Research Article

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A comprehensive RP-HPLC technique for analyzing disodium edetate and its degradation products in eye drops using AQbD and Six Sigma perspective

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Abstract: The study presents the development of a novel, sustainable, and analytical quality by design (AQbD) driven RP-HPLC method to simultaneously identify disodium edetate in eye drops and its degradation products. The goal of this method is to meet the growing demand for robust, eco-friendly methods of quality control for pharmaceutical products. AQbD ensured method robustness, while minimizing solvent consumption and waste generation to ensure sustainability. Several sustainable tools were investigated to assess their ecological impact in the study. Using Box–Behnken design, three chromatographic parameters were optimized: column oven temperature, flow rate, and buffer pH. An ethanol and tetrabutylammonium hydroxide solution mobile phase at pH 7.0 in gradient mode was found to be optimal. An Avantor Hichrom C18 (4.6 mm × 150 mm, 5 µm) column was used with UV detection at 258 nm and pumped at 1.1 mL/min. The linearity of disodium edetate occurred in the 2–40 µg/mL range with an R^2 of 0.9999. Disodium edetate was subjected to acidic, basic, oxidative, photolytic, and thermal stress conditions in accordance with ICH guidelines. Degradation caused by bases, acids, and oxidation provided the highest degradation rates. Incorporating quality by design principles with sustainability considerations makes this method robust, eco-friendly, and cost-effective for quality control in pharmaceutical products. Having been successfully applied to eye drop formulations, its utility in the pharmaceutical industry is unquestionable.

Keywords: disodium edetate; RP-HPLC; Six Sigma; analytical quality by design; accelerated stability study; sustainability

1 Introduction

The chelating agent disodium edetate (Figure 1) is frequently used in ophthalmic formulas to enhance stability and shelf life. There are however conditions under which disodium edetate may degrade, resulting in degradation products that may compromise eye drops' safety and effectiveness. As the pharmaceutical industry faces ever-increasing pressure to develop analytical methods that are not only robust and reliable but also environmentally sustainable, such methods are becoming increasingly important [1, 2]. The safety and efficacy of pharmaceutical products are greatly enhanced by quality control, particularly that of identifying active ingredients and their degradation products. Environmental concerns arise from traditional methods for detecting disodium edetate and its degradation products, which often require heavy solvent consumption and generate significant waste. Analytical method development has increasingly incorporated green chemistry and QbD principles. As part of the QbD approach, robustness, reproducibility, and regulatory compliance are emphasized. Meanwhile, green chemistry advocates minimizing solvent and waste generation, energy consumption, and other environmental impacts of analytical methods. The combination of these approaches offers a path to developing sustainable and efficient analytical methods that meet both quality control and environmental concerns [3, 4].

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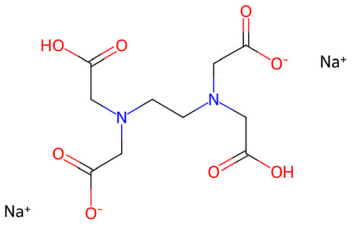

Figure 1: Chemical formula of disodium edetate.

An impressive reputation has been earned by HPLC for its high accuracy, groundbreaking precision, and remarkable responsiveness in detecting various elements on a variety of substrates. The versatility and reliability of this technique make it a preferred choice across a wide variety of industries. Green analytical chemistry (GAC) uses low solvent levels, short analysis times, and high accuracy to make it an attractive choice [5]. As part of our analysis methodologies, we need to measure their potential environmental influence to make certain they are practical and environmentally friendly. We have a variety of resources available to us, including HPLC-EAT, a well-known solution. The AGREEprep and BAGI procedures have also been developed to promote sustainable analytical practices. As long as these resources are integrated into our study endeavors, we can enhance our method and uphold our ethical obligations at the same time [6].

Box–Behnken design (BBD) improves the accuracy of experiments significantly. Using BBD for optimizing chromatographic conditions, HPLC analytical techniques can be developed and validated more accurately. HPLC procedures can be made more efficient by using optimized conditions for analyses. Additional adjustment is made to the peak parameter until it meets the required level. Regardless of the number of parameters to analyze, the BBD method can be easily managed and is regarded as a reliable method regardless of how many parameters are analyzed [7].

As part of our experimental work, we selected the BBD based on its efficiency, practicability, and suitability for our study's objectives. With BBD, fewer experimental runs are required than when using CCD, which makes it a more resource-efficient design when optimizing multiple variables. Moreover, BBD does not allow combinations of factors at extreme levels, which is useful for preventing potentially hazardous or unstable conditions, especially when working with sensitive pharmaceutical compounds. This design allows us to optimize the response surface accurately by capturing the curvature of the response surface. Furthermore, the structure's rotatable and balanced characteristics enhance the accuracy of predictions across the design space. By combining these benefits, BBD provided a statistically robust and experimentally feasible approach to optimizing our chromatography method [8, 9].

Degradation products are produced more rapidly by forced degradation tests than by long-term stability assessments. Stress accelerates the degradation of drug ingredients and products. Drug stability can be assessed by evaluating stress parameters. A force-degradation study revealed that disodium edetate was most susceptible to degradation under acidic, basic, and oxidative conditions, with noticeable changes in chromatographic peak area and retention time. Degradation is also measurable when it occurs due to thermal and photolytic stress, although they are less detrimental. As a result of these studies, not only is the RP-HPLC method validated but also its robustness is demonstrated and the necessity of periodically monitoring disodium edetate degradation products in pharmaceutical formulations is highlighted. The purpose of this study is to provide valuable data for optimizing formulation stability and maintaining product quality over its shelf life by identifying the primary degradation pathways [10]. The study of accelerated stability is essential for evaluating the behavior of active ingredients and excipients in pharmaceutical products under stress conditions. These studies are conducted according to ICH guidelines for assessing pharmaceutical stability. We investigated how disodium edetate reacts with eye drop formulations under accelerated stability conditions [11].

Over the last few years, the Six Sigma approach has gained considerable recognition in the pharmaceutical industry as a powerful tool for improving process efficiency, reducing errors, and improving overall product quality. The methodology is particularly attractive because it can achieve high capability indices (Cpk), which measure the output quality of a process. In industries such as pharmaceuticals, where precision and consistency are paramount, Six Sigma is an ideal choice because it has a high Cpk. In addition to improving the reliability of analytical methods, Six Sigma reduces resource consumption and environmental impact, aligning it with sustainability goals. As a result of integrating Six Sigma into pharmaceutical processes, operational efficiency, cost-effectiveness, and regulatory compliance have significantly improved. Quality and sustainability continue to drive innovation and excellence in the industry, so the adoption of Six Sigma is expected to play an increasingly vital role [12].

Each pharmacopoeia, BP and USP, describes a titration method for quantifying disodium edetate separately [13, 14]. The comprehensive literature review offered that only a limited number of HPLC methods are available for the measurement of disodium edetate [15–19].

AQbD framework was adopted to ensure both environmental and operational sustainability of the proposed RP-

HPLC method throughout its development. Through careful selection of method parameters including buffer pH, flow rate, and column oven temperature, the method minimized solvent consumption, reduced hazardous waste, and optimized energy consumption. BBD was used to optimize these parameters, which allowed for efficient experimentation with fewer runs and reduced environmental impact. Moreover, the method eliminates toxic reagents and utilizes eco-friendly mobile phase components, aligning with green analytical chemistry principles. Choosing the three response variables-retention time, tailing factor, and theoretical plates-was not only based on their relevance to analytical performance, but also on their value in assessing method efficiency and robustness, both of which are crucial to ensuring long-term sustainability. By integrating statistical optimization, green chemistry principles, and AQbD strategies, the proposed methodology provides a modern, sustainable, and scientifically rigorous method for analyzing pharmaceuticals. The study therefore has a significant impact and relevance in the current analytical science field, thus positioning it as a meaningful contribution [20–22].

As far as we know, there is no HPLC method available for evaluating disodium edetate and its degraded products simultaneously. Furthermore, accelerated stability studies and forced degradation tests are carried out in conjunction with greenness and whiteness appraisal techniques such as HPLC-EAT, AGREEprep and BAGI. Traditional analytical methods used to detect disodium edetate are often lacking in specificity, sensitivity, and sustainability. An innovative RP-HPLC technique is presented in this study that uses BBD principles and sustainability practices to overcome these limitations. Using BBD, this method optimizes critical chromatographic parameters -column oven temperature, flow rate, and buffer pH – to ensure robustness and efficiency. Using ethanol for mobile phase reduces environmental impact. Linearity, accuracy, precision, and sensitivity were validated for the method. Disodium edetate was tested under various stress conditions in accordance with ICH guidelines. This method makes it possible to develop eco-friendly, cost-effective, and reliable quality control tools for pharmaceuticals by integrating BBD principles with sustainability considerations.

2 Experimental

2.1 Chemicals and Reagents

Disodium edetate with 99.0 % purity was procured from Imperial Chemical Corporation (Taiwan). HPLC-grade ethanol was obtained from Scharlau (Barcelona, Spain). Analytical grades of HCl, NaOH, and H_2O_2 were acquired from Chem-Lab (Zedelgem, Belgium). While tetrabutylammonium Hydroxide Solution 40 wt% in water was obtained from Sigma-Aldrich (Missouri, USA).

2.2 Instruments and software

This Shimadzu HPLC 20A system is designed for analytical laboratories. It features a photodiode array (PDA) detector (Maryland, USA), autosampler, column oven, and pump system that are compatible with Empower 3 software which meets FDA 21 CFR Part 11 regulations.

The stability chamber used for the test was the Vötsch VT1,300 stability chamber (Balingen, Germany). Temperature and humidity can be controlled precisely with this chamber, which was designed for stability tests based on ICH guidelines. Temperature and humidity data are monitored using SIMPATI software.

For the investigation of response surface methodology, Stat-Ease, Inc. (Minnesota, USA) used its Design-Expert version 13. Minitab 2018 evaluated the process capability index using Quality by Design and Six Sigma, all from Minitab, LLC (Pennsylvania, USA). A software tool called HPLC-EAT evaluates methods for liquid chromatography using HPLC-EAT. http://www.biotek.lu.se/hplc-eat/provides a free version of the software.

2.3 Mobile phase preparation

The mobile phase operates using a gradient mode consisting of ethanol: tetrabutylammonium hydroxide solution at pH 7.0, see Table S1.

2.4 Diluent preparation

Dissolve about 0.25 gm of copper sulfate pentahydrate in 1,000 mL water.

2.5 Chromatographic system

A gradient-mode RP-HPLC system was set up for chromatographic analysis. The separation was conducted on a C18 column (Avantor Hichrom, 4.6 mm × 150 mm, 5 µm). A 5 °C sample temperature and a 30 °C column oven temperature was maintained. Using gradient mode, ethanol and tetrabutylammonium hydroxide solution at pH 7.0 were used as mobile phases. There was a 14-min runtime for the RP-HPLC

method, a 20 µL injection volume, a flow rate of 1.1 mL/min, and UV detection at 258 nm.

2.6 Stability studies

The purpose of the study was to investigate degradation pathways and estimate the lifetime of the product based on an accelerated stability study. For this study, samples were exposed to extreme conditions of high temperature and humidity to simulate long-term stability within a shorter period of time. It was aimed at identifying potential degradation mechanisms and assessing product stability under stress [23].

Upon determining that the accelerated stability study has detected a substantial change, for example, a drop in assay results by more than 5 %, an intermediate stability study would be initiated. A 1-year monitoring period will be conducted on intermediate stability samples stored under controlled conditions. The product's stability will be evaluated under more realistic storage conditions. Maintaining quality and effectiveness over the course of a product's shelf life is guaranteed by this step [24].

Stability profiles can be comprehensively understood through accelerated and intermediate stability studies. By doing so, compliance with regulatory requirements can be ensured while establishing proper storage conditions and expiration dates [25].

2.7 Standard stock solutions (200 µg/mL)

Using an accurate weight, weigh about 50 mg of disodium edetate working standard into a 250 mL volumetric flask. Add 175 mL of water, then sonicate for about 10 then mix well.

2.8 Working standard solutions

Pipette out 1.0 mL of the standard stock solution into a 10 mL volumetric flask, diluent to volume, mix well, and filter through a PVDF syringe membrane filter (0.45 µm).

2.9 Preparation of sample solution

Mix five bottles of Plegica 1 % eye drops. Pour accurately 5 mL (each mL contains 0.5 mg disodium edetate) of Plegica 1 % eye drops into a 25-mL volumetric flask. Fill the flask with water to about 70 % of volume then sonicate for 10 min. Add more water if needed and mix well. Make sure a 2 mL sample stock solution is transferred accurately into a 10-mL volumetric flask. It is diluted to volume with diluent, combined well, and filtered through PVDF 0.45 m syringe membrane filters.

2.10 Creating calibration curves

We prepared a stock solution for the drug at a concentration of 200 µg/mL. Serial dilutions of this stock solution were then carried out using the appropriate diluent to achieve concentrations ranging from 2 to 40 µg/mL. This diluted solution was used to construct calibration curves for disodium edetate. Based on the measured response (peak area) within the specified range, calibration curves were established.

3 Degradation sample preparation procedures

3.1 Acid hydrolysis

Transfer 5 mL of the Standard Stock Solution of disodium edetate, then add 3 mL of 1 N HCl, mix well and heat for 30 min at 50 °C. Neutralizing acid hydrolysis by adding 3 mL of 1 N NaOH. After that add 30 mL of solvent and fill up the 50 mL volumetric flask with solvent.

3.2 Base hydrolysis

Transfer 5 mL of the Standard Stock Solution of disodium edetate, then add 3 mL of 1 N NaOH, mix well and heat for 30 min at 50 °C. Neutralizing acid hydrolysis by adding 3 mL of 1 N HCl. After that add 30 mL of solvent and fill up the 50 mL volumetric flask with solvent.

3.3 Light decomposition

Transfer 5 mL of the Standard Stock Solution of disodium edetate, keep in sunlight for 2 h. After that add 30 mL of solvent and fill up the 50 mL volumetric flask with solvent.

3.4 Thermal decomposition

Transfer 5 mL of the Standard Stock Solution of disodium edetate, and heat for 30 min at 50 °C in the water bath.

Finally, add 30 mL of solvent and fill up the 50 mL volumetric flask with solvent.

3.5 Oxidation

Transfer 5 mL of the Standard Stock Solution of disodium edetate, then add 3 mL of 3.0 % H_2O_2, mix well and heat for 30 min at 50 °C. In the following step, add 30 mL of solvent and fill up the 50 mL volumetric flask with solvent.

3.6 Using Six Sigma methods

Using the Process Capability Index (Cpk), industries can significantly improve their production processes. Measuring product quality contributes to the elimination of scrap, improving product consistency, and reducing production costs. Process behavior can be used to determine the Cpk value, which is a key indicator of process capability. Process control is robust if the Cpk value is high, even in the presence of significant variations. Cpk values below 10 are indicative of a process that is not performing optimally, requiring improvement to meet quality targets. To achieve high-quality output and meet customer expectations, industries should aim to achieve a Cpk of 1.33 (4 sigma) or higher. A key aspect of this target is to reduce variability and defects in production processes, which is in line with Six Sigma principles [26].

3.7 Establishing comprehensive analytical target profiles (ATP) and critical quality attributes (CQA)

As part of the developed method, ATP must be constructed before the AQbD process can be applied. A basic attribute that determines technique performance is identified in this section. A reliable result was achieved by identifying CQAs based on ATP definitions. The implementation of a CQA is crucial for ensuring a successful outcome.

An overview of ATP components used in RP-HPLC for disodium edetate is provided in Table S2. Based on retention time, tailing factor, and theoretical plates, several CQAs were conducted for ATP [27].

3.8 Analyzing FMEA in the development of RP-HPLC methods

Based on a comprehensive FMEA (Figure S1) risk assessment, Table S3 identifies critical parameters that could compromise RP-HPLC's analytical performance. In the evaluation of factors impacting retention time variability, flow rate fluctuations due to pump wear or air bubbles pose the greatest risk, with Risk Priority Number (RPN) 192. A pH drift in the buffer can reduce peak resolution if the buffer is unstable or improperly mixed, so an RPN of 105 is obtained. Instability in the column's temperature, commonly caused by oven malfunctions or ambient changes, results in inconsistent peak shapes and has a moderate RPN of 48. The presence of errors in the composition of the mobile phase, such as incorrect solvent ratios caused by poor preparation, can reduce the separation efficiency but are not as critical (RPN 30). Last but not least, wavelength miscalibration caused by detector errors has the lowest RPN, meaning its impact on sensitivity is minimal.

The **Risk Priority Number (RPN)** is calculated as:

RPN = Severity × Occurrence × Detectability RPN = Severity × Occurrence × Detectability.

3.9 Experiment design for enhancing RP-HPLC technique

An RP-HPLC method based on AQbD was developed by selecting method parameters and response variables that influenced analytical performance and aligned with the defined ATP. Three key chromatographic parameters, column oven temperature, flow rate, and buffer pH, were optimized using the BBD. In particular, we selected these parameters because of their well-established impact on chromatography behavior, such as peak symmetry, separation efficiency, and retention characteristics.

As CQAs, retention time, tailing factor, and theoretical plates are representative of the method's precision and efficiency. By integrating these parameters and responses into the BBD framework, we were able to conduct a systematic evaluation of their interactions and optimize the method statistically. By employing this approach, a robust, reliable, and quality-driven analytical method was developed in accordance with AQbD principles [28].

In this study, a BBD with response surface methodology (RSM) was used for the optimization of an RP-HPLC method. A combination of three critical variables-flow rate, buffer pH, and column oven temperature-was evaluated at three levels to enhance chromatographic separation. Study objectives included minimizing retention time and maintaining parameters such as tailing factor and theoretical plates. Using 17 experimental runs, a second-order polynomial equation representing quadratic models was developed. It is clear from the equation that the independent variables are related to the responses in a meaningful

way. An analysis of the interactions between variables and their impacts on chromatographic outcomes was undertaken by generating 3D response surface plots. As a result of these plots, we were able to optimize the method. The optimal conditions were also predicted using overlay plots and the desirability function. By balancing resolution, retention time, and system suitability, we ensured that the system was suitable [29].

An analysis of this study demonstrated the effectiveness of BBD and RSM in optimizing RP-HPLC methods, offering an efficient and systematic approach to enhancing the performance of RP-HPLC. This method was fine-tuned to deliver reliable and reproducible results, tailored specifically to the analytes under investigation, using statistical tools and visualization techniques. As a result, the method development process takes less time and resources, which enhances analytical performance [30].

4 Evaluating sustainability

4.1 AGREEprep metric

It is essential to prepare samples correctly for analytical methods, and to acquire strength is crucial. AGREEprep is extremely important to us so that our sample preparation processes do not negatively impact the environment. Several evaluation steps are incorporated into this cutting-edge technique, along with sample preparation techniques that are environmentally friendly. To prepare sustainable and environmentally friendly samples. Our AGREEprep measure offers a revolutionary way of assessing sample preparation's environmental impact. With AGREEprep, the sample preparation process is simplified through the incorporation of 10 fundamental principles [31].

4.2 HPLC-EAT tool

HPLC-EAT (Environmental Assessment Tool) provides a way of evaluating how HPLC methods impact the environment, health, and safety. HPLC solvents are evaluated using this tool for their toxicity, flammability, and environmental persistence. By calculating a score, the tool can make comparisons between different HPLC methods easier. This program also comes with a free software component that facilitates these calculations. Consequently, HPLC-EAT can be a valuable resource for researchers and practitioners trying to develop more sustainable and eco-friendly analytical methods [32].

4.3 BAGI tool

Additionally, BAGI examines the practical aspects of White Analytical Chemistry in addition to existing green metrics, in order to evaluate the applicability and practicality of analytical methods. It identifies 10 key attributes such as the type of analysis, the number of analytes that are measured at the same time, the number of samples analyzed per hour, the type of reagents and materials used, the required, the number of samples that are treated simultaneously, whether preconcentration is required, whether automation is necessary, the type of sample preparation, and the number of samples involved. Visually depicting the practicality of the method is accomplished by generating a pictogram of an asteroid, which is accompanied by a score. Using BAGI is free and open source, and anyone can use it to evaluate analytical methods' practicality [33].

5 Results and discussion

5.1 Initial study

Among its many advantages are its versatility, wide application, and ability to separate a wide range of compounds, including nonpolar and moderate polar compounds. The C18 stationary phase is highly hydrophobic, which makes it ideal for retaining and separating target analytes. Our method development goals align with the robustness and reproducibility provided by C18 columns in reverse-phase HPLC. Analytes of interest were separated using ethanol and tetrabutylammonium hydroxide (TBAH) at pH 7.0, which provided the best peak shapes for optimal separation. It was decided that ethanol would be used as an organic modifier as it is less toxic than acetonitrile and can be detected by ultraviolet light at 258 nm. As a common ion-pairing agent, TBAH interacts with the charged species of ionizable analytes to enhance retention and resolution. Choosing a pH of 7.0 maintains the analytes partially ionized, thereby enhancing the interactions between them and the stationary phase and ion-pairing agent. C18 columns are highly dependent on the ionization states of the analytes for their retention and separation. An acidic analyte tends to deprotonate at alkaline pH (pH 7.0 and above), while a basic analyte remains neutral or partially protonated. As a result of this ionization behavior, they interact differently with the stationary phase and with the ion-pairing agent (TBAH). When acidic analytes are deprotonated at alkaline pH, their polarity increases, reducing their retention on the hydrophobic C18 column. In contrast, TBAH enhances the retention of negative analytes by forming ion pairs with them. Basic analytes are retained more efficiently with the C18 stationary

phase at pH 7.0 due to stronger interactions with neutral or partially protonated species. All analytes were ionized at the same pH to ensure sufficient solution resolution and peak symmetry. By using this approach, peak tailing is minimized, and overall chromatographic performance is improved. The diluent selected in this method is copper sulfate pentahydrate, which enhances the analytical performance of disodium edetate and its degradation products in ophthalmic formulations. Disodium edetate is particularly useful as a chelating agent, and copper sulfate pentahydrate is suitable for using as a chelating agent instead of ethanol, which is a general organic solvent. Analytes and degradation products are stabilized by its presence, which prevents unwanted interactions with trace metal ions in the sample.

The copper sulfate also improves peak symmetry, resolution, and reproducibility of reversed-phase chromatography under reversed-phase conditions due to its buffering capacity and ionic strength. Eye drop formulations are especially sensitive to trace metal contamination and pH sensitivity, which can affect method accuracy and robustness. In preliminary trials, copper sulfate pentahydrate provided greater precision and stability than ethanol as a diluent for multiple runs, while ethanol produced inconsistent peak shapes and reduced sensitivity.

Thus, copper sulfate pentahydrate was chosen both for its analytical necessity and for its method optimization.

5.2 Design of experiments (DoE)

The chromatographic conditions were optimized by applying a Design of Experiments approach using BBD. A quadratic effect and an interaction between the selected variables and responses were evaluated using this design. A variety of factors were investigated, including pH, column temperature, and flow rate, and retention time, tailing factor, and theoretical plates were measured (Table 1).

A linear polynomial equation was derived and analyzed to provide a better understanding of the relationships between variables and responses. As a result of ANOVA, the following equations were generated:

$$\begin{aligned}\text{Retention time} = {} & +6.09 - 0.2212A - 0.1411B - 0.0654C \\ & + 0.0050AB + 0.0305AC + 0.0653BC \\ & - 0.0737A^2 - 0.0084B^2 + 0.0571C^2 \end{aligned} \quad (1)$$

$$\begin{aligned}\text{Theoretical plates} = {} & +8,339.60 - 88.25A - 30.87B \\ & + 17.13\,C - 61.00AB + 7.00AC \\ & - 5.75BC - 159.18\,A^2 - 67.93B^2 \\ & - 99.92\,C^2 \end{aligned} \quad (2)$$

$$\begin{aligned}\text{Tailing factor} = {} & +1.32 + 0.0225A + 0.0100B + 0.0175C \\ & + 0.0050AB + 0.0000AC + 0.0000BC \\ & + 0.0525\,A^2 + 0.0025B^2 - 0.0125C^2 \end{aligned} \quad (3)$$

Observations from Equation (1) show that independent variables with a negative sign adversely affect retention time. The contour plot of the response surface illustrates positive interactions between variables if the interaction terms indicate a positive sign. Equation (1) shows that flow rate (A), pH (B), and column temperature (C) negatively influence retention time. A quadratic relationship has a positive influence, suggesting that even a slight increase in column temperature results in a reduction in retention time, as pictured in.

Equation (2) reveals that independent variables with a negative sign adversely impact theoretical plates, such as flow rate and pH. In contrast, theoretical plates decrease with an increase in flow rate and pH. Conversely, theoretical plates are directly affected by column temperature factors with positive sign. In this case, the positive sign precedes the interacting terms, denoting that both components behave favorably. Additionally, this term implies another noteworthy effect. Figures 2–4 illustrate how the two predictor variables behave antagonistically in the negative result.

The tailing factor response is influenced by the flow rate, pH, and column temperature. Graphs, contour plots, and polynomial equation (3) illustrate the relationships between these variables with significant accuracy. There are several factors that influence tailing factor response. Flow rate and pH are important factors to consider when determining the best results. When these parameters are increased, the tailing factor can increase as well.

The ANOVA data for theoretical plates, retention time, and tailing factor responses, presented in Tables S4–S6, indicate that the model and its terms are significant, with a probability (*P*-value) of < 0.05. Among the model parameters, R-squared and adjusted R-squared values were > 0.9, with a standard deviation < 0.1.

Using numerical optimization, we predicted the responses and obtained the most desirable separation parameters. Figure 5 shows that a retention time of less than 6.0 min, theoretical plates > 7,000, and tailing factor less than 1.5 were the objectives. Plots of overlays (Figure S2) highlight the optimal variable combinations in order to obtain the desired results. A laboratory validation was conducted on these predicted parameters.

According to the statistical analysis, flow rate and pH have the greatest influence on theoretical plates, tailing factor, and retention time. In contrast to theoretical plates and retention time, column temperature significantly affects

Table 1: Experimental setup variables and responses for Box–Behnken experiments.

Std	Run	Factor 1 A: Flow rate	Factor 2 B: pH	Factor 3 C: Column temperature	Response 1 Retention time	Response 2 Tailing factor	Response 3 Theoretical plates
16	1	1	6	30	6.023	1.32	8,350
12	2	1	7.0	35	5.905	1.34	8,180
3	3	0.8	7.0	30	6.166	1.36	8,187
7	4	0.8	6	35	6.213	1.35	8,195
9	5	1	4.8	25	6.506	1.28	8,152
10	6	1	7.0	25	5.945	1.29	8,158
4	7	1.1	7.0	30	5.719	1.42	7,920
8	8	1.1	6	35	5.846	1.39	8,001
17	9	1	6	30	6.021	1.32	8,341
2	10	1.1	4.8	30	5.843	1.38	8,160
5	11	0.8	6	25	6.365	1.33	8,174
14	12	1	6	30	6.123	1.32	8,330
15	13	1	6	30	6.178	1.32	8,342
6	14	1.1	6	25	5.876	1.37	7,952
1	15	0.8	4.8	30	6.31	1.34	8,183
13	16	1	6	30	6.113	1.32	8,335
11	17	1	4.8	35	6.205	1.33	8,197

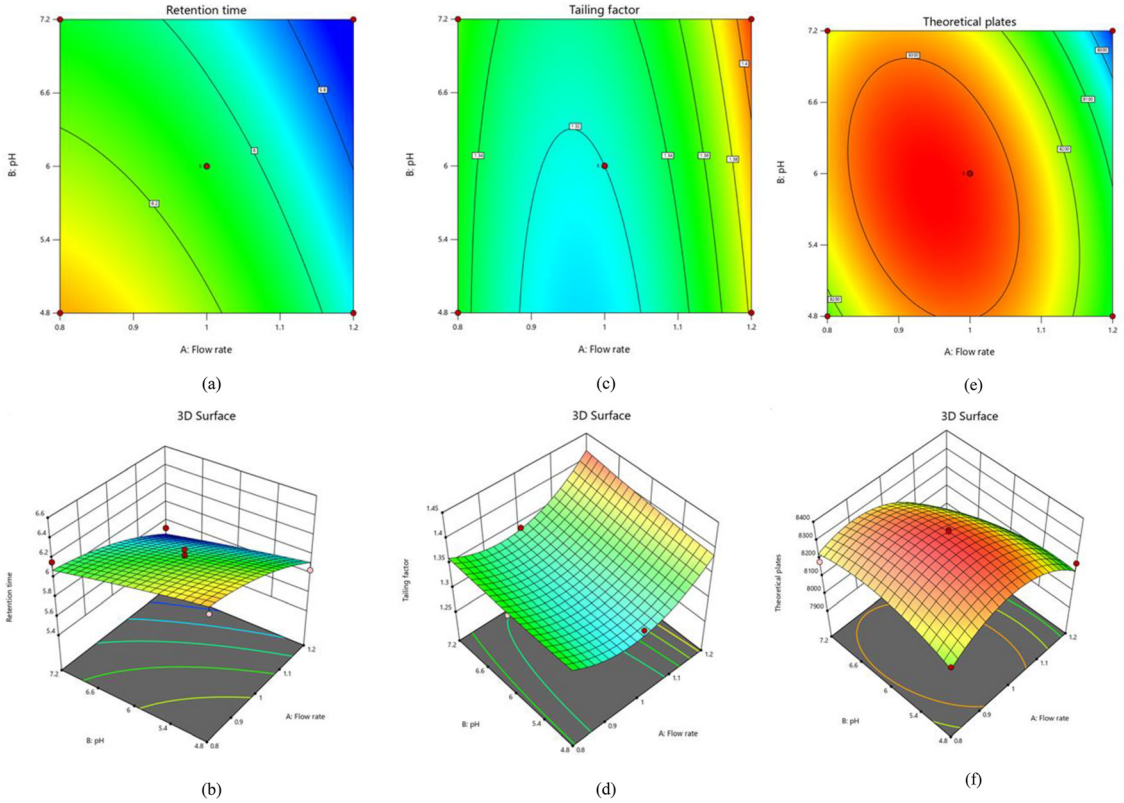

Figure 2: Contour and 3D response plots showing how flow rate and pH influence disodium edetate (a, b) retention time, (c, d) tailing factor, and (e, f) theoretical plates.

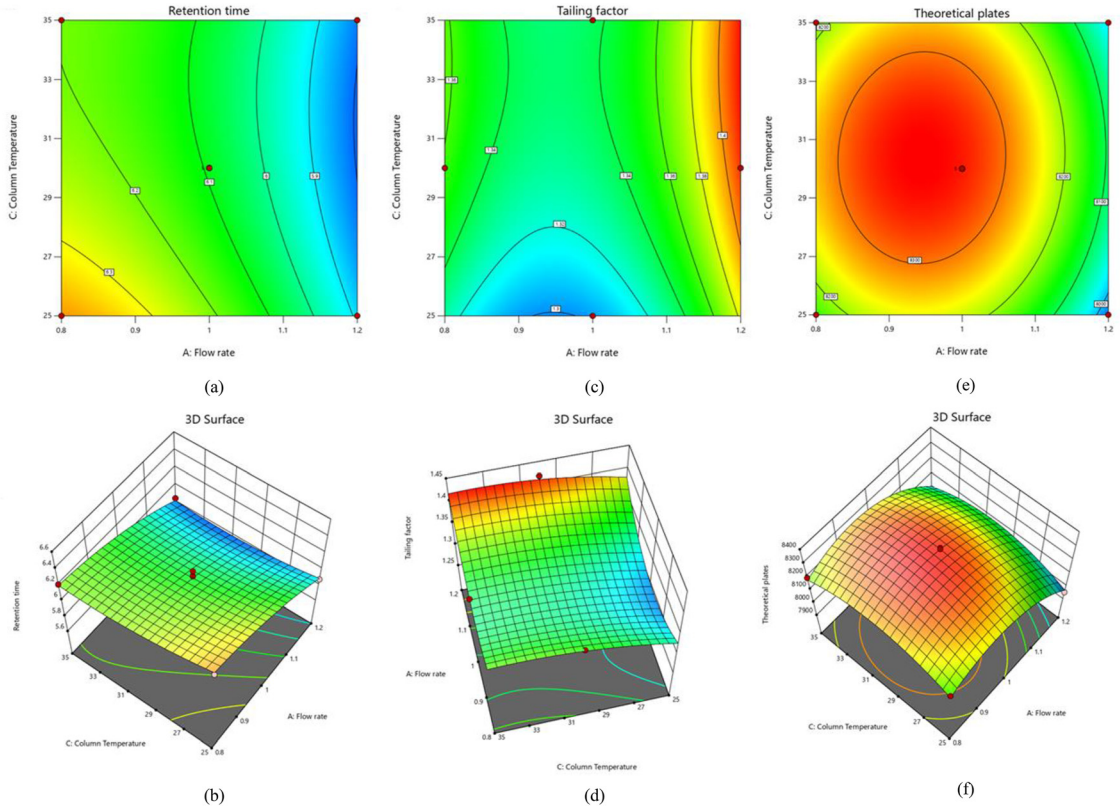

Figure 3: Contour and 3D response plots showing how flow rate and column temperature influence disodium edetate (a, b) retention time, (c, d) tailing factor, and (e, f) theoretical plates.

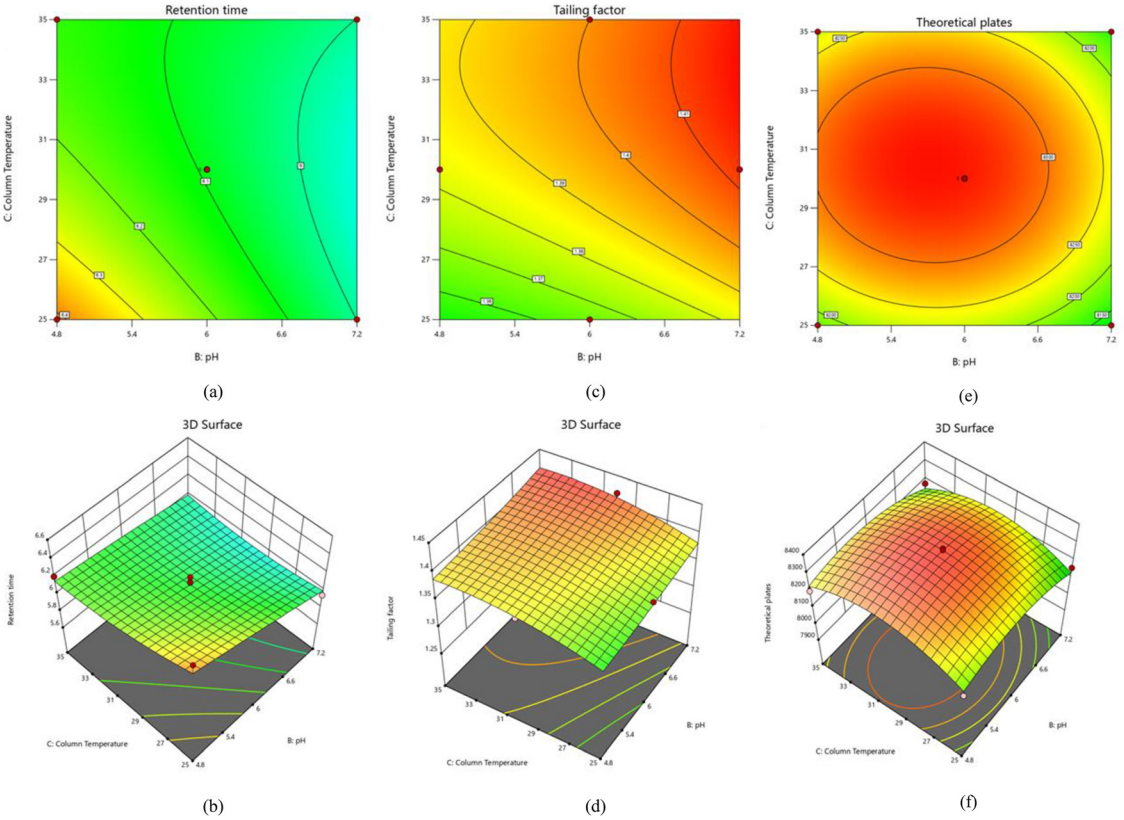

Figure 4: Contour and 3D response plots showing how pH and column temperature influence disodium edetate (a, b) retention time, (c, d) tailing factor, and (e, f) theoretical plates.

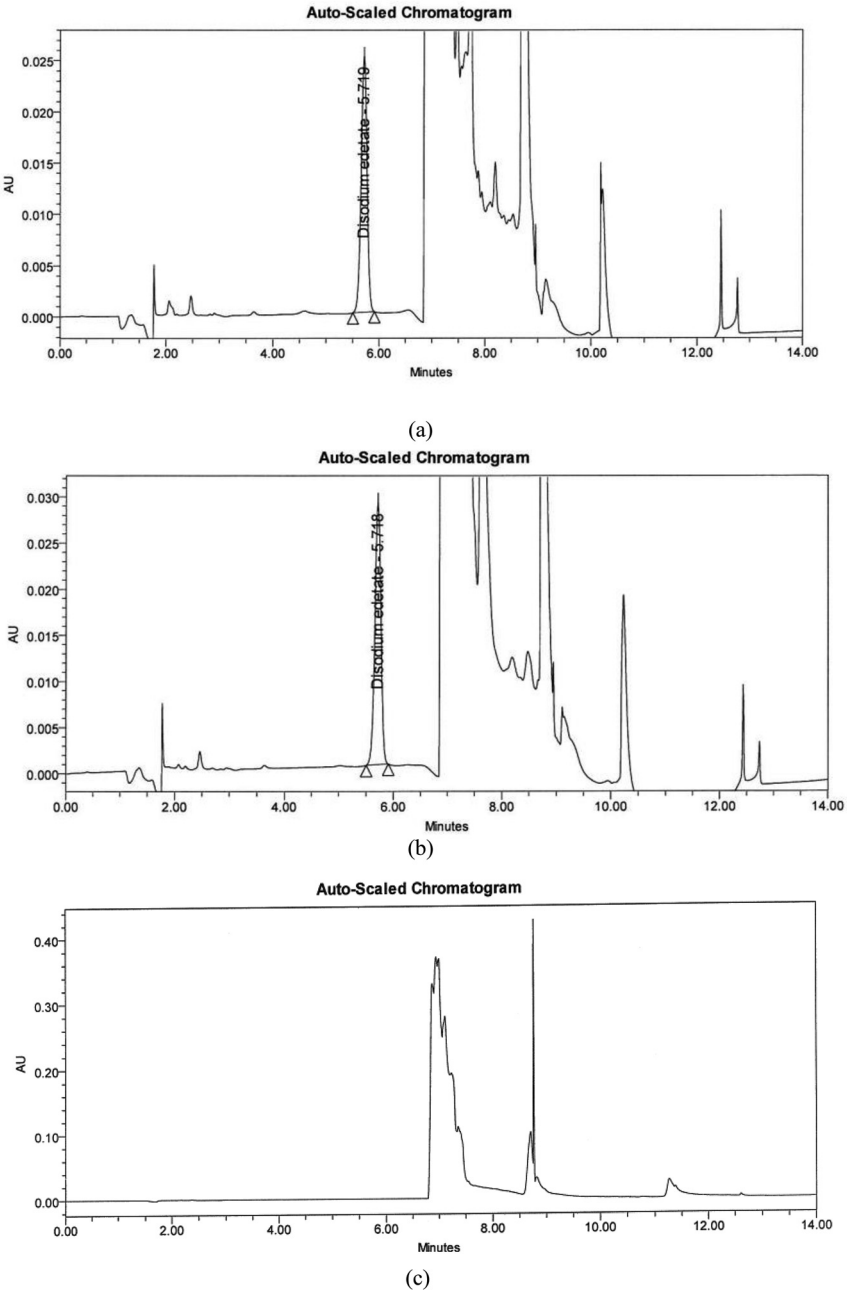

Figure 5: HPLC charts of (a) disodium edetate standard, (b) disodium edetate in Plegica eye 1 % drops, and (c) blank.

tailing factor. Flow rate and pH interact significantly, highlighting the importance of optimizing these parameters simultaneously.

As a result of this expanded interpretation, a clearer understanding of the relationships between chromatographic parameters and response is provided, which will assist in the optimization of the RP-HPLC method for the analysis of disodium edetate and its degradation products in eye drops.

At pH 7.0, 1.1 mL/min flow rate, and the column temperature increased to 30 °C to achieve the optimal chromatographic conditions. A short retention time was achieved under these conditions, resulting in excellent accuracy, symmetric peaks, and short retention times.

5.3 Implementing lean Six Sigma

Increasing process efficiency and quality requires an index of process capability, also known as the Cpk. Cpk effectively identifies and addresses inefficiencies caused by ineffective quality control. Cpk measures how closely a process aligns with the target center limit when natural variability is considered. It indicates that the process meets or exceeds

specifications consistently and reliably. Cpk values that are below a certain level indicate that improvements are necessary, as the process is not performing as intended.

On pharmaceutical batch analyses, disodium edetate assay testing was performed using the proposed method. Process capability six-pack reports were generated using Minitab software. There is no doubt that the investigation approach employed is of the highest quality based on the results. There were no outliers on the X-bar and R charts. As well, the data in the 20 most recent subgroups are homogeneous and random, closely centered around the method mean.

Based on the statistical techniques applied-capability charts, mean probabilities and histograms-the process aligns well with the desired outcomes. A notable finding was the fact that the Cpk values for disodium edetate exceeded the acceptance threshold of 1.33, providing further support for the suggested methods' effectiveness. Although higher Cpk values were demonstrated by the proposed method, it showed superior precision. An impressive Cpk values using the proposed method was 4.74 (Figure 6).

The results show that our process is performing exceptionally well, with very few variations and a high degree of consistency (Table 2).

5.4 Accelerated stability study

Table S7 summarizes the assay results for the accelerated stability study. The results reveal a significant degree of drug stability at 0, 1, 3, and 6 months. As determined by the data, there is no significant change or degradation exceeding 5 % from the initial value of the drug assay. Figure 7 support the findings that the API is stable and within acceptable limits during the study period.

5.5 Method validation

A rigorous validation was conducted in compliance with ICH Q2(R1) guidelines to ensure reliability, reproducibility, and suitability for its intended use. Specificity, linearity,

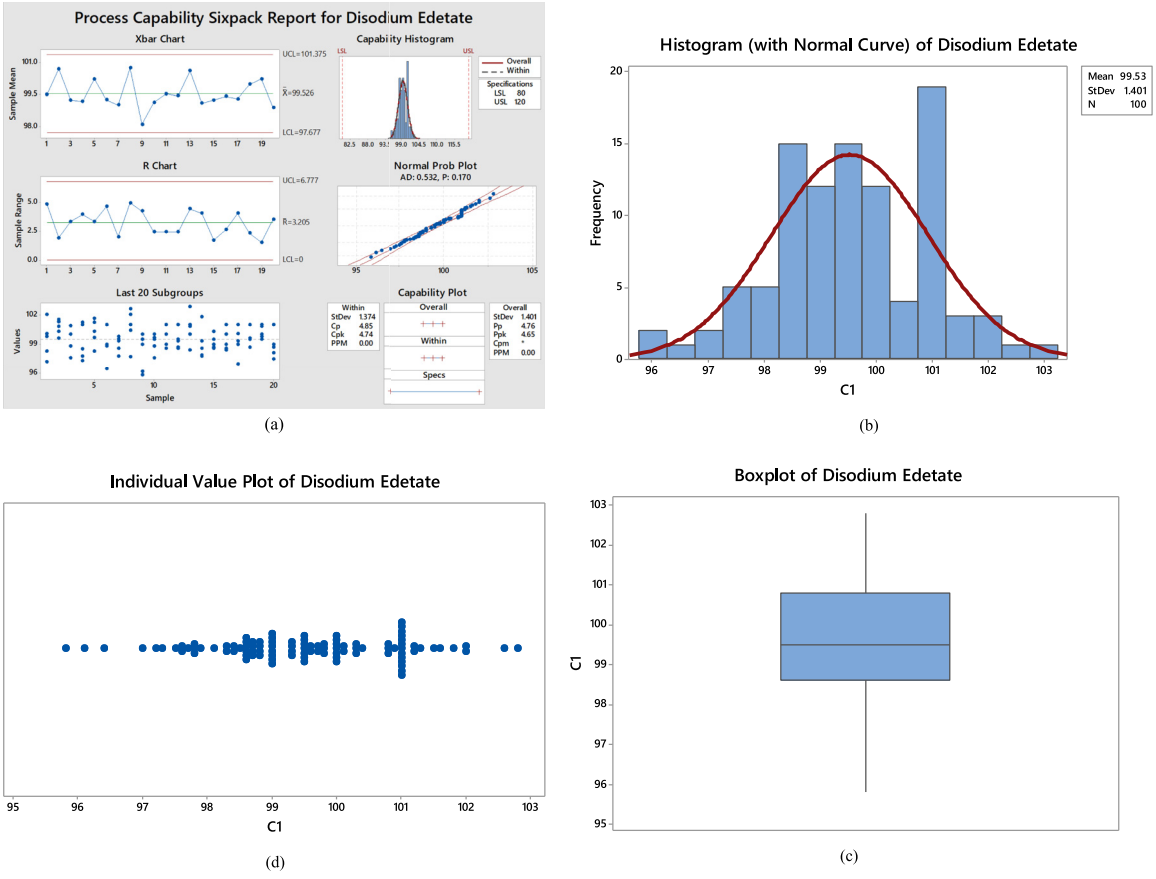

Figure 6: Process capability and distribution analysis for disodium edetate. (a) Process capability six-pack report. (b) Histogram with fitted normal curve. (c) Boxplot of disodium edetate. (d) Individual value plot.

Table 2: Statistical analysis of accuracy measurements for the proposed HPLC technique for disodium edetate.

Variable	*N*	*N*[a]	Mean	SE mean	StDev	Variance	CoefVar	Sum	Sum of squares
Disodium edetate	100	0	99.526	0.140	1.401	1.962	1.41	9,952.600	990,736.700

Variable	Minimum	Q1	Median	Q3	Maximum	Range	Mode	*N* for Mode	Skewness	Kurtosis
Disodium edetate	95.800	98.600	99.500	100.800	102.800	7.000	101	13	−0.15	−0.11

[a]Indicates the number of missing or excluded observations in the dataset.

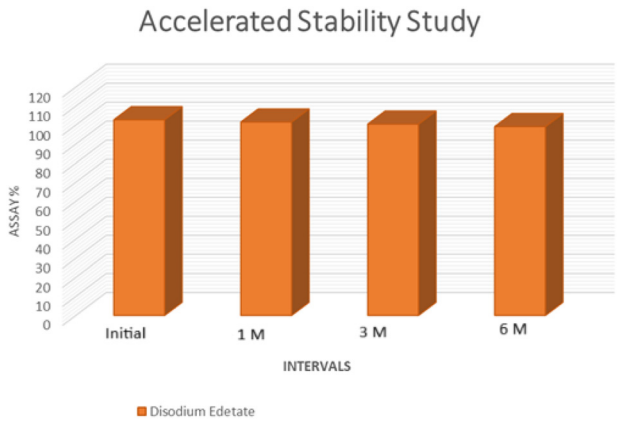

Figure 7: Assay results for disodium edetate after a 6-month accelerated stability study.

accuracy, precision, sensitivity (limits of detection and quantification), robustness, and system suitability were evaluated during validation [34].

5.6 Linearity

An analysis of several standard solutions at concentrations ranging from 2-40 µg/mL of the target concentration was used to evaluate linearity. Using the peak area (or response) to plot the concentration, a calibration curve was created. An assessment of the linear relationship was made by calculating the correlation coefficient (R^2) [35]. Using this method, a strong linear relationship for the tested range was demonstrated by R^2 = 0.999 (Table 3).

5.7 Detection and quantitation limits

The method's sensitivity was determined by establishing detection and quantification limits. The LOD was defined as the lowest concentration of the analyte that could be detected (signal-to-noise ratio ≥ 3), while the LOQ was defined as the lowest concentration that could be quantified (signal-to-noise ratio ≥ 10). Observational results demonstrate that this method

Table 3: An analysis of regression coefficients and variables from disodium edetate calibration curves.

Parameters	HPLC Disodium edetate
Linear	
Range (µg mL^{-1})	2–40
Wavelength (nm)	258
Intercept (a)	−15.546
Slope (b)	881.07
S_a	7.24
S_b	0.78
$S_{y/x}$	28.81
Correlation coefficient	0.9999
System precision	0.2
LOD[a] (µg mL^{-1})	0.03
LOQ[a] (µg mL^{-1})	0.09

[a]Limit of detection (3.3 × σ/slope) and a limit of quantitation (10 × σ/slope).

is capable of detecting low concentrations of analytes at LOD and LOQ, respectively (Figure S3) and quantify it (Table 3).

5.8 Precision

We considered repeatability (intraday precision) and intermediate precision (interday precision) in evaluating precision. We measured repeatability by injecting the analyte six times on the same day at 100 % concentration. In order to achieve intermediate precision, three separate analyses were conducted on three different days using three different instruments. Results were calculated based on their relative standard deviation (RSD). As for repeatability and intermediate precision, both methods demonstrated excellent precision with RSD values of less than 2.0 % (Table 3).

5.9 Accuracy and recovery

Analytes were spiked into two blank matrixes (e.g., placebo or sample matrix) at three concentration levels to determine

Table 4: Accuracy and recovery findings of the suggested approach for computing disodium edetate.

Parameters	HPLC
Relative concentrations %	Disodium edetate
	Recovery (%)
50	100.08
	100.46
	100.73
	99.33
	99.85
	98.79
Mean ± RSD	99.87 ± 0.72
100	99.16
	99.47
	99.26
	100.79
	100.43
	100.49
Mean ± RSD	99.93 ± 0.71
150	100.59
	98.15
	101.22
	101.77
	99.42
	98.19
Mean ± RSD	99.89 ± 1.55

accuracy (50 %, 100 %, and 150 % of the target concentration). Analytes were measured and theoretical concentrations were compared to calculate recovery percentages. A recovery rate between 98 % and 102 % was found to be acceptable using this method (Table 4).

5.10 Robustness

A robust assessment was conducted by deliberately introducing small variations in critical parameters such as 0.1 pH, 0.1 mL flow rate, and 1 nm detection wavelength. Analysis of system suitability parameters (such as retention time, peak area, and resolution) was performed to assess the impact of these variations on the method's performance. It was found that all system suitability parameters remained within acceptable limits despite the introduced variations, proving that the method is robust (Table S8).

5.11 System suitability

During system suitability testing, the analytical system was ensured to be suitable for the intended application. A standard solution was used to evaluate critical parameters such as retention time, theoretical plates, tailing factor, and resolution. As a result, the system met the predefined acceptance criteria, confirming its correct and consistent operation (Table 5).

Table 5: System suitability data of the HPLC approach.

Item	HPLC	Reference values
	Disodium edetate	
Tailing factor	1.45	$T \leq 2.0$
Injection precision	0.3	RSD ≤ 2.0 %
Number of theoretical plates (N)	7,920	$N > 2{,}000$
Retention time (%RSD)	0.1	RSD ≤ 10 %

5.12 Specificity and selectivity assessment

As part of our evaluation of the method's specificity, we ensured that the analyte of interest can be measured accurately even with potential interferences, such as impurities, degradation products, and matrix components. The method was evaluated by forcing degradation (acid, base, oxidation, thermal, and photolytic stresses) on the analyte to determine its ability to differentiate between the analyte and its degradation products (Table 6).

As a result, the method confirmed that chromatographic peaks of analytes and degradation products were well-resolved from impurities and degradation products (Figure 8).

6 Assessment tools for environmental sustainability

6.1 AGREEprep evaluation

Figure S4a provides a clear and easy-to-read overview of each of the 10 criteria through distinct graphic symbols. In addition, Figure 9a shows the technique's environmental practicality, with a value of 0.62 confirming its effectiveness.

Table 6: Degradation findings for disodium edetate using the suggested approach.

	Condition	% Degradation	% Assay	Purity angle	Purity threshold
Disodium edetate	Sunlight	1.0 %	99.0 %	0.123	0.277
	Heat	1.5 %	98.5 %	0.156	0.296
	Acid	3.5 %	96.5 %	0.139	0.296
	Base	15 %	85 %	0.314	0.355

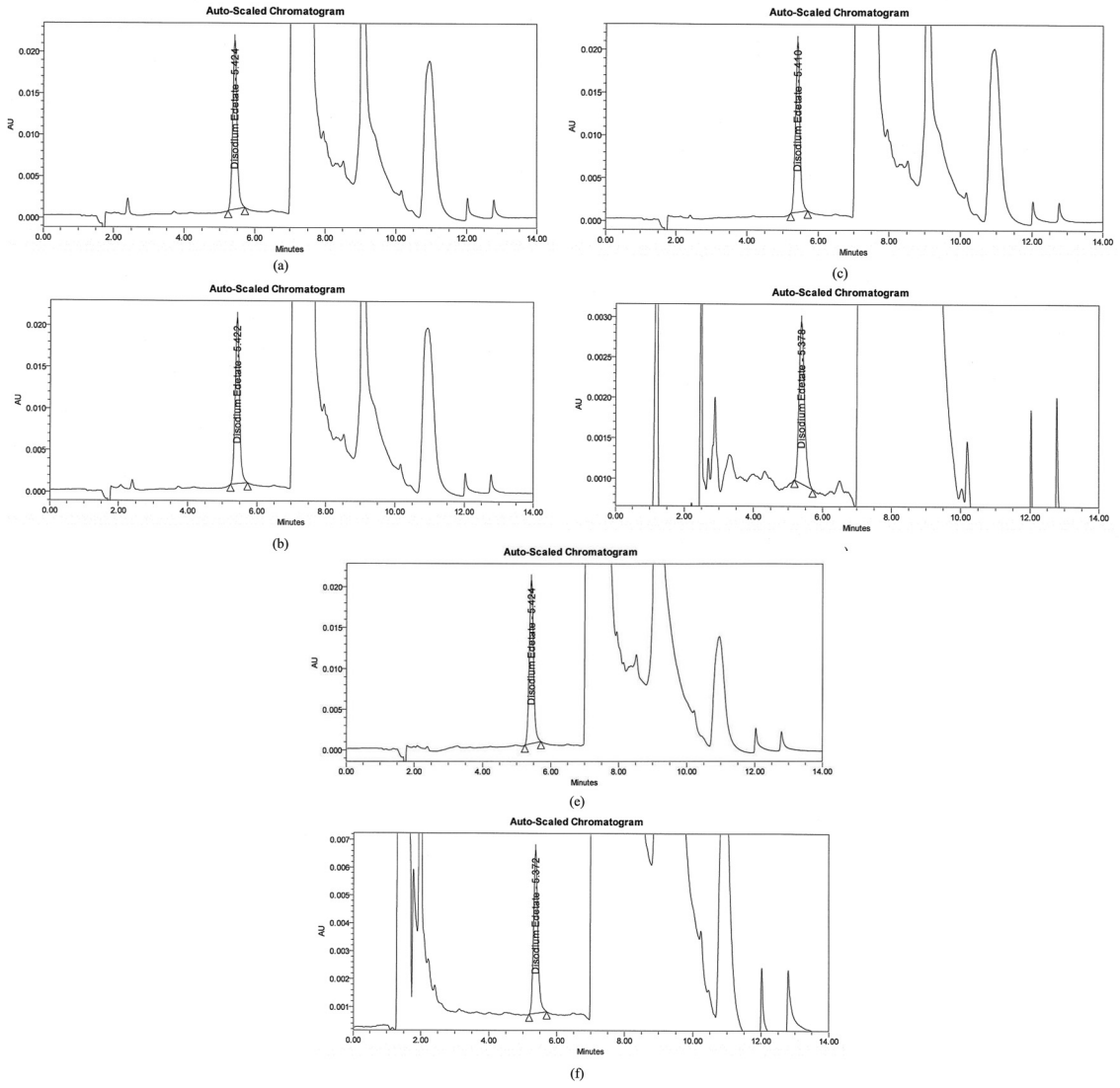

Figure 8: Disodium edetate standard of (a) no degradation and after exposure to (b) sunlight, (c) heat, (d) 1.0 N NaOH, (e) 1.0 N HCl, and (f) 3.0 % H_2O_2 degradation.

6.2 HPLC-EAT evaluation

Our assessment of the software was that it was user-friendly and intuitive, as shown in Figure 9b. It is important to know, however, that the metric only considers the environmental impact of solvents and no other factors, such as energy consumption and sample preparation conditions, in GAC. A lower score indicates greater environmental sustainability, as determined by the software's evaluation of the data provided. Its environmental sustainability was confirmed by a score of 8.631, as shown in Figure S4b.

6.3 BAGI evaluation

In industries such as pharmaceuticals and environmental testing, BAGI helps select reliable and sustainable methods. Furthermore, BAGI scores can also be used to demonstrate environmental commitment as part of sustainability reports. An analytical technique that produces a precise pictogram and score that follows the 10 BAGI criteria demonstrates its value and practicality. The color spectrum represents the final HPLC score of 77.5 that ranges from dark blue to light blue and back to dark blue as shown in Figure S4c. According to these colors, the method meets the

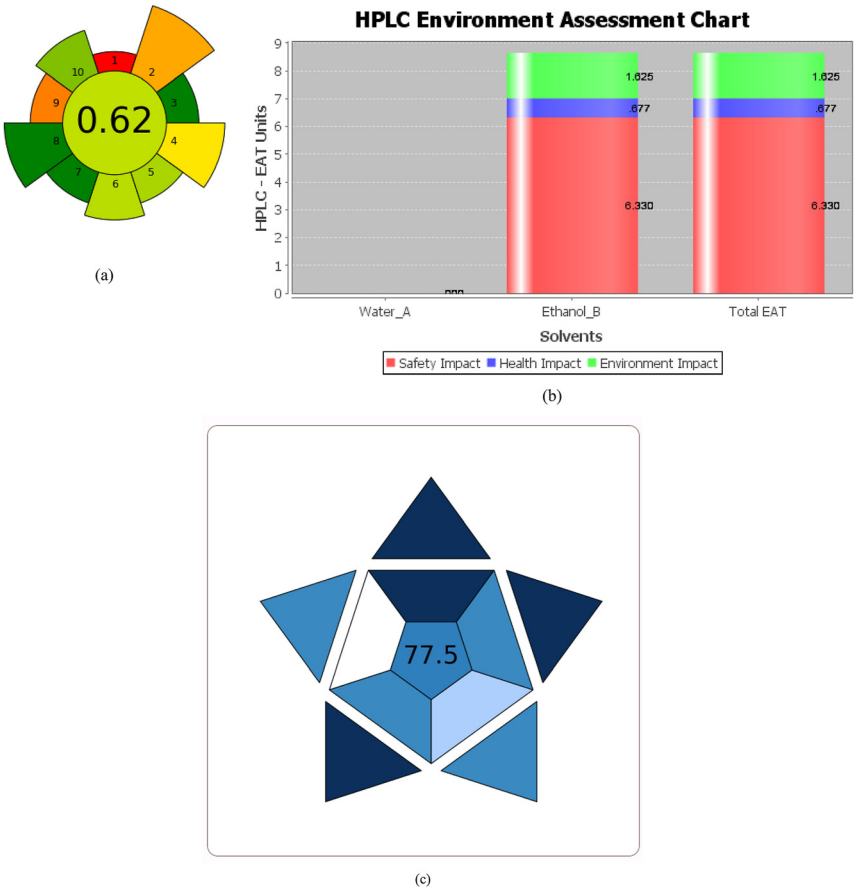

Figure 9: Assessment tools (a) AGREEprep, (b) HPLC-EAT, and (c) BAGI diagrams are designed to measure the ecological impact of recommended techniques.

criteria to a greater or lesser extent. A score of more than 60 deems the analytical method to be applicable. Figure 9c illustrates how each proposed BAGI index pictogram can represent the strategy.

6.4 GAC and WAC assessments

As demonstrated by the comparative analysis, the proposed RP-HPLC method offers superior sustainability and analytical efficiency to previous methods. Accordingly, it has the highest AGREEprep score (0.62), proving a better alignment with GAC. This method reduces environmental impact and operator risk significantly compared with previously reported methods that rely heavily on solvents such as acetonitrile and methanol. A further benefit of the method is its excellent sensitivity, as it achieves a LOD of 0.03 µg/mL and LOQ of 0.09 µg/mL, which is significantly higher than that of other methods that either lack this data or report lower detection limits. As well as maintaining a moderate flow rate (1.1 mL/min), the method has a short run time. This leads to decreased energy consumption.

As compared with existing analytical methods, the proposed RP-HPLC method for analyzing disodium edetate and its degradation products in eye drops stands out significantly. A sustainable approach is first emphasized by minimizing solvent consumption and waste generation by using an eco-friendly solution of ethanol in the mobile phase. The existing methods, however, often use hazardous solvents like methanol and acetonitrile, which affect the environment more severely. A second advantage is that the proposed method uses QbD principles along with BBD to optimize chromatographic parameters, ensuring robustness and reliability compared with existing methods that do not provide such systematic optimizations. Moreover, the proposed method uses a gradient mode mobile phase at pH 7.0 at a flow rate of 1.1 mL/min, unlike existing methods that utilize varying flow rates and mobile phase compositions. Comparatively to existing methods [15–19], which have generally higher LOD and LOQ values, this method is more sensitive with a sensitivity of 0.03 µg/mL and 0.09 µg/mL. Additionally, the proposed method provides comprehensive degradation studies under a wide range of conditions (acid, base, photo, thermal, and oxidation), which are generally not covered by existing techniques. Based on these differences, the proposed method is a superior choice for pharmaceutical quality control due to its sustainability, robustness, sensitivity, and comprehensive degradation analysis (Table S9).

A whiteness profile assessment shows that this method integrates all 12 principles of WAC, including minimal sample preparation, cost-effectiveness, and full ICH compliance. Through this holistic approach, environmental protection, robustness, reproducibility, and suitability for routine pharmaceutical analysis are also assured. Contrary to these findings, the reported methods often compromise either greenness or validation completeness in order to meet whiteness criteria. Hence, both scientific rigor and environmental responsibility are aligned with the proposed method, making it an effective, efficient, and environmentally friendly analytical approach.

Despite the BAGI score of 77.5 being equal between the proposed and reported RP-HPLC methods [19], our approach clearly demonstrates superior GAC and WAC principles. Efficiencies in sustainability, robustness, and reproducibility are enhanced by the use of sustainable solvents (ethanol-based buffer), systematic optimization using BBD, and AQbD strategies. Most other reported methods [15–18] use conventional solvents such as acetonitrile and methanol, involve higher flow rates or longer run times, and do not assess whiteness or risk comprehensively. The AQbD and BBD methodologies are not incorporated into Method [19], even though it achieves a BAGI score of 77.5. Consequently, the proposed method not only meets but exceeds the standards implied by the BAGI score, offering a more balanced, validated, and environmentally responsible analytical approach than the existing method.

In contrast to the reported methods [15–19], which often use conventional solvents such as acetone and methanol, the proposed method uses ethanol-based buffers, thus reducing toxicity, environmental burden, and operator risk. In addition to making the method green, it is aligned with WAC, which emphasizes eco-friendliness, safety, and sustainability.

Further, the proposed method ensures robustness, reproducibility, and minimal resource consumption thanks to AQbD) and BBD. In contrast, the reported methods are typically based on trial-and-error approaches that do not reflect these advanced design strategies.

7 Conclusions

Our study aimed to develop a novel, sustainable, and BBD-driven RP-HPLC method for the simultaneous identification of disodium edetate in eye drops and its degradation products. QC in the pharmaceutical section needs a robust and eco-friendly analytical method. Our approach is aligned with sustainability goals by integrating BBD principles to ensure method robustness and minimize solvent consumption and waste generation. Environmentally friendly tools including HPLC-EAT, BAGI, and AGREEprep were used to assess the method's ecological impact. BBD was used to optimize three critical chromatographic parameters, including column oven temperature, flow rate, and buffer pH. The three responses included retention time, theoretical plates, and tailing factor. The method was tested under acidic, basic, oxidative, photolytic, and thermal conditions, in accordance with ICH guidelines. A combination of acidic, basic, and oxidative conditions led to the highest degradation rates. A validation study ensured the method's reliability for routine quality control by measuring specificity. The BBD method combines sustainability with robustness, making it a cost-effective, eco-friendly method for analyzing pharmaceuticals. By providing regulatory compliance with greener analytical practices, it has been applied to eye drop formulations successfully, demonstrating its practicality and utility in the pharmaceutical industry.

7.1 Study limitations and future plan

Despite identifying potential degradation pathways and confirming the resolution of chromatographic peaks, unknown degradants cannot be fully characterized through forced degradation. As a result, we were limited in time and resources. Analytes and their degradation products were evaluated using standard chromatographic methods. Although NMR and mass spectrometry are considered advanced methods for identifying and characterizing impurities, they were not fully used to determine all known impurities. MS and NMR should be used as advanced analytical techniques for identifying and characterizing unknown degradants in the future. It will provide a more comprehensive understanding of analyte degradation. This method will also be applied to injectables, topical creams, and oral tablets in the future. To explore its relevance in regulatory environments beyond ICH guidelines, the future plan intends to investigate its applicability to a variety of pharmaceutical formulations, such as injectables, topical creams, and oral tablets. As part of the plan, the method will also be evaluated in relation to regulatory frameworks in different areas, including the Food and Drug Administration's (FDA's) and European Medicines Agency's (EMA's). A continuous improvement and innovation process will be pursued by gathering feedback, investing in research, and keeping track of advancements. It will ensure the method remains cutting-edge and effective.

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Conflict of interest: Authors state no conflict of interest. The authors declare that Hikma Pharmaceuticals PLC had no influence on the design, execution, interpretation, or reporting of the results presented in this study.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supporting Information: Supporting information includes desired parameters and overlay plots for the predicted response, in addition to AGREEprep, HPLC-EAT, and BAGI diagrams, which are used to measure the ecological impact of recommended techniques. As well as LOD and LOQ chromatograms of disodium edetate, A comparison of degradation and chromatographic conditions according to the proposed approach and the reported method using eco-friendly tools, chromatographic analysis conducted using a gradient-mode RP-HPLC system, ANOVA for the quadratic model of theoretical plates, tailing factor, and retention time responses are included. Additionally, it summarizes the results of the accelerated stability study and provides intraday and interday robustness and stability data for HPLC analytical solutions.

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