Design and Optimization of LC-MS Bioanalytical Method for Efonidipine and Chlorthalidone Using Quality by Design Strategy

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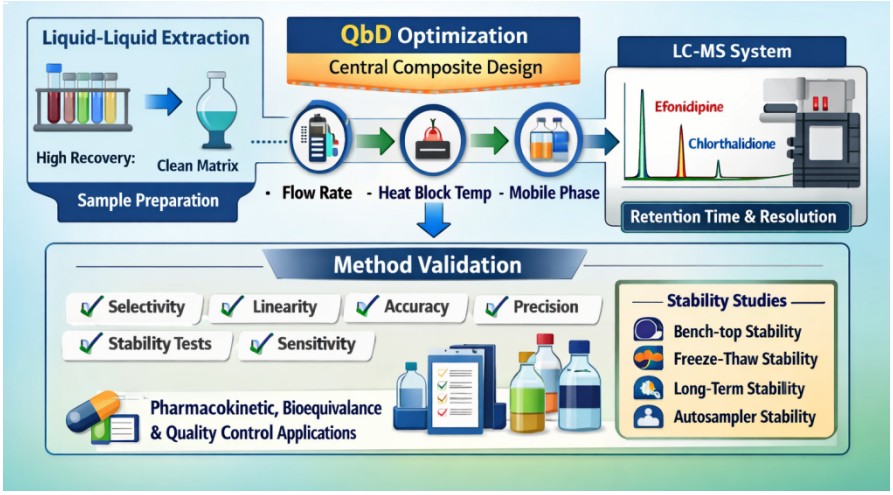
Graphical Abstract:



ABSTRACT

Objective

The Quality by Design (QbD) technique was used in this work to design and validate a novel LC-MS method for the simultaneous measurement of Efonidipine Hydrochloride, Ethanolate, and Chlorthalidone in artificially generated human plasma. To guarantee the method achieved the right analytical performance, the parameters of flow rate, heat block temperature, and mobile phase composition in methanol were optimised in an analytical science, systematic approach employing a central composite design. Resolution and retention time were two of the analytical method's most important characteristics.

Methods

The chromatographic separation was achieved using a Shimadzu capcell pak C18 (150×4.6mm, 3µm), and a mobile phase consisting of Phosphate buffer (pH 3.0) (40:35:25% v/v/v): methanol and acetonitrile. The mobile phase was allowed to flow at a rate of 1.0 mL/min. A mass spectrometer coupled with an electrospray ionization (ESI) source operating in the positive ion was used for detection. Data were obtained in the multi-reaction monitoring (MRM) acquisition mode. The best recovery and maximum matrix cleanliness were obtained from the liquid-liquid extraction sample preparation.

Results

The retention time for Efonidipine and Chlorthalidone were 6.38 and 8.60 min respectively. Sample extraction was performed using liquid-liquid extraction (LLE), and this technique produced very pure extracts with good recovery rates. A linear calibration curve was found in the range of 18.75-65.61 µg/mL for Efonidipine and 60-210 µg/mL for Chlorthalidone with a correlation coefficient $r^2 > 0.99$. The validation process confirmed the LC-MS analytical method's selectivity, sensitivity, linearity, accuracy, and precision for both Chlorthalidone and Efonidipine. Bench-top stability, freeze-thaw stability, short-term stability, long-term stability, and autosampler stability data showed good recovery and proved the method's stability.

Conclusion

Overall, it was shown that the optimised LC-MS analytical approach was dependable and might be helpful for routine work in pharmacokinetic, bioequivalence, and quality control investigations for medications like Chlorthalidone and Efonidipine.

Keywords: LC-MS, QbD, Efonidipine Hydrochloride Ethanolate, Chlorthalidone, Artificial Human Plasma.
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Conflict of interest: None

1.Introduction:

Often prescribed to treat cirrhosis, hypertension, congestive heart failure, renal impairment, and oedema, chlorthalidone (CHL), sometimes called 2-chloro-5-(1-hydroxy-3-oxo-2H-isoindol-1-yl) benzene sulphonamide, is a thiazide-like diuretic. Its primary pharmacodynamic effect is caused by its reduction of sodium and chloride reabsorption at the distal convoluted tubule of the nephron, which increases the excretion of water, sodium, and chloride ions. Its diuretic action reduces blood pressure and volume, indicating its importance as a therapy for cardiovascular and renal condition[1-3].

(±)-2-(N-benzyl-N-phenylamino) phosphinan-2-yl.2) 2,6-dimethyl-4-(3-nitrophenyl)Efonidipine Hydrochloride Ethanolate (EFD), 1,4-dihydropyridine-3-carboxylate hydrochloride ethanoate, is a dihydropyridine calcium channel blocker having a variety of uses in the management of hypertension and angina pectoris. By inhibiting L-type and T-type calcium channels in cardiac and vascular smooth muscle, EFD reduces peripheral vascular resistance (PVR) and increases vasodilation. The pharmacological properties of CHL and EFD complement each other, and the simultaneous use of both agents allows for controlling the degree of hypertension through two mechanisms: a diuretic and a calcium channel blocker[4-5].

An extensive review of relevant analytical literature demonstrates both products have been dosed alone or in combination using a variety of chromatographic and spectroscopic techniques. Some examples of the various techniques include stability-indicating HPLC[6], Central Composite Design (CCD)-assisted RP-HPLC[7], eco-friendly HPLC and UV-spectrophotometry[8], UV spectroscopy (synthetic mixture)[9,] Efonidipine alone analysed by RP-HPLC[10], LC-MS/MS [11], Chiral LC-MS/MS[12]and LC-Q-TOF[13]. Efonidipine combined with other drugs, RP-HPLC (Synthetic mixture)[14] has been reported. Chlorthalidone alone analysed by RP-HPLC use of Experimental Design in Forced Degradation Experiments,[15] UV spectroscopy (different order derivative methods),[16] RP-HPLC stability-indicating (process related impurity),[17] Chlorthalidone combined with other drugs, Stability-indicating RP-LC method,[18] HPTLC and HPLC with photodiode array detector method,[19] RP-HPTLC,[20] HPTLC[21] has been reported.

Although there have been studies detailing procedures to assess CHL and EFD independently, to date no chemo metrically optimized LC-MS method exists to assess CHL and EFD concurrently in biological matrices. Our objective in this workspace is to develop and validate a quick, dependable, and sensitive LC-MS method for the simultaneous detection of Efonidipine Hydrochloride Ethanolate and Chlorthalidone drug in artificial human plasma using the Analytical Quality by Design (AQbD) method, which is a methodical, risk-based approach to developing analytical methods and supporting the validity of the method.

The Quality by Design (QbD) framework has received considerable recognition in pharmaceutical research especially in the field of Analytical Method Development (AMD). Within QbD, Critical Analytical Attributes (CAAs) and Critical Method Parameters (CMPs) are based on scientific experimentation and risk assessment[22]. The implementation of Response Surface Methodology (RSM) in a Central Composite Design (CCD) can establish a reliable, strong design space for analytical methods and analytical methods robustness[23].

Thus, the objective of this study is to apply QbD approaches to the development of LC-MS methods for a scientifically robust, reproducible, and efficient analytical method. The developed methods are able to be suitable for applications and are more scientifically advanced than simply relying on methodological empiricism, for pharmacokinetic, bioequivalence, and quality control studies of both analytes.

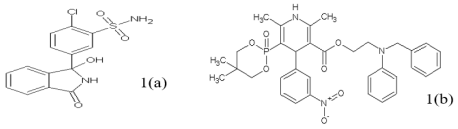

Figure 1(a-b): Chemical structures of Chlorthalidone and Efonidipine Hydrochloride Ethanolate

2. Experimental Section:

2.1 Instrumentation and Chromatographic Conditions:

The method was developed and validated using a Shimadzu LC-MS 8040 system that had a triple quadrupole mass spectrometer and an electrospray ion source (ESI). The interface voltage was maintained at 4.5 kV, the drying gas flow rate was set at 12.0 L/min, and the nebulizing gas flow rate was set at 2.7 L/min. The desolation line and heat block were set at 250 °C and 400 °C, respectively, while the cooler was set at 15 °C. Argon was used as the collision gas and nitrogen as the nebulizing gas.

A low-pressure differential four solvent delivery system was employed, with a flow rate range of 0.0001 to 10 mL/min. There were five degassing lines in the system: one 400 µL for rinse solution and four for mobile phases. The autosampler may inject volumes ranging from 0.1 to 100 µL. Six columns of 10 cm or three columns of 10–30 cm might fit in the oven. Between 190 to 700 nm in wavelength, the UV detector staged detection.

A Shimadzu Capcell Pak C18 column (150 × 4.6 mm, 3 µm) was employed as the stationary phase in the chromatographic separation. The mobile phase consisted of methanol, acetonitrile, and potassium dihydrogen phosphate buffer in a 30:30:40 (v/v/v) ratio at pH 3.0 with orthophosphoric acid. The flow rate of 1.0 mL/min was selected. Data processing and collection were done using Shimadzu LC-Solution software version 6.42.

2.2 Chemicals and Reagents:

We received gift samples of efonidipine hydrochloride, ethanol, and chlorthalidone from Madras Pharmaceuticals in Chennai, India. For reference, a commercial formulation called EFONTA-T, which contains 20 mg of efonidipine and 40 mg of telmisartan, was bought locally. We purchased HPLC-grade methanol, glacial acetic acid, and acetonitrile from Merck (India). We purchased Milli-Q water (HPLC quality) from Qualigens in Mumbai, India.

2.3 Software Tools:

Design-Expert (Version 12.0.0) was utilized to fit quadratic response surfaces and carry out Central Composite Design (CCD) for statistical optimization and experimental design. Statistics such as mean, standard deviation, and percent relative standard deviation (%RSD) were calculated and evaluated using Microsoft Excel.

2.4 Preparation of Mobile Phase:

A new mobile phase was made by combining 30 mL of phosphate buffer (pH 3.0), 40 mL of acetonitrile, and methanol. Before being used, the mixture was filtered through a 0.22 µm membrane filter. The solvent mixture's ratios were consistently maintained at 30:30:40 (v/v/v).

2.5 Preparation of Calibration and Quality Control (QC) Samples:

Standard stock solutions of efonidipine (1000 µg/mL) and chlorthalidone (1000 µg/mL) were prepared in methanol and stored at 4°C. Drug-free plasma was spiked with appropriate aliquots of stock solutions to achieve the final concentrations of 60–210 µg/mL for chlorthalidone and 18.75–65.61 µg/mL for efonidipine.

Quality Control (QC) samples were similarly prepared at three levels:

- Low QC (LQC): 60 µg/mL (CHL), 18.75 µg/mL (EFD)
- Medium QC (MQC): 120 µg/mL (CHL), 37.5 µg/mL (EFD)
- High QC (HQC): 180 µg/mL (CHL), 56.25 µg/mL (EFD)

2.6 Artificial Plasma Sample Preparation:

Before analysis, artificial human plasma was kept at -20°C. QC and calibration samples were mixed with aliquots (50–100 µL) of plasma and vortexed for one minute. The proteins were precipitated using 2 mL of methanol, vortexed for 60 seconds, and centrifuged at 5000 rpm for 15 minutes. After the supernatant was filtered via 0.45 µm PTFE syringe filters, 10 µL of it was introduced to the LC-MS for analysis.

2.7 Screening Design:

Three critical analytical parameters methanol percentage (%), flow rate (mL/min), and heat block temperature (°C) were optimized based on their effects on chromatographic performance and ionization efficiency. The factors and responses were arranged using a Central Composite Design (CCD):

Factors:

Methanol composition in mobile phase (A): (25–35% v/v).

Flow rate (B) : (0.8–1.2 mL/min)

heat block temperature (C) : (300–500 °C)

Responses:

Retention time for first peak (Efonidipine)-(Rt1)

Resolution between Efonidipine and Chlorthalidone-(Rs1,2)

2.8 Optimization Design:

CCD was used to evaluate how the independent variables affected the response variables, retention time, and resolution. Each independent variable was assessed at five levels: low −1, medium 0, high +1, and the axial points −α and +α. Twenty studies in all were conducted in a randomized fashion to lessen bias. The desirability function and numerical optimisation were then utilised to examine the operating circumstances that maximised analytical performance after the model's performance was evaluated using Analysis of Variance (ANOVA).

2.9 Environmental Impact Assessment:

To achieve environmental sustainability, the Analytical Eco-Scale (AES) technique was used to assess the method's ecological footprint. AES assigns penalty points (PP) based on a number of factors, including chemical toxicity, solvent volume, energy consumption, waste production, and occupational hazards. These scores promote environmentally friendly analytical chemistry techniques and show how green the approach is overall.

3. Results and Discussion:

A comprehensive study has been carried out to create a dependable and repeatable LC-MS method for the simultaneous detection of ethanol, chlorthalidone, and efonidipine hydrochloride in human plasma. In order to determine which mobile phase composition would best separate the two analytes, several were first tested. The effects of the concentration of potassium hydrogen phosphate buffer and the ratios of methanol to acetonitrile as organic modifiers were investigated. The factors were selected for further study based on experimental optimization, and those that significantly affected chromatographic performance were evaluated in further detail.

The resulting chromatographic parameters are listed in Table 1. In every analysis, a DAD was utilized for detection at 254 nm. The optimal chromatographic conditions were obtained using a Shimadzu Capcell Pak C18 column (150 × 4.6 mm, 3 µm) with a mobile phase made up of acetonitrile, methanol, and potassium hydrogen phosphate buffer (pH 3.0, adjusted with orthophosphoric acid) in a ratio of 30:35:35 (v/v/v). The flow rate was maintained at 1.0 mL/min throughout the analysis. Under these optimal conditions, efonidipine and chlorthalidone eluted in 6.38 and 8.60 minutes, respectively (Figure 2).

Table 1: LC-MS Operating Conditions

Parameter	Operating Conditions
Instrument	Shimadzu LC-MS 8040 Triple Quadrupole System
Column	Shimadzu Capcell Pak C18, 150×4.6 mm (3 µm)
Interface Type	ESI
Interface Voltage	4.5 kV
Heat Block Temperature	400°C
Detector Wavelength	254 nm
Drying Gas Flow	12.0 L/min
Nebulizing Gas Flow	2.7 L/min
Polarity	Positive and Negative
Scan Range	100–200 m/z
Cooler Temperature	15°C
Column Temperature	25°C
Flow Rate	1.0 mL/min
Total Analysis Time	15 min
Injection Volume	20 µL
Mobile Phase	Phosphate buffer: methanol: acetonitrile (40:35:25 % v/v/v)

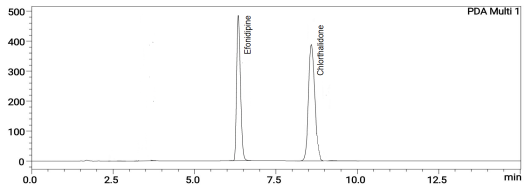

Figure 2: Optimized RP-HPLC chromatogram of Efonidipine and chlorthalidone at 254 nm with Retention times of 6.38 min and 8.60 min, respectively

Mass Spectral Analysis:

The LC-MS spectra confirmed the molecular identity and integrity of both analytes. In the case of chlorthalidone, there was a very prominent molecular ion peak at m/z 281.0 [see Figure 3(a)] for the [M+H]+ ion. In the Efonodipine mass spectrum [see Figure 3(b)], several ion fragments were observed at m/z 206.1, 252.1, 298.2, 357.1, 464.1, and 611.3 suggesting further fragmentation occurred. As such, those unique

ion peaks confirm both the molecular identity and the purity of each analyte.

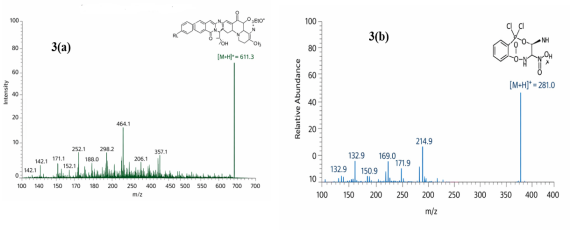

Figure 3(a-b): Mass Spectrum for Efonidipine and Chlorthalidone

Experimental Design and Optimization (CCD Approach):

A Central Composite Design (CCD) was used to evaluate the effects of the flow rate (mL/min), heat block temperature (°C), and % v/v of methanol on the significant responses of retention time (Rt1) and resolution (Rs1,2) in order to improve the method performance. The design included twenty factorial and axial point trials to assess the concept's validity. The experimental design and response values are shown in Table 2.

Table 2: CCD Experimental Runs and Responses

Run	Methanol (%)	Flow Rate (mL/min)	Heat Block Temp (°C)	Retention Time (Rt1)	Resolution (Rs1,2)
1	30	1.0	400	6.28	2.17
2	30	1.0	400	6.28	2.17
5	30	1.0	400	6.28	2.17
7	30	1.0	400	6.28	2.17
11	30	1.0	400	6.28	2.17
17	30	1.0	400	6.28	2.17
6	30	1.0	568.18	5.13	3.45
9	30	1.33	400	5.86	3.98
10	30	0.66	400	6.01	2.13
13	30	1.0	231.82	6.16	3.44
14	21.59	1.0	400	5.75	3.72
19	38.40	1.0	400	6.23	2.54
3	25	0.8	500	5.45	3.51
4	35	0.8	500	5.37	3.63
8	35	1.2	300	6.09	3.30
12	25	1.2	300	6.11	2.80
15	35	1.2	500	5.59	2.32
16	25	1.2	500	6.12	3.11
18	25	0.8	300	5.97	2.73
20	35	0.8	300	6.13	2.65

mL/min- millilitre/minute, %- percentage, °C-degree celusius,Rt1- Retention time for first peak, Rs1.2- Resolution between peak one and two

Model Fitting and Statistical Validation:

The CCD model was assessed using ANOVA, lack of fit measurement, corrected R2 coefficient of variation (CV), and sufficient precision. The quadratic model had an excellent fit and was statistically significant ($p<0.0001$), with adjusted R2 consistently >0.80. A comparatively high signal to noise ratio was suggested by the sufficient precision values (9.73-14.10), which were higher than the tolerable value (4). High precision and repeatability were demonstrated by the CV<10%.

The optimized quadratic equations for the two responses were:

Retention Time (Rt1):

$$Rt1 = +6.28 - 0.0247A + 0.0540B - 0.2464C - 0.0787AB - 0.0937AC + 0.0098BC - 0.0977A^2 - 0.1172B^2 - 0.2197C^2$$

Resolution (Rs1, 2):

$$Rs1,\ 2 = +2.18 - 0.1636A + 0.1553B + 0.0810C - 0.0412AB - 0.1363AC - 0.3038BC + 0.2831A^2 - 0.2566B^2 + 0.3945C^2.$$

The developed polynomial models for retention time (Rt1) and resolution (Rs1,2) demonstrate the significant influence of methanol concentration (A), flow rate (B), and heat block temperature (C) on chromatographic performance in the LC-MS method. For Rt1, methanol concentration and heat block temperature show negative effects, indicating that increasing these factors reduces retention time, with temperature having the most pronounced impact, while flow rate slightly increases Rt1; interaction and quadratic terms confirm a nonlinear relationship and the presence of an optimum region. In contrast, the resolution model (Rs1,2) reveals that increasing methanol concentration decreases resolution, whereas flow rate and temperature improve it to some extent, although their combined interaction (especially BC) adversely affects separation. The quadratic effects further indicate that optimal levels of methanol concentration and temperature enhance resolution, while excessive flow rate reduces it. Overall, both models highlight the complex interplay of variables and emphasize the importance of systematic optimization to achieve a balance between faster analysis (lower Rt) and better separation (higher Rs).

(Table 3) summarizes the ANOVA validation parameters.

Table 3: ANOVA Model Summary

Response	Adjusted R^2	Model p-value	C.V. (%)	Adequate Precision
Rt1	0.8828	<0.0001	2.71	9.7345
Rs1,2	0.8108	<0.0001	7.42	14.100

C.V. (%)- Coefficient of variation percentage

Response Surface and Perturbation Analysis:

The obtained 3D surface and contour (2D) contour plots for the tests (Figs. 4 and 5) clarified that varying the flow rate, concentration of the methanol, and heat block temperature influenced both the tR and resolution; increasing the methanol percentage and increasing %v/v methanol (and flow rate) reduced tR and did compromise and slightly reduce the resolution, while reducing the heat block temperature (to the point of not ionizing) increased the retention, seemingly due to the ionization efficiency. These interactions between the different factors justified the multivariate optimisation of the extractant composition, flow, and heat block temperature.

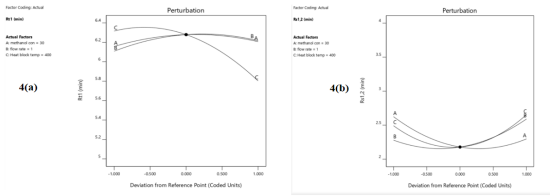

Fig.4(a) Perturbation plot showing the effect of methanol concentration, flow rate, block heat temperature on for first peak Retention Time (Rt1) of first peak (Efonidipine) 5(b). Perturbation plot depicting the effect of chromatographic variables on Resolution (Rs1,2) between Efonidipine and chlorthalidone

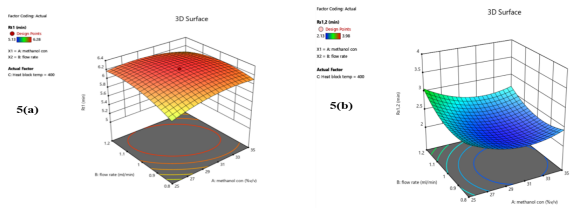

Fig. 5 (a) 3D Surface plot depicting the effect of methanol concentration on Retention Time (Rt1) for first peak (Efonidipine) shown in chromatogram at constant flow rate (b) 3D Surface plot showing the effect of methanol concentration and flow rate on Resolution (Rs1,2) between Efonidipine and chlorthalidone peaks at constant flow rate

Optimization Using Derringer's Desirability Function:

The desirability method of Derringer was employed to achieve simultaneous optimization of the responses. The composite desirability value (D) provides a summary measure of two or more response objectives, where D could range from 0 (completely undesirable) to 1 (completely desirable). The optimization objectives were to minimize retention time and maximize resolution.

Under the optimized conditions of 35% (v/v) methanol, 1.0 mL/min flow rate, and 500°C heat block temperature, the model estimated the Efonidipine retention time to be 6.541 min and a resolution of 2.303 for the response of interest and an overall desirability of 0.673. Following experimental verification, the experimental values were closely related to the model predictions, with an average error of 3.44% for Rt1 and 2.99% for Rs1, 2 (Table 4, Figure 7).

Table 4: Comparison of Predicted and Experimental Results

Parameter	Methanol (%)	Flow Rate (mL/min)	Heat Block Temp (°C)	Rt1 (min)	Rs1,2	Avg. Error (%)
Predicted	35.00	1.0	500	6.541	2.303	—
Experimental	35.00	1.0	500	6.323	2.234	3.22
Desirability (D)						**0.673**

mL/min- millilitre/minute, %- percentage, °C-degree celusius,Rt1- Retention time for first peak, Rs1.2- Resolution between peak one and two

It was demonstrated that the LC-MS approach developed for the simultaneous separation and quantification of chlorthalidone and efonidipine worked well. Additional guarantees of approach robustness, accuracy, and dependability were given by the QbD and CCD optimization. The study successfully developed the critical method parameters that affected chromatographic performance, which was

a scientifically justified, reproducible, and environmentally conscious method with broader potential application to routine bioanalytical methods.

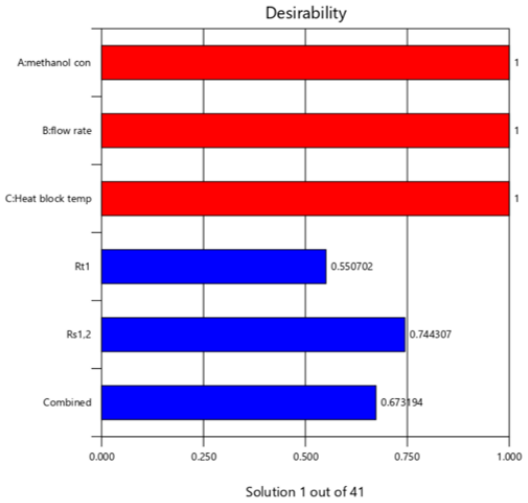

Figure 6: Individual and combined Desirability scores of chromatographic parameters.

3.1 Environmental impact assessment of the Developed method:

The Analytical Eco-Scale (AES) and the Analytical Greenness Metric (AGREE), two complementary assessment methods that help measure the environmental and safety performance of analytical methodology, were used to evaluate the environmental sustainability of the developed LC-MS method.

A semi-quantitative evaluation method called the Analytical Eco-Scale (AES) allocates penalty points (PP) for the toxicity, waste generation, operator risk, and energy consumption of each reagent. The final ecological score is calculated by deducting the total penalty points from a possible score of 100. The designed approach in this investigation obtained an AES score of 75, or 25 penalty points (14 + 4 + 1 + 3 + 3).

The scores on the Eco-Scale give the following interpretations, values over 75 suggests an excellent green analysis, values between 50-75 are good green analysis and values less than 50 are considered poor green performance. Thus, a score of 75 suggests the proposed LC-MS method has excellent ecological compatibility indicating that the method leaves minimal toxic reagent residues, reduces waste, and is energy efficient. Therefore, this proves that the method is suitable to what is proposed by Green Analytical Chemistry (GAC) principles and sustainability.

3.2 Analytical Greenness Metric (AGREE):

In order to further distinguish the environmental assessment, a tool was utilized based on AGREE software. AGREE captures all twelve principles of Green Analytical Chemistry (GAC) in one overall assessment of greenness of their method(s). Each principle is assessed qualitatively between 0 (no compliance) and 1 (complete compliance). Thus, an overall average value is calculated and indicative of the method environmental impact. The developed LC-MS method achieved a score of 0.68 on AGREE, demonstrating a meaningful adherence to the principles for sustainability or respect for green analytical chemistry. Specifically, this demonstrates an efficient use of solvent, minimal waste, low energy cost, and operator safety benefit.

The AGREE diagram, depicted in Figure 7, provides a clock-like visual representation of the principles. When using the AGREE software program, a score greater than 0.60 is typically qualified as a strong indication of environmental stewardship in analytical practice. The LC-MS method used in this research study demonstrated an overall high degree of greenness making it applicable to use in laboratories for the near and distant future with little impact on the environment. While it does not imply the method is entirely “green” in every sense, the score indicates that real progress is being made toward more sustainable practices in analytical approaches i.e., less hazardous solvents and reduced efforts on energy.

Figure 7: AGREE-Based Eco-Scale Visualization of Analytical Greenness

3.3 Method Validation:

The FDA's bioanalytical technique validation guidelines were used to validate the developed LC-MS method[24–27]. Under the ideal chromatographic circumstances, the validation parameters (linearity, accuracy, precision, recovery, carryover, and stability) were assessed. According to the chromatographic analysis, there was no endogenous interference and both efonidipine and chlorthalidone showed clear, interference-free peaks at 6.382 and 8.609 minutes, respectively.

Chromatographic Performance:

The optimized chromatographic system produced symmetrical peaks with tailing factors ranging from 0.8 - 1.2, a capacity factor (k′) ranging from 1.0 - 1.6, and resolution values greater than 2, indicating high separation efficiency and system suitability. There was a theoretical plate number (>3500) indicating a high column efficiency and consistent performance. The system suitability parameters are presented in Table 5.

Table 5: System Suitability Parameters

Criteria	Chlorthalidone	Efonidipine
Retention time (tR, min)	8.60	6.38
Capacity factor (k′)	1.58	1.58
Peak Asymmetry (Tailing Factor, T)	1.00	0.92
Theoretical Plates (N)	3620	3587

3.3.1 Calibration and Linearity:

Samples of spiked plasma taken from blood transfusion bags were used to create calibration curves. Over the investigated concentration ranges, the technique demonstrated exceptional linearity:

- Efonidipine: 18.75–65.61 µg/mL
- Chlorthalidone: 60–210 µg/mL

Linear regression analysis yielded the following equations:

- Efonidipine: y = 678.99x – 20950 (r = 0.9993)
- Chlorthalidone: y = 1042.3x – 8583.1 (r = 0.9996)

The high correlation coefficients confirm a strong linear relationship between concentration and detector response, demonstrating the method's reliability for quantitative analysis.

Figure 8 and Figure 9 illustrate the calibration plots for both analytes.

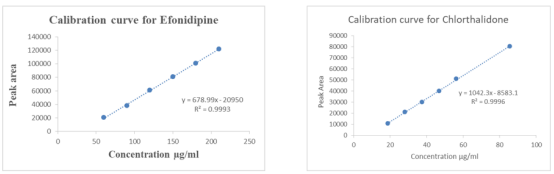

Figure 8&9: Calibration Curve for Efonidipine and Chlorthalidone

3.3.2 Accuracy and Precision:

Accuracy and Precision: Six replicates were used to evaluate the developed methods' accuracy and precision at low (LQC), medium (MQC), and high (HQC) concentration levels. The CV% (Coefficient of variation%) and RSD% (Relative standard deviation%) were used to calculate the interlay and intraday reproducibility. The great precision of the approach was confirmed by the %RSD values of 0.187% and 0.267% for efonidipine and chlorthalidone, respectively. They are well within the FDA-accepted range of less than 2%.

3.3.3 Recovery:

Peak areas of extracted plasma samples were compared to areas of unextracted standards made in the mobile phase to assess the extraction efficiency. Efonidipine (%RSD of 0.232%) and chlorthalidone (%RSD of 0.464%) had average recoveries of 99.76% and 99.53%, respectively.

3.3.4 Carryover Study:

Carryover from analytes in the system was estimated as % deviation following six replicate injections of low and high QC samples and injecting blank matrix samples. The RSD values indicated no significant carryover. For extracted samples, %RSD was between 0.076%–0.236% (Efonidipine) and 0.237%–0.442% (Chlorthalidone); for unextracted samples, RSD was between 0.226%–0.565% (Efonidipine), and 0.446%–0.888% (Chlorthalidone).

3.3.5 Stability Studies:

All analytes in plasma were subjected to stability testing under various conditions, including short-term, long-term, freeze-thaw, bench-top, and dry extract. Both short-term and bench-top stability (sample stability 24–48 hours at room temperature, +4 °C) and long-term stability (samples held at -20 °C for 14 days) were established. Stability was observed at RSD %47< after three freeze-thaw cycles. The analytes can be regarded as stable in every situation. Results summarised in Table 6.

Table 6: Percentage RSD Values of Efonidipine and Chlorthalidone Under Various Stability Conditions

Stability Condition	Concentration Level	Efonidipine (%RSD)	Chlorthalidone (%RSD)
Long-Term (7 days)	LQC / MQC / HQC	0.306 / 0.250 / 0.102	1.576 / 0.801 / 0.610
Long-Term (14 days)	LQC / MQC / HQC	0.665 / 0.466 / 0.362	0.110 / 0.224 / 0.600

Short-Term	LQC / MQC / HQC	0.058 / 0.138 / 0.127	1.754 / 0.483 / 0.431
Bench-Top	LQC / HQC	0.837 / 0.127	1.201 / 0.445
Dry Extract	LQC / MQC	0.647 / 0.327	1.412 / 0.374
Freeze-Thaw	LQC / MQC	0.705 / 0.416	1.184 / 0.438

%RSD- Percentage relative standard deviation

The validated LC-MS method showed excellent selectivity, linearity, accuracy, precision, recovery and stability, meeting all regulatory validation criteria. The AGREE and AES evaluations also affirm that the method is environmentally sustainable and provides a balance between analytical practicality and green chemistry.

In summary, the established method is suitable for routine bioanalytical applications including pharmacokinetics, bioequivalence and quality assurance studies of Efonidipine Hydrochloride Ethanoate and Chlorthalidone.

4. Conclusion:

The present study successfully designed and validated an effective, robust, and environmentally friendly approach based on LC-MS bioanalysis that simultaneously quantifies Efonidipine Hydrochloride Ethanolate and Chlorthalidone within the Quality by Design (QbD) framework. The QbD framework provided a method to identify to manage critical parameters ultimately it aided in understanding the performance of the method and provided a methodology to perform optimally under all critical (and non-critical) analytical parameters.

The method demonstrated thorough validation according to the FDA bioanalytical method validation criteria which will allow the method to perform at a high level of quality with respect to the following parameters: linearity, accuracy, precision, sensitivity, selectivity, recovery, and stability. The overall method exhibited a reproducible, robust performance indicating potential utility in pharmacokinetics, bioequivalence, therapeutic monitoring.

Implementation of a green analytical chemistry (GAC) study using the Analytical Eco-Scale and AGREE metrics confirmed the method and environmental commitment. The reported greenness score indicates a high level of sustainability was achieved, including minimizing hazardous reagents, minimal waste, and energy in order to meet so-called "green" analytical performance and to achieve goals to benefit the environment. In summary, the QbD LC-MS method presents a sensitive, accurate, and sustainable analytical structure able to quantify both Efonidipine and Chlorthalidone from the biological matrix.

The methodological developments achieved will assist future applications in the bioanalytical space while supporting the need for sustainability in the modern biopharmaceutical analytical space. Through this process, perhaps the bioanalytical field can achieve a high-level quality while maintaining an environmental mission in pharmaceutical analysis.

Conflict of Interest

No conflicts of interest are disclosed by the authors.

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