RESEARCH ARTICLE

Green Analytical HPLC Method Development of Acyclovir Loaded Floating Raft Formulation in Spiked Rabbit Plasma

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ABSTRACT

Objective: The need of this investigation is to design eco-friendly, sensitive, and precise bioanalytical high-performance liquid chromatography (HPLC) techniques that leverage green analytical tools such as green analytical procedure index (GAPI), analytical eco scale (AES), and analytical greenness metric (AGREE) to analyze ACV-spiked rabbit plasma and ACV-loaded floating raft formulation (FRF) because The literature survey revealed insufficient data for the HPLC approach to FRF.

Method: Methods involving plasma spikes mirroring the *in-vivo* models provide insights into potential real-life scenarios and the functionality of floating raft formulations loaded with ACV. The Design Expert tool optimized critical process parameters and identified significant factors influencing the chromatographic technique.

Results: The CCD was used to optimize the flow rate (0.6 mL/min) and buffer solvent ratio (99:1) for separation. The C18 column (250 X 4.6 mm) was utilized to achieve the elution of ACV. The developed method was thoroughly validated through ICH guidelines. The ACV, lower limit of detection (LLoD), and lower limit of quantitation (LLoQ) retention times were established at 2.3 minutes, 15, and 20 ng/mL, respectively.

Discussion: In summary, the study demonstrated the exceptional performance sensitivity, specificity, and robustness of the bioanalytical method. The method proved highly effective in quantifying ACV in plasma samples in terms of revalidatability, transferability, reproducibility, and the product development cycle. The developed method proves that using both design of experiments (DoE) and green analytical chemistry together creates a valid, eco-friendly, and reliable way to analyze the making of an ACV-loaded floating raft formulation.

Keywords: Acyclovir, Green analytical chemistry, HPLC, Spiked rabbit plasma, Floating raft formulation.

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INTRODUCTION

Acyclovir, a potent antiviral agent and characterized as an acyclic guanosine analog (9-((2-hydroxyethoxy)-methyl)-guanosine), is primarily treated for herpes simplex virus, shingles and chickenpox. Additionally, it serves as a preventive measure against CMV infections post-transplantation and Epstein-Barr virus infections.[1]

A comprehensive examination of the available literature reveals that a limited number of developed methods for ACV spiked rabbit plasma are only for conventional dosage forms and not for novel drug delivery systems such as FRF. The majority of these documented methods focus on estimating Acyclovir levels in human serum and plasma. However, it is worth noting that many of the existing reversed-phase high performance liquid chromatography (RP-HPLC) approaches suffer from hazardous solvents, extended run times, peak tailing, limited sensitivity, selectivity, and not feasible.[2,3] With consideration of the advantages presented by previously established methods in the literature, an endeavor has been undertaken to develop an innovative RP-HPLC approach.[4]

The objective of this research to be simple, accurate, rapid, feasible, precise, cost-effective, and eco-friendly, employing a readily obtainable mobile phase for the quantification of acyclovir in floating raft formulation. The proposed technique meets the requirements of ICHQ14.[5] This research finds a suitable approach for the quantification of ACV to ensure sensitive and selective plasma samples through a high-performance liquid chromatography (HPLC) sensitive bioanalytical procedure with green analytical method.[6] Because the existing method has potentially harmful and non-degradable solvents have adverse effects on the ecosystem and pose potential harm to the analyst. The utilization of spiked rabbit plasma as a model for *in-vivo* assessment supports a

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layer of practical relevance to this research, bridging the gap between analytical methodologies and real-world applications. By exploring the potential of acyclovir-loaded floating raft formulations in this context, this work strive to contribute in the progression of pharmaceutical science and pave the way for improved antiviral drug delivery systems.[7,8] Consequently, this method offers a more "sensitive, less time-consuming and less intricate means of quantification" in comparison to other available methods, along with an enhanced recovery rate from human plasma (98.0%) using eco-friendly solvent such as ethanol. The Design Expert 12 software were utilized to optimize the critical process parameters such as flow rate, solvent-buffer ratio and injection volume.[9] DoE was not been employed so far in method development and validation. DOE restricts unnecessary use of organic solvents, cost effective and high reproducibility.[10,11]

The green analytical chemistry tool utilised in this study to develop a non-hazardous, and safe analytical technique to assess the quantity and quality of ACV.[12,13] The green analytical approach is evaluated using the analytical eco scale (AES), green analytical procedure index (GAPI), and the analytical greenness metric (AGREE), along with considerations for cumulative energy demand (CED) and environmental hazard safety (EHS).[14,15]

MATERIAL AND METHODS

Material

A pure sample of ACV standard was gifted from Sterile Gene Laboratory Pvt. Ltd., Pondicherry, India. The analytical grade chemicals and solvents were utilized in the current investigation. HPLC grade ethanol, orthophosphoric acid, and triethylamine were obtained from the SISCO Research Laboratory in India.

Instruments Used

The method development and validation was conducted in isocratic separation using high-performance liquid chromatography performed at Agilent Technologies, 1220 Infinity II LC. Which included binary solvent system with auto-injector and photodiode array detector. The samples were eluted in the C18 Column (mentioned in Table 1). pH of the buffer was modified with orthophosphoric acid measured with pH meter (ELICO INDIA model LI 120). Filtration and degassing was done by a vacuum filtration unit and ultrasonicator (bath sonicator, model ATS-1).

Mobile Phase

The choice of solvents for the mobile phase is decided by carrying out an overview of existing literature. A standard flask containing 3.4 g of potassium dihydrogen orthophosphate was solubilized in double-distilled water to make a total volume of 1000 mL. The pH was subsequently modified using 1% orthophosphoric acid. The solution underwent filtration and degassing. The prepared phosphate buffer solution has a pH of 3.5, and ethanol was used as the mobile phase in a ratio of 99:1% v/v, respectively.[16]

Preparation of Stock Solution

To develop the ACV stock solution, the substance was solubilized in the combination of methanol and water (50:50 v/v), which was utilized as the sample's diluent. Following the precise amount of 100 mg of ACV being placed into a 100 mL standard flask, the substance was thoroughly dissolved with sonication for 10 minutes. The volume was subsequently adjusted to the desired level by adding diluent. The aforementioned is the primary stock standard solution of acyclovir with a µg/mL concentration. The necessary dilution was subsequently performed in order to obtain the desired concentrations of 2, 4, 6, 8, and 10 µg/mL. A similar diluent was utilized to prepare the stock solution for the acyclovir-loaded floating raft formulation.[17,18]

Sample Preparation

Male albino rabbits were utilised to spike plasma. The IAEC of the SRM College of Pharmacy at SRMIST, Kattankulathur, Chennai, India, approved the experimental protocol (IAEC/271/2021). Blood samples were acquired from the marginal ear vein of the rabbit in a cuvet containing EDTA in a blood collection unit. Plasma was extracted from the blood samples using a centrifugation process at 2500 to 3000 rpm. Plasma protein precipitation was done by adding orthophosphoric acid, which vortexed for a few minutes and centrifuged for 10 minutes. The supernatant solution (blank plasma) was collected and stored at deep freezer for future. The 10 mcL of ACV spiked in the rabbit plasma in the appropriate working dilutions and vortexing for proper mixing. The plasma sample concentrations ranged from 100 to 1000 ng/mL. The spiked blood samples were validated using high-performance liquid chromatography. Plotting the standard calibration curve for spiked plasma using the peak *vs.* concentration area as the reference.[19,20]

Procedure for Acyclovir loaded Floating Raft Formulation

The solid ingredients, namely sodium bicarbonate, calcium carbonate, sodium alginate, and sodium chloride, were thoroughly ground using a mortar and pestle and subsequently sifted through a No. 40 sieve. Afterwards, sodium benzoate was solubilized in distilled water. The natural mucilages, specifically guar gum, have been hydrated by adding a portion of the vehicle. Carbopol 974p was dispersed in water, and sodium chloride was used to balance the pH. The acyclovir was dispersed in water. Sequentially, aspartame and sodium saccharin were dissolved in hot distilled water. The natural mucilage was gradually mixed with a combination of sodium bicarbonate, calcium carbonate, sodium alginate, and sodium chloride under constant conditions. Next, the medication was added to the mixture and aggressively stirred for a duration of 5 minutes. Additional sweeteners and flavoring were incorporated, and the overall volume was modified using purified water.[21]

Optimization by Central Composite Design (CCD)

The bioanalytical validation of ACV present in floating raft formulation was frequently overlooked in past publications.

This research focused on developing a method and validating a reliable and sensitive bioanalytical method that requires less organic solvents with short retention time. These parameters were optimized through central composite design.[22]

The optimization of the pH of the buffer, injection volume, ACN concentration, buffer concentration, flow rate, and temperature-critical parameters was determined through the trial-and-error method. The rate of injection volume and flow rate affect the peak area and retention time. The CCD selected as a suitable design for optimization analyses the significant effects of selected factors and variables.[22, 23]

Validation of Developed Method

The ICH Q14 guidelines followed throughout the method development and validation process. The developed and optimized method was validated to ensure the accuracy, linearity, sensitivity, precision, selectivity, specificity and system suitability with LoD and LoQ.[24, 25]

Linearity

The linearity was evaluated for spiked plasma and standard ACV, and serial dilutions were prepared using appropriate diluents in concentrations from 100 to 1000 ng/mL. The standard calibration curve was plotted against amount of drug and peak area. The intercept value and regression correlation coefficient were utilised to evaluate linearity. LLoD and LLoQ determined the sensitivity of the procedure.[26]

System suitability

In order to assess the system suitability of the proposed HPLC method for the floating raft formulation of the ACV, the peak area of the middle concentration (middle quantity control) of standard ACV from a predetermined range (500 ng/mL) was measured. The peak area were compared with the mean value obtained for middle concentration.[27]

Sensitivity, selectivity and specificity

This was evaluated by determining its detection and quantitation limits. The LLoQ is lower concentration of the drug can be quantified and validated. Also, the variance of the nominal and measured concentrations at LLoQ must not exceed ± 20%. The LLoD represents the concentration that is detectable but unquantifiable. A comparison was made between spiked and neutral plasma samples with regard to their selectivity. In order to denote the separation of primary components from other analytes in the sample matrix, representative chromatographs were generated.

Plasma recovery

The drug's recovery from plasma that had been spiked was assessed at three distinct levels (n = 3), and the extraction efficiency of ACV was examined, reporting the mean value along with the standard deviation.[28]

Precision and accuracy

The developed method was validated through both intra and interday analytical days to analyse the "precision and accuracy" for the devised HPLC method. To assess this, the method's intra-day variability was determined by analysing five validation samples multiple times in one single day. Similarly, the interday variability was examined by doing replication analysis of different validation samples on five distinct days. Precision was quantified by calculating the relative standard deviation (RSD) as a percentage. The accuracy was determined by calculating the relative error (R.E.) as a percentage. The average precision and accuracy must fall within a range of 15.0% as per the acceptance standards. Nevertheless, the lower limit of quantification may deviate by approximately ±20.0% from the nominal concentration. (LLoQ).

Stability

The ACV solution was maintained at ambient temperature and monitored for stability for a period of 72 hours. A comparison was made between the assay value percentages of the standard samples and the newly prepared samples. Short-term stability was assessed at 30°C for 2 hours. Freeze-thaw cycles were conducted at 4°C for 24 hours. Long-term stability was evaluated for a period of 30 days at a temperature of 20°C. The ACV plasma stability was assessed at two distinct concentrations (500 and 1000n g/mL). This experiment was

Table 1: The chromatographic conditions maintained in Instruments

Parameter	*Chromatographic technique*
Instrument	Agilant 1220 infinity II LC
Column	C18 Column (4.6 X 250 mm, 5 µm Particle size)
Detector	Photodiode array detector
Diluents	Ethanol:Water (1:1)
Ratio of the mobile phase	Ethanol:Phosphate buffer (1: 99)
pH of the buffer	pH 3.5
Run time	05 minutes
Flow rate	0.6 mL/min
Injection volume	15 µL
Detection wavelength	230 nm
Temperature	31°C

Table 2: Central composite design and statistical analysis

Design run	*Response A*	*Response B*	*Responses*
1	0	-1	
2	-1	1	
3	0	0	
4	0	0	
5	1	0	Retention Time
6	-1	-1	
7	1	-1	Peak Area
8	-1	0	
9	0	0	
10	0	0	
11	1	1	
12	0	1	
13	0	0	

conducted in triplicate (n = 3) to analyze the average relative standard deviation (RSD).

Statistical Analysis

The HPLC procedure was replicated three times to get the average and standard deviation. This was established using a significance level of $p < 0.05$. ANOVA was utilised anytime data analysis was necessary for computation aggregation. The Expert program (state-ease version 6.0.0) was utilized to optimise the process variables.

RESULTS AND DISCUSSION

Response Surface Methodology (Optimization)

Optimization was accomplished through the implementation of the CCD design, which unveiled two components across three different levels (Table 2). The statistical quadratic equation was derived to evaluate the relationship between the important factors and their respective answers.

Y Responses) = β0 + β1X1 + β2X2 + β3X1X2 + β4X12 + β5X22

The dependent variable Y is affected by two coefficients (β1 and β2) associated with the independent variables X1 and X2. The word β0 represents the constant term or intercept in the mathematical model. The coefficient β3 represents the interaction effect between the components X1 and X2, whereas β4 and β5 correspond to the quadratic terms for X1 and X2. Figure 1 illustrates the investigation of the correlation between variables and outcomes, using three-dimensional and two-dimensional graphs to represent key characteristics. Figures 1(a) and 1(b) demonstrate that when the flow rate is low and the injection volume is high, the retention time (RT) decreases and the peak area increases. On the other hand, a higher flow rate results in a longer retention duration accompanied by peak tailing. In order to develop a reliable measurement technique, the flow rate was precisely calibrated to a maximum value of 0.6 mL/min. The 2D and 3D data illustrate an increasing trend in injection volume values, whereas a significant decrease is noticed as the flow rate lowers. The studies were performed in triplicate (n = 3) to minimize the influence of uncontrolled variables.

One-way ANOVA was utilized for statistical analysis to evaluate the practicality of the model. Table 2 displays the statistical evidence, indicating that both X1 and X2 have *p-values* of 0.0001. This confirms that the model employed to analyze Y1 and Y2 has the highest level of fitness. The objective of the optimization was to minimize the duration of retention time (Y1) while maximizing the area under the peak (Y2). The desirability parameter, selected as the dependent variable within a range of 0 to 1, resulted in a chromatographic condition with a desirability value of 0.9865. Furthermore, the R2 value, which represents the adjusted correlation coefficient, closely approached the observed value, suggesting a high level of agreement between the model and the data. The optimized parameters for the robust bioanalytical approach of ACV on HPLC are specified in Tables 3 (a) and 3 (b).

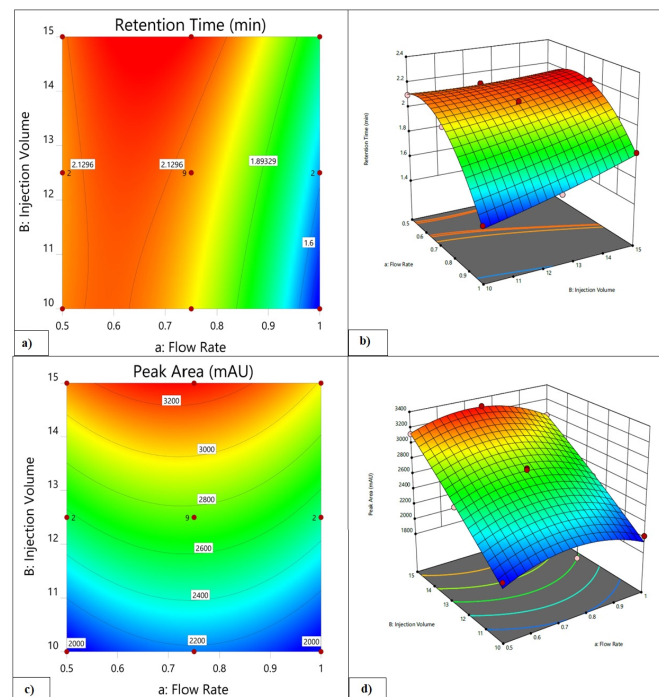

Figure 1: Contour plot and 3D response surface curve of retention time (a & b) and peak area (c & d)

Bionalytical Method Validation

Standard curve and linearity

A calibration curve for ACV in plasma was developed using 200, 400, 600, 800, and 1000 ng/mL concentrations. The correlation coefficient (R2) value of 0.997, as shown in Table 4, indicates an effective relationship with concentrations and peak area of measured value. The calibration curve's regression equation is expressed as Y = 5.6541x + 170.43.

Table 3 (a): Assay results of acyclovir formulation

Assay Parameter	*Results*
Formulations	Acyclovir loaded FRF
Dose	200 mg/10 mL (Suspension)
Amount found (mg) (mean ± SD, n = 3)	198.87 ± 0.35 mg
Assay ±% RSD	99.435 ± 0.25

Table 3 (b): Specificity study for acyclovir

Solutions	*Retention time (RT)*
Mobile phase	Peak not observed
Blank plasma	1.872
Acyclovir standard	2.037
Acyclovir loaded formulation	2.049

Table 4: Calibration data of the proposed method for the estimation of acyclovir

Concentration of ACV (ng/mL)	*Peak area, mV.s*
0	0
200	1415
400	2514
600	3625
800	4721
1000	5710

Table 5: Summary of validation parameters

Validation	*Results*
Linearity range (ng/mL)	200–1000
Regression equation (Y=A + BX)	Y = 5.6541x + 170.43
Intraday-precision	2.82% RSD
Interday-precision	2.77% RSD
Recovery	98.4–99.8
Specificity and selectivity	
LLoD (µg/mL)	15
LLoQ (µg/mL)	20

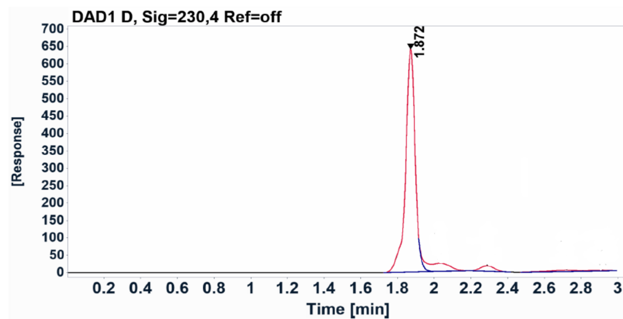

Figure 2: Chromatogram of rabbit plasma

LLoD and LLoQ

The LLoQ and LLoD values that were estimated for ACV are 15 and 20 ng/mL, correspondingly. The results prove that the novel bioanalytical method for detecting drugs in plasma is highly sensitive, rendering it feasible for future developments.

Drug plasma recovery

A concise summary of the plasma recovery values is provided in Table 3(a). The range of average plasma clearance for ACV was between 98.4 and 99.8%. As illustrated in Table 3(b), Figures 2-5, and the maximal retention period of the eluted ACV, there was no discernible interference.

Precision and accuracy

The optimized bioanalytical method was evaluated for accuracy and precision, both intra and interday. The RSD values were found to be less than 5% in three different concentration levels. The measured values for percentage accuracy ranged from 90.3 to 97.5%. The intraday and interday precision values were determined to be 2.82 and 2.77%, respectively, as listed in Table 5. The results clearly demonstrate the established HPLC approach is appropriate and precise and exhibits a high level of accuracy for quantifying ACV in plasma samples

Stability

The sample's stability was analyzed under various conditions that are expected to be encountered during the process of method development and storing the sample. The results are displayed on Table 6, demonstrating the selected drugs maintained its stability in plasma while stored at freezing temperatures.

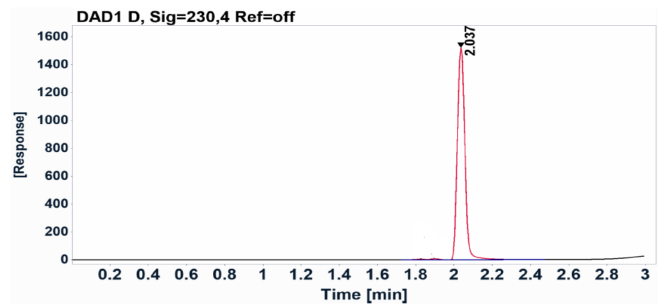

Figure 3: Standard chromatogram of acyclovir

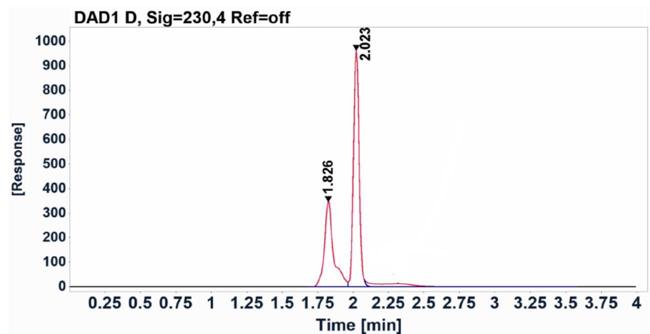

Figure 4: ACV spiked rabbit plasma sample

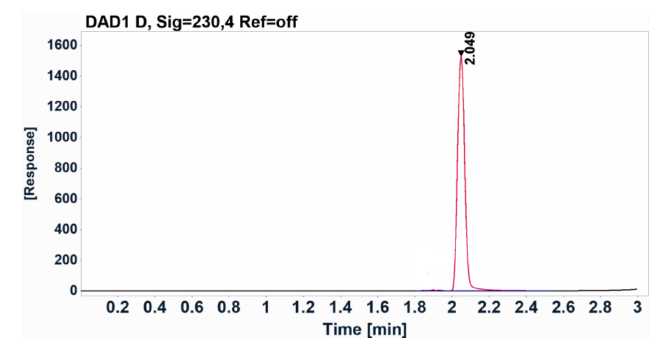

Figure 5: Chromatogram of acyclovir loaded FRF

Table 6: Stability studies of acyclovir for developed method

	Observed concentration (ng/mL)
Freeze-thaw cycle	472.4 ± 8.9
Short term	484.2 ± 4.5
Long term	479.5 ± 2.1

Green analytical tool

The proposed and existing method's green principles were compared through green analytical tools like GAPI, AGREE and AES depicted in Figure 6.

- *GAPI*

The pictogram of GAPI has five major parameters are sample preparation, solvents and reagents, instrumentation and energy efficiency, waste minimization and solvent recycling. The "GAPI indicated through different colors like green, yellow, and red". Green indicates an eco-friendly solvent and safe procedure while red shows non-eco-friendly and hazardous

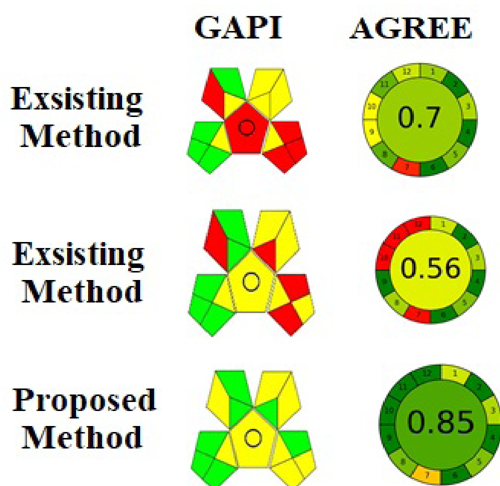

Figure 6: Greenness comparison with proposed and existing method

methods. The proposed method has maximum green and yellow because it contains ethanol, whereas the existing method has red color because of hazardous solvents like acetonitrile methanol.

- *AES*

The AES score is based on hazard penalty points for chemicals or reagents, instrumental energy, waste produced, and occupational risk. AES score above 75 indicates green method, 50–75 reasonably green, below 50 inadequate green analyses. The proposed method has a higher AES score of 93 than the existing method (AES score 74 and 71), which indicates a highly green analytical method and is less harmful to the environment.

- *AGREE*

AGREE is a recent software-based green analytical tool with a clock shape pictogram. Which contains twelve sections and center of the pictogram shows the ecological score has 1. Closer to 1 indicates higher greenness and less value, indicating the method has harmful solvents and is hazardous to analysts. The current method has a numerical value of 0.85 showing safe approach and existing method has 0.7 and 0.56.

CONCLUSION

This research analyzed the development of a bioanalytical technique for ACV-loaded FRF using HPLC to precisely quantify the content of the drug in biological materials, specifically plasma. The implementation of essential process elements involved the utilization of screening and optimization techniques, namely the application of CCD, to identify the most influential features that impact the performance of the developed bioanalytical procedure. The plasma recovery technique demonstrated both simplicity and a high degree of accuracy, with a recovery rate of 98%. The ACV elution in plasma was fully accomplished within a maximum duration of 3 minutes. Moreover, validating the bioanalytical method is essential in terms of "selectivity, linearity, and sensitivity" with a low limit of detection (LLoD) value of 15 ng/mL for assessing ACV plasma samples. Regarding the principles of green analytical chemistry, ethanol was employed as a non-hazardous organic solvent instead of a potentially risky one. Ultimately, the results from green evaluation tools indicated that the method was highly safe and easily applicable for the pharmaceutical industry.

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