

# Development and Validation of a Bioanalytical Method for Estimating Ellagic Acid in Wistar Rat Plasma by RP-HPLC Using a Gradient Elution Technique

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## Abstract

**Background:** Ellagic acid (EA) is a natural polyphenolic compound recognized for bioactive and pharmacologic properties that is found abundantly in various plant groups, particularly eudicotyledons. In the current study a novel, reliable, and cost-effective bioanalytical method was developed for quantifying EA in Wistar rat plasma using RP-HPLC.

**Methods:** Zorbax SB C18 (5 $\mu$ , 4.6150 mm) and Ascentis C18 (5 $\mu$ , 1004.6 mm) columns were used in the stationary phase, while the mobile phase consisted of water with 0.1 % formic acid (A) and acetonitrile (ACN) with 0.08% formic acid (B).

**Results:** Optimized parameters were as follows: temperature, 30°C; flow rate, 1.0 ml/min; and PDA detectors, 254 nm. The method exhibited high linearity ( $r^2 = 0.9993$ ) between 5 and 300  $\mu$ g/ml. Precision, both intra- and inter-day, was within acceptable limits (relative standard deviation <2%), and the mean recovery was 99.73%. The LOD and LOQ were  $1.564 \pm 0.026$   $\mu$ g/ml and  $5.015 \pm 0.025$   $\mu$ g/ml, respectively. Stability tests, including short- and long-term evaluations, demonstrated stability under various conditions.

**Conclusion:** The developed method met the necessary criteria and holds promise for application in clinical laboratories for assessing EA levels, either alone or with analytes.

## Keywords

Ellagic acid, HPLC, ICH, plasma, validation.

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## Introduction

Ellagic acid (EA) is a natural polyphenolic compound recognized for bioactive and pharmacologic properties that is found abundantly in various plant groups, particularly eudicotyledons [1]. EA holds significance in the pharmaceutical and cosmetics industries, which has prompted exploration of diverse plant species to identify new sources for human nutrition and raw materials for functional supplements and nutraceuticals. Renowned for its antioxidant, anti-mutagenic, and anti-cancer attributes, EA stands out as a highly stable molecule with lipophilic and hydrophilic domains composed of four rings, four phenolic, and two lactone groups. The potent antioxidant, anti-inflammatory, anti-mutagenic, and anti-proliferative characteristics coupled with therapeutic potential across various human diseases have garnered considerable research attention in recent years [2].

Utilizing thoroughly validated and well-characterized bioanalytical methods is crucial for generating reliable and interpretable results. These methods continually evolve and improve, often at the forefront of technological advancements. It is important to note that each bioanalytical technique possesses unique characteristics, which may vary depending on the analyte being studied. Consequently, specific validation criteria might need development for individual analytes. Furthermore, the suitability of a technique can be influenced by the overarching objectives of the current study [3].

It is imperative to validate the bioanalytical methods at each site and furnish appropriate validation data to ensure inter-laboratory reliability in studies in which sample analysis occurs across multiple sites. A limited number of UV and HPLC techniques exist for estimating EA alone or in combination with different

dosage forms [4]. Notably, there is still a gap in the literature with respect to the development of a bioanalytical technique for measuring EA in rat plasma [5]. To close this gap, a simple, accurate, and sensitive bioanalytical RP-HPLC method is needed.

The objective of this work was to develop and test a new bioanalytical HPLC technique for measuring EA in rat plasma. The approach is intended to be simple, economical, sensitive, sturdy, and long-lasting.

## Materials and methods

### Materials

Standard EA was gifted by Sigma-Aldrich (Mumbai, Maharashtra, India). HPLC grade formic acid, methanol, and acetonitrile (ACN) were obtained from Merck (Mumbai, Maharashtra, India). The remaining chemicals used in experiments were of analytical grade.

### Plasma sample preparation

A 5% EDTA solution was added to centrifuge tubes with the blood from Wistar rats. Following 1 min of vortex agitation, the material was centrifuged for 10 min at 10,000 rpm and 4 °C. The supernatant was collected and kept at -80°C until further use [6].

### EA standard stock solution (SSS) preparation

An EA SSS was prepared by accurately weighing 400 mg of pure EA and transferring the EA into a 100-ml volumetric flask. After adding 25 ml of methanol, the mixture was sonicated for 5 min. Methanol was then added to a volume to

100 ml. The solution was filtered using Whatman No. 41 filter paper. The resulting average stock solution had a concentration of 4000 µg/ml.

### Preparation of EA working standard solutions

SSS (0.3125, 0.625, 1.563, 3.125, 6.25, 9.375, 12.5, 15.625, and 18.75 ml [4000 µg/ml]) was placed into separate 25 ml volumetric flasks and diluted with methanol to achieve 50, 100, 2500, and 3000 µg/ml concentrations.

### Sample preparation for linearity and quality control

Blank plasma (2.7 ml) was mixed with 0.3 ml of working standard solutions. Each concentration was prepared by transferring 0.3 ml of the standard solution into separate 10-ml Eppendorf tubes, followed by spiking with 2.7 ml of blank plasma. Subsequently, 3 ml of methanol was added to each tube and the contents were vortex-agitated for 1 min. The solutions were then centrifuged at 10,000 rpm for 20 min at 5°C using a Remi centrifuge (Mumbai, Maharashtra, India). The resulting clear supernatant (3 ml) from each tube was transferred to new Eppendorf tubes. The clear supernatants were passed through a 0.42-mm membrane filter paper for filtration prior to HPLC loading. The final linearity and quality control samples were prepared at concentrations ranging from 5–300 µg/ml.

### Optimized chromatographic conditions

The optimized chromatographic conditions were derived based on several trials. The amount of EA in plasma was determined (Waters 2695 Alliance; Milford, MA, USA) using HPLC. Columns from Ascentis (C18, 5 µ,

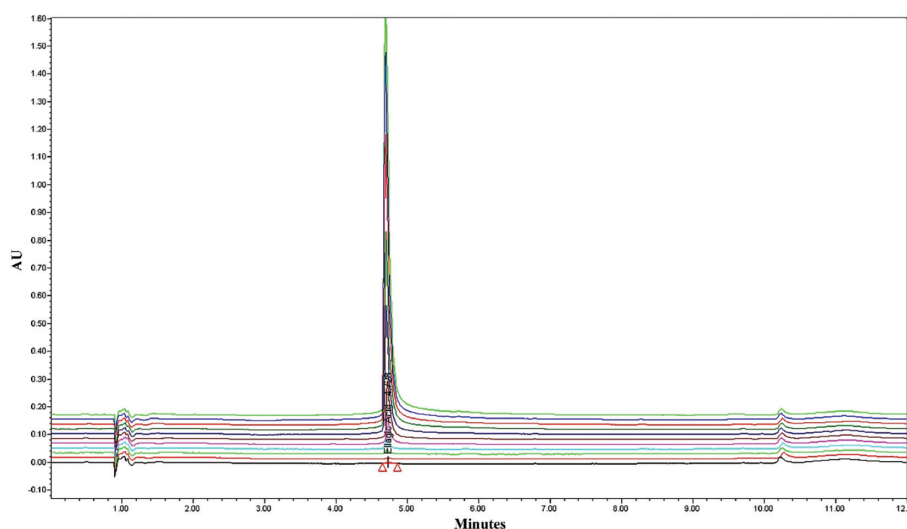


Figure 1 Overlay spectra of ellagic acid linearity at different concentrations.

**Table 1** Linearity Observations with RSD and Accuracy

Conc (µg/ml)	Measured Conc (n = 3; µg/ml)	RSD (%)	Accuracy (%)
5	4.96 ± 0.076	1.528	99.2
10	10.06 ± 0.1758	1.747	100.6
25	24.93 ± 0.4371	1.754	99.72
50	50.06 ± 0.8403	1.679	100.12
100	99.94 ± 0.9218	0.9224	99.94
150	149.8 ± 0.7271	0.4855	99.86
200	199.8 ± 0.7612	0.3810	99.9
250	249.8 ± 0.5953	0.2384	99.92
300	300.1 ± 0.3570	0.1190	100.03

**Table 2** Optimized Conditions of the Bioanalytical Method

Parameters	Optimized Parameters
Chromatograph	Waters 2695 alliance
Software	Empower 2
Method	Gradient
Column	Zorbax SB C18 (5µ, 4.6 × 150 mm) and Ascentis C18 (5µ, 100 × 4.6 mm)
Flow rate	1.0 ml/min
Temp.	30°C
Detector	W2996 PDA
Wavelength	254 nm
Injection volume	5.00 µl
Mobile phase	Water with 0.1% FA: ACN with 0.08 % FA
Run time	12 min

100 x 4.6 mm; Bellefonte, PA, USA) and Zorbax SB (C18, 5 µ, 4.6 x 150 mm; Santa Clara, CA, USA) were utilized. The column temperature was maintained at 30 °C. The mobile phase flow rate was fixed at 1.0 ml/min. A PDA-style detector was used at 254 nm. The experiments were performed using gradient chromatographic methods. ACN containing 0.08% formic acid (B) and water containing 0.1% formic acid (A) made up the mobile phase. The components of the

mobile phase were changed while maintaining a constant flow rate.

## Validation

### Linearity

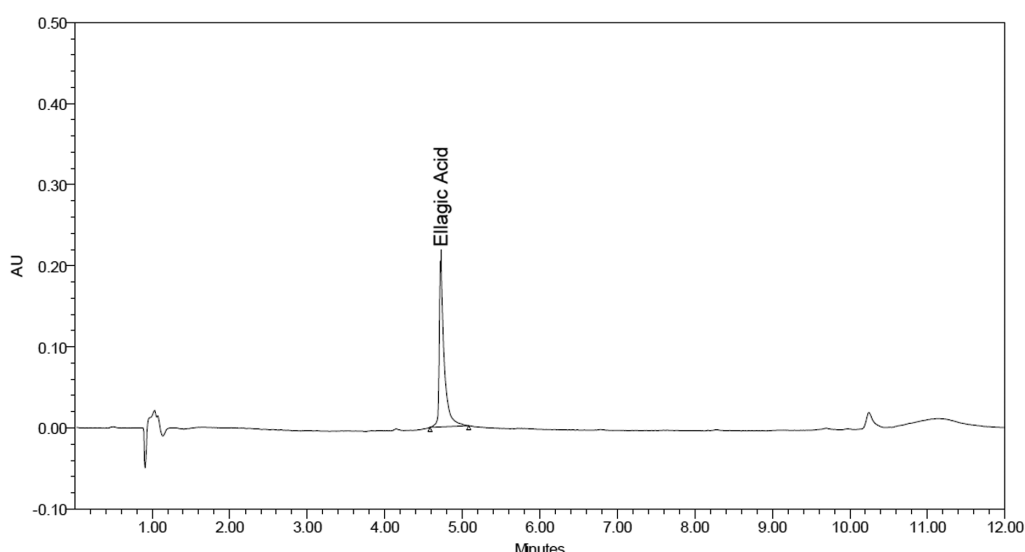
Nine control samples for linearity and quality were created by mixing different volumes of working standard solutions with 2.7 ml of blank rat plasma. After agitation for 1 min on a vortex shaker, the solutions underwent centrifugation to obtain clear supernatants, which were then transferred to Eppendorf tubes. These samples had EA concentrations ranging from 5–300 µg/ml. Plotting peak area versus concentration using three replicates for every sample allowed linearity assessment. The concentration of EA in each sample was determined using the resulting regression equation. The linearity curve was considered legitimate if all the answers showed a relative standard deviation (RSD) <15% [7].

### Specificity or selectivity

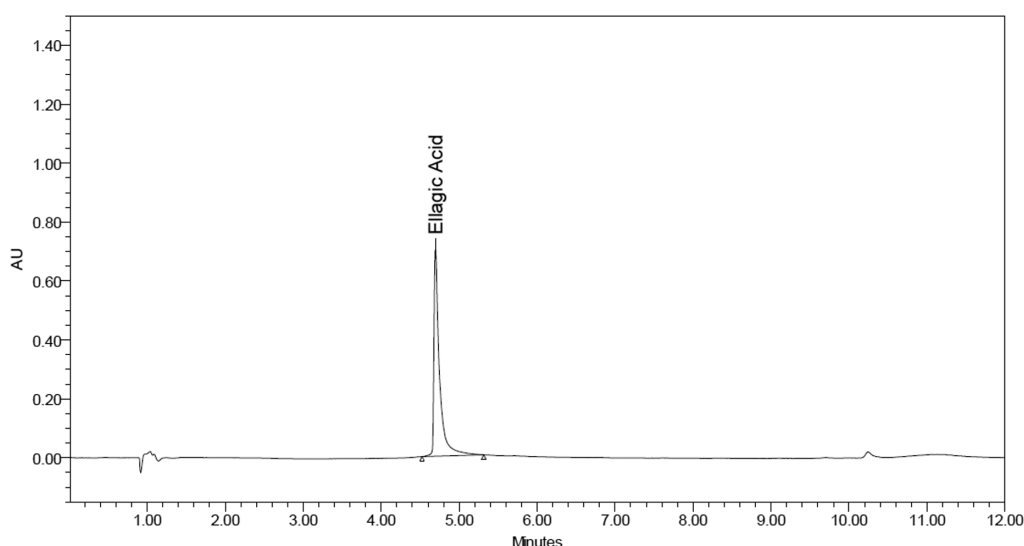
Specificity and selectivity were assessed by analyzing the chromatogram of blank Wistar rat plasma in triplicate without EA. Plasma samples spiked with EA were then examined to identify interfering co-eluting peaks in the rat plasma chromatogram. The retention time (RT) of the EA chromatographic peak was determined. To determine method specificity, adjustments were made to the HPLC method, including altering gradient slopes, to detect interference from endogenous substances causing co-eluting peaks in the chromatogram [8].

### Accuracy and precision

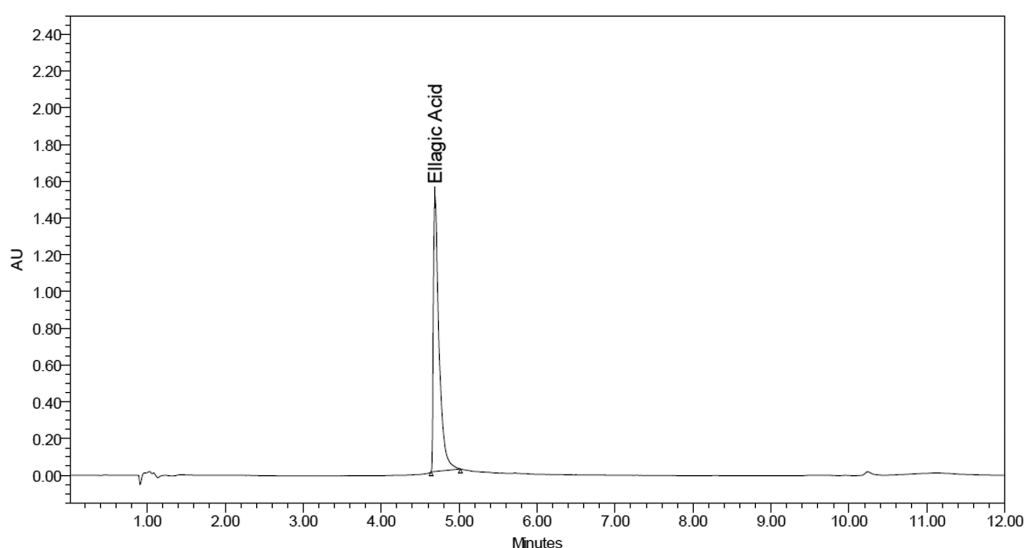
Accuracy and precision were evaluated through five replicates for intra- and inter-day assessments. This evaluation included 4 different quality control (QC) levels: 300 µg/ml



**Figure 2** Standard EA chromatogram at 50 µg/ml; Rt = 4.728 min.



**Figure 3** Chromatogram of standard EA at 150 µg/ml; Rt = 4.728 min.



**Figure 4** Chromatogram of standard EA at 300 µg/ml; Rt = 4.728 min.

**Table 3** Intraday Study Results

Conc (µg/ml)	Measured Conc (µg/ml)	RSD (%)	Accuracy (%)
Morning			
5 (LLOQ)	5.040 ± 0.05568	1.105	100.8
50 (LQC)	50.40 ± 0.4474	0.888	100.8
150 (MQC)	149.8 ± 1.201	0.802	99.86
300 (HQC)	300.3 ± 0.6067	0.202	100.1
Afternoon			
5 (LLOQ)	5.217 ± 0.09074	1.739	104.34
50 (LQC)	49.27 ± 0.6429	1.305	98.54
150 (MQC)	148.2 ± 1.041	0.703	98.8
300 (HQC)	297.1 ± 0.8505	0.286	99.03

**Table 4** Interday Study Results

Spiked Plasma Conc (µg/ml)	Measured Conc (µg/ml)	RSD (%)	Accuracy (%)
First day			
5 (LLOQ)	4.960 ± 0.08888	1.792	99.2
50 (LQC)	49.50 ± 0.6611	1.335	99
150 (MQC)	149.7 ± 0.6506	0.435	99.8
300 (HQC)	299.9 ± 0.4008	0.134	99.96
Second day			
5 (LLOQ)	4.787 ± 0.09018	1.884	95.74
50 (LQC)	48.29 ± 0.7954	1.647	96.58
150 (MQC)	147.3 ± 0.8622	0.585	98.2
300 (HQC)	298.4 ± 1.573	0.527	99.46

(high-quality control [HQC]); 150 µg/ml (medium-quality control [MQC]); 50 µg/ml (low-quality control [LQC]); and 5 µg/ml (lower limit of quantification [LLOQ]). The amount

of plasma retrieved was calculated utilizing a regression equation. Precision was expressed as the % RSD and accuracy was expressed as the % recovery [9].

## Recovery study

A recover study was undertaken across three distinct QC concentrations: 300 µg/ml (HQC); 150 µg/ml (MQC); and 50 µg/ml (LQC). Initially, EA was introduced into blank plasma at the three QC levels and the concentrations were determined utilizing the established HPLC bioanalytical method chromatogram. Identical standard concentration solutions were subsequently prepared without the biological matrix. To determine the EA extraction efficiency, the EA concentration ratio with and without the biological matrix was then calculated [10].

## Limit of detection (LOD)

The LOD involves determining the amount of an analyte in a sample at the lowest detectable level, although the LOD is seldom quantified. By injecting 1.5 µg/ml of EA 3 times into a plasma sample and comparing the chromatograms to the blank plasma, the LOD was determined [11].

## Limit of quantification (LOQ)

The LOQ was accomplished by comparing the peak areas of plasma samples that were spiked with EA at the LLOQ level with the blank plasma samples in triplicate [12].

## Ruggedness

Two separate operators evaluated the proposed process using separate columns of the same manufacturer with different reagents and solvents. EA was spiked into rat plasma at 2 quality control concentration levels: HQC at 300 µg/ml; and LQC at 50 µg/ml [12].

## Robustness

Robustness gauges the ability to withstand slight, intentional variations in method parameters. In the current study various critical parameters, like mobile phase composition, flow rate, and column temperature, varied by ± 5%, ± 10%, and ± 10% respectively, with the resulting chromatograms compared to

the normal chromatograms. EA was spiked into rat plasma at 300 µg/ml (HQC) and 50 µg/ml (LQC) [13].

## Stability study

Before analysis, LQC and HQC samples were kept for 6 h and 10 d at room temperature (25°C) to test short- and long-term stability, respectively. In addition, the samples were kept in an auto sampler tray at 5°C for a full day to assess auto sampler stability. LQC and HQC samples were kept at room temperature for benchtop stability testing. To evaluate the freeze-thaw stability of EA in plasma, LQC and HQC samples underwent three cycles of freezing and thawing. After each cycle, the samples were removed from the deep freezer at regular intervals and allowed to thaw at room temperature for 1 h. The stability of EA at LQC and HQC levels was then measured and compared to typical content in plasma [14].

# Results and discussion

## Validation of the bioanalytical method

### Linearity

The EA linearity study was studied by plotting calibration curves in rat plasma. The linearity was excellent over a 5–300 µg/ml concentration with an R<sup>2</sup> of 0.9993. The overlay spectra and calibration curve are presented in **Figure 1**. The linear equation was  $Y = 25683.09 X - 199830.75$  with an intercept of -199830.75 and a slope of 25683.09. The RT was 4.728 min. The resulting standard deviation (SD) was <2%. The % RSD was <2% with an accuracy between 99% and 101%, as shown in **Table 1**. The optimized chromatographic conditions of the bioanalytical method are presented in **Table 2**.

### Specificity or selectivity

The method was shown to be highly specific and selective without interference of impurity.

**Figures 2-4** displayed blank plasma and EA-treated plasma at three QC levels.

**Table 5** Recovery Study Results

Replicate Number	LQC (Area)		MQC (Area)		HQC (Area)	
	Unextracted	Extracted	Unextracted	Extracted	Unextracted	Extracted
1	1027000	1232896	3496369	3645697	7579593	7756792
2	1035000	1245623	3498570	3698756	7598649	7798654
3	1039123	1221344	3545979	3698654	7698745	7895643
Mean	1033708	1233288	3513639	3681036	7625662	7817030
SD	6164	12144	28.29	30604	64005	71226
% RSD	0.596	0.985	0.798	0.831	0.839	0.911
% Mean recovery	101.41		98.19		99.59	
Overall % mean recovery	99.73					
Overall SD	1.615					
Overall % RSD	1.619					

No peaks interfered with the EA retention period ( $R_t = 4.728$  min) based on a comparison of the peaks. This finding showed that there was no interference from other solvents or chemicals used in the development process. It is clear from these findings and chromatograms that rat plasma containing EA does not include any interference from co-eluting peaks. As a result, the technique that was developed was specific in identifying and evaluating EA in plasma [15].

## Accuracy and precision

Tables 3 and 4 show the intra- and inter-day precision and accuracy findings for EA in rat plasma at four QC levels based on the developed bioanalytical approach. The accuracy ranged from 98.54%–104.34% and the intraday % RSD was <2%. There was a  $\leq 2\%$  interday % RSD and the accuracy ranged between 95.74% and 99.96%. These results showed that the suggested bioanalytical approach was accurate, precise, and repeatable [16].

## Recovery study

Peak areas with and without the plasma were compared to compute the EA recovery. Table 5 presents the results of the recovery study. The recovery rates were 99.59%, 98.19%, and 101.41%. The overall recovery rate for EA was 99.73%, with a total RSD of 1.619%. Endogenous substances inside the biomatrix usually obstruct the examination of drugs in biological specimens. Nevertheless, examination of the gathered information showed that the EA in the plasma sample was not affected by the matrix peak [17].

## LOD and LOQ

The LOD ( $1.564 \pm 0.026$   $\mu\text{g/ml}$ ) indicated that the developed bioanalytical method is very sensitive and able to quantify very low levels of EA from plasma samples with an RSD of 1.654% [18]. The LOQ was  $5.015 \pm 0.025$   $\mu\text{g/ml}$ . This finding demonstrates that the system can detect

**Table 6** Ruggedness EA Data

Condition	Plasma Concentration ( $\mu\text{g/ml}$ )	RT (Min.)	RSD (%)	Measured Concentration ( $\mu\text{g/ml}$ )	RSD (%)	Accuracy (%)
Operator - 1	50 (LQC)	$4.707 \pm 0.019$	0.398	$48.40 \pm 0.882$	1.821	96.8
	300 (HQC)	$4.679 \pm 0.034$	0.722	$300.0 \pm 0.812$	0.270	100
Operator - 2	50 (LQC)	$4.775 \pm 0.073$	1.523	$49.31 \pm 0.781$	1.584	98.62
	300 (HQC)	$4.752 \pm 0.072$	1.521	$300.5 \pm 1.163$	0.387	100.17
Column - 1	50 (LQC)	$4.667 \pm 0.078$	1.672	$49.41 \pm 0.958$	1.939	98.82
	300 (HQC)	$4.671 \pm 0.084$	1.802	$300.2 \pm 0.965$	0.322	100.07
Column - 2	50 (LQC)	$4.703 \pm 0.037$	0.776	$50.01 \pm 0.719$	1.438	100.02
	300 (HQC)	$4.733 \pm 0.073$	1.550	$299.5 \pm 1.450$	0.484	99.83
Source of reagent and chemicals (Merk, Mumbai)	50 (LQC)	$4.749 \pm 0.024$	0.506	$50.45 \pm 0.741$	1.468	100.9
	300 (HQC)	$4.717 \pm 0.065$	1.377	$300.3 \pm 0.700$	0.233	100.1
Source of reagent and chemicals (Qualigens Fine Chemicals, Mumbai)	50 (LQC)	$4.677 \pm 0.030$	0.650	$50.36 \pm 0.303$	0.601	100.72
	300 (HQC)	$4.713 \pm 0.003$	0.065	$298.7 \pm 1.836$	0.615	99.56

**Table 7** Robustness Data of EA

Condition	Plasma Conc ( $\mu\text{g/ml}$ )	RT (Min.)	RSD (%)	Measured Conc ( $\mu\text{g/ml}$ )	RSD (%)	Accuracy (%)
Flow rate (-10%)	50	$4.742 \pm 0.012$	0.260	$50.45 \pm 0.794$	1.574	100.9
	300	$4.747 \pm 0.092$	1.945	$302.1 \pm 1.502$	0.497	100.7
Flow rate (+10%)	50	$4.706 \pm 0.015$	0.322	$47.89 \pm 0.809$	1.688	95.78
	300	$4.673 \pm 0.019$	0.396	$299.0 \pm 1.642$	0.549	99.67
Temperature (-10%)	50	$4.779 \pm 0.076$	1.601	$52.42 \pm 0.669$	1.276	104.84
	300	$4.714 \pm 0.047$	0.993	$302.5 \pm 0.852$	0.282	100.83
Temperature (+10%)	50	$4.686 \pm 0.057$	1.209	$48.91 \pm 0.278$	0.568	97.82
	300	$4.666 \pm 0.049$	1.051	$300.9 \pm 0.612$	0.203	100.3
Organic mobile phase (-5%)	50	$4.823 \pm 0.082$	1.702	$51.40 \pm 0.995$	1.935	102.8
	300	$4.789 \pm 0.095$	1.975	$299.1 \pm 1.299$	0.435	99.7
Organic mobile phase (+5%)	50	$4.722 \pm 0.061$	1.299	$48.67 \pm 0.565$	1.162	97.34
	300	$4.635 \pm 0.071$	1.532	$298.7 \pm 1.834$	0.614	99.57

**Table 8** Stability Study Results

Type of Stability	Spiked Plasma Conc (µg/ml)	Measured Conc (µg/ml, n = 3; mean ± SD)	RSD (%)	Accuracy (%)
Short-term stability (6 h at 25°C)	50	49.73 ± 0.70	1.39	99.46
	300	296.4 ± 0.88	0.29	98.80
Long-term stability (10 days at 25°C)	50	46.21 ± 0.90	1.96	92.42
	300	294.0 ± 1.59	0.54	98.00
Auto-sampler stability (24 h at 5°C)	50	48.18 ± 0.84	1.73	96.36
	300	289.9 ± 1.79	0.62	96.63
Freeze-thaw stability (cycle 3, 4 h at -30°C)	50	48.61 ± 0.76	1.56	97.22
	300	288.7 ± 1.20	0.42	96.23
Benchtop stability (Old solution)	50	45.16 ± 0.86	1.91	90.32
	300	289.5 ± 1.06	0.364	96.50
Benchtop stability (Fresh solution)	50	48.46 ± 0.85	1.76	96.42
	300	297.7 ± 1.52	0.51	99.23

even small amounts of EA in rat plasma with an RSD of 0.505%.

### Ruggedness

**Table 6** presents the results of the ruggedness study for EA in rat plasma. Ruggedness was assessed to determine the impact of different operating conditions on the RT and peak area. Spiked EA standard chromatograms at these two concentrations in Wistar rat plasma were analyzed under several operating conditions. No significant differences were detected in RT or concentrations. Additionally, the % RSD was <2%. Thus, the developed method was sufficiently robust to withstand variations in chromatographic conditions, including changes in operators, columns, and sources of reagents and chemicals [15].

### Robustness

**Table 7** presents the robustness study for EA in rat plasma. To assess robustness, several parameters were changed and the effects on the RT and concentration were measured by peak area. The RT was shortened and the concentration was determined based on peak regions by increasing the flow rate, temperature, and composition of the organic mobile phase. The RT decreased by 1–2 s for every incremental degree increase in column temperature. Temperature has a major impact on solvent viscosity and vapor pressure, yet pressure loss is proportionate to mobile phase thickness [19]. As the temperature rises, the mobile phase density decreases, which led to an increased flow rate. Consequently, with increasing temperature and flow rate, the RT and measured concentration from the peak areas decreased and vice versa. At concentrations of 50 and 300 µg/ml, the RT decreased from 4.728 min to 4.673, 4.706, and 4.635 min with a +10% flow rate and a +5% organic mobile phase. The percent RSD for each parameter variation was approximately 2%. The RT and concentration did not significantly change from the parameters of the conventional chromatographic technique.

Therefore, the established bioanalytical technique may be applied in routine laboratory settings and is dependable under the given conditions [20].

### EA stability in rat plasma

**Table 8** presents the stability analysis results for EA in rat plasma under various conditions. The % RSD ranged from 0.29% (short-term stability) to 1.96% (long-term stability). The % RSD was <2% of the true value in every stability study. No discernible EA degradation was detected, even after 3 freeze-thaw cycles (4 h at -30°C), a 24-h storage period at 5°C in the auto-sampler tray, and a 10-day storage period at 25°C. These results showed that EA in rat plasma does not change when stored in different ways [15].

### Conclusion

The current developed and validated a novel buffer-free RP-HPLC method for quantifying EA in rat plasma, which is characterized by simplicity, precision, accuracy, sensitivity, and repeatability. The method met all validation criteria and showed no matrix effect on RT and concentration. Stability tests confirmed that EA remains stable during storage and sample preparation. The method is linear with a high correlation coefficient, making EA ideal for pharmacokinetic, bioequivalence, and therapeutic drug monitoring studies. Advantages include the minimal plasma volume requirement, low limits of quantification and detection, high accuracy, cost-effectiveness, and straightforward preparation. This gradient elution technique effectively analyses complex samples within a 20-min RT free from interference by late-eluting peaks.

### Conflicts of interest

The authors declare that they have no competing interests.

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