Green Chromatographic Method for Determination of Active Pharmaceutical Ingredient, Preservative, and Antioxidant in an Injectable Formulation: Robustness by Design Expert

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ABSTRACT: We report an efficient HPLC method for simultaneous qualitative and quantitative analysis of lincosamide antibiotic injectable formulations containing Clindamycin phosphate (CMN), benzyl alcohol (BA), and ethylenediaminetetraacetic acid (EDTA) as major ingredients. The three components were separated by Phenomenex prodigy C8 (250 mm × 4.6 mm, 5 μm) HPLC column, flow rate 1.1 mL/min, injection volume 30 μL, and column temperature 35 °C, using 0.05 M sodium acetate buffer (pH 4.5) with acetonitrile (ACN) in the ratio of 80:20 (v/v). The detection wavelength was set as 240 nm. The method was validated as per International Conference on Harmonization (ICH) guidelines and was confirmed to be specific, precise, accurate, and linear. Method robustness was executed by utilizing quality in the design of the experiment. Accuracy results were found to be 99.3−100.5% for CMN, 99.3−100.8% for BA, and 99.1−100.3% for EDTA. Precision results were obtained as % relative standard deviation (RSD): 0.6% for CMN, 0.4% for BA, and 0.4% for EDTA. Correlation coefficient (r²) values were obtained as >0.999 for the three components. Analytical solutions are stable for 48 h at benchtop and refrigerator conditions. The greenness of the analytical method was evaluated by the Green Analytical Procedure Index (GAPI), National Environmental Method Index (NEMI), analytical eco-scale, and Analytical Greenness (AGREE) tools to confirm that the method is eco-friendly.

1. INTRODUCTION

Clindamycin phosphate (CMN) is an injectable lincosamide antibiotic to prevent bacterial infections. The CMN injectable formulation contains two other primary ingredients, benzyl alcohol (BA) and ethylenediaminetetraacetic acid (EDTA). It also contains hydrochloric acid and sodium hydroxide as minor components for pH adjustment purposes. Specifically, CMN is a water-soluble ester of lincomycin, formed by the 7(S)-chloro-substitution of the parent antibiotic 7(R)-hydroxyl, and CMN is available as a phosphate salt. The molecular formula and molecular weight are C_{18}H_{34}ClN_{2}O_{8}PS and 505.0, respectively. The compound BA consists of benzene with a hydroxymethyl substitution. It is a metabolite, a solvent, a fragrance, an antioxidant, and an insect repellent.

EDTA is an aminopoly(carboxylic acid) with the formula [CH₂N(CH₂CO₂H)]₂. As a hexadentate chelating agent, this white, water-soluble solid is typically used to bind iron and calcium ions. EDTA is available in several salt forms, including sodium calcium edetate, disodium EDTA, and tetraysodium EDTA. EDTA is used as a chelating agent in this formulation.

The chemical structures of CMN (active), BA (preservative), and EDTA (antioxidant) are shown in Figure 1.

As per the current Committee for Proprietary Medicinal Products (CPMP), the Committee for Veterinary Medicinal Products (CVMP) regulatory guidance, document numbers CPMP/CVMP/QWP/115/95,¹ CPMP/QWP/419/03,² USP general chapter (341),³ and ICH topic Q6 A,⁴ antioxidants and antimicrobial preservative levels in the formulation should be qualitative and quantitatively monitored periodically during the shelf life of the finished product. The present formulation had both preservatives and antioxidants. In the stability study of the finished product, the content levels of BA and EDTA are monitored using two different analytical methods. There is no...
method available for combined determination with the presence of CMN. Many analytical techniques are available for individual analysis of BA.\textsuperscript{5–8} EDTA is a UV inactive component, and few authors made the analyte UV active by a derivatization process and determined by using different techniques.\textsuperscript{9–16} Several kinds of literature are available for the CMN content determination in different formulations.\textsuperscript{17–21} Two reports are available to determine EDTA and BA by HPLC and MS analyses.\textsuperscript{22,23}

Assay determination of CMN, BA, and EDTA by three diverse methods is time-consuming, expensive, and not viable in regular pharmaceutical quality control laboratories. The current research targeted the single analytical method for all three analytes in a simple RP-HPLC isocratic way; simultaneously, the technique should be eco-friendly and cost-effective. The added derivatizing agent to make EDTA UV sensitive should not interfere with the BA and CMN peak shapes and recoveries.

Methods and techniques of analysis play a significant role in QbD. DoE (Design of Experiment) is commonly used to find ranges for operating parameters of the equipment, understand sample preparation variations, and evaluate method robustness. Design Expert software ver. 13 is used for design space trails with identified critical quality attributes.\textsuperscript{24} The QbD tool is used to study the method’s robustness.

Green Analytical Procedure Index (GAPI), National Environmental Method Index (NEMI), and Analytical Eco-scales are employed for green chemistry evaluations. GAPI is a pictogram design, and it is a valuable tool to evaluate the method’s greenness in all aspects, including sample collection, sample preparation, technique, and determination. NEMI is also a pictogram that assesses the process by calculating the penalty points and expresses the method’s eco-friendliness. These three green metrics were utilized to confirm the method’s greenness.\textsuperscript{25–34} The modern tool Analytical Greenness (AGREE) is also used to assess greenness. The approach was devised by the Gdańsk University of Technology, Poland, and expresses the greenness of the procedure based on 12 green chemistry principles.\textsuperscript{35}

2. RESULTS AND DISCUSSION

2.1. Method Development. Generic injectable formulations do not require clinical studies to be submitted to the regulatory agency. The contents of the formula with the reference listed drugs have to be attested as being qualitatively and quantitatively identical for the dosage form submissions. The current formulation contains two major excipients, BA and EDTA. For regulatory compliance, it is essential to prove that these components are the same as the reference sample. Moreover, as per the regulatory body requirements, the levels of antioxidants and antimicrobial preservatives should be monitored in the stability study of the formulation. In small-scale laboratories, determining these contents using different methods and high-end techniques is unreliable. HPLC is the most widely used simple technique in the pharmaceutical industry. Current research targeted a single and straightforward isocratic RP-HPLC method to determine these excipients and the active drug. CMN is the therapeutically active ingredient, and BA is used as a preservative in the current formulation. BA protects the formulation from microbial growth throughout the lifetime of injection. Both are UV active, so no special treatment is required to quantify in sample preparation.

EDTA stabilizes the formulation by chelating free ions and metals present in the formulation, which may trigger the degradation reaction of the active component. EDTA is commonly used as a chelating agent in biochemistry, cellular biology, and molecular biology to bind divalent metal ions (such as calcium and magnesium). Metals are bound to EDTA through amine and carboxylate groups. Mn (II), Cu (II), Fe (III), Pb (II), and Co (III) form strong EDTA complexes. HPLC analysis of EDTA is complicated due to its UV inactivity and strong metal ion binding tendency. The method’s sensitivity is increased by using copper acetate as a derivatizing agent. Cu (II) ions react with EDTA to form a stable complex: \textsuperscript{36–37}

\begin{equation}
\text{Cu}^{2+} + 4\text{EDTA} \rightleftharpoons \text{CuEDTA}_{4}^{2-} + 2\text{H}^+\n\end{equation}

Figure 1. Structures of clindamycin phosphate (A), EDTA (B), and benzyl alcohol (C).
Based on the literature review, we initiated chromatography optimization trials with C18 and C8 stationary phases with potassium phosphate, sodium phosphate, and orthophosphoric acid buffers with the ACN combination at pH 4.5. Based on compatibility with the drug, the copper acetate reaction was chosen based on its nontoxic nature, eco-friendliness, cost-effectiveness, and UV-active complex. Copper II acetate was chosen. With the derivatizing agent, the EDTA peak was not detected. No impact if the derivatizing agent on the CMN and BA, samples were prepared with and without Copper II acetate, and the analyte assay values were calculated. Table 1 summarizes the results.

The results express the impact of the derivatizing agent on EDTA clearly. With the derivatizing agent, the EDTA peak eluted with recovery >98.9%. Without the agent, the EDTA peak was not detected. No impact if the derivatizing agent on CMN and BA was observed. The % difference between with and without derivatizing agent CMN and BA was 0.1% and 0.3%, respectively.

3. CONCLUSIONS

A green reverse-phase liquid chromatographic method is developed for the quantitative analysis of active ingredient (Clindamycin phosphate), preservative (benzyl alcohol), and chelating agent (ethylenediaminetetraacetic acid) in Lincosamide injectable antibiotic formulations. This RP-HPLC method could not reduce the EDTA retention time or elute CMN and BA. The results concluded that the ion-pairing reagent concentration was affecting the retention time of the EDTA. The minimum amount of 8 mL was selected per 1 L of buffer, the organic ratio changed to 20%, and BA and CMN samples were injected individually. CMN, BA, and EDTA in separate samples eluted under the same chromatographic conditions. Lambda max 240 nm was selected based on the EDTA spectrum due to the low amount of EDTA present in the formulation. Using single chromatographic conditions and preparing the different samples for different analytes was not the objective. The CMN and BA samples were prepared with the derivatizing agent (Copper II acetate) and injected to overcome the problem. BA and CMN peaks were eluted, but CMN eluted with a broader peak, and peak fronting was observed. The diluent’s concentration of derivatizing agent was varied to improve the CMN peak shape. The diluent was prepared with the derivatizing agent at different concentrations (5, 10, and 20 mg/mL). The standard and sample solutions are injected into HPLC. All the peaks were eluted with good peak shape with 10 mg/mL of derivatizing agent and met the system suitability requirements. Chromatographic conditions were optimized with a Phenomenex C8 (250 mm × 4.6 mm, 5 μm) HPLC column with a 1.1 mL/min flow rate. The mobile phase ratio fixed buffer:acetonitrile at 80:20 (v/v), lambda max was attained at 240 nm, and the column compartment temperature was set at 35 °C. The total run time of 20 min was finalized based on the robustness and specificity results. The final optimized chromatogram is shown in Figure 2.

To check the effect of the derivatizing agent on the CMN and BA, samples were prepared with and without Copper II acetate, and the analyte assay values were calculated. Table 1 summarizes the results. The results express the impact of the derivatizing agent on EDTA clearly. With the derivatizing agent, the EDTA peak eluted with recovery >98.9%. Without the agent, the EDTA peak was not detected. No impact if the derivatizing agent on CMN and BA was observed. The % difference between with and without derivatizing agent CMN and BA was 0.1% and 0.3%, respectively.

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A green reverse-phase liquid chromatographic method is developed for the quantitative analysis of active ingredient (Clindamycin phosphate), preservative (benzyl alcohol), and chelating agent (ethylenediaminetetraacetic acid) in Lincosamide injectable antibiotic formulations. This RP-HPLC
method was validated per the ICH and proved specific, precise, accurate, robust, and stability-indicating. The developed and validated process was simple, sensitive, and economical. A single determination of all three components is a significant asset of this method. Therefore, the proposed method is reliable, suitable for routine analysis, and an excellent approach to obtaining reliable results. This protocol can be used even for the individual determination of CMN, EDTA, and benzyl alcohol. The proposed method is environmentally friendly.

4. MATERIALS AND METHODS

4.1. Chemicals and Reagents. Grade A glassware was used in the complete development and validation. In the mobile phase preparation, buffer salt sodium acetate, acetonitrile (HPLC grade), and pH adjustment chemical glacial acetic acid (AR grade) were procured from Merck (Merck Chemicals (Shanghai) Co., Ltd.). The ion pairing reagent tetrabutylammonium hydroxide 10% in water (HPLC grade) was procured from JT Baker. The EDTA UV activation derivatizing agent, copper II acetate (AR grade), was obtained from Sino Pharm Chemical Reagents, China. EDTA and BA were obtained from Merck (Merck Chemicals (Shanghai) Co., Ltd.), and CMN was purchased from Hubei Yitai Pharmaceutical Co., Ltd., China. For stress studies, hydrogen peroxide, sodium hydroxide, and hydrochloric acid (AR grade) were procured from Sino Pharm Chemical Reagents, China. Milli-Q water was used throughout the studies. Formulation and placebo samples were prepared in-house.

4.2. Equipment, Software, and Column. The chromatography method was developed with a Prodigy C8 (250 mm × 4.6 mm, 5 μm) column from Phenomenex. Agilent Technologies, Model 1100 series with UV and DAD detectors, was used for liquid chromatography. We used Open lab CDS software to run the HPLC. Shimadzu AP225SD semi-microbalance was used for weighing chemicals. Buffer pH 4.5 was prepared by using the Mettler pH meter model no FE-28. The buffer was filtered with a vacuum pump from HA diaphragm model no. HPD-2SB. Olavo water bath (Model DHG-9250A) and vacuum oven (HS-56) were used for the forced degradation study with model numbers. Photo stress was performed on the Yesi drug photostability chamber model no. SHH-100GD-2. Design Expert software ver. 13 was used for a robustness study by DoE (design of experiments).

4.3. Analytical Solution Preparation. The mobile phase was prepared by mixing the buffer (8 mL of tetrabutylammonium hydroxide 10% in water diluted with 0.05 M pH 4.5 sodium acetate buffer, pH adjusted with acetic acid) and ACN in the ratio of 80:20 (v/v). Diluent for the sample and standard preparation was prepared by mixing the buffer, ACN, and copper II acetate solution (10 mg/mL) in the ratio of 78:20:2 (v/v/v). Standard solution concentrations of EDTA 5 μg/mL, BA 95 μg/mL, and CMN 1500 μg/mL were prepared by transferring 10.0 mg of EDTA and 19.0 mg of BA in a 20 mL diluent, then equivalent to 15.0 mg of CMN, and 1 mL of the previous solution diluted to 10 mL. Test sample solutions were prepared by diluting 1.0 mL of the injection sample solution into 50 mL with diluent and injecting all samples into the HPLC system.

4.4. Chromatography Conditions. A single and straightforward isocratic method developed with Phenomenex prodigy C8 (250 mm × 4.6 mm, 5 μm) column was used. 0.05 M pH 4.5 buffer mixed with acetonitrile in the ratio of 80:20 (v/v) was used as the mobile phase and was filtered through a 0.45 μm membrane filter. Flow rate was 1.1 mL/min, injection

| Parameter name | EDTA | BA | CMN |
|----------------|------|----|-----|
| Tailing factor (≤2.0) | 1.3 | 1.2 | 0.9 |
| Plate count (>2000) | 50906 | 100154 | 75963 |
| RSD% (n = 6 < 2.0) | 0.3 | 0.4 | 0.8 |
| Diluent/Mobile phase Interference (Should be absent) | No | No | No |
| Placebo interference (Should be absent) | No | No | No |
| Peak purity (Should be passed) | Passed | Passed | Passed |
| Range (μg/mL) | 1.2–7.5 | 23.6–141.8 | 375–2250 |
| Slope | 139668 | 197415 | 239230 |
| Intercept | 102.05 | 349.85 | 756.46 |
| Correlation coefficient | 0.9999 | 0.9999 | 0.9994 |
| LOQ (μg/mL) | 0.04 | 0.03 | 0.02 |
| Accuracy (n = 6 avg Percentage) | | | |
| LOQ level ± SD | 100.5 ± 0.3 | 100.8 ± 0.6 | 100.3 ± 0.9 |
| 80% mean ± SD | 100.1 ± 0.5 | 99.6 ± 0.2 | 99.1 ± 0.6 |
| 100% mean ± SD | 99.3 ± 1.1 | 99.9 ± 0.9 | 99.5 ± 0.4 |
| 120% mean ± SD | 100.3 ± 1.1 | 99.3 ± 0.1 | 99.6 ± 1.5 |
| Precision (Intraday, n = 6 RSD % < 2.0) | 0.5 | 0.4 | 0.6 |
| Intermediate Precision (Intraday, n = 6 RSD% < 2.0) | 0.8 | 0.8 | 0.8 |
| Ruggedness (n = 12 RSD% < 2.0) | 0.6 | 0.6 | 0.7 |
| Solution stability B.T (0 and 24 h % difference <2.0) | 0.3 | 0.5 | 0.8 |
| Solution stability 2–8 °C (0 and 24 h % difference <2.0) | 0.6 | 0.4 | 1.3 |
| Solution stability B.T (0 and 48 h % difference <2.0) | 1.3 | 0.5 | 1.6 |
| Solution stability 2–8 °C (0 and 48 h % difference <2.0) | 1.6 | 1.1 | 1.4 |

Table 3. Forced Degradation Data of Clindamycin

| Condition | % Degradation sample | Assay (% w/w) in degradation sample | Purity index | Single point threshold | Peak purity |
|-----------|----------------------|-----------------------------------|--------------|-----------------------|------------|
| Control sample (unstressed) | 1.15 | 98.8 | 1.000 | 0.999 | Passed |
| Acid degradation 1 M; HCl 120 min at @ 80 °C | 11.50 | 86.6 | 1.000 | 0.998 | Passed |
| Base degradation 1 M; NaOH 1 min at @ 80 °C | 16.50 | 82.5 | 1.000 | 0.996 | Passed |
| Peroxide Stress 0.5% H₂O₂ | 11.30 | 85.5 | 1.0000 | 0.9999 | Passed |
| Thermal Stress @ 80 °C 2 h | 13.50 | 85.6 | 1.000 | 0.999 | Passed |
| Photo Stress Fluorescent 1.2 M Lux and UV-200 W h⁻¹ (Sample in ampule) direct exposure | 1.29 | 100.9 | 1.000 | 0.999 | Passed |
volume was 30 μL, column temperature was 35 °C, and the detection was done at 240 nm. The total isocratic run was 20 min.

4.5. Analytical Method Validation. ICH guidelines Q2 (R1) and USP general chapter (1225) were followed in the validation of the current method. All the validation results were tabulated in Table 2.

4.5.1. Specificity. Specificity is the capability to quantify analyte characteristics in the presence of other components expected to subsist. The specificity of the method was verified by injecting the mobile phase, placebo, individual impurities of CMN, and standard and sample solutions in a PDA system. No optical interference was noticed with mobile phase, placebo, and impurity solutions. Peak purity was passed for three analytes, which expresses the specificity of the method.

4.5.2. Forced Degradation Study. The idea behind forced degradation is that a substance or product was subjected to different stress conditions before its degradation. In the industry context, a stability-indicating method is an analytical procedure that eliminates the interference of process impurities, excipients, and degradation products with the desired analyte. The current method’s stability-indicating nature was proven by conducting acid, base, peroxide, thermal, and light degradation studies.

Table 4. Method Ruggedness Analysis Data (F and T-test)

| Test         | Parameter                                         | Acceptance Criteria | EDTA  | BA    | CMN   |
|--------------|---------------------------------------------------|---------------------|-------|-------|-------|
| Method       | Precision                                         | Recovery at each level should be 98–102%  | 99.3  | 100.2 | 100.1 |
|              | n = 6 (6 determinations at 100% specification level) |                     | 100.2 | 100.1 | 100.6 |
|              |                                                   |                     | 99.4  | 100.2 | 100.3 |
|              |                                                   |                     | 100.2 | 100.1 | 99.8  |
|              |                                                   |                     | 99.6  | 101.2 | 100.1 |
|              |                                                   |                     | 101.1 | 100.7 | 101.6 |
| Mean         |                                                   |                     | 99.8  | 100.4 | 100.4 |
| RSD          |                                                   |                     | 0.4   | 0.4   | 0.6   |
| Intermediate | Precision                                         | Recovery at each level should be 98–102%  | 100.9 | 100.9 | 99.8  |
|              | n = 6 (6 determinations at 100% specification level) |                     | 100.2 | 99.6  | 100.8 |
|              |                                                   |                     | 100.9 | 100.1 | 98.9  |
|              |                                                   |                     | 99.6  | 98.7  | 100.1 |
| Mean         |                                                   |                     | 98.9  | 100.8 | 101.1 |
| RSD          |                                                   |                     | 100.2 | 99.6  | 100.3 |
| F-Test       | Degrees of freedom                                |                     | 5     | 5     | 5     |
| F-Value      |                                                   |                     | 0.8595| 0.2861| 0.5871|
| P-Value      |                                                   |                     | 0.4361| 0.0979| 0.2865|
| F-Critical   | One tail                                          |                     | 5.0503| 5.0503| 5.0503|
| T-Test       | Degrees of freedom                                |                     | 5     | 5     | 5     |
| T-Value      |                                                   |                     | −0.9363| 1.5349| 0.6459|
| P-Value One  | tail                                              |                     | 0.196 | 0.0927| 0.2734|
| T-Critical   | One tail                                          |                     | 2.015 | 2.015 | 2.015 |
| P-Value Two  | tail                                              |                     | 0.3921| 0.1854| 0.5468|
| T-Critical   | Two tail                                          |                     | 2.5706| 2.5706| 2.5706|

“Indicates reject null hypothesis, i.e., no statistical significance (differences) observed between the data performed on different days.

Figure 3. Precision chromatogram: 6 individual sample preparations from a homogeneous sample expressing the closeness of agreement between series of measurements.
that the formulation product was sensitive to all the conditions. The major degradants were CMN EP impurity E and CMN EP impurity F. These peaks clearly separated from the three analytes. All the requirements for peak purity were passed, and no interference was observed with degradant peaks. No interference was observed at the retention of EDTA, CMN, and BA. Forced degradation data were summarized in Table 3.

**4.5.3. Precision.**

4.5.3.1. **Method Precision.** Precision reflects how closely measurements of the same homogeneous sample taken under specified conditions agree across numerous measurements obtained from multiple samplings. The six replicate prepared samples from the same formulation batch were injected into HPLC, and the RSD% of 6 sample analyte assays was calculated. The analyte assay RSD% was agreeable, and the current method is precise. The chromatogram is shown in Figure 3.

4.5.3.2. **Intermediate Precision (after 4 days).** Variations within laboratories can be expressed by intermediate precision: different days, different analysts, or different equipment. Six test samples were prepared and injected into diverse HPLC columns on different days. In analytical procedures, precision is usually measured as variance, standard deviation, or the coefficient of variation. The RSD% of 6 samples of analyte assay was calculated and the results found to be within the limit.

The $F$ value and $F$ critical value for both the method precision and intermediate precision and the USP monograph method and current method (Method Precision data) were calculated, and we observed that the $F$ value < $F$ critical value. Similarly, the $T$ value and $T$ critical value for both the method precision and intermediate precision and the USP monograph method and current method (Method Precision data) were calculated, and we observed that the $T$ value < $T$ critical value (one tail and two tail). The statistical data reject the null hypothesis and indicate that the two different sets of data and the two different methods of data are not statistically significant. Table 4 and Table 5 show the $T$ and $F$ test data.

**4.5.4. Linearity.** Analytical procedures that are linear are those in which the response is directly proportional to the analyte concentration present in the sample. The linearity samples were prepared at 25%, 50%, 75%, 100%, 125%, and 150% levels of target concentration of three analytes and injected in triplicate to calculate the correlation coefficient value for three analytes. The correlation coefficient value was >0.999, and the method obeys the Beer–Lambert law. Hence the current process is linear. The chromatogram is shown in Figure 4.

**4.5.5. Accuracy.** In analytical procedures, trueness is how the value found accedes to the true conventional value or accepted reference value. The trueness of the method was

| Test Parameter | Acceptance Criteria | Results (Recovery (%), Precision (% RSD)) |
|----------------|---------------------|-----------------------------------------|
| USP Method     | Recovery at each level should be 98−102% | CMN 99.3 100.2 99.4 100.2 99.6 100.1 |
| Current Method | Recovery at each level should be 98−102% | 100.9 100.2 100.9 99.6 98.9 100.6 |
|                | Mean                | 99.8                                    |
|                | RSD                 | 0.4                                     |
| $F$-test       | Degrees of freedom | 5                                       |
| $F$-Value      | 0.8595              |
| $F$-Critical One tail | $F < F$ critical | 5.0503                                 |
| $T$-Test       | Degrees of freedom | 5                                       |
| $T$-Value      | -0.9363             |
| $T$-Critical One tail | $T < T$ critical | 2.015                                  |
| $P$-Value One tail | 0.196             |
| $P$-Value Two tail | 0.3921             |
| $T$-Critical Two tail | $T < T$ critical | 2.5706                                 |

"Indicates reject null hypothesis, i.e., no statistical significance (differences) observed between the current and USP method.

Figure 4. Linearity Chromatogram: EDTA linearity from 0.1 μg/mL to 0.8 μg/mL, BA linearity from 2.4 μg/mL to 14.2 μg/mL, and CMN linearity from 37.5 μg/mL to 225.0 μg/mL.
established by preparing the LOQ (LOQ of the three analytes was determined by injecting low concentrations and considering the ICH guideline S/N ratio method and determined the LOQ), 80%, 100%, and 120% level samples. For each level, four determinations of a total of nine samples are prepared by spiking the analytes at LOQ, 80%, 100%, and

Table 6. DoE Trails HPLC Runs and Results

| Std | Run | F1 | F2 | F3 | F4 | R1  | R2  | R3  | R4  | R5  | R6  | R7  | R8  | R9  |
|-----|-----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1   | 1   | 180| 4.3| 0.9| 30 | 15.852| 1.02| 14985| 9.885| 1.01| 18612| 4.018| 1.22| 8691|
| 2   | 2   | 180| 4.3| 0.9| 40 | 15.431| 1.11| 14961| 9.695| 1.02| 18672| 3.990| 1.29| 8591|
| 3   | 3   | 220| 4.3| 0.9| 40 | 10.785| 1.10| 14895| 7.861| 1.10| 18912| 3.109| 1.22| 8599|
| 4   | 4   | 220| 4.3| 1.2| 40 | 10.236| 1.10| 14695| 7.618| 1.09| 18961| 3.011| 1.22| 8609|
| 5   | 5   | 220| 4.3| 1.2| 30 | 10.432| 1.02| 14985| 9.654| 1.10| 18931| 3.128| 1.26| 8651|
| 6   | 6   | 200| 4.5| 1.05| 35 | 13.310| 1.01| 14795| 8.701| 1.08| 18662| 3.516| 1.22| 8656|
| 7   | 7   | 220| 4.7| 0.9| 30 | 10.985| 1.11| 14886| 7.951| 1.06| 18966| 3.210| 1.26| 8599|
| 8   | 8   | 220| 4.7| 0.9| 40 | 10.426| 1.11| 14785| 9.881| 1.08| 18625| 3.995| 1.28| 8596|
| 9   | 9   | 200| 4.5| 1.05| 35 | 13.310| 1.01| 14795| 8.701| 1.08| 18662| 3.516| 1.22| 8656|
| 10  | 10  | 220| 4.3| 1.2| 40 | 10.236| 1.10| 14869| 7.618| 1.09| 18931| 3.128| 1.26| 8651|
| 11  | 11  | 180| 4.7| 0.9| 40 | 15.426| 1.11| 14785| 9.881| 1.08| 18625| 3.995| 1.28| 8596|
| 12  | 12  | 180| 4.7| 0.9| 40 | 10.881| 1.17| 14889| 7.856| 1.07| 18967| 3.090| 1.22| 8597|
| 13  | 13  | 220| 4.7| 1.2| 40 | 10.231| 1.11| 14869| 7.610| 1.06| 18639| 3.010| 1.22| 8596|
| 14  | 14  | 200| 4.5| 1.05| 35 | 12.412| 1.02| 14795| 8.691| 1.08| 18639| 3.520| 1.21| 8601|

F1: Organic ACN mL, F2: pH, F3: Flow rate mL min⁻¹, and F4: Column temperature °C. R1: CMN retention time (Rt), R2: CMN Tailing factor (Tq), and R3: CMN Plate count (N). R4: BA retention time (Rt), R5: BA Tailing factor (Tq), and R6: BA Plate count (N). R7: EDTA retention time (Rt), R8: EDTA Tailing factor (Tq), and R8: EDTA Plate count (N).

Table 7. ANOVA Table for Response Factors of CMN, BA, and EDTA

| Source       | Sum of squares | df | Mean Square | F-value | p-value |
|--------------|----------------|----|-------------|---------|---------|
| R1 Model     | 16676.96       | 7  | 2382.42     | 6762.12 | <0.0001 |
| A-Organic ACN| 16551.60       | 1  | 16551.60    | 46979.06| <0.0001 |
| C-Flow rate  | 25.35          | 1  | 25.35       | 71.96   | <0.0001 |
| D-Column temperature | 25.35 | 1  | 25.35       | 71.96   | <0.0001 |
| AD           | 36.01          | 1  | 36.01       | 102.20  | <0.0001 |
| AC           | 1.48           | 1  | 1.48        | 4.20    | 0.0651  |
| CD           | 8.47           | 1  | 8.47        | 24.04   | 0.0005  |
| ACD          | 11.56          | 1  | 11.56       | 32.80   | 0.0001  |
| Residual     | 3.88           | 11 | 0.3523      |         |         |
| Lack of Fit  | 0.5985         | 9  | 0.0665      | 0.0406  | 0.9998  |
| Pure Error   | 3.28           | 2  | 1.64        |         |         |
| Cor total    | 16680.84       | 18 |             |         |         |
| R4 Model     | 15.88          | 2  | 7.94        | 2206.94 | <0.0001 |
| A-Organic ACN| 15.65          | 1  | 15.65       | 4349.40 | <0.0001 |
| C-Flow rate  | 0.2302         | 1  | 0.2302      | 63.98   | <0.0001 |
| Residual     | 0.0576         | 16 | 0.0036      |         |         |
| Lack of Fit  | 0.0511         | 14 | 0.0037      | 1.13    | 0.5650  |
| Pure Error   | 0.0065         | 2  | 0.0032      |         |         |
| Cor total    | 15.91          | 18 |             |         |         |
| R7 Model     | 3.07           | 7  | 0.4389      | 11222.39| <0.0001 |
| A-Organic ACN| 2.99           | 1  | 2.99        | 76408.12| <0.0001 |
| C-Flow rate  | 0.0696         | 1  | 0.0696      | 1778.53 | <0.0001 |
| D-Column temperature | 0.0056 | 1  | 0.0056      | 142.86  | <0.0001 |
| AD           | 0.0009         | 1  | 0.0009      | 21.87   | 0.0076  |
| AC           | 0.0013         | 1  | 0.0013      | 33.60   | 0.0001  |
| CD           | 0.0042         | 1  | 0.0042      | 107.19  | <0.0001 |
| ACD          | 0.0025         | 1  | 0.0025      | 64.56   | <0.0001 |
| Residual     | 0.0004         | 11 | 0.0000      |         |         |
| Lack of Fit  | 0.0003         | 9  | 0.0000      | 0.6165  | 0.7497  |
| Pure Error   | 0.0001         | 2  | 0.0001      |         |         |
| Cor total    | 3.07           | 18 |             |         |         |
120% levels into the placebo. The sample is injected into HPLC, with six replicates, and recoveries are found between 98.0% and 102.0% at target concentrations of 99.3% to 100.5% for EDTA, 99.3% to 100.8% for BA, and 99.1% to 100.3% for CMN. The results prove the accuracy of the method.

4.5.6. Robustness. A method’s robustness can be defined as its competency to preserve its performance while undergoing minuscule but deliberate variations in method parameters and can be habituated to determine its reliability. The current method’s robustness was verified by using the QbD concept with Design Expert software ver. 13. Critical quality attributes were identified as pH of the buffer, flow rate (mL/min), organic ratio (mL), and column compartment temperature (°C) changed to lower and higher levels by the suggestion of ICH Q2 (R1)\textsuperscript{26} and USP general chapter (621).\textsuperscript{28} Sample preparation, column, and wavelength variations did not affect the proposed method and conditions. As evidenced by the method’s precision and intermediate precision results, the instrument variation did not impact the protocol. Four factorials with two levels, including three center points without any blocks designed, were created, and 19 experimental runs were conducted, and results are tabulated in Table 6.

Three analytes’ retention time (R1, R4, and R7), tailing factor (R2, R5, and R8), and theoretical plates (R3, R6, and

Figure 5. (A) Half normal plot, Contour plot, and 3D plot of CMN Retention time. (B) Half normal plot, Contour plot, and 3D plot of BA Retention time. (C) Half normal plot, Contour plot, and 3D plot of EDTA Retention time.
R9) were monitored. For the statistical evaluation, the significant factors were selected and analyzed. Results visualize the variations in retention time and no impact on tailing and plate count are seen. Constructing the results of the 3D plot was rolled into the Design Expert software ver. 13. The ANOVA (analysis of variance) table (Table 7) exhibited the design significance and passed the model for R1, R4, and R7. Box-Cox diagnostics current lambda was equal to 1, recommending there be no data transformation. Organic ratio and flow rate changes showed significant changes in the retention times of the three analytes, but pH and column temperature changes are not impacting the chromatography. Organic ratio and flow rate changes showed variations, but variations met the system suitability criteria and concluded that the method is robust. Figure 5 shows the half-normal, 2D contour, and 3D surface plots.

4.5.7. Analytical Solution Stability. Establishment of analytical solution stability is crucial for this method because EDTA chromophore activation is the reason the derivatizing agent (Copper II acetate) added to the solution. This study shows the derivatizing agent’s impact on other analytes. To confirm the solution stability under benchtop and refrigerator conditions, the prepared standard and sample solutions were injected to HPLC at 0, 24, and 48 h as per the method. The difference between the analyte assays in all time points was <2.0%. At 0 h, the values were 1.3%, 0.5%, and 1.6% for EDTA, BA, and CMN, respectively, on the benchtop, and 1.6%, 1.1%, and 1.4% for EDTA, BA, and CMN, respectively, at 2–8 °C. Standard and sample solutions were stable for 48 h on benchtop and refrigerator conditions.

5. METHOD FOR GREENNESS ASSESSMENT

Green chemistry is the design of chemical products and processes that minimize or eliminate hazardous substances’ utilization or generation. The greenness of the current method was assessed by utilizing the analytical eco-score tool, calculating the penalty points. The eco-score value of 80 is in the range of excellent greenness. The penalty points table is tabulated in Table 8.

Other tools like NEMI and an advanced tool, GAPI, were used to express the method greenness in the pictogram (Figure 6). AGREE is a modern tool that evaluates all 12 green principles using appropriate software. The AGREE circle is divided into 12 parts; each part describes one green principle, and the estimated AGREE value of the current method is 0.68 (Figure 6). All four tools signify the current method is green and eco-friendly.

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Table 8. Penalty Points Table for the Current Method

| Sample No | Reagents/Instruments          | Penalty points |
|-----------|-------------------------------|----------------|
| 1         | Acetate buffer                | 0              |
| 2         | Acetonitrile                  | 4              |
| 3         | Copper acetate                | 1              |
| 4         | Acetic acid                   | 2              |
| 5         | Tetra butyl ammonium hydroxide 10% in water | 3 |
| 6         | HPLC                          | 1              |
| 7         | Occupational hazard           | 0              |
| 8         | Waste                         | 5              |
| 9         | Total penalty points          | 16             |
| 10        | Analytical Eco-Scale          | 84             |

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Figure 6. NEMI, GAPI, and AGREE pictograms of the current method. In NEMI, GAPI and AGREE are marked green, yellow, or red depending on the impact on the environment. Waste disposal was not discussed; hence waste was not addressed in NEMI.
Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED
ANOVA, Analysis of Variance; DoE, Design of Experiments; GAPI, Green Analytical Procedure Index; NEMI, National Environmental Method’s Index; QbD, Quality by design; AGREE, Analytical GREENess

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