew the calibration curve from this. Calculations were conducted to determine the equation of the line and the correlation coefficient (R^2). Within the used concentration range, R^2 indicated a functional linear relationship between analyte concentration and area under the peak was greater than 0.999. A correct equation for y is y = 32123657x + 406568 and R^2 is 0.9997 (Figure 2).

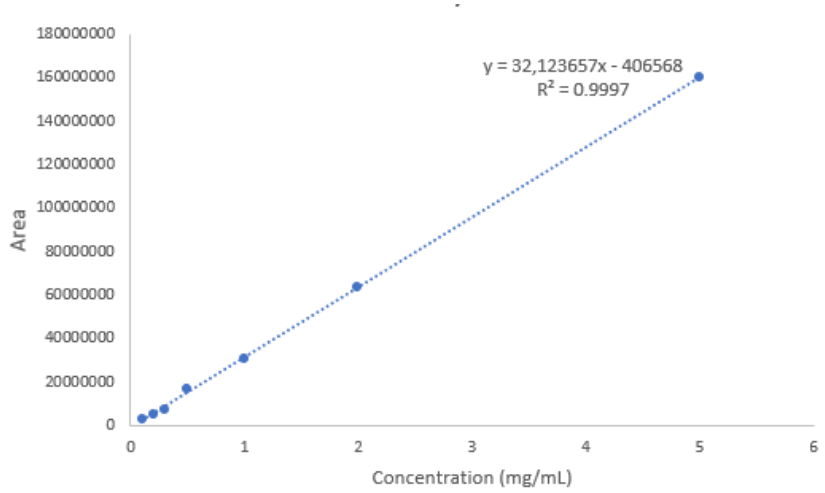


Figure 2. Rimantadine calibration curve

For an analytical procedure, precision studies are performed to evaluate the proximity between measurements obtained from homogeneous samples under similar conditions. A 9-times consecutive measurement of the solution at the low, middle, and high concentrations of the working range (0.15 mg/mL, 1.5 mg/mL, and 3 mg/mL) was conducted to validate the analytical method by ICH guidelines, and the mean, standard deviation, coefficient of variation (RSD), accuracy, and precision values were calculated (Table 1).

Table 1. Analysis of HPLC data to calculate accuracy and precision values at the lowest and highest concentrations of the working range (p>0.05)

Injection numbers	**Concentration in theory (mg/mL)**	**Area**	**Concentration in practically (mg/mL)**
1		3949854	0.141
2		3875190	0.139
3		3790333	0.136
4		3549854	0.150
5	0.15	3475190	0.148
6		3490333	0.149
7		3532857	0.154
8		3457686	0.151
9		3374526	0.149
		Mean	**0.146**
		SD	**0.002**
		%RSD	**1.464**
		%Accuracy	**98.599**
Injection numbers	**Concentration in theory (mg/mL)**	**Area**	**Concentration in practically (mg/mL)**
1		50655307	1.548
2	1.5	51138100	1.563
3		50986038	1.558
4		51655307	1.566

5		51138100	1.550
6		51986038	1.576
7		47902201	1.535
8		49395338	1.581
9		47246317	1.515
		Mean	**1.554**
		SD	**0.019**
		%RSD	**1.259**
		%Accuracy	**102.638**
Injection numbers	**Concentration in theory (mg/mL)**	**Area**	**Concentration in practically (mg/mL)**
1		95890405	2.947
2		95315910	2.929
3		96026926	2.951
4		95890405	2.941
5	3.0	95315910	2.923
6		96026926	2.945
7		92372597	2.919
8		90809592	2.871
9		90506387	2.861
		Mean	**2.922**
		SD	**0.031**
		%RSD	**1.067**
		%Accuracy	**98.362**

The rimantadine HPLC analysis demonstrates excellent accuracy (98.6, 102.64, and 98.36%) and precision (standard deviation <2% which is the threshold for acceptance) across all tested concentrations (0.15, 1.5, 3 mg/mL) (Table 1). These results indicate the method's effectiveness for quantitative analysis of rimantadine, further confirmed by its low standard deviation values showcasing good repeatability and reproducibility [21, 22]. Additionally, because the standard deviation values for the measurements were low, the repeatability of the measurements was also determined to be high. The results of the study indicate that the equipment used for the study was of high quality, and therefore the developed analytical method is highly repeatable and reproducible. To verify the accuracy of the method used in this calculation, the average recovery values (%) were calculated to confirm its accuracy. To find the concentration that corresponds to each area value, the areas of three parallel samples were measured at three different concentrations (0.2, 1, and 5 mg/mL) and the concentration that corresponds to each area value was calculated by placing it into the calibration equation (Table 2).

Table 2. Results of the HPLC study relating to accuracy and recoverability (p>0.05)

Concentration in theory (mg/mL)	**Area (run 1)**	**Area (run 2)**	**Area (run 3)**	**Average Area**	**Concentration in practically (mg/mL)**	**%Recovery**
0.2	4826866	4804160	4963586.4	4864870.67	0.1995	99.735
0.2	4730328.29	4708076.8	4864314.67	4767573.25	0.198	98.966
0.2	5309552.16	5284576	5459945.04	5351357.73	0.202	101.254
					Mean	**99.985**
					SD	**0.95**
					%RSD	**0.832**
Concentration in theory (mg/mL)	**Area (run 1)**	**Area (run 2)**	**Area (run 3)**	**Average Area**	**Concentration in practically (mg/mL)**	**%Recovery**
1	32250924.16	32798153.60	31267549.44	32105542.400	1.012	101.2
1	29505906	31062191	31522198	30696764.89	0.994	99.42
1	30414668.48	29310640.8	27161920.32	28962409.87	0.988	98.83
					Mean	**99.816**
					SD	**1.01**
					%RSD	**0.998**
Concentration in theory (mg/mL)	**Area (run 1)**	**Area (run 2)**	**Area (run 3)**	**Average Area**	**Concentration in practically (mg/mL)**	**%Recovery**

5	160110152.00	160016019.5	160400601.28	160175591	4.973	98.626
5	159825312	160397420	160400601	160207777.6	5.021	100.01
5	160397420	157800601	160740661	159646227	5.012	100.09
					Mean	**99.575**
					SD	**0.672**
					%RSD	**0.596**

It can be seen that RIM recovered 99.985, 99.816, and 99.575 % of its original concentration from samples, respectively. Based on this analysis, the mean RSD (%) was found to be 0.832, 0.998, and 0.596, which are acceptable for this study (Table 2). The Limit of Detection (LOD) and Limit of Quantitation (LOQ) are critical parameters in analytical method validation, particularly in drug development. LOD represents the lowest concentration of an analyte that can be reliably detected but not necessarily quantified, while LOQ is the lowest concentration that can be both reliably detected and quantified with acceptable precision and accuracy. These parameters are essential for assessing the sensitivity and performance of analytical methods, ensuring that the methods are capable of detecting and quantifying low levels of analytes in complex matrices such as biological fluids and pharmaceutical formulations. The determination of LOD and LOQ is by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, which provide a standardized approach to method validation in drug development, ensuring the reliability and robustness of analytical procedures.

Table 3. LOD and LOQ values of the data (ANOVA, p>0.05)

REGRESSION STATISTICS				
Multiple R		0.99985		
R^2		0.99969		
Adjusted R^2		0.99962		
Observations		63		
ANOVA				
	df	**SS**	**F**	**Significant F**
Regression	1	1.92561E+16	1.92561E+16	16126.62292
Residual	62	5.97028E+12	1.19406E+12	
Total	63	1.92621E+16		
LOD and LOQ values				
Intercept	**Slope**	**SD**	**LOD (µg)**	**LOD (µg)**
Total	63	1.92621E+16	5.42	16.45

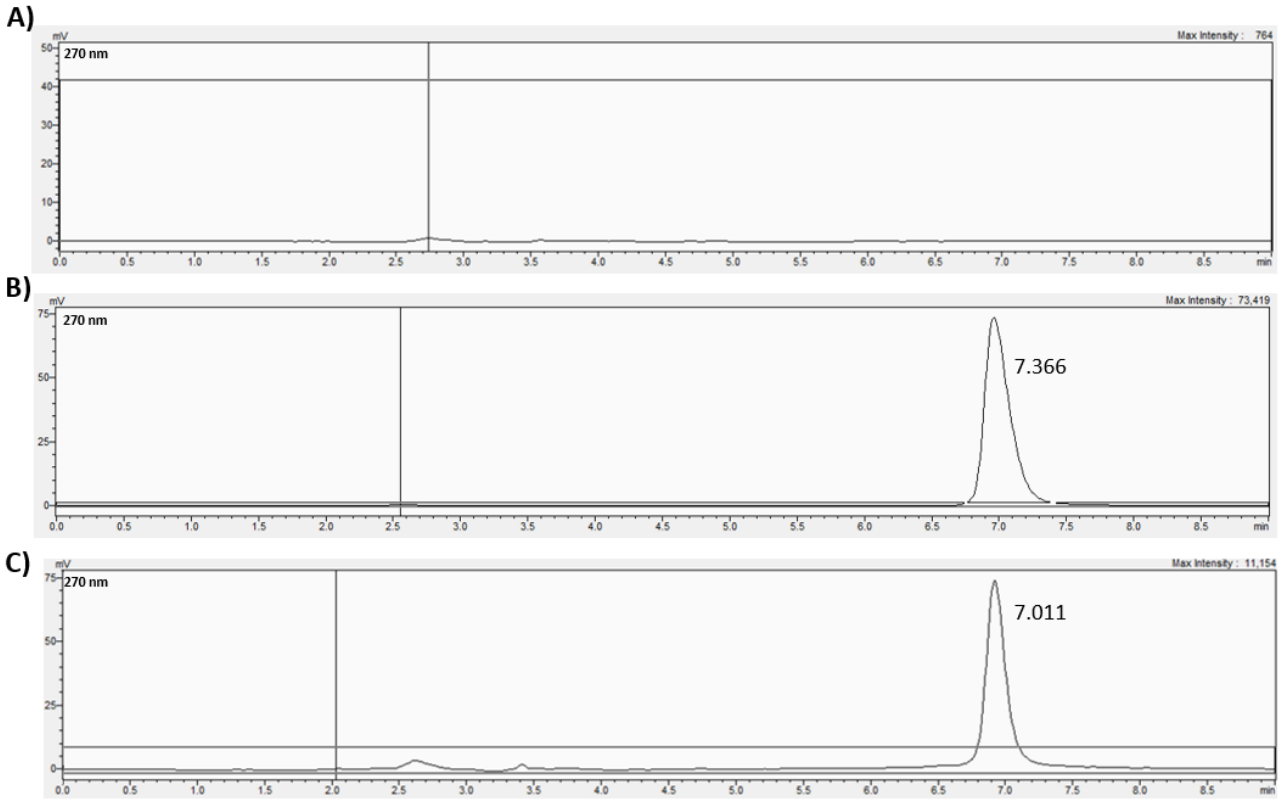
In our study, LOD and LOQ values were determined by regression analysis of three days and three different time intervals within the same day (15 minutes, 90 minutes, and 240 minutes) (Table 3).

The stability assessment of the rimantadine HPLC method (Table 4), as evidenced by the testing results, demonstrates a robust analytical procedure with minor variance over time. The measured concentration of rimantadine, initially at 2.045 mg/mL, exhibited a nominal decline to 2.042 mg/mL over 72 hours, indicating a marginal decrease of approximately 0.03%. This slight concentration decrement is corroborated by consistent area values under the curve, reinforcing the method's precision. The standard deviation (SD) for concentration is notably low, with values of 0.053 at 15 minutes and maintaining an equivalent level of precision at 0.011 at both 48 and 72 hours, further substantiating the method's reproducibility. The stability data collectively suggests that the rimantadine remains chemically stable in the mobile phase across the assessed duration, implying the method's suitability for reliable long-term analytical application.

All three chromatograms (Figure 3) meet the acceptance criteria for tailing factor, plates, %RSD (n=6)(area), and %RSD (n=6)(retention time). Comparing the rimantadine solution and marketed drug chromatograms reveals highly similar profiles. Both present sharp, symmetrical peaks for rimantadine at identical retention times (7 minutes). However, subtle differences exist: the marketed drug peak area is slightly higher for plates (4123456 vs. 3949854), suggesting a slightly higher concentration, and/or a minor impurity peak is present at 2.5 minutes, absent in the solution. This indicates that the rimantadine HPLC method is stable and reliable. Specifically, the chromatogram for the dissolved rimantadine tablet shows a sharp, symmetrical peak for rimantadine, with no significant interfering peaks. The peak area and retention time are consistent with those of the standard rimantadine solution, indicating that the rimantadine tablet contains the expected amount of rimantadine.

Table 4. Testing results for the stability of 2 mg/mL concentrations (p> 0.05)

Time	15 min	90 min	240 min	48 h	72 h
Area (run 1)	63655692.88	63374520.84	63212963.77	64292249.81	64008266.05
Concentration (run1)	2.025	2.017	2.012	2.045	2.036
Area (run 2)	66227640.07	65935107.55	65767022.92	64903087.27	64616405.39
Concentration (run 2)	2.105	2.096	2.091	2.064	2.055
Area (run 3)	63012706.09	62734374.17	62574448.99	64272960.21	63989061.65
Concentration (run 3)	2.005	1.997	1.992	2.045	2.036
Area (average)	64298679.68	64014667.52	63851478.56	64489432.43	64204577.7
Concentration (average)	2.045	2.037	2.032	2.051	2.042
SD (Concentration)	0.053	0.052	0.045	0.011	0.011



Parameter	Rimantadine solution	Marketed product	Acceptance Criteria
Tailing	1.29	1.43	≤2.0
Plates	8112.25	7966.91	≥2000
%RSD (n=6)(area)	0.54	0.49	≤1.0
%RSD (n=6)(retention time)	0.42	0.24	≤1.0

Figure 3. Chromatograms of mixtures and chromatographic interpretations according to acceptance criteria A)Blank solution B)standard RIM solution and C)Dissolved RIM tablet

3. CONCLUSION

In conclusion, the study successfully developed and validated a high-performance liquid chromatography (HPLC) method for quantifying rimantadine hydrochloride in a marketed pharmaceutical formulation. The method employed a reverse-phase column and UV detection at 270 nm, optimizing conditions for the effective separation and quantification of rimantadine. Validation parameters, including system suitability, linearity, precision, accuracy, and specificity, met the regulatory requirements, confirming the method's reliability. Notably, the method demonstrated good accuracy, with recovery rates closely aligning with the true value across different concentrations. Stability tests showed that rimantadine remains stable in the mobile phase over time, with no significant changes in concentration observed up to 72 hours post-preparation. The low

standard deviation and %RSD values across repeated measurements underscored the method's precision and reproducibility. These results affirm the developed HPLC method as a robust and reliable tool for the routine quality control analysis of rimantadine, ensuring the consistency and safety of the pharmaceutical product

4. MATERIALS AND METHODS

4.1 Materials

The marketed product of the rimantadine HCl tablet was kindly gifted from Patrin Pharmaceuticals (Skokie, IL, US). Several substances from Sigma Aldrich (St. Louis, MO, US) can be used in this study, including rimantadine, HPLC quality acetonitrile, methanol, chloroform, and analytical grade potassium dihydrogen phosphate. All of the reagents used in the study were of excellent analytical quality, and double distilled water was prepared in the laboratory.

4.2 Methods

4.2.1 Measurement

It was conducted using an HPLC system from Agilent 1260 Infinity I (Agilent, Columbia, MO, US) that included a solvent pump, a thermostable column, an injection valve, and a UV detector. A SunFire C18 150 x 4.6mm, 5m analytical column (Quincy, WA, US) was used to achieve the separation of the compounds as well as a guard column, a SunFire C8 100 x 5 m 4.6 mm integrated into the system, which served as a guard column before the analytical column.

4.2.2 UV Condition

RIM samples were analyzed using a UV spectrometer (Accuris MR9610 SmartReader Microplate Reader UV-Vis) that was configured to analyze UV spectral data. During the analysis of the RIM sample, the range of 200–400 nm was used. This study showed that the optimum wavelength of the RIM in the mobile phase was 270 nm (Figure 4) and that at this wavelength, the maximum response of the RIM in the mobile phase can be determined, as well as the related impurities of the RIM.

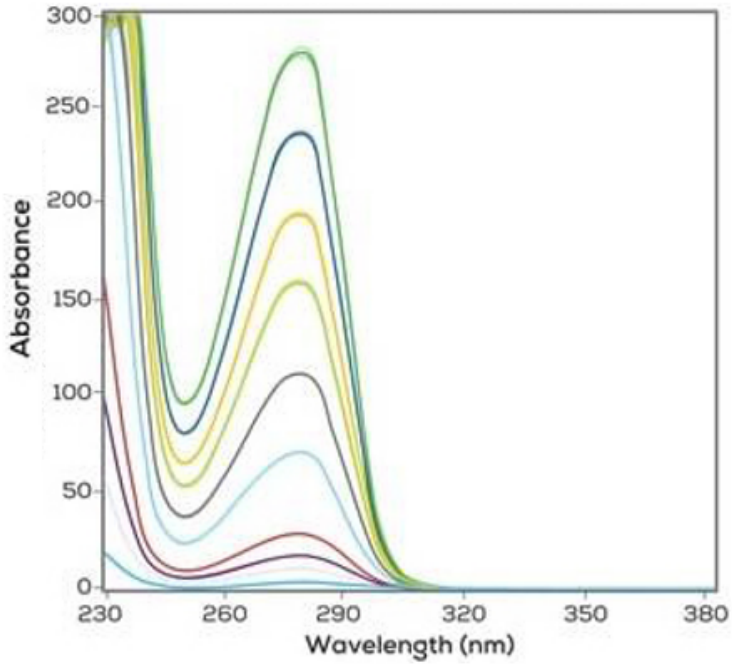


Figure 4. Rimantadine calibration curve in terms of interday data

Using UV spectroscopy to determine the optimal absorbance value, this absorbance was applied to the HPLC. The detection wavelength was set at 270 nm with a mobile phase comprised of 15mM phosphate buffer pH was adjusted to 3.0 using orthophosphoric acid and acetonitrile in the ratio of 65:35 with a flow rate of 1.2 ml min-1 to achieve a detection wavelength of 270 nm [23]. After filtering the mobile phase through a

0.22 m nylon filter, the ultrasonic bath was used for a 5-minute degassing process. The sample injection volume was maintained at 10µL, and the temperature of the column oven was kept at 30°C throughout the process.

4.2.3 Stock solution

To prepare a stock solution of RIM, 10 mg of the compound was dissolved in 100 mL of 0.9% NaCl solution to prepare a 5 mg/mL stock solution of RIM. For preparation of the stock solutions, a clear glass volumetric flask with a tight-fit lid and aluminum foil cover was used to store the solution in the refrigerator at 4°C. Aluminum foil is used to prevent light from entering the flask. To draw calibration curves, concentrations of 0.1, 0.2, 0.3, 0.5, 1, 2, and 5 mg/mL of RIM were selected as the concentrations in working solutions. Using dilutions of the stock solution in the mobile phase, fresh working solutions were prepared daily based on dilutions of the stock solution. All of the solutions that were going to be filtered before injection were treated with an aqueous membrane filter with a pore size of 0.2 µm.

4.2.4 Validation

There were several parameters tested for validation, which included suitability of the system, linearity, limit of detection (LOD) and limit of quantitation (LOQ), precision, accuracy, specificity, and selectivity [24].

It was used to determine whether the linear relationship between peak area and concentration can be established using the calibration curve. To draw the calibration curve, samples were prepared by diluting a stock solution in a mobile phase to concentrations of 0.1, 0.2, 0.3, 0.5, 1, 2, and 5 mg/mL in parallel, and these samples were examined in parallel in three experiments. Observers took measurements of the area of each injection and plotted them against the concentration to create a calibration curve. There is a calculation that takes place to determine the equation of the line and the R^2 value. After injecting standard solutions into the HPLC, the peak area of each concentration was calculated to determine the retention time and maximum absorption of each concentration at which the retention time and maximum absorption were obtained. A graph of RIM concentrations versus peak areas was drawn and the calibration equation was determined based on the graph (n=3)

An analytical method is defined as accurate if the results obtained by the method are close to the true value as determined by the test results obtained by the method [25, 26]. During the experiment, three parallel samples of different concentrations (0.15, 1.5, and 3 mg/mL) were measured in the field. This area value was placed in the calibration equation to find the concentration amount that corresponds to the area value.

4.2.5 Evaluation of stability, selectivity, and repeatability

A solution was prepared with a concentration of 2 mg/mL using the mobile phase of the active ingredient of the RIM, which was then measured in HPLC at various minutes (15, 90, and 240) and 48 and 72 hours after the preparation, to determine if there were any changes in the values (n=3) after the preparation of the solution.

Using the stock solution prepared to produce the calibration equation, a concentration of 5 mg/mL was selected from the stock solution, and the solutions prepared on two different days at this concentration were injected into the HPLC ten times on consecutive days. Based on the area value, the mean, standard deviation, and RSD % of the concentrations were calculated [27].

4.3 Analysis of data

An analysis of the data was conducted using Stat-Ease's Design Expert software version 13.0.5.0 (Minneapolis, MN, USA). To determine whether the models fit the data (R^2), the coefficient of determination was calculated. Statistical analysis was performed using the analysis of variance (ANOVA) and F test.

Author contributions: Concept – BU, ADE.; Design – BU, ADE; Supervision – BU, ADE; Resources – BU, ADE; Materials – BU, ADE.; Data Collection and/or Processing- BU, ADE; Analysis and/or Interpretation – BU, ADE.; Literature Search – BU, ADE.; Writing – BU, ADE.; Critical Reviews – BU, ADE

Conflict of interest statement: There were no conflicts of interest declared by the authors.

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