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Method Development and Validation of Sustainable RP-HPLC Analytical Methods for Clonidine Hydrochloride Using AQBD Optimization

Vineetha Rosireddy[1]*, Manikandan Krishnan[1]*, Fariya.M[1], Madhumitha.J[1], Sujal Saurav[1], Parimala.D[1]

[1]Department of Pharmaceutical Analysis, SRM College of Pharmacy, Faculty of Medicine and Health Sciences, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu district., Tamil Nadu-603203.

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KEYWORDS

Clonidine Hydrochloride, RP-HPLC, Analytical Quality by Design (AQbD), Sustainable Analytical Method, Method Validation

ABSTRACT

A sustainable and robust reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the estimation of clonidine hydrochloride using green analytical principles with Analytical Quality by Design (AQbD) optimization. Chromatographic separation was achieved on a ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5 µm) using an Agilent 1220 Infinity II HPLC system equipped with a binary pump, autosampler, and PDA detector. The mobile phase consisted of water and phosphate buffer (50:50 v/v), adjusted to pH 5, and was eluted in isocratic mode at a flow rate of 1 mL/min. The detection wavelength was set at 245 nm with an injection volume of 10 µL, and the column oven temperature was maintained at 35°C.The developed method possessed good linearity over the concentration range of 5–15 µg/mL with a regression equation of y = 372.84x + 145.62 and a correlation coefficient (R^2) of 0.999. The limit of detection and limit of quantification were found to be 0.52 µg/mL and 1.58 µg/mL, respectively. The method exhibited accuracy (98.7–100%) and high precision, with %RSD values of 0.64 (interday) and 0.49 (intraday). The developed method is simple, precise, accurate, and environmentally considerate, aligning with sustainable analytical chemistry principles. It is suitable for routine quality control analysis of clonidine hydrochloride in pharmaceutical formulations.

1.Introduction

Clonidine, also known as 2-[2,6-dichlorophenyl) imino] imidazoline HCl, is an imidazoline derivative that has hypotensive properties. Clonidine is a member of the alpha-adrenergic central agonist drug class. As a hypotensive, it is used to treat hyperactivity disorders, blood pressure conditions, dependency headaches caused by migraines, and the symptoms of alcohol and opium withdrawal. Additionally, it reduces the central nervous system's sympathetic nerve activity. Clonidine is associated with several adverse effects, most frequently sedation, dry mouth, and dizziness. It can also produce cardiovascular effects such as hypotension and bradycardia, along with gastrointestinal discomfort. Sudden withdrawal of clonidine should be avoided, as it may trigger rebound hypertension accompanied by headache and restlessness [1–3]. **Figure 1 shows the structure of Clonidine Hydrochloride.**

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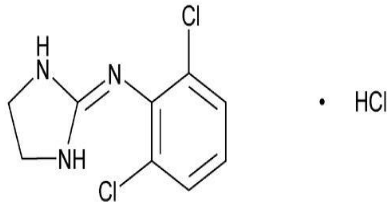

Through the integration of process and product control, Quality by Design (QbD) promotes a methodical approach to method development. It is based on predefined objectives, sound scientific understanding, and proactive risk management. Consequently, QbD concepts are increasingly adopted in analytical method development to enhance method robustness and performance. QbD addresses the limitations of the traditional approach by considering multiple variables that influence the method's responses [4-5].

Designing chemical products and procedures that reduce the use and production of hazardous compounds is the focus of the field of green chemistry. The concept of "environmentally friendly chemistry" covers the complete life cycle of chemicals, including their production, application, and final disposal. Green chemicals are designed to break down either into harmless by-products or into substances that can be recovered and reused. Environmental problems such as smog formation, ozone layer depletion, and climate change lead to the release of toxic pollutants that negatively affect plants and animals. Green analytical chemistry (GAC) aims to develop analytical techniques that are safer for the environment and more user-friendly for analysts by reducing the consumption of toxic reagents, promoting energy-efficient instruments, and minimizing waste generation.

This is the first study to combine clonidine with green analytical chemistry principles along with the AQbD approach. This integrated strategy offers substantial advantages, making the developed method highly efficient, scientifically significant, and environmentally safe.

2.Materials and methods

2.1Chemicals and reagents

Sigma-Aldrich provided the 99.5% pure API clonidine hydrochloride. A Milli-Q filtration system from ELGA Lab Water (UK) was used to produce HPLC-grade water. The potassium hydroxide and potassium dihydrogen orthophosphate from Rankem (India). Pall Life Sciences (Bengaluru, India) supplied the 0.45 µm membrane filter. The clonidine hydrochloride was purchased from local pharmacy.

2.2. Instrument and software used

2.2.1 Instruments

An Agilent 1220 Infinity II HPLC system with a binary pump, autosampler, and PDA detector was used for the chromatographic study. Agilent Open Lab CDS Chem software, version 2.6, was used for instrument control, data collection, and processing.

2.2.2 Software

Design-Expert® software (version 13.0) was used to create a central composite design for the experimental design. The GAPI Chart Maker (version 0.1 beta) was used to assess the analytical method's environmental friendliness. Additionally, the AGREE tool and AMGS calculator were used to evaluate greenness metrics**.**

2.3 Solution preparation

2.3.1Preparation of phosphate buffer pH 5.0

The 6.8 g of potassium dihydrogen orthophosphate was precisely weighed before being moved to a 1000 mL volumetric flask, where it was dissolved in distilled water and diluted to the appropriate level. 10 M potassium hydroxide was used to change the solution's pH. After sonicating the resultant solution, a membrane filter was used to filter it.

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2.3.2 Standard preparation

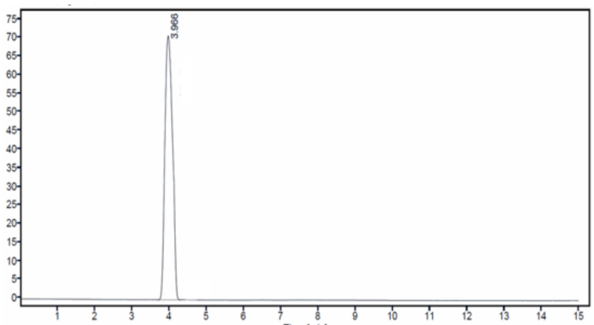
A precisely weighed quantity of clonidine was dissolved in ethanol, and the volume was adjusted to provide a standard stock solution with a concentration of 1000 µg/mL. After 15 minutes of sonication, the solution was passed through a membrane filter. After that, appropriate dilutions were made using ethanol to reach a final working concentration of 10 µg/mL.

2.3.3 Clonidine test preparation

Weigh accurately twenty tablets of Clonidine hydrochloride. After that, the tablets were ground into a fine powder. An equivalent amount Arakamin tablet containing 100mg/mL was transferred in a standard flask. To dilute the drug powder, the required amount of water was added. Then the solution was kept in the sonicator for approximately 20 minutes and then adjusted to the mark. The membrane filter was used to filter the mixture, and then a concentration of 10 µg/mL was achieved by diluting the filtrate with water.

2.4 Chromatographic conditions

An Agilent 1220 Infinity II HPLC system with a binary solvent supply pump, an autosampler injector, and a PDA detector was used for method development and validation. A ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5 µm) was used to accomplish chromatographic separation. A 0.45 µm membrane filter from Pall Life Sciences in Bengaluru, India, was used to filter the solutions, and an ultra sonicator from Lab Man in Maharashtra, India, was used to degas the solvents. Water and phosphate buffer in a 50:50 ratio that was adjusted to pH 5 made up the mobile phase. With an injection volume of 10 µL and a detection wavelength of 245 nm, the flow rate was kept at 1 mL/min. The analyte was eluted using an isocratic elution mode while the column oven temperature was kept at 35°C. Open LAB CDS Agilent software, version 2.6, was used for data collection and processing. The standard chromatogram of Clonidine Hydrochloride is displayed in **Figure 2 shows the Standard Chromatogram of Clonidine Hydrochloride.**



2.5 System suitability studies

The standard clonidine solution was injected six times to ascertain the system appropriateness characteristics. For retention time (Rt), peak area, tailing, and resolution, the % RSD was computed. The discovered percentage RSD value was less than 2. **The system suitability for clonidine hydrochloride are displayed in Table 1.**

Parameter	Mean	SD	%RSD
Retention Time	3.9	0.002	0.05
Tailing Factor	1.5	0.011	0.73
Theoretical Plates	2250	37.35	1.66

3.Results

3.1Quality by design

Quality by Design (QbD) represents a structured and science-driven strategy focused on assuring pharmaceutical product quality by integrating statistical evaluation, analytical methodologies, and risk-based principles throughout the stages of drug design, development, and manufacturing. Adoption of the QbD paradigm facilitates a deeper understanding of critical process parameters, thereby allowing deliberate and accurate modification of specific process variables, provided

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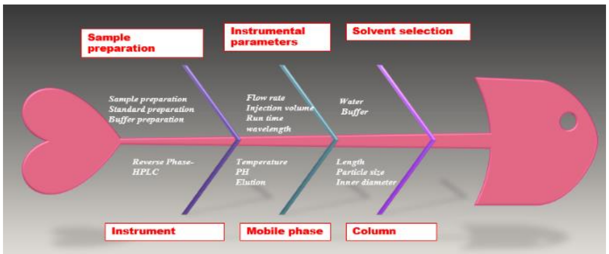
these parameters are proactively defined during development and document with the relevant regulatory authorities [6-10].

3.2 Design-based analytical quality

By design, AQbD stands for analytical quality by design. This method is frequently used for method development procedures and encompasses all facets of the analytical process, including risk assessment and product lifecycle management. By lowering the number of trials and resources needed to create robust analytical processes, the enhanced (AQbD) approach can save time. Preliminary experiments were conducted using trial and error to identify important independent parameters and their impacts on dependent variables in order to increase the method's effectiveness. The important variables that could affect the development of a good method were identified and evaluated using the Ishikawa-Fishbone Diagram. The adopted parameters are graphically displayed in the Ishikawa fishbone figure. To make sure that every aspect of the methodology functioned optimally in its particular area, the trial design process was employed.

3.3 Central composite design (CCD):

The Central Composite design adds 2k points to the two-level factorial design, representing the number of independent variable points between the axes and repeat points around the centre. Like all excellent experimental designs, the experiments are characterized by randomization. There are five different levels for each factor in the core composite design: low, the designating point, higher, an extreme low star point, and extreme high, also known as a star point. Central composite designs reduce the variability in the regression coefficients by generating orthogonal blocks that allow the estimate of block effects and model components separately. Rotatable designs guarantee that the prediction variance stays the same at all locations that are equally spaced from the design's centre. **Figure 3 shows the Ishikawa fish bone diagram.**



3.4 Execution of central composite design:

Among the designs used in the response surface methodology is CCD. Because of its versatility, CCD can produce nonlinear responses with ease, which offer useful information on the primary collected responses and the variable interactions. It chooses alpha-values that can be reached with the right efficiency with fewer runs. The mobile device and the flow rate B (-alpha at 0.8, mid value 1, and +alpha 1.2) Phase A (-alpha at 45, mid value 50, and +alpha 55) was chosen as factors 1 and 2, respectively.

4.Discussion

4.1 AQbD-based development and optimization:

Numerous solvent combinations and buffer systems, such as phosphate, acetate, and formate buffers, were assessed at various pH levels. The best buffer and solvent combination were chosen following methodical testing [11]. Throughout the investigation, ecologically friendly solvents were used in compliance with the principles of green chemistry.

A ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5 µm) was used to accomplish chromatographic separation. Phosphate buffer at pH 5 made up the aqueous phase, whereas water and phosphate buffer adjusted to pH 5 An injection volume of 10 µL and a flow rate of 1.0 mL/min were used for isocratic elution.

The temperature of the column was kept at 35°C, and detection was carried out at 245 nm. Design-Expert® software (Version 22.0) was used to create and optimize the method utilizing a Central Composite Design approach [11-12].

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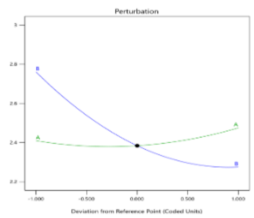
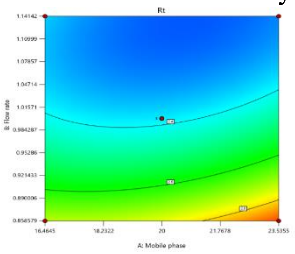
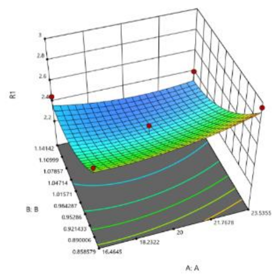
Implementation of the Analytical Quality by Design (AQbD)strategy significantly reduced experimental trials, minimized solvent consumption, shortened analysis time, and improved method reliability.



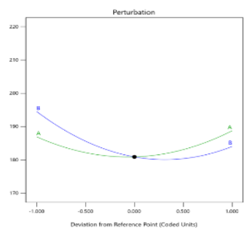
FACTOR 1: RT: PERTUBATION



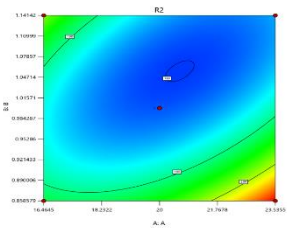
FACTOR:1: RT: CONTOUR



FACTOR:1: RT 3 D SURFACE



FACTOR:2: AREA: PERTUBATION



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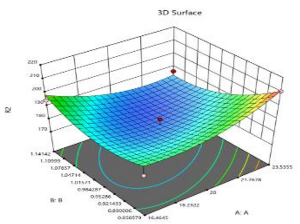
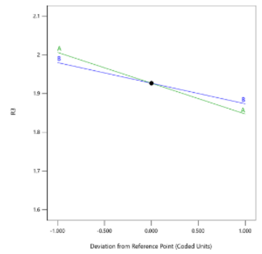
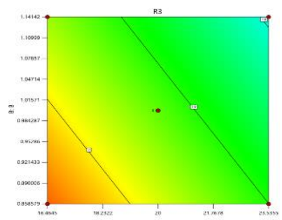


FACTOR:2: AREA 3 D SURFACE



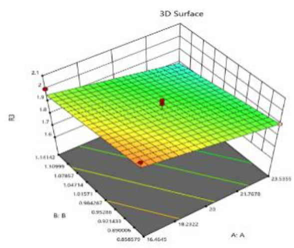
FACTOR:3: TAILING PERTUBATION



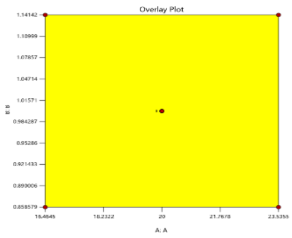
FACTOR:3: TAILING CONTOUR

Moreover, the AQbD approach contributed to enhanced robustness of the developed method.

Figure 4 shows the factors and responses of QbD.



FACTOR:3: TAILING 3 D SURFACE



OVERLAY PLOT FOR FACTOR 1,2,3

4.2 The solution stability

The stability of the standard solutions was examined for 72 hours at room temperature. The standard and test samples were compared in order to get the assay value percentages.

4.3 Parameters for validation

Getting written proof that a process continuously satisfies quality standards and requirements is known as validation. Determining a drug's suitability for its intended use is the main task of drug validation. By following the ICH Q14 criteria, the reliability of the proposed technique was demonstrated.

4.3.1 Accuracy

A recovery study was conducted following ICH guidelines. To analyse the tablet formulation, a solution with known concentrations of 80%, 100%, and 120% of the total drug content was added to a previously analysed solution. The percentage of recovery was then calculated.

4.3.2 Precision

The test solution was performed on multiple days and times to determine intra- and inter-day precision. The procedure was repeated at least six times with the test solution. The method was done six times on the same day for intraday precision and

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six times on a different day for interday precision in order to guarantee reproducibility.

4.3.3 LOD, LOQ, and linearity

The assessment of clonidine's linearity was assessed across a concentration range of 5–15 µg/mL. Water was used as the solvent to create the standard solutions, and each concentration was examined three times. Plotting peak area against the relevant clonidine concentration allowed for the creation of a calibration curve. The method's correlation coefficient (R^2) was 0.99, indicating acceptable linearity. These results show how exact and accurate the suggested procedure is. The standard deviation (σ) of the response and the slope of the calibration curve were used to compute the limits of detection (LOD) and quantification (LOQ). **Table 2 shows the validation parameters for Clonidine Hydrochloride.**

S. No	Parameter	Result
1	Linearity Range (µg/mL)	5 – 15
2	Regression Equation	y = 372.84x + 145.62
3	Correlation Coefficient (R^2)	0.999
4	LOD (µg/mL)	0.52
5	LOQ (µg/mL)	1.58
6	Accuracy (%)	98.7 – 100.1
7	Interday Precision (%RSD)	0.64
8	Intraday Precision (%RSD)	0.49

4.4 HPLC’s benefits for solvent reduction

Polar solvents are well suited for method development and validation in HPLC, particularly RP-HPLC, as they enhance analyte solubility and chromatographic performance. The use of cross-linked stationary phases with smaller particle sizes facilitates faster elution and improves separation efficiency. Operating the column at elevated temperatures increases analyte polarity and decreases mobile-phase viscosity, which in turn lowers back pressure and reduces the need for higher proportions of organic modifiers. Additionally, the application of high-pressure conditions in HPLC enables efficient separations with reduced solvent consumption, thereby minimizing waste generation and supporting environmentally sustainable analytical practices

4.5 Green evaluation techniques

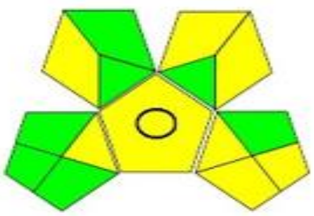
Ensuring environmental sustainability requires the careful evaluation of every analytical approach before its adoption and future implementation. A number of evaluation instruments have been created to determine how environmentally friendly analytical techniques are under the framework of GAC [13-15]. Each of these tools possesses inherent limitations, as they emphasize different aspects of environmental impact and sustainability. Consequently, no single tool can comprehensively address all criteria of GAC. These evaluation strategies are designed in alignment with Green Analytical Chemistry assessment principles, enabling systematic and transparent comparison of analytical methods with respect to their environmental performance.

4.5.1 Green Analytical Procedure Index [GAPI]

GAPI, or the Green Analytical Procedure Index covers range of process involved in the handling of samples, collection, transportation, storage, preparation, analysis, and the various operations that occur before the actual analysis takes place [16]. GAPI incorporates all aspects of an analytical technique. The NFPA list classifies the solvents used in method development [17]. The pictogram uses red, yellow, and green colour indicators.

Yellow should be considered moderately dangerous. Green should be considered the least toxic, while red should be classified as the most hazardous component. The GAPI's guiding concepts are based on ecological scale analysis. The methodology used is qualitative. The outcome depends on how colour is depicted. To obtain the complex GAPI, combine the regular GAPI with the E-factor [18]. The E-factor includes the GAPI, which measures chemical yield and consumption. The research primarily focuses on developing and accessing analytical methods, including their environmental sustainability [19]. The technique utilizes ethanol, a solvent known for its environmental benefits [19]. GAPIs provides a green symbol indicating environmental safety.

Figure 5 shows the Pictogram of GAPI for Clonidine Hydrochloride.



4.5.2 Analytical Greenness [AGREE]

A well-known instrument for assessing the environmental sustainability of analytical techniques is AGREE. The twelve principles of green analytical chemistry served as the foundation for this software's creation, and each principle is assigned a score between 0 and 1 A value closer to 1 indicates a greener and more environmentally friendly method. The evaluation takes into account a number of variables, such as the amount and toxicity of chemicals used, waste production, and energy requirements, number of analytical steps, level of automation, and degree of miniaturization. To satisfy greenness requirements, the application of advanced and expert analytical technologies is essential. The evaluation approach is comprehensive, flexible, and transparent, ensuring analytical reliability. The primary objective is to develop a method that is simple in design while delivering effective and reliable results.

Figure 7 shows the Pictogram of AGREE for Clonidine Hydrochloride.

4.5.3 Analytical Method Greenness Score [AMGS]

A spreadsheet-based model designed especially for chromatographic processes is called AMGS. This method is composed of two components: cumulative energy demand (CED) and analytical mass volume intensity (AMVI), which evaluates solvent waste for health and safety, and environmental assessment (SHE), which determines solvent safety using the geometric mean.

The spreadsheet is used throughout the process to identify the most environmentally friendly options with the lowest output. AMGS comprises three parts: instrument energy, fluid energy, as well as solvent safety and environmental health. To preserve the environment, the method's overall score—which is determined by adding the three scores—should be as low as possible.

Based on the total AMGS score, the proposed procedure appears to be environmentally friendly. This appears to be a better way to achieve things. The ACS Green Institute helped the statistics into perspective. This implies a greener approach. The score on the AMGS spreadsheet was **85**.

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4.5.4 Carbon Footprint Analysis

Carbon footprint analysis is a top priority. Estimating carbon dioxide emissions is critical. This study focuses on the RP-HPLC of Clonidine. The HPLC utilizes approximately 1.5 kWh, significantly reducing energy usage in contrast to other pieces of equipment. The tools utilized for method development and validation emit additional CO_2. It demands more energy to perform. Using energy in HPLC greatly decreases carbon dioxide emissions, having no effect on the environment. This demonstrated that the evolved technology had significant environmental benefits. This signifies an eco-friendlier approach.

5.Conclusion:

A rapid, automated, and highly reliable the HPLC technique was effectively created and approved for the determination of clonidine, demonstrating excellent analytical performance with minimal environmental impact. The method enables precise identification and quantification of the analyte while contributing to reduced greenhouse gas emissions through optimized solvent usage and efficient chromatographic conditions. Notably, this study represents the first reported analytical method for clonidine developed and validated by integrating AQbD principles with GAC concepts. The combined application of AQbD ensured systematic method robustness and lifecycle management, while GAC principles enhanced environmental sustainability. Overall, the proposed method is accurate, precise, robust, and environmentally benign, which qualifies it for regular regulatory and quality control applications.

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CONFLICT OF INTEREST

We declare that there were no competing interests

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