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Predictive analytical workflow for rapid structure elucidation and *in silico* toxicological qualification of an unidentified impurity in cefixime granules for oral suspension

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ABSTRACT

During in-use stability testing of cefixime granules for oral suspension, an impurity with a relative retention time of 0.19 was consistently detected and increased during storage. To ensure regulatory compliance and patient safety, the impurity was structurally identified and toxicologically qualified. A laboratory-scale formulation and commercial products were studied under refrigerated and ambient conditions. The impurity was isolated by automated fraction collection and characterised by liquid chromatography-mass spectrometry and tandem mass spectrometry. Kinetic evaluation showed pseudo-first-order formation, with faster accumulation at ambient temperature. The impurity was identified as a γ-lactone degradation product of cefixime, present as multiple stereoisomers stabilised under acidic conditions. *In silico* toxicological assessment using complementary platforms indicated no additional structural alerts, no mutagenic potential, and negligible acute toxicity. The impurity forms only after prolonged storage of reconstituted suspensions and is classified as an ICH M7 Class 5 impurity, requiring no further genotoxicity testing. The applied analytical-computational workflow provides an efficient approach for impurity qualification in β-lactam antibiotics.

Keywords: cefixime, degradation impurity, in-use stability, LC/MS, *in silico* toxicology, γ-lactone

INTRODUCTION

Cefixime is an oral, semisynthetic, third-generation cephalosporin with a broad antibacterial spectrum. Unlike other antibiotics, cefixime possesses a vinyl group at the C-3 position and a (*Z*)-2-(2-amino-4-thiazolyl)-2-(carboxymethoxyimino) acetyl moiety at the C-7 position (Fig. 1), both of which contribute to its enhanced activity against Gram-negative bacteria and favorable pharmacokinetic properties (1, 2).

The presence of impurities in pharmaceutical products necessitates their identification and quantitative determination in order to assess the efficacy and stability of the products, which are essential parameters of quality and safety (3–5). Insufficient stability

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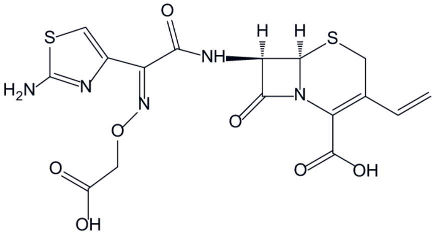

Fig. 1. Molecular structure of cefixime.

of a drug product may lead to changes in physical properties (*e.g.*, hardness, dissolution rate, phase separation) as well as in chemical properties (*e.g.*, formation of potentially toxic degradation products) (6, 7). Cefixime is known to degrade under certain storage conditions (temperature and relative humidity), and its degradation products may cause undesirable side effects in patients (8, 9).

During stability testing of the finished product, an impurity with a relative retention time (RRT) of 0.19 was detected using a previously validated high-performance liquid chromatographic (HPLC) method. In the course of in-use stability testing (10), this impurity showed a progressive increase, reaching levels above the ICH identification threshold of 0.2 % (11, 12) after 14 days from reconstitution of the granules in water, which corresponds to the maximum in-use period recommended in the patient information leaflet (PIL) for cefixime oral suspensions.

The qualification of degradation products in pharmaceutical drug products is a critical step in ensuring patient safety, especially when such impurities exceed the identification threshold specified by regulatory guidelines (*e.g.*, ICH Q3A/B, ICH M7). The impurity with a RRT of 0.19, identified as a γ-lactone derivative of cefixime, was evaluated using complementary *in silico* methodologies to assess its potential mutagenic and acute toxicological risks. This approach aligns with the ICH M7 principle that the absence of structural alerts in both expert rule-based and statistical QSAR systems is sufficient to conclude that an impurity is of no mutagenic concern, thereby eliminating the need for experimental mutagenicity testing. According to ICH M7 (13) and related regulatory guidelines (14–19), the qualification of impurities involves the acquisition and evaluation of toxicological data to ensure that degradation products present at or above specified thresholds are biologically safe. For impurities with potential genotoxic concern, *in silico* methodologies provide an accepted non-testing alternative for initial hazard assessment, particularly in line with REACH principles for minimising animal testing. The absence of structural alerts from two complementary (Q)SAR approaches, one expert rule-based and one statistical, is considered sufficient evidence to conclude that the impurity is of no mutagenic concern, negating the need for further genotoxicity testing. Moreover, if a structural alert present in an impurity is identical to one in the parent drug or a structurally related compound with a negative bacterial mutagenicity result, the impurity may also be qualified as non-mutagenic.

For structural elucidation, a sufficient quantity of the impurity was isolated and analysed using mass spectrometry. The aim of this study was to identify the unknown degradation product and to investigate the mechanism of cefixime degradation in aqueous solution through kinetic studies and degradation product analysis. Accordingly, this work integrates analytical characterisation and toxicological qualification of the impurity with an RRT of 0.19, with the objective of providing a comprehensive assessment of its structural identity, formation pathway, and potential clinical relevance.

EXPERIMENTAL

Chemicals and reagents

Cefixime trihydrate reference standard was obtained from the European Pharmacopoeia (Ph. Eur.). Acetonitrile and methanol (HPLC grade) were purchased from Merck (Germany), while tetrabutylammonium hydroxide 30-hydrate was obtained from Sigma-Aldrich (USA). Ultrapure water was generated using a Milli-Q system (Millipore, USA). All other chemicals were of analytical grade.

Instrumentation and chromatographic conditions

The chromatographic analysis was performed on a validated HPLC system in accordance with ICH Q3B (R2) and EMA guidelines for setting specifications for related impurities in antibiotics (11, 12). Separation was achieved using a C18 column (250 × 4.6 mm, 5 µm) under isocratic elution with a mobile phase consisting of acetonitrile and tetrabutylammonium hydroxide buffer pH 6.5 in a ratio 23:77 (*V*/*V*) (20, 21). The flow rate was set at 1.0 mL min^{-1}, and detection was performed at 254 nm. The injection volume was 20 µL, and the column temperature was maintained at 25 °C.

Preparation of standard and test solutions

A standard solution containing 0.01 mg mL^{-1} cefixime was prepared in the mobile phase (acetonitrile/buffer pH 6.5, 23:77, *V*/*V*), while the test solution contained 1 mg mL^{-1} cefixime. Solutions were freshly prepared and filtered through 0.45 µm membrane filters prior to injection (20, 21).

Stability in-use study

The continued integrity of pharmaceutical products in multidose containers after first opening is a critical quality attribute. For this reason, an in-use stability study was conducted for cefixime granules for oral suspension to simulate real-world conditions, considering the container filling volume and the dosing regimen. The study design included both the test drug product, consisting of a candidate formulation manufactured at laboratory scale at Alkaloid AD Skopje, and reference marketed products obtained from several European markets, including the United Kingdom, Germany, and Italy. All products were tested in their original marketed packaging, in accordance with EMA guidance on in-use stability evaluation (10). Aliquots corresponding to the minimal prescribed dose (2.5 mL suspension) were withdrawn at daily intervals throughout the in-use period, and each sample was analysed for potential changes in physical, chemical, and microbiological characteristics as defined in the finished product specifications. Two storage conditions were evaluated: ambient conditions (25 ± 5 °C), with sampling performed on days 0, 3, 7, and 14, and refrigerated conditions (2–8 °C), with sampling conducted on days 0, 7, and 14.

In line with the Patient Information Leaflet (PIL) of marketed Cefixime suspensions, which specifies a maximum in-use period of 14 days post-reconstitution, the study was designed to fully cover this timeframe. Comparative analysis of the impurity profiles revealed the emergence of an unidentified impurity with an RRT of 0.19 in both the test and reference products. This finding enabled the application of a qualification-by-use approach, consistent with ICH Q3B(R2) requirements, thereby supporting the toxicological qualification of the impurity based on real-world in-use stability conditions (11).

Fraction collection and isolation of the target impurity

The target impurity with an RRT of 0.19 was isolated from both the test and reference drug products after 14 days of in-use stability testing, in accordance with EMA in-use stability testing guidelines (10). Fraction collection was performed using an automated fraction collector (Thermo Scientific AFC-3000) connected to a Thermo Scientific Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, USA). The automated fraction collector was operated in time-based collection mode with a programmable retention time window targeting the peak of interest (RRT 0.19), which had been established by replicate injections under identical chromatographic conditions. Fractions were collected into 2 mL amber glass vials (Thermo Scientific) to minimise potential photodegradation. The system was calibrated to achieve a time accuracy of ± 0.1 min and volumetric accuracy of ± 1 %, with a minimum programmable fraction volume of 100 μL. To avoid carry-over, the sampling needle was automatically rinsed using a dual-solvent wash cycle (water/acetonitrile, 1:1, *V*/*V*). Collected fractions containing the target peak were concentrated under a gentle stream of dry nitrogen at 50 °C to a final volume of 1,500 μL. The integrity of the concentrated material was verified by re-injection using a validated in-house HPLC method for related substances in the finished product. This method was originally developed in alignment with the European Pharmacopoeia monograph for cefixime drug substance (22), but subsequently adapted and fully validated for the analysis of cefixime oral suspension. The re-injection confirmed the presence of the peak corresponding to the target impurity with an RRT of 0.19, ensuring that the collected fractions indeed originated from the same degradation product (23). For all samples containing impurity RRT 0.19, DAD-based peak purity evaluation was performed. The purity angle was consistently lower than the purity threshold (purity factor > 980), confirming no co-eluting UV-absorbing species.

LC/MS analysis for structural identification

Structural identification of impurity with an RRT of 0.19 was performed using LC/MS in accordance with established methodologies for β-lactam degradation products (23–25). Analyses were conducted under electrospray ionisation (ESI) in both positive and negative modes. For direct infusion experiments, the temperature of the heated electrospray vaporiser was optimised following recommendations for cephalosporin analysis to minimise thermal degradation during ionisation (21). The HPLC system was coupled to a Thermo Scientific LTQ XL linear ion trap mass spectrometer with a heated electrospray ionisation (HESI) source. Instrument control and data acquisition were managed using Chromeleon 5.0 and Xcalibur 5.0 software. Nitrogen served as the nebulising gas at 50 psi with a flow rate of 12 L min^{-1}. For hyphenated detection, the HPLC effluent first passed through the UV detector and was subsequently directed to a post-column micro-flow splitter. Approximately

10 % of the total flow (around 0.10 mL min^{-1}) was diverted to the mass spectrometer, while the remaining portion proceeded to waste. This setup ensured that the MS source operated within optimal flow capacity, while maintaining identical chromatographic conditions between UV and MS detection. The heated capillary temperature was maintained at 400 °C, while the spray voltage was set to 3.5 kV. The full-scan LC/MS (*m*/*z* 50–500) was performed on isolated fractions of the targeted impurity with RRT of 0.19. Only ions corresponding to the three stereoisomeric γ-lactone components were observed. No additional molecular species were detected above background noise. The mobile phase consisted of acetonitrile and 0.5 % formic acid in water (20:80, *V*/*V*).

In silico *toxicological evaluation*

The toxicological qualification of impurity with an RRT of 0.19 was conducted according to the ICH M7 guideline on assessment and control of DNA-reactive impurities (24). Three complementary computational tools were used: Toxtree (v. 2.6.13) (15–17), an expert rule-based system; TEST (v. 4.1) (18, 19), a statistical QSAR platform; OECD QSAR Toolbox (v. 3.3) (20), for mechanistic profiling and read-across analysis. Acute toxicity classification was performed using CASE Ultra models (GHS categories) (21–23).

Toxtree is a Java-based, open-source application developed by IDEA Consult Ltd. under contract with the Institute for Health and Consumer Protection as part of the Joint Research Centre (JRC) of the European Commission. The software estimates toxicological hazards using a decision-tree approach. Several decision tree modules are implemented, among which the following are relevant to mutagenicity and carcinogenicity assessment:

Benigni/Bossa Rulebase for Mutagenicity and Carcinogenicity: The Benigni/Bossa Rulebase for Mutagenicity and Carcinogenicity, described in the European Commission Report EUR 23241 EN, applies a defined set of structural alerts and quantitative structure-activity relationship (QSAR) models to predict the carcinogenic and mutagenic potential of chemical entities. The QSAR models incorporated in this module include predictions of the mutagenic activity of aromatic amines in *Salmonella typhimurium* TA100 (Ames test), the carcinogenic activity of aromatic amines in rodents (based on summary datasets from rats and mice), and the mutagenic activity of α,β-unsaturated aldehydes in *S. typhimurium* TA100. These models are developed using Canonical Discriminant Analysis, as described by Franke (1984) and Franke and Gruska (2003), with performance evaluated through accuracy, sensitivity, specificity, and squared canonical correlation. *Decision Tree for Estimating In Vitro Mutagenicity (Ames Test)*: Developed by Benigni *et al.* (2013), this module provides predictive models for bacterial mutagenicity based on curated datasets.

Structural Alerts for the In Vivo Micronucleus Assay in Rodents: Based on *European Commission Report EUR 23844 EN*, these alerts identify structural features linked to genotoxic potential in rodent micronucleus assays.

The Toxicity Estimation Software Tool (TEST) was developed by the U.S. Environmental Protection Agency as a statistical tool for estimating toxicity from molecular structure using multiple QSAR methodologies. The current study used TEST v.4.1, an open-source Java library for computational toxicology. The Ames mutagenicity endpoint was selected for assessment. TEST offers several predictive methods: hierarchical clustering, FDA method, group contribution, nearest neighbour, consensus method, and random forest. For statistical external validation of Ames mutagenicity, the consensus method achieved the highest prediction accuracy and coverage. Multiple validated prediction methods were applied to increase confidence in the results, with priority given to endpoints yielding consistent predictions across models.

The OECD QSAR Toolbox, developed by LMC ASIS in collaboration with the Organisation for Economic Co-operation and Development (OECD) and the European Chemicals Agency (ECHA), is an *in silico* platform designed to support hazard assessment by identifying structural features of chemicals, exploring potential mechanisms or modes of action, and selecting structurally or mechanistically similar analogues to enable data gap filling through read-across. In the present study, both the parent compound (cefixime) and the impurity with an RRT of 0.19 were evaluated using profilers relevant to genetic toxicity and carcinogenicity. These included carcinogenicity classifiers (genotoxic and non-genotoxic) developed by ISS, DNA alerts for the Ames test from the OASIS profiler, DNA alerts for chromosome aberration and the micronucleus test (OASIS), *in vitro* mutagenicity predictions (Ames) by ISS, *in vivo* chromosomal mutation alerts, and the OncoLogic™ primary carcinogenicity classification system. When experimental data were unavailable, chemical categories and subcategories were defined to enable read-across using structurally and mechanistically appropriate analogues. Toxicological information was sourced from curated databases, including the RepDose Fraunhofer ITEM database and the Repeated Dose Toxicity HESS database. The QSAR Toolbox operates in accordance with OECD principles for chemical grouping and QSAR model validation and integrates regulatory inventories, experimental datasets, and structure–activity profilers, including molecular initiating event (MIE) tools within the Adverse Outcome Pathway (AOP) framework.

RESULTS AND DISCUSSION

The in-use stability study was conducted in accordance with EMA guidance on in-use stability testing for human medicinal products (10). The study included both the candidate formulation (laboratory-scale cefixime granules for oral suspension) and commercial reference products sourced from several markets (UK, DE, IT). According to the product information leaflets (PILs) of the reference products, the recommended in-use period after reconstitution is 7 to 14 days when stored at 2–8 °C. To reflect realistic patient use, our study investigated both refrigerated and ambient conditions.

As shown in Table I, the impurity was not detected in freshly reconstituted suspensions but became prominent after 14 days of storage at ambient conditions (25 °C/60 % RH). Under these conditions, the impurity with an RRT of 0.19 exceeded the ICH Q3B identification threshold (0.2 % area) in both the candidate and reference products. Under refrigerated in-use conditions (2–8 °C), impurity RRT 0.19 increased only marginally and remained below the ICH identification threshold (0.2 %) at all sampling points (days 0, 7, and 14) across both the candidate formulation and all reference products. This contrasts with ambient storage, where the impurity exceeded the threshold after 14 days. These findings confirm that refrigerated storage effectively slows the acid-driven degradation pathway responsible for γ-lactone formation. Refrigerated storage markedly reduced the formation rate of the impurity with an RRT of 0.19, consistent with the temperature dependence of pseudo–first-order kinetics and with PIL-recommended in-use storage at 2–8 °C. Isolation was achieved by automated fraction collection from multiple HPLC runs, as described in

Table I. In-use stability study of cefixime granules for oral suspension: impurity with an RRT of 0.19 levels compared across candidate and reference products

Product type	Market/ Origin	PIL recommended in-use period	Tested storage condition	Day 0 (% area RRT 0.19)	Day 7 (% area RRT 0.19)	Day 14 (% area RRT 0.19)
Candidate formulation (lab batch)	Internal (test)	n/a (lab scale)	25 °C/60 % RH	n.d.	0.12	0.24[a]
Candidate formulation (lab batch)	Internal (test)	n/a (lab scale)	2–8 °C	n.d.	0.08	0.16
Reference product A	UK	14 days (2–8 °C)	25 °C/60 % RH	n.d.	0.15	0.27[a]
Reference product A	UK	14 days (2–8 °C)	2–8 °C	n.d.	0.09	0.18
Reference product B	DE	7–14 days (2–8 °C)	25 °C/60 % RH	n.d.	0.14	0.25[a]
Reference product C	IT	14 days (2–8 °C)	25 °C/60 % RH	n.d.	0.13	0.22[a]

n.d. – not detected (< 0.05 % area);

[a] Values above 0.20 % exceed the ICH Q3B identification threshold.

the Experimental section, and concentrated under controlled conditions to prevent secondary degradation. Under the acidic conditions of the LC/MS mobile phase, the peak corresponding to RRT 0.19 was resolved into three chromatographic components (Fig. 2).

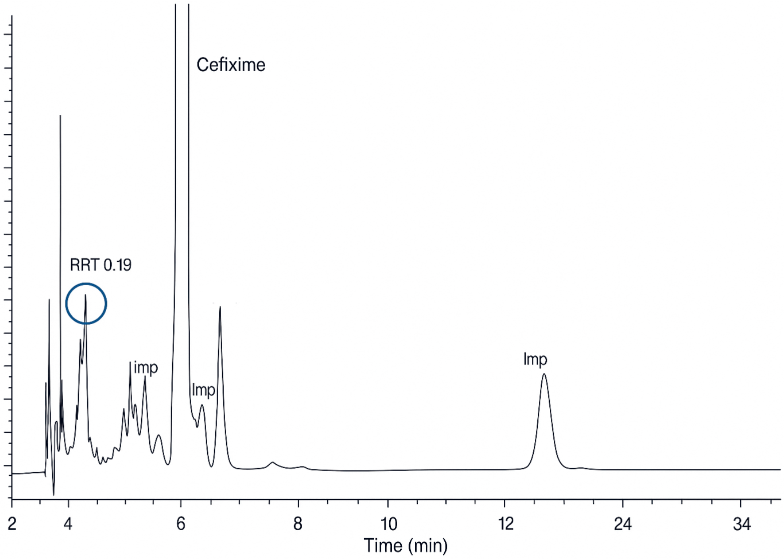

Fig. 2. UV chromatogram of cefixime and related impurities recorded at 254 nm using the UV detector under the chromatographic conditions applied for the LC/MS method of tested drug products stored 14 days at room temperature.

Although three partially separated peaks were observed under LC/MS conditions, full chromatographic resolution was not achieved. Given their overlapping nature, both UV and MS/MS spectra must be interpreted with caution, as the three components cannot be treated as independently characterised species. The similar UV spectral profiles and the identical protonated molecular ion at *m*/*z* 214 obtained for each of the three chromatographic shoulders strongly suggest that these signals originate from stereoisomeric or conformational variants of the same degradation product. The comparison of UV spectra and mass spectral parameters is summarised in Table II, where all three components exhibit matching spectral characteristics, supporting their interpretation as forms of a single γ-lactone impurity rather than three chemically distinct entities. Because the peaks are not fully resolved, the structural assignment cannot be confirmed without either (i) isolation of each chromatographic component or (ii) analysis of an authentic standard known to generate this pattern. Therefore, the γ-lactone structure proposed here represents the most plausible interpretation based on MS/MS fragmentation pathways and alignment with established cephalosporin degradation mechanisms described in the literature (26–28).

Importantly, in the context of kinetic evaluation, the entire asymmetric peak area was treated as a single impurity with an RRT of 0.19. This approach ensured that quantitative monitoring of its growth over the 14-day in-use stability period remained valid, despite the underlying chromatographic heterogeneity. The observed splitting of the target impurity peak into three separate chromatographic peaks is most probably promoted by the acidic conditions of the mobile phase used in the MS-friendly HPLC method. This observation is in line with previous literature reports on acid-promoted formation of γ-lactone derivatives of cefixime (26–28), where similar peak splitting under acidic conditions was described. In contrast, the more basic conditions of the routine HPLC method, based on phosphate buffer, consistently revealed a single asymmetric peak at the corresponding retention time. This corroborates the proposed theory that the apparent multiplicity of impurity with an RRT of 0.19 observed under LC/MS conditions reflects acid-induced stabilisation of different stereoisomeric or conformational forms of the same γ-lactone impurity. For quantification purposes, however, the impurity was always integrated as a single component under the validated HPLC method for related substances. The concentrated samples obtained from the collected fractions were analysed using the routine validated HPLC method and compared with the retention time and UV spectral profile of the impurity with an RRT of 0.19 from Cefixime granules for oral suspension. The results confirmed that the collected fractions contained the same target compound, as demonstrated by

Table II. Retention, UV-absorbance and mass spectral data for three components of impurity with an RRT of 0.19 detected in the test drug product in samples stored 14 days at room temperature

Peak	t_R (min)	UV_{max} (nm)	[M+H$^+$] ion[a] (*m*/*z*)	MS^2 ions (*m*/*z*)
Peak 1	3.71	262, 228	365 (70 %), 214	191, 173, 158
Peak 2	3.90	264, 232	365 (50 %), 214	191, 173, 158
Peak 3	4.08	264, 232	365 (35 %), 214	191, 173, 158

[a] Molecular ion obtained using positive ionisation mode (protonation).

matching retention times and superimposable UV spectra (Fig. S1). In addition, full-scan LC/MS analysis (*m*/*z* 50–500) was performed on the isolated fractions of the impurity (Fig. S2). The spectra showed only ions corresponding to the three stereoisomeric γ-lactone components. No additional molecular species were detected above background noise, indicating the absence of co-eluting impurities and supporting the purity of the isolated material. Comprehensive LC/MS investigations of the tested drug product after 14 days of in-use stability testing showed that the impurity with an RRT of 0.19 was not a single homogeneous peak but appeared as three partially resolved chromatographic components (RT = 3.71, 3.90, and 4.09 min). The UV spectra of these three components were virtually identical, and all were characterised with the same protonated molecular ion $(M+H)^+$ at *m*/*z* 214. MS/MS spectra of each peak revealed identical fragmentation patterns, demonstrating that the three species share the same chemical connectivity and differ only in stereochemical or conformational arrangement. The *m*/*z* 214 ion is known from literature as a stable fragment originating from the cefixime structure (26). It can be considered that the ion with mass 365 *m*/*z* is unstable at high temperature when the sample passes through heat electrospray ionisation, and it is fragmented into a more stable fragment of the molecular ion with mass 214 *m*/*z*. This is in accordance with the fact that cefixime is a very sensitive compound, which easily decomposes under different environmental conditions, especially at elevated temperatures. Analysis of the full MS spectra showed that this ion most likely originates from the main peak at *m*/*z* 365, which was detected with lower relative abundance (Table III and Fig. 3).

To address the observed variations between the MS/MS spectra obtained from the reference product and the candidate drug product (Figs. 3a,b), it should be noted that these differences derive from quantitative signal intensity rather than structural divergence. The reference product presented a higher precursor ion abundance, resulting in more pronounced secondary fragments, while the candidate formulation exhibited a weaker signal due to lower impurity concentration. Importantly, both spectra share the same diagnostic ions at *m*/*z* 454 → 365 → 214, confirming identical fragmentation pathways and supporting a single structural identity. No unique fragments were detected in either product. Siegel *et al.* have described the changes of the experimental condition, such as lowering of the temperature profile of the thermospray vaporiser during direct injection in to the mass spectrometer in order to avoid the degradation of β-lactam antibiotics. For that reason, in our study, dedicated experiments were conducted, where the temperature of the heated electrospray vaporizer was reduced from 400 to 200 °C, and the isolated impurity an RRT of 0.19 fraction was directly infused into the mass spectrometer. Under these milder conditions, the base peak shifted from *m*/*z* 214 to *m*/*z* 365, and an additional ion at *m*/*z* 454

Table III. Mass spectral data for unidentified impurity with an RRT of 0.19 in fractions of drug products obtained by direct injection in the MS spectrometer (at 200 °C)

Compounds	$[M+H^+]$ ion[a] (*m*/*z*)	MS^2 ions (*m*/*z*)
Candidate formulation (lab batch)	454 (~10 %), 365	203, 185
Reference product A	454 (~10 %), 365	203, 185

[a] Molecular ion obtained using positive ionisation mode (protonation).

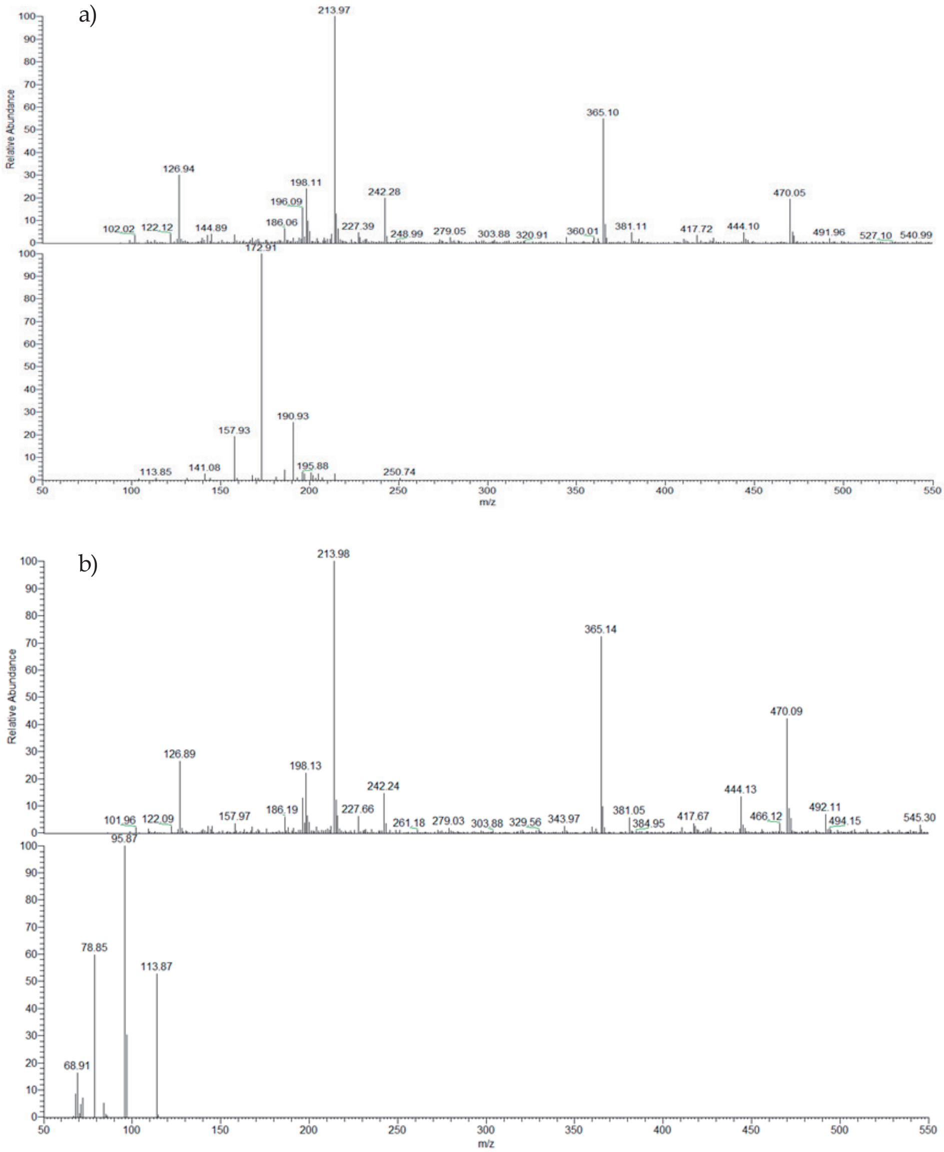

Fig. 3. a) MS and MS^2 data of target unidentified impurity with an RRT of 0.19 in reference drug product, obtained by LC/MS method; b) MS and MS^2 data of target unidentified impurity with an RRT of 0.19 in candidate drug product, obtained by LC/MS method.

appeared at lower intensity (Fig. 4). The appearance of *m*/*z* 454 is consistent with the proposed γ-lactone structure of the impurity, providing strong evidence for its structural assignment.

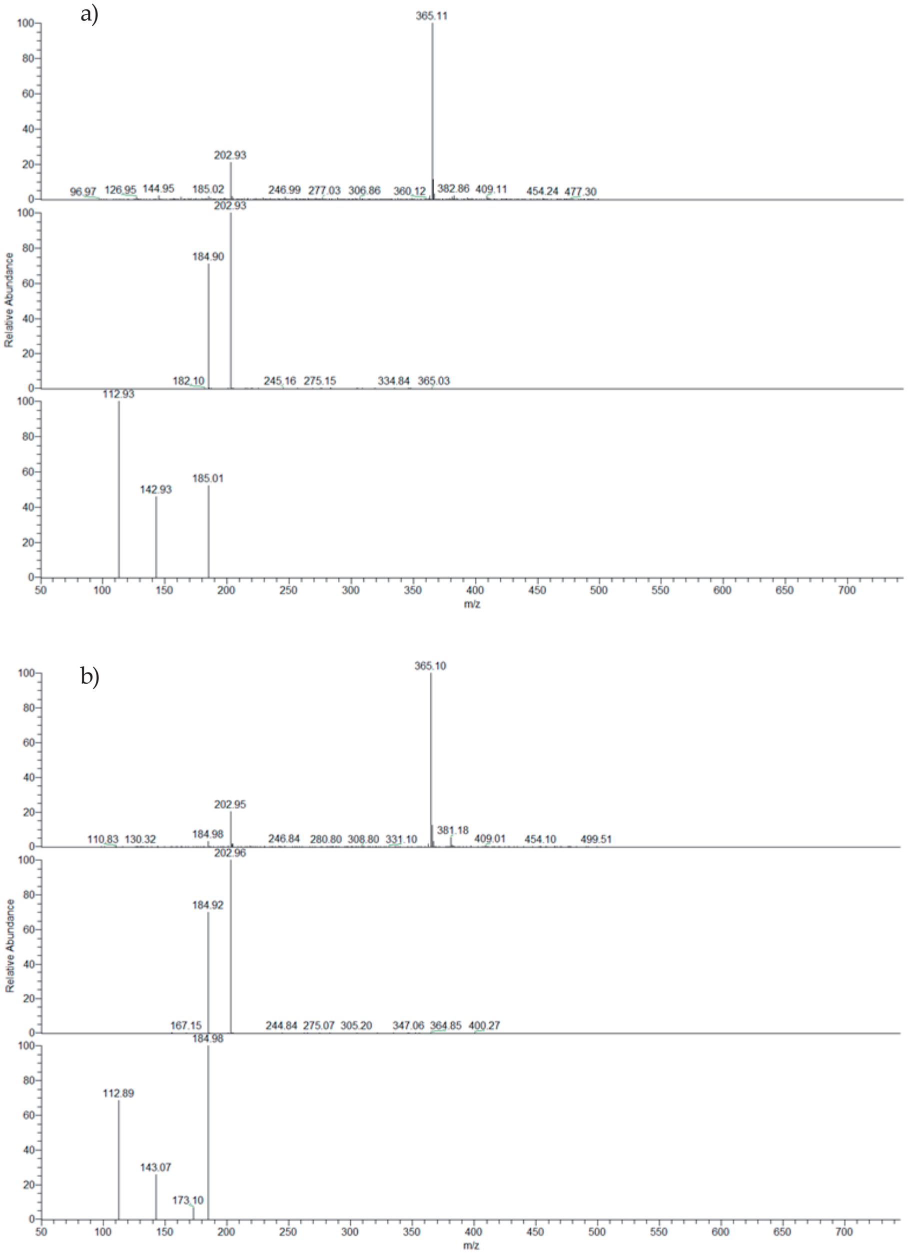

Fig. 4. a) MS, MS^2 and MS^3 data of unidentified impurity with an RRT of 0.19 in fractions of test drug product obtained by direct injection in MS spectrometer (at 200 °C); b) MS, MS^2 and MS^3 data of unidentified impurity with an RRT of 0.19 in fractions of reference drug product obtained by direct injection in MS spectrometer (at 200 °C).

As can be seen in the full MS spectra (Fig. 4) base peak with mass 365 *m*/*z* and the ion with 454 *m*/*z* with lower abundance were obtained. This leads to the conclusion that the molecular ion with mass 365 *m*/*z* is a fragment which originates from the ion with mass 454 *m*/*z*, previously detected and described in the prepared reconstituted suspension. This ion with mass 454 *m*/*z* and the corresponding fragmentation patterns are in agreement with the lactone structure of degradation impurity 3 previously detected and characterised by Namiki *et al.* The ions with mass 365 *m*/*z* (~15 % abundance) were also detected in the corresponding MS spectra of the unspecified impurities observed during the initial analysis of the reconstituted suspension. This fact strongly supports the experimental observation about the acid-induced growth of impurity with an RRT of 0.19, during the stability in use testing. Impurity with RRT of 0.19 is most probably obtained by transformation of the two unspecified impurities after 14 14-day stability in-use testing period. This is in accordance with the fact that after 14 days, the peaks from the two unspecified impurities (RT = 5.86 and 7.51) have completely disappeared. Studying the molecular ions, fragments in the MS^2 spectra and UV spectra as well, leads to the conclusion that the three components of RRT 0.19 (in reconstituted samples after 14 days) are stereoisomers of degradation impurity 3 described by Namiki *et al.*, with fragmentation patterns shown in Table III.

The Toxtree software (v. 2.6.13) was used to screen both cefixime and the impurity with an RRT of 0.19 for mutagenicity and carcinogenicity alerts. Both compounds triggered identical structural alerts (Table S1), primarily related to the presence of a primary aromatic amine (mutagenicity-related) and H-acceptor-path3-H-acceptor (SA 34). Importantly, the impurity did not generate any novel structural alerts beyond those already inherent to cefixime.

The QSAR-based TEST software (v. 4.1) was employed using the Ames mutagenicity endpoint. Both cefixime and impurity with a RRT of 0.19 were consistently predicted to be negative for mutagenic potential across all statistical methodologies incorporated in the consensus model (Table S2). Mechanistic toxicity assessment was conducted using the OECD QSAR Toolbox, employing a suite of relevant profilers including DNA alerts for Ames bacterial mutagenicity, micronucleus (MN) formation, chromosomal aberrations (CA), carcinogenicity alerts (both genotoxic and non-genotoxic), and repeated-dose toxicity indicators. The impurity with an RRT of 0.19 exhibited no additional structural or mechanistic alerts beyond those identified for the parent compound cefixime, confirming the absence of new mutagenic structural features. To further substantiate the toxicological profile, a read-across analysis was performed using structurally analogous compounds identified within the Toolbox's curated chemical categories. The analogue selection was based on structural similarity, mechanistic plausibility, and availability of high-quality toxicological data. This analysis yielded predicted thresholds for systemic toxicity: a No Observed Effect Level (NOEL) of 58.8 mg kg^{-1} per day and a Lowest Observed Effect Level (LOEL) of 119 mg kg^{-1} per day for repeated oral exposure. Acute oral toxicity was predicted for both cefixime and RRT 0.19 using the CASE Ultra platform, applying the Globally Harmonized System (GHS) acute toxicity classification models (Table S1). In all tested scenarios, the predicted median lethal dose (LD_{50}) values exceeded 2000 mg kg^{-1} body mass, placing both substances in GHS Category 5, the lowest hazard classification for acute toxicity. Taken together, this converging evidence from three independent and complementary platforms, expert rule-based (Toxtree), statistical QSAR (TEST), and mechanistic/

read-across approaches (OECD QSAR Toolbox, CASE Ultra), provides a consistent conclusion that impurity with an RRT of 0.19 poses no additional genotoxic or acute toxicity risk beyond that of cefixime itself.

Kinetic interpretation of impurity with an RRT of 0.19. – The results obtained from the in-use stability study showed that the impurity with an RRT of 0.19 follows an apparent first-order formation pattern under both ambient (25 ± 5 °C) and refrigerated (2–8 °C) conditions. Linearised plots of $\ln(C\infty - C)$ *vs.* time provided straight-line fits with correlation coefficients ($R^2 \approx 0.87$), confirming pseudo-first-order kinetics within the studied period (Supplementary Fig. S3–S6). The calculated rate constant under ambient storage ($k \approx 0.099$ day^{-1}) corresponded to a half-life of ~7 days, whereas refrigerated storage produced lower asymptotic concentrations ($C\infty \approx 0.16$ % *vs.* 0.24 % for ambient). These findings confirm the stabilising effect of refrigeration on cefixime suspensions. Importantly, the impurity remained undetectable immediately after reconstitution but exceeded the ICH Q3B identification threshold (0.2 %) after 14 days under ambient conditions. This observation is consistent with the patient information leaflet (PIL) recommendations limiting in-use periods to 7–14 days, providing an analytical justification for such restrictions.

Mechanistic proposal for γ-lactone formation. – LC/MS and MS/MS fragmentation studies rationalise the formation of impurity with an RRT of 0.19 via an acid-catalysed intramolecular lactonisation pathway, previously reported for cephalosporins (25–28). The pH of reconstituted cefixime suspensions (3.2–3.5) creates an acidic environment favouring *β*-lactam hydrolysis, which initiates intramolecular cyclisation between the C-3 vinyl group and the neighbouring C-4 carboxyl group. This results in a *γ*-lactone ring system, consistent with cephalosporin degradation mechanisms described by Talebpour *et al.* (24) and Namiki *et al.* (26). The complete molecular structure of degradation impurity 3 was identified as (4*S*)-4-[(2*Z*)-2-[(2-amino-1,3-thiazol-4-yl)amino]-2-oxoethyl]-3-[(carboxymethoxy)imino]-7-oxo-2,3-dihydro-1*H*-thieno[2,3-*e*][1,4]diazepine-5-carboxylic acid. This structural assignment corresponds to a *γ*-lactone derivative arising from intramolecular cyclization of cefixime under acidic aqueous conditions (Fig. 5).

The impurity has a molar mass of 455.46 g mol^{-1}, with an exact monoisotopic mass of 455.0569 Da, consistent with the elemental composition $C_{16}H_{17}N_5O_7S_2$. Elemental distribution analysis showed that carbon accounts for 42.19 % of the molecular mass, hydrogen for 3.76 %, nitrogen for 15.38 %, oxygen for 24.59 %, and sulfur for 14.08 %, values that are in full agreement with the proposed chemical formula. These physicochemical parameters support the identity of the impurity and corroborate the mass spectral data obtained during structural elucidation.

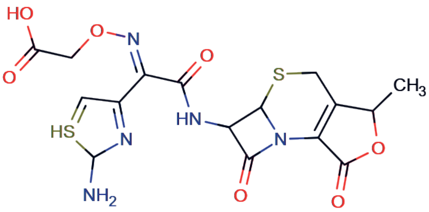

Fig. 5. Molecular structure of “degradation impurity 3” (γ-lactone of cefixime).

Targeted ionisation experiments revealed a consistent and diagnostically meaningful fragmentation pattern for the precursor ion at *m/z* 454. The spectra showed an initial neutral loss of CO_2 (44 *m/z*) from the carboxyl group adjacent to the *β*-lactam region, followed by cleavage of the *β*-lactam ring, generating characteristic fragment ions associated with the thiazine and aminothiazole moieties. A further neutral loss of 98 *m/z*, corresponding to elimination of the *γ*-lactone unit, was observed across all MS/MS replicates. Importantly, the spectra retained aminothiazole-related fragment ions, confirming preservation of the C-7 substituent and excluding degradative pathways involving side-chain scission. Taken together, these fragmentation characteristics strongly support the proposed *γ*-lactone structure of the impurity (Fig. 6). The observed MS/MS behaviour is fully consistent with previously reported degradation pathways for cephalosporins forming lactone derivatives under acidic conditions (24–28).

Although LC/MS data strongly support the *γ*-lactone structural hypothesis, full structural confirmation would require chromatographic isolation of each isomeric component

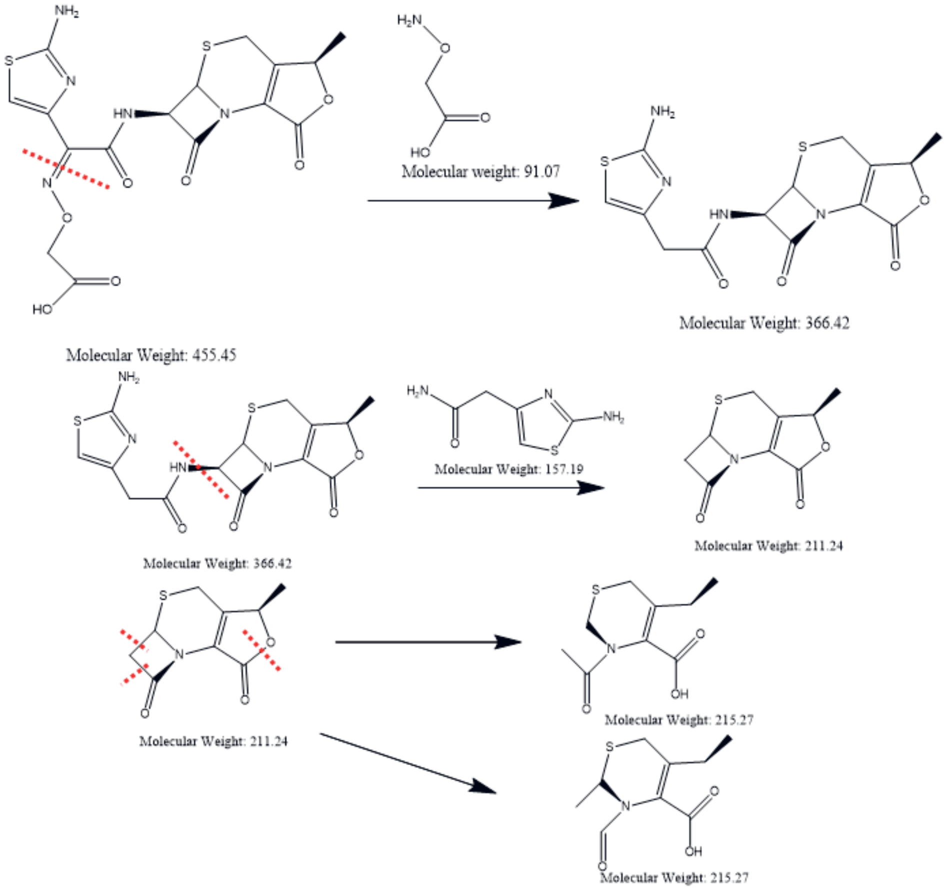

Fig. 6. Proposed fragmentation pathway of the unidentified impurity with an RRT of 0.19.

followed by NMR analysis or synthesis of an authentic reference standard. Due to the limited yield of the isolated impurity and insufficient peak resolution, such confirmation was not feasible in the present work. Therefore, the proposed γ-lactone structure should be regarded as the most plausible interpretation, supported by MS/MS fragmentation behaviour and previously reported cephalosporin degradation mechanisms.

Toxicological and regulatory interpretation. – The toxicological qualification of the impurity with an RRT 0.19 was supported by converging evidence obtained from three complementary computational approaches. The rule-based system Toxtree identified no new structural alerts beyond those already associated with the parent compound cefixime. Statistical QSAR evaluation using the TEST v4.1 consensus model consistently predicted negative Ames mutagenicity outcomes. Mechanistic assessment through the OECD QSAR Toolbox demonstrated the absence of novel genotoxicity alerts and provided favourable systemic toxicity thresholds, including a no-observed-effect level (NOEL) of 58.8 mg kg^{-1} per day and a lowest-observed-effect level (LOEL) of 119 mg kg^{-1} per day. Acute toxicity predictions generated using CASE Ultra classified both cefixime and the impurity with RRT 0.19 in GHS Category 5 ($LD_{50} > 2000$ mg kg^{-1}), indicating the lowest level of acute toxicity concern. The predicted NOEL for the g-lactone impurity (58.8 mg kg^{-1} per day) is approximately half of the reported NOEL for cefixime itself (≈120 mg kg^{-1} per day in rodents), yet still over 300-fold higher than the maximum patient exposure to the impurity (Table S4). Collectively, these results demonstrate that the γ-lactone impurity does not introduce additional mutagenic liability relative to cefixime, lacks high-concern structural alerts under the ICH M7 classification system, exhibits wide safety margins in comparison with patient exposure, and presents negligible acute toxicity potential.

From a regulatory toxicology standpoint, the integration of kinetic, structural, mechanistic, and computational evidence provides a coherent scientific justification for the qualification of the RRT 0.19 impurity under ICH M7 principles. Its identification as a γ-lactone derivative of cefixime, formed exclusively under in-use conditions and only at low concentrations, supports its classification as an ICH M7 Class 5 impurity (Table IV). Accordingly, no additional in vitro or in vivo genotoxicity studies are required, and the impurity can be considered qualified for acceptance in cefixime oral suspensions.

Comparable degradation and impurity profiling studies have been reported for other β-lactam antibiotics, particularly cefuroxime and cefpodoxime. For instance, Wang and Notari (28) studied the hydrolysis kinetics of cefuroxime in aqueous solution as a function of pH and temperature, demonstrating that hydrolysis under acidic conditions follows predictable rate constants.

Similarly, Uzunović *et al.* evaluated the stability of cefuroxime axetil oral suspension after reconstitution and found that under room temperature storage, degradation becomes noticeable within ~10 days (29). In the case of cefpodoxime proxetil, Li *et al.* characterised multiple impurities, including several degradation products, using LC/MS/MS, which mirrors the analytical approach employed in this study (30). These comparisons reinforce that the mechanistic pathway (β-lactam hydrolysis followed by intramolecular lactonisation) and the observed first-order kinetic behaviour are not unique to cefixime but are shared among several cephalosporins. Hence, the proposed predictive workflow has potential applicability beyond cefixime, increasing its relevance for generic and newly developed β-lactam antibiotics.

Table IV. Integrated in silico *genotoxicity and acute toxicity predictions for cefixime and the impurity with an RRT of 0.19*

Tool/version	Model type	Endpoint(s)	Key alerts/ notes	Prediction/ quantitative output	ICH M7 regulatory interpretation	Recommended action
Toxtree v2.6.13	Expert rule-based (+ QSAR plug-ins)	Mutagenicity & Carcinogenicity	Same structural alerts as cefixime: primary aromatic amine; H-acceptor-path3-H-acceptor (SA-34). No new alerts in impurity.	Qualitative: No additional concern beyond parent.	Meets "two complementary methods with no concerning alerts specific to impurity" criterion (half of the M7 requirement).	No further genotox testing triggered by Toxtree alone.
TEST v4.1	Statistical QSAR (consensus: hierarchical, NN, GC, RF)	Ames bacterial mutagenicity	–	Negative (non-mutagenic) for RRT 0.19 (and parent).	Complements expert system; paired with Toxtree, fulfils M7 (expert + statistical) → No mutagenic concern.	No additional Ames testing recommended for qualification.
OECD QSAR Toolbox	Read-across (category/analogue) + mechanistic profilers	Genotoxicity profilers (Ames, MN, CA), carcinogenicity; repeated-dose tox	No new alerts *vs.* cefixime; consistent mechanistic profile.	NOEL = 58.8 mg/kg/day; LOEL = 119 mg/kg/day (read-across).	Wide margin *vs.* trace exposure levels → Low toxicological concern.	Use outputs to support risk rationale in dossier.
CASE Ultra (GHS acute tox models)	Statistical + expert	Acute oral toxicity (rat, LD_{50} categories)	–	GHS Category 5 ($LD_{50} > 2000$ mg/kg) → lowest acute hazard class.	Not directly part of M7 mutagenicity, but supports the overall safety narrative.	No acute toxicity testing indicated for qualification at observed levels.

CONCLUSIONS

An impurity with an RRT of 0.19 was detected during in-use stability testing of reconstituted cefixime granules and structurally assigned as a γ-lactone derivative of cefixime. It appeared only after storage beyond the recommended in-use period, consistent with an acid-catalysed intramolecular lactonisation mechanism, and remained below toxicologically relevant thresholds during the intended in-use timeframe. These findings underline the need to consider both direct hydrolysis and subsequent rearrangement reactions when evaluating degradation pathways of β-lactam antibiotics.

The integrated LC/MS-*in silico* workflow demonstrated that this γ-lactone impurity does not introduce additional mutagenic, carcinogenic or acute toxicity risk compared with cefixime and fulfils the criteria for ICH M7 Class 5, obviating further genotoxicity testing. The approach provides a robust, resource-efficient framework for impurity identification and qualification that can be readily extended to degradation impurities in other cephalosporins, such as cefuroxime and cefpodoxime, and more broadly across β-lactam antibiotics.

Abbreviations and acronyms. – API – active pharmaceutical ingredient, CA – chromosomal aberration, C_∞ – asymptotic concentration, EP – European Pharmacopoeia, ESI – electrospray ionization, GHS – Globally Harmonized System of Classification and Labelling of Chemicals, HESI – heated electrospray ionization, HPLC – high-performance liquid chromatography, ICH – International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use, LD_{50} – median lethal dose, LOEL – lowest observed effect level, LC/MS – liquid chromatography mass spectrometry, MN – micronucleus, MS/MS – tandem mass spectrometry, *m*/*z* – mass-to-charge ratio, NOEL – no observed effect level, PIL – patient information leaflet, QSAR – quantitative structure activity relationship, RRT – relative retention time, RH – relative humidity, $t_{1/2}$ – half-life.

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Author's contribution. – Conceptualisation, I.M.; methodology, I.M. and M.C.; investigation, I.M. and M.C.; writing, original draft preparation, I.M.; writing, review and editing, M.C. and G.P.; supervision, G.P. All authors have read and agreed to the published version of the manuscript.

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Supplementary materials

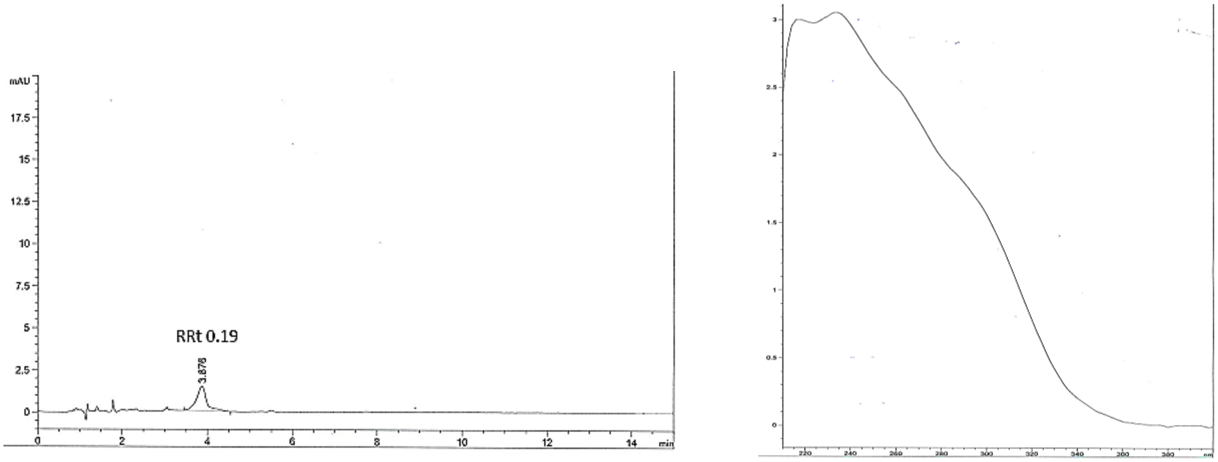


Fig. S1. Chromatogram and UV spectra obtained from the fractions of impurity with relative retention time (RRT) of 0.19 from Cefixime granules for oral suspension, collected by routine HPLC method in absorbance measurements at 254 nm.

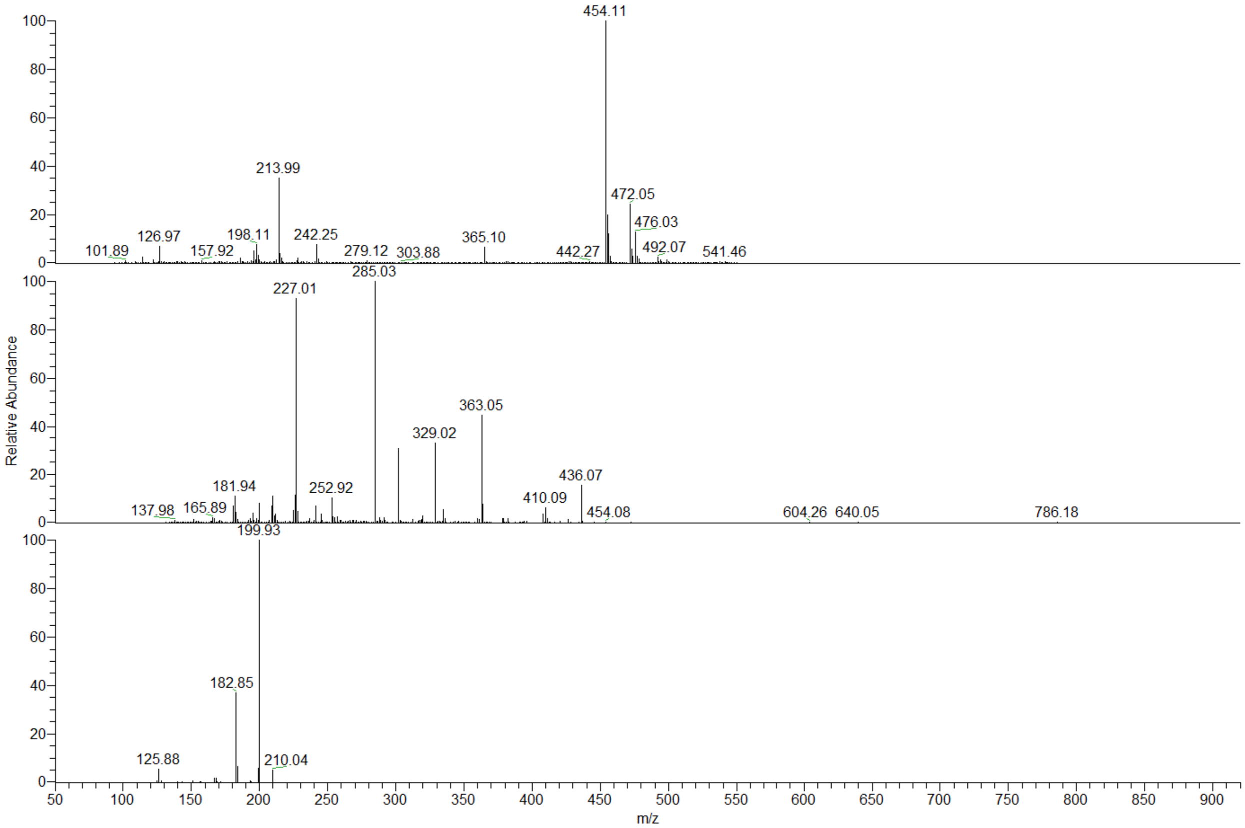


Fig. S2. Full-scan LC/MS (*m/z* 50–500) on isolated fractions of RRT 0.19.

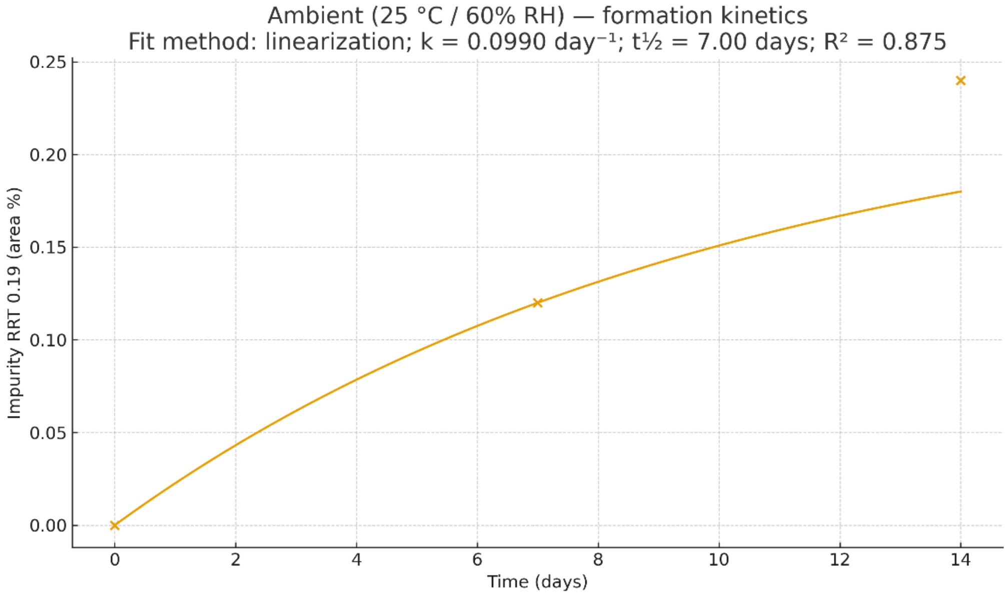


Fig. S3. Ambient formation kinetics.

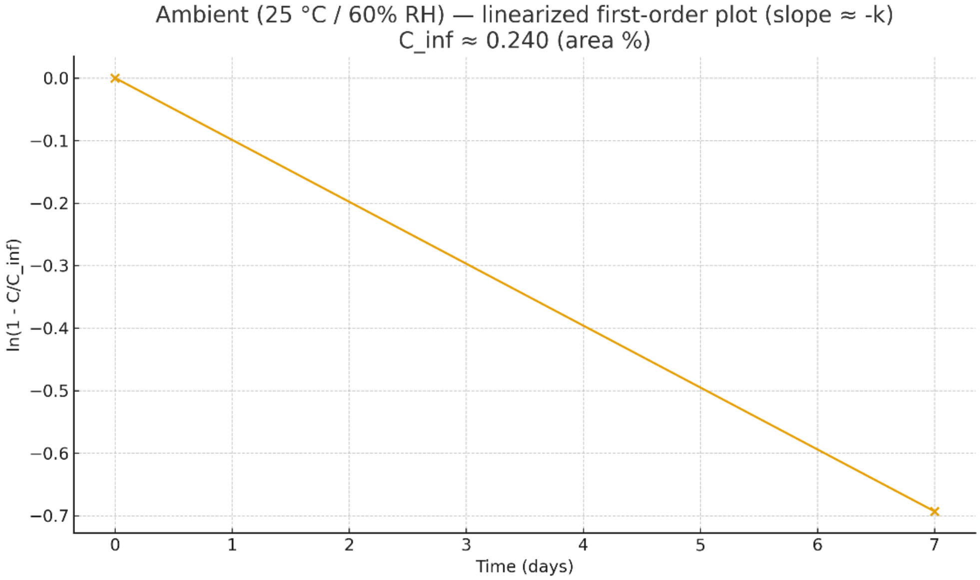


Fig. S4. Ambient linearized first-order plot.

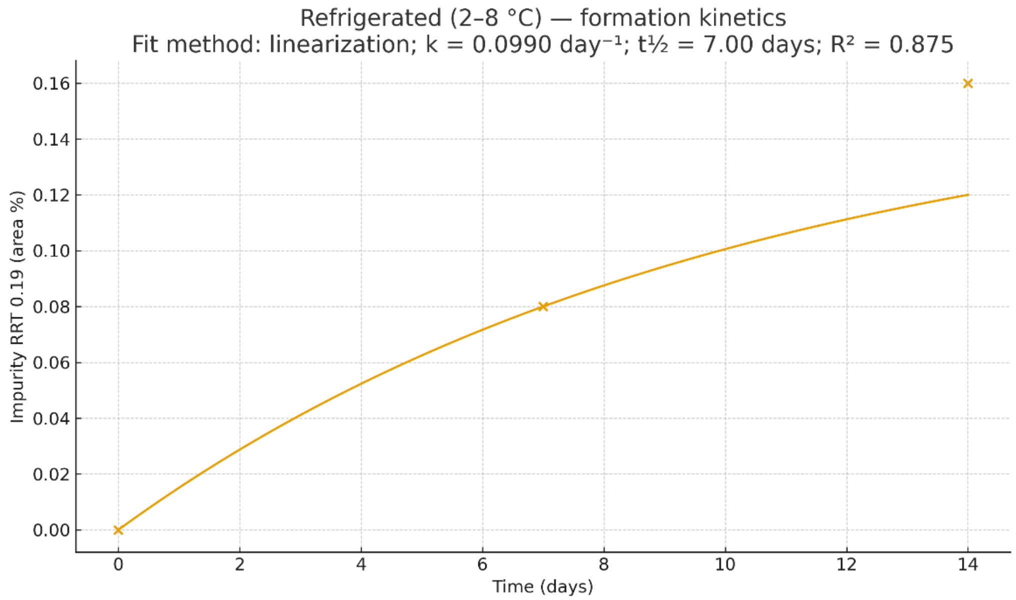


Fig. S5. Refrigerated formation kinetics.

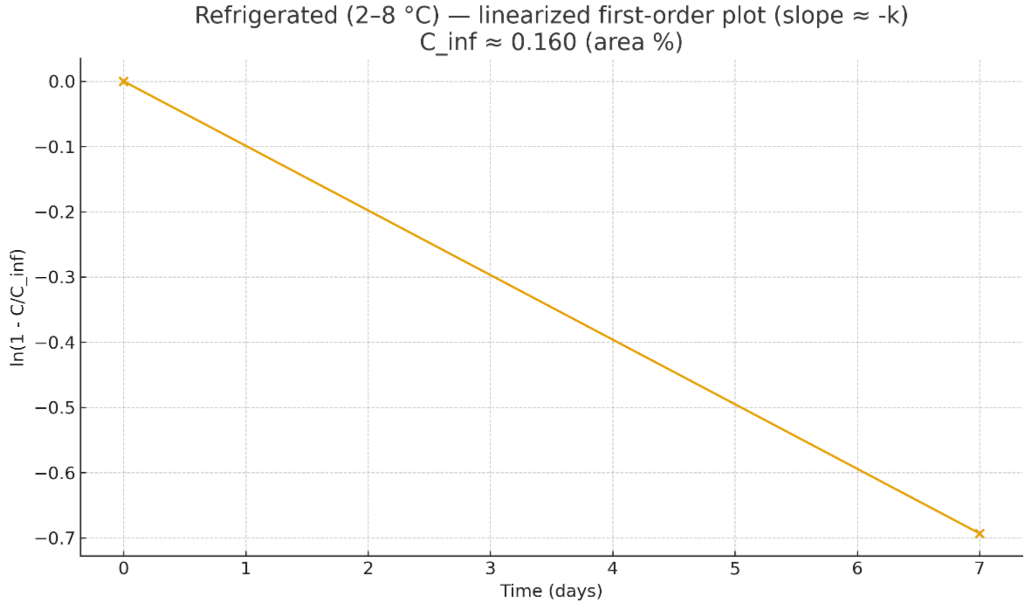


Fig. S6. Refrigerated linearized first-order plot.

Table S1. Results from the Toxtree-analysis of the parent compound cefixime impurity with relative retention time (RRT) of 0.19

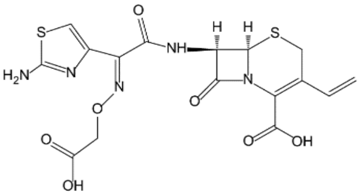
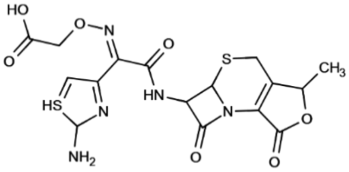
Beningni/Bossa rulebase for estimating carcinogenicity and mutagenicity	Structure Alerts for the *in vivo* micronucleus assay in rodents	A decision tree for estimating *in vitro* mutagenicity (Ames test)	Test chemical
(a) cefixime			
SA 28_ames primary aromatic amine, hydroxyl amine and its derived ester	SA 28_ primary aromatic amine, hydroxyl amine and its derived ester SA 34 H-acceptor-path3-H-acceptor	SA 28_ames primary aromatic amine, hydroxyl amine and its derived ester	
(b) impurity with relative retention time (RRT) of 0.19			
SA 28_ames primary aromatic amine, hydroxyl amine and its derived ester	SA 28_ primary aromatic amine, hydroxyl amine and its derived ester SA 34 H-acceptor-path3-H-acceptor	SA 28_ames primary aromatic amine, hydroxyl amine and its derived ester	

Table S2. Results from the TEST software using Ames mutagenicity endpoint with the consensus method for the parent compound cefixime and impurity with relative retention time (RRT) of 0.19

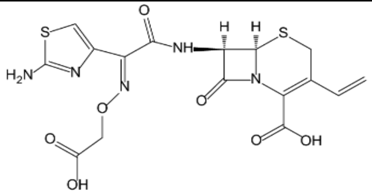
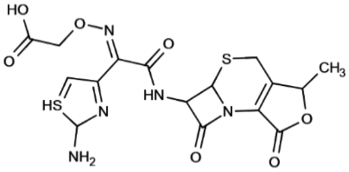
Endpoint	Experimental value	Predicted value	Test chemical
(a) cefixime			
Mutagenicity value	N/A	0.32	
Mutagenicity result	N/A	Mutagenicity negative	
(b) impurity with relative retention time (RRT) of 0.19			
Mutagenicity value	N/A	0.04	
Mutagenicity result	N/A	Mutagenicity negative	

Table S3. Results of tests with the acute toxicity set for the parent compound cefixime and impurity with relative retention time (RRT) of 0.19

Compound	CASE ULTRA LD50 (mg/kg-bw)							Final conclusion
	CAT1	CAT2	CAT3	CAT4	CAT5	GHS category	Range of toxicity mg/kg-bw	
cefixime	Known Neg	Known Neg	Known Neg	Known Neg	Known Neg	Below CAT5	> 5000	Negative
impurity with relative retention time (RRT) of 0.19	Neg	Neg	Neg	Inc	Pos	CAT5	> 2000 and ≤ 5000	Negative

Table S4. Toxicity thresholds and margin-of-safety comparison between cefixime and the γ-lactone impurity (RRT 0.19)

Parameter	cefixime (parent compound)	γ-lactone impurity (RRT 0.19)	Notes
NOEL (mg/kg/day)	~120 mg/kg/day	58.8 mg/kg/day	Predicted using OECD QSAR Toolbox (read-across).
LOEL (mg/kg/day)	~240 mg/kg/day	119 mg/kg/day	Impurity LOEL ≈ half of parent drug.
Acute toxicity (LD_{50})	>2000 mg/kg (GHS Category 5)	>2000 mg/kg (GHS Category 5)	Both substances fall into lowest acute hazard category.
Structural alerts (ICH M7)	Primary aromatic amine; no mutagenic concern	No new alerts; identical to cefixime	Compliant with ICH M7 requirement for no additional alerts.
Ames mutagenicity prediction	Negative	Negative	Toxtree + TEST consensus.
Maximum daily human exposure to impurity	—	≈0.18 mg/day (based on worst-case batch, day 14 in-use)	Calculated from maximum 0.27% area and daily dose.
Margin of Safety (MOS)	—	>300-fold	MOS = NOEL/Human Exposure. Regulatory acceptable.

- Acute Rat oral LD50 < 5 mg/kg-bw GHS Category 1 (LD50CAT1, 12262 records)
- Acute Rat oral LD50 > 5 and < 50 mg/kg-bw GHS Category 2 (LD50CAT2, 12262 records)
- Acute Rat oral LD50 > 50 and < 300 mg/kg-bw GHS Category 3 (LD50CAT3, 12262 records)
- Acute Rat oral LD50 > 300 and < 2000 mg/kg-bw GHS Category 4 (LD50CAT4, 12262 records)

- Acute Rat oral LD50 > 2000 and < 5000 mg/kg-bw GHS Category 5 (LD50CAT5, 12262 records)

GHS models were used to evaluate a range of LD_{50} values and to assign the tested compounds to one of the GHS categories.

For the γ-lactone impurity, the estimated MOS exceeds 300, indicating a wide safety margin relative to patient exposure and supporting its qualification as an ICH M7 Class 5 impurity.