Natural Resources for Human Health
eISSN: 2583-1194

Original Research

Received: 02 August 2026
Revised: 06 October 2026
Accepted : 22 October 2026

Natural Resources for Human Health 2026, Vol. 6 (6): 436–448

KEYWORDS:
Cefazolin sodium; contamination control; liquid chromatography; Karl Fischer titration; water for injections; sterile products.

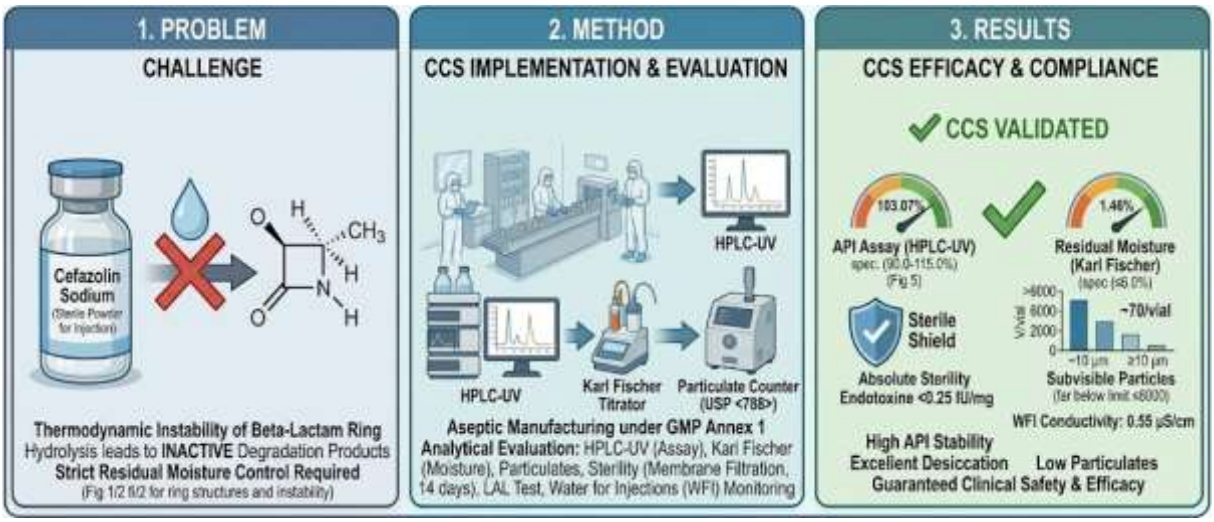
Physicochemical and Microbiological Evaluation of Cefazolin Sodium under a Contamination Control Strategy

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ABSTRACT: Sterile injectable antibiotics such as cefazolin sodium require strict control of moisture, particles, sterility and process water, an expectation reinforced by the 2022 revision of European good manufacturing practice Annex 1, which requires a contamination control strategy. This study assessed the physicochemical and microbiological quality of a commercial sterile cefazolin sodium powder for injection (Mirazolin® 1 g) and of the purified water and water for injections of the manufacturing site. Analyses were performed in the manufacturer's quality control laboratory following European Pharmacopoeia and United States Pharmacopeia procedures. Cefazolin was assayed by isocratic liquid chromatography with ultraviolet detection at 254 nm using salicylic acid as internal standard. Residual water (Karl Fischer titration), pH, identity, subvisible particles (light obscuration), mass uniformity (20 vials), sterility (membrane filtration, 14 days) and bacterial endotoxins (gel-clot, lysate sensitivity 0.125 EU/mL) were determined, together with conductivity, nitrates, oxidizable substances and microbial counts in the waters. Cefazolin content was 103.07% of the labelled amount (specification 90.0-115.0%), residual water 1.46% (≤6.0%) and pH 5.0. Mean particle counts were 70.25 (≥10 µm) and 5.25 (≥25 µm) per vial, and the maximum mass deviation was -9.57%. No microbial growth or gel formation occurred (endotoxins <0.25 EU/mg). Water for injections showed a conductivity of 0.55 µS/cm and 0 CFU/100 mL. For the batch examined, all measured attributes met the applied specifications. Such release-type results are consistent with good control of moisture, particles and water quality but cannot, by themselves, demonstrate the effectiveness of a complete contamination control strategy.

Natural Resources for Human Health
eISSN: 2583-1194



Highlights

- Strict moisture control (1.46%) ensures cefazolin thermodynamic stability.
- Validated HPLC-UV analysis confirms optimal active ingredient assay at 103.07%.
- Minimal subvisible particulates eliminate clinical vascular micro-embolism risks.
- High-purity process water (0.55 µS/cm) ensures total aseptic clinical safety.

1. INTRODUCTION

Injectable medicines bypass the natural barriers of the body, so that microbial, endotoxin or particulate contamination reaches the circulation directly. The resulting hazards include systemic sepsis, pyrogenic reactions and vascular occlusion by particles (Sandle, 2016; Aulton and Taylor, 2022). The quality of sterile injectable products therefore depends on strict control of both the product and the environment in which it is made.

Cefazolin sodium ($C_{14}H_{13}N_8NaO_4S_3$; 476.5 g/mol; **Figure 1**) is a first-generation cephalosporin with time-dependent bactericidal activity. It inhibits the synthesis of bacterial cell-wall peptidoglycan by binding to penicillin-binding proteins (Brunton and Knollmann, 2023). It is a semi-synthetic product derived from a fermentation product (European Directorate for the Quality of Medicines & HealthCare, 2023).

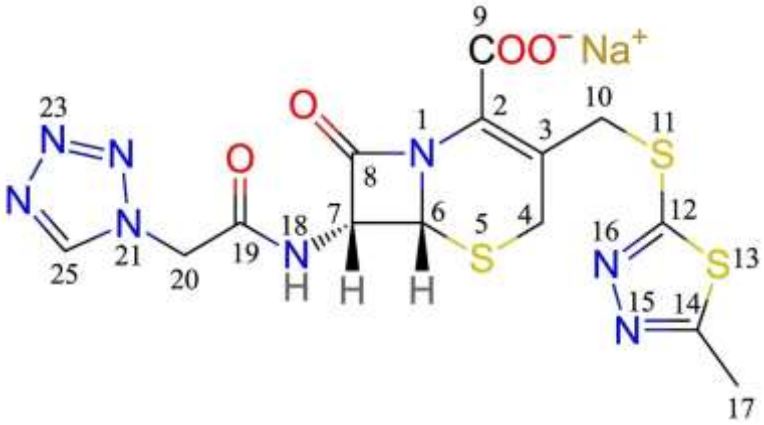


Figure 1. The detailed chemical structure of cefazolin sodium, showing the beta-lactam and dihydrothiazine rings

Its activity depends on the integrity of the β-lactam ring, a strained four-membered cyclic amide. In water, the electrophilic carbonyl carbon of this ring is attacked by water, which opens the ring through a tetrahedral intermediate and gives inactive products (**Figure 2**). One of these ring-opened products, cefazoloic acid, is controlled as an impurity in the European Pharmacopoeia (European Directorate for the Quality of Medicines & HealthCare, 2023).

Since hydrolysis requires water, cefazolin sodium is supplied as a sterile powder for injection. Water content, however, is not a single quantity for this substance: hydrated, amorphous and dehydrated solid forms take up moisture differently as relative humidity changes (Osawa et al., 1988). The published analytical literature on cefazolin sodium has largely concerned the development and validation of stability-indicating liquid chromatography methods, with forced degradation under hydrolytic, oxidative and thermal stress (Hasan et al., 2013). Published release-testing and quality-control studies of cefazolin sodium powders have principally reported assay or potency results close to the labelled strength; for example, a validated turbidimetric microbiological assay applied to lyophilised cefazolin sodium for injection showed accuracy of 99.92% and was proposed as a rapid, low-cost alternative for routine quality control (Pedroso and Salgado, 2014). Pharmacopoeial release specifications for sterile cefazolin sodium powder further require a water content not greater than 6.0% w/w, bacterial endotoxins not more than 0.15 USP EU mg^{-1}, and compliance with the particulate-matter requirements applicable to small-volume injections (United States Pharmacopeial Convention, 2019).

In Europe, the revised Annex 1 of the Good Manufacturing Practice guidelines was published in 2022 and has applied since August 2023 (European Commission, 2022). It requires manufacturers of sterile products to document a Contamination Control Strategy, a facility-wide approach to preventing microbial, particulate and pyrogen contamination built on quality risk management (European Commission, 2022). Utilities are part of this strategy. Purified water and water for injections are critical utilities, and Annex 1 addresses the production of water for injections, including by reverse osmosis, and the minimisation of biofilm (European Commission, 2022).

This work is consistent with our laboratory's sustained efforts in pharmaceutical quality assurance, the evaluation of bioactive compounds, and analytical rigor. These efforts have encompassed the chromatographic quantification of active molecules and antioxidant capacity assessment in complex matrices (Aliouche et al., 2007, 2022), the structural elucidation of bioactive constituents (Boudjerda et al., 2008; López-Rodríguez et al., 2009; Hamimed et al., 2018; Mokhtari et al., 2020), as well as the antimicrobial screening and biological evaluation of medicinal plant extracts (Zater et al., 2014, 2016, 2021), together with studies on botanical distribution (Zater et al., 2019a, 2019b). Building on this analytical expertise, the present study extends these principles to parenteral drug manufacturing. It highlights the essential role of high-performance liquid chromatography and stringent contamination-control strategies in preserving the structural integrity and clinical safety of sensitive anti-infective agents, particularly cefazolin sodium.

Release testing of a finished powder and monitoring of the waters used at the same site are normally reported separately. Whether such data, taken together, can support statements about contamination control is seldom examined explicitly. This study therefore asked whether the physicochemical and microbiological quality attributes of a commercial sterile cefazolin sodium powder for injection, and of the purified water and water for injections of its manufacturing site, meet pharmacopoeial criteria, and what such release-type data can and cannot tell us about the effectiveness of a contamination control strategy.

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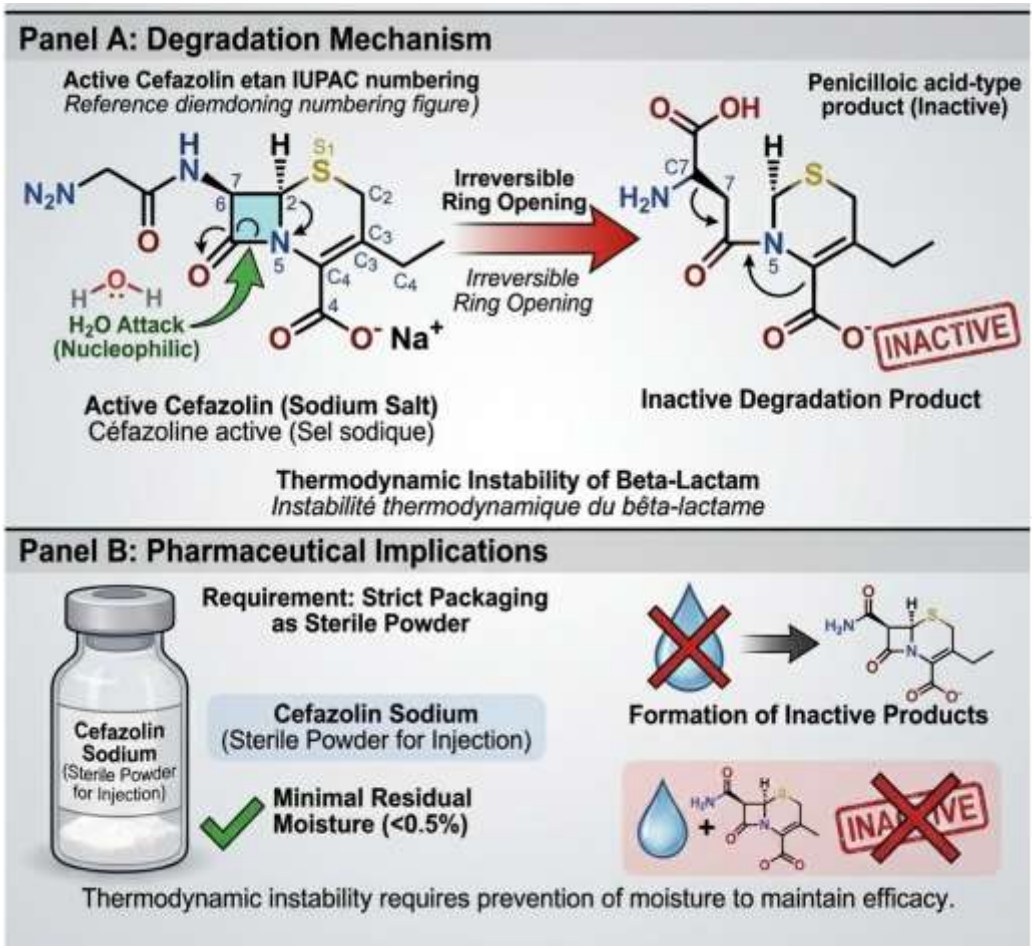


Figure 2. Hydrolytic degradation mechanism of the cefazolin beta-lactam ring. The nucleophilic attack leads to the irreversible opening of the ring. This thermodynamic instability necessitates strict packaging as a sterile powder and the maintenance of minimal residual moisture to prevent the formation of inactive degradation products (Aulton and Taylor, 2022).

2. MATERIALS AND METHODS

2.1. Study Setting and Analyzed Matrices

The experimental protocols were conducted within the quality control laboratories of the IMGSA Pharma industrial complex (Aïn M'lila, Algeria), a facility specializing in sterile parenteral antibiotics. The geographical location of the industrial complex is illustrated in **Figure 3**. The analytical investigation evaluated three interdependent matrices: the finished product (Mirazolin® 1 g), Purified Water (PW), and Water for Injections (WFI). All procedures strictly complied with the monographs of the European Pharmacopoeia (11th Ed.) (European Directorate for the Quality of Medicines & HealthCare, 2023) and the United States Pharmacopeia (USP 43-NF 38) (United States Pharmacopeial Convention, 2020). Reference standards included Cefazolin CRS and salicylic acid ($C_7H_6O_3$), utilized as an internal standard.

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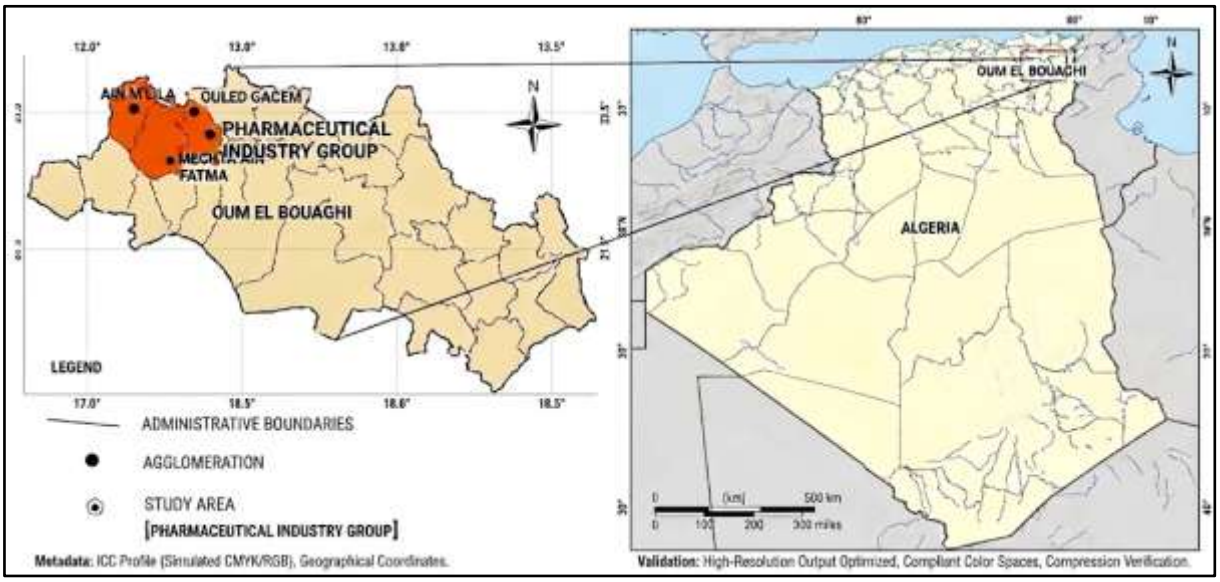


Figure 3: Geographical Location of the IMGSA Unit in Aïn M'lila

2.2. Reagents and reference standards

Cefazolin CRS (USP reference standard) and salicylic acid ($C_7H_6O_3$), used as the internal standard, were employed for the quantitative analysis of cefazolin sodium. Methanol and acetonitrile were of high-performance liquid chromatography (HPLC) grade. Karl Fischer titrations were performed using a standardised HYDRANAL® reagent.

For the bacterial endotoxin test, limulus amebocyte lysate (LAL) reagent with a labelled sensitivity (λ) of 0.125 EU mL^{-1} was used. Control standard endotoxin (CSE) and LAL reagent water (LRW) were used for the preparation of endotoxin standards and sample dilutions, respectively. Sterility testing was performed using fluid thioglycollate medium (FTM) and tryptic soy broth (TSB). Prior to use, the culture media were verified for their growth-promoting properties in accordance with the applicable pharmacopoeial requirements.

2.3. Physicochemical analysis

2.3.1. Cefazolin assay by HPLC-UV

Cefazolin was assayed using a Shimadzu LC-2050C system equipped with a diode-array UV-visible detector. Separation was achieved on a reversed-phase L1 (C18) column (30 cm × 4.0 mm, 10 µm particle size) using an isocratic mobile phase consisting of acetonitrile and an acidic buffer (pH 3.6) in a 10:90 (v/v) ratio. The flow rate was maintained at 2.0 mL min^{-1} at 25 °C. The injection volume was 10 µL, and detection was performed at 254 nm. Salicylic acid served as the internal standard.

System suitability was confirmed using replicate injections of the standard solution, which yielded a relative standard deviation (%RSD) of 0.933% for the cefazolin peak. Baseline resolution was obtained between cefazolin (retention time ≈ 3.35 min) and the internal standard (retention time ≈ 6.00 min). The cefazolin content was calculated from the ratio of the peak areas of cefazolin and the internal standard in both the sample and reference standard solutions.

2.3.2. Residual water, pH and identity

Residual water was determined by volumetric Karl Fischer titration using a ZSD-2 titrator equipped with a double platinum electrode. An accurately weighed amount of powder (300.2 mg) was dissolved in 30 mL of methanol and titrated to a defined end-point signal of 60 µA. The titration consumed 2.25 mL of reagent, with a titre factor of 1.95.

The pH was measured potentiometrically using an INESA pH meter in a reconstituted solution prepared by dissolving 1 g of cefazolin powder in 10 mL of purified water. Sodium identity was confirmed chemically by precipitation with potassium pyroantimonate ($K_2H_2Sb_2O_7$), which produced a characteristic white crystalline complex. The identity of cefazolin was further confirmed by comparing the ultraviolet–visible absorption spectrum of the reconstituted sample solution with that of

a reference standard solution prepared in the same manner. Both spectra showed complete overlap, with absorption maxima at 223 and 271 nm.

2.3.3. Subvisible particles and mass uniformity

Subvisible particulate matter of 10 μm or greater and 25 μm or greater was quantified by the light-obscuration method using a TAN particle counter, in accordance with the requirements of USP General Chapter <788>. Pooled, ultrasonically degassed sample solutions were used for the analysis.

For mass uniformity, 20 randomly selected units were weighed full and then empty. The individual deviation from the mean was calculated as follows: **$[(x_i - \bar{x}) / \bar{x}] \times 100$.**

$$\text{Deviation (\%)} = \frac{(x_i - \bar{x})}{\bar{x}} \times 100$$

where x_i is the individual net mass and $\bar{x}$ is the mean net mass of the 20 units.

2.4. Microbiological analysis and water utilities

Sterility testing was performed under ISO 5 laminar-flow conditions. Samples from 10 reconstituted vials were filtered through 0.45 μm membranes and incubated in FTM at 32 ± 2.5 °C and in TSB at 22 ± 2.5 °C for 14 days. Positive and negative controls were included to verify the growth-promoting capacity of the media and the sterility of the test environment, respectively.

Pyrogenicity was evaluated using the gel-clot LAL assay. Serial dilutions of the samples (1/1, 1/10 and 1/100) were incubated at 37 °C for 60 min in a water bath, together with positive and negative controls.

Pharmaceutical water utilities, including purified water (PW) and water for injection (WFI), were characterised by conductivity measurement at 25 °C using an INESA conductivity meter and by pH measurement. Colorimetric limit tests were performed for nitrates (limit: 0.2 ppm) and oxidisable substances, the latter based on potassium permanganate reduction.

The total aerobic microbial count (TAMC) was determined by aseptic filtration of 1 L of PW and 200 mL of WFI through 0.45 μm membranes, followed by incubation on R2A and TSA agars at 32.5 °C for 5 days. WFI was additionally tested for bacterial endotoxins using the LAL method and was confirmed to be negative.

2.5. Statistical analysis

Unless otherwise stated, analytical determinations were performed in quintuplicate (n = 5). For mass uniformity, 20 units were analysed (n = 20). Results are expressed as the mean ± standard deviation (SD). The relative standard deviation (RSD) was calculated to assess the precision of the analytical methods and the variability of the results; for example, the %RSD obtained for system suitability was 0.933%.

3. RESULTS

3.1. Physicochemical parameters of Mirazolin® 1 g

Visual inspection showed that the finished product was a uniform, white to off-white crystalline powder, free from visible agglomerates. Mass uniformity testing of 20 vials gave a mean mass of 1.0228 g. Individual deviations ranged from -9.57% (unit 18; net mass, 0.9252 g) to +5.46% (unit 13; net mass, 1.0787 g). Eighteen of the 20 units were within the pharmacopoeial ±5% specification; the batch was therefore considered compliant with the predefined acceptance criterion.

The pH of the reconstituted solution was 5.0, within the specified range of 4.0-6.0. Volumetric Karl Fischer titration of a 300.2 mg sample consumed 2.25 mL of reagent, with a titre factor of 1.95, corresponding to a residual water content of 1.46%, below the specified limit of 6.0% **(Figure 5)**. Sodium identity was confirmed chemically, forming a characteristic white crystalline complex upon precipitation with potassium pyroantimonate **(Figure S1, Supplementary Material).**

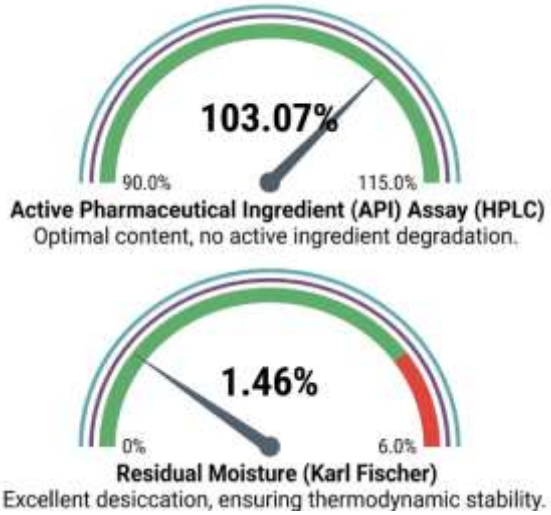


Figure 5. Determination of active pharmaceutical ingredient content by HPLC and residual moisture by Karl Fischer titration. *Theoretical acceptance limits are represented by the lower and upper bounds of each gauge (90.0-115.0% for the API; 0-6.0% for moisture). HPLC: High-Performance Liquid Chromatography. API: Active Pharmaceutical Ingredient.*

Subvisible particulate counting gave mean levels of 70.25 particles per vial for particles ≥10 µm and 5.25 particles per vial for particles ≥25 µm, below the corresponding limits of 6000 and 600 particles per vial, respectively. UV-visible spectrophotometry was used as an orthogonal identity test. The absorption spectrum of the sample overlapped with that of the reference standard, with absorption maxima at 223 and 271 nm **(Figure 6)**.

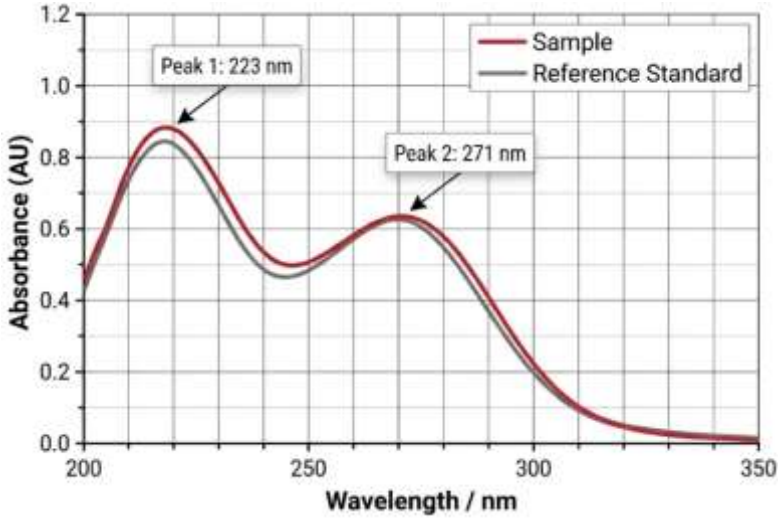


Figure 6. Comparative UV–Vis overlay spectrum confirming the identity of cefazolin sodium.

HPLC–UV analysis showed separation of cefazolin and the salicylic acid internal standard, with retention times of approximately 3.35 min and 6.00 min, respectively **(Figures 7 and 8)**.

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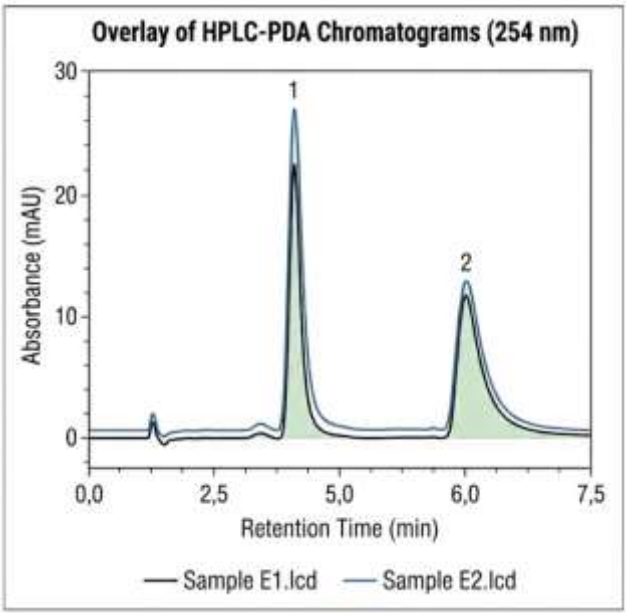


Figure 7. HPLC-PDA chromatograms of samples E1 and E2. Separation of cefazolin (1, Rt ≈ 3.35 min) and compound S.A. (2, Rt ≈ 6.00 min). Conditions: injection volume, 10 µL; detection, 254 nm.

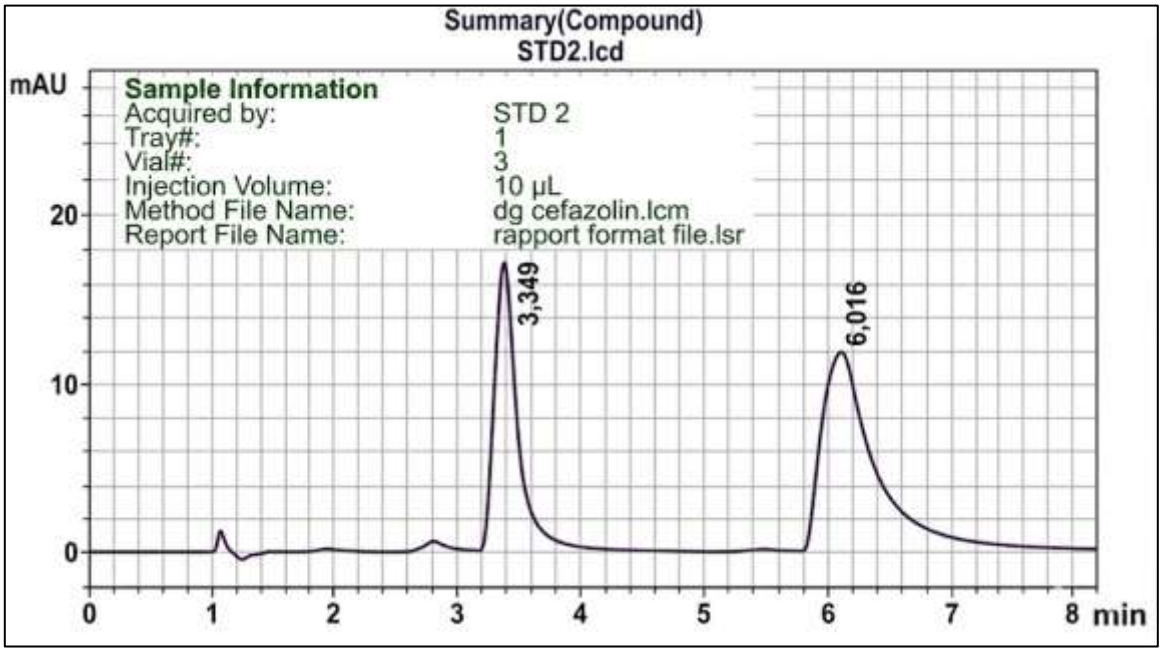


Figure 8. Chromatographic profile of the test solution by HPLC-UV at 254 nm. The principal peak corresponds to cefazolin (Rt ≈ 3.35 min) and is baseline-resolved from the salicylic acid internal standard peak (Rt ≈ 6.00 min).

The RSD for five consecutive injections of the standard solution was 0.933% for cefazolin, indicating acceptable system suitability and repeatability **(Figure 9)**. Under these chromatographic conditions, the assay of the active pharmaceutical ingredient was 103.07%, within the specified range of 90.0-115.0%.

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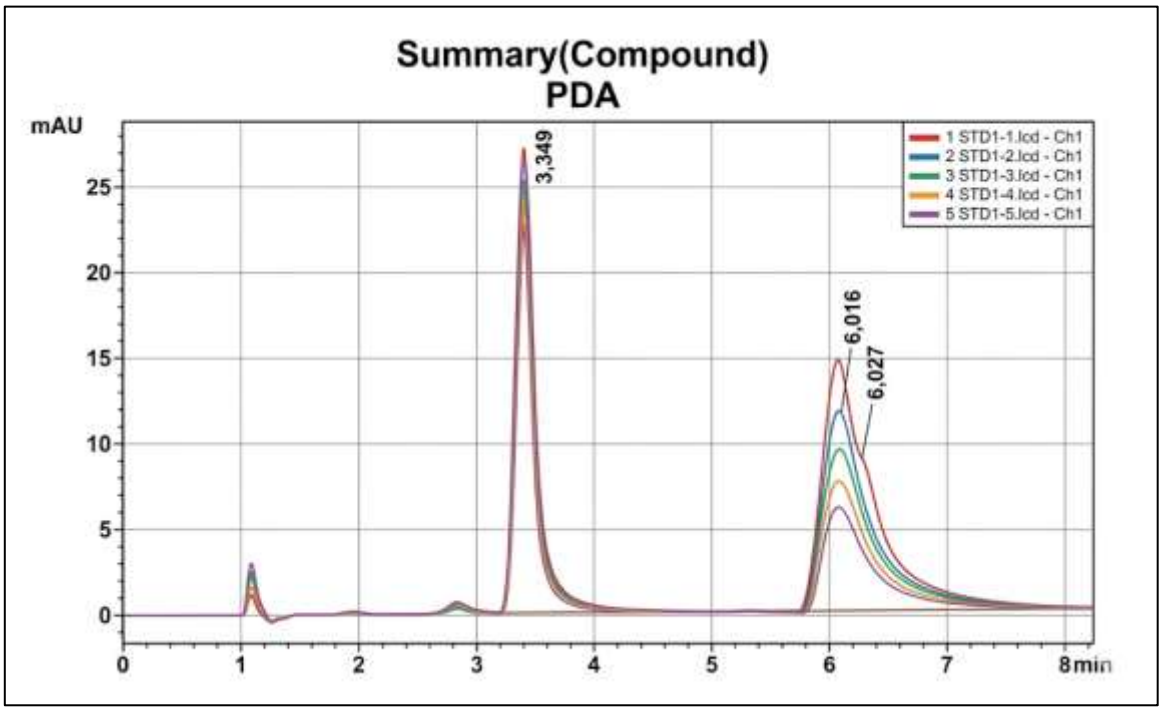


Figure 9. Overlay of HPLC-PDA chromatograms obtained from five consecutive injections of the standard solution at 254 nm. The overlay shows repeatability and stable retention times for cefazolin and the internal standard.

A summary of the physicochemical evaluation is presented in Table 1.

Table 1. Physicochemical parameters of Mirazolin® 1 g.

Analytical Parameter	Method	Specification (USP/Ph. Eur.)	Result (Mean)	Compliance
API Assay	HPLC-UV (254 nm)	90.0%–115.0%	103.07%	Compliant
Residual Moisture	Karl Fischer Titration	≤6.0%	1.46%	Compliant
pH (1 g/10 mL)	Potentiometry	4.0–6.0	5.0	Compliant
Particles (≥10 µm)	Light obscuration	≤6000/vial	70.25/vial	Compliant
Particles (≥25 µm)	Light obscuration	≤600/vial	5.25/vial	Compliant
Mass Uniformity	Gravimetry (n = 20)	Max 2 units >5%, 0 >10%	Max dev.: −9.57%	Compliant

3.2. Microbiological safety and critical utilities

The 14-day membrane-filtration sterility test showed no microbial growth or turbidity in either FTM or TSB. Positive and negative controls confirmed the growth-promoting capacity of the media and the sterility of the test environment, respectively.

The gel-clot LAL test was negative for all sample dilutions tested (1/1, 1/10 and 1/100). Positive controls reacted as expected, whereas no gel formation was observed in the sample dilutions. These results indicate that the bacterial endotoxin content was below the detection limit of the assay and compliant with the specified limit of <0.25 IU mg^{-1}.

Analysis of the critical water utilities gave conductivity values of 0.55 µS cm⁻¹ for WFI and 0.82 µS cm⁻¹ for PW at 25 °C. Both water types tested negative for nitrates, with no blue coloration observed, and for oxidisable substances, with persistence of the pink permanganate colour. The TAMC of WFI was 0 CFU per 100 mL, and WFI tested negative for bacterial endotoxins by the LAL method. The microbiological results are summarised in Table 2.

Table 2. Microbiological control of the finished product and critical utilities.

Matrix	Test	Acceptance Limit	Observed Result	Compliance
Mirazolin® 1 g	Sterility (14 days)	No growth	No turbidity	Compliant
Mirazolin® 1 g	Endotoxins (LAL)	<0.25 IU/mg	No gelation	Compliant
WFI	Conductivity (25 °C)	≤1.3 µS/cm	0.55 µS/cm	Compliant
WFI	TAMC	≤10 CFU/100 mL	0 CFU/100 mL	Compliant
PW	Conductivity (25 °C)	≤5.1 µS/cm	0.82 µS/cm	Compliant

4. DISCUSSION

The results obtained for Mirazolin® 1 g were compliant with the relevant requirements of the European Pharmacopoeia and the USP monograph for cefazolin sodium (European Directorate for the Quality of Medicines & HealthCare, 2023; United States Pharmacopeial Convention, 2020). Taken together, the physicochemical and microbiological data support the suitability of the analysed batch for parenteral use and reflect effective control of the critical quality attributes of the product.

Cefazolin sodium is particularly sensitive to hydrolysis because of the strained β-lactam ring. Nucleophilic attack by water can open this ring and lead to the formation of inactive cephalosporoic acid derivatives (Brunton et al., 2023). In this context, the residual water content of 1.46%, well below the compendial limit of 6.0%, is an important quality attribute. A low moisture content reduces the amount of water available for hydrolytic degradation and therefore contributes to preserving the integrity of the active substance during storage. The HPLC-UV assay result of 103.07%, within the 90.0-115.0% specification, further supports the conclusion that the declared content of cefazolin was maintained in the analysed batch.

The mass uniformity data also merit attention from a process-control perspective. Although the batch met the predefined acceptance criterion, one unit showed a deviation of -9.57%. This result remains within the permitted limits but indicates a degree of variability in the filling operation. In an aseptic powder-filling process, such variability may arise from the behaviour of the powder, the performance of the dosing system or environmental conditions. Within a Quality by Design approach, monitoring this parameter and investigating the causes of individual deviations can support continued improvement of process capability (International Council for Harmonisation, 2023; Aulton and Taylor, 2022).

The subvisible particulate results were substantially below the pharmacopoeial limits, with mean values of 70.25 particles per vial for particles ≥10 µm and 5.25 particles per vial for particles ≥25 µm. Control of particulate contamination is clinically relevant because particles administered intravenously may contribute to vascular irritation, phlebitis or, in certain circumstances, microvascular obstruction (Sandle, 2016). The observed results therefore support the effectiveness of the filtration, filling and environmental controls applied during manufacture.

The quality of the water used in the facility is closely linked to the microbiological and endotoxin quality of sterile products (Baird et al., 2000). In this study, WFI showed a conductivity of 0.55 µS cm⁻¹, a TAMC of 0 CFU per 100 mL and a negative LAL result. These findings are consistent with effective purification and monitoring of the water system. Similarly, the negative sterility and endotoxin results obtained for the finished product indicate that the aseptic process and the associated controls maintained the microbiological quality of the product at the time of testing.

These results are consistent with the principles of the Contamination Control Strategy required by the revised GMP Annex 1, which emphasises a facility-wide, risk-based approach to controlling microbial, particulate and pyrogenic contamination (European Commission, 2022). Nevertheless, the present data represent a release-testing evaluation of one batch and its

associated water utilities. They demonstrate compliance at the time of analysis but do not, on their own, establish the long-term performance of the contamination control system or the full capability of the manufacturing process.

4.1. LIMITATIONS AND FUTURE PERSPECTIVES

Several limitations should be acknowledged. The analytical evaluation relied mainly on conventional HPLC-UV methods, which are validated and compendial but may provide limited sensitivity and resolution for trace degradation products compared with more recent chromatographic approaches. Future work could include the use of ultra-performance liquid chromatography coupled with photodiode-array or mass-spectrometric detection to obtain more detailed impurity and degradation profiles.

From a microbiological perspective, the sterility test requires a 14-day incubation period, which delays the availability of release results. The implementation of rapid microbiological methods, such as ATP bioluminescence or solid-phase cytometry, could provide earlier information on microbial contamination. However, such methods would require appropriate validation and regulatory consideration before being used as alternatives to compendial sterility testing.

Finally, extending the evaluation to several batches, longer storage periods and additional water-system sampling points would strengthen the assessment of process capability and provide a more comprehensive picture of the performance of the contamination control strategy.

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Natural Resources for Human Health
eISSN: 2583-1194

SUPPLEMENTARY MATERIAL



Figure S1. Visual appearance of the crystalline precipitate obtained during the sodium identification test for Mirazolin® 1 g. Sodium identity is confirmed by the formation of a white crystalline precipitate upon reaction with potassium pyroantimonate ($K_2H_2Sb_2O_7$).