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PREVER framework for specification of related substances based on in silico prediction and forced degradation studies

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Abstract

Establishing impurity specifications during pharmaceutical development is challenging due to the limited stability information available in the early stages of the product lifecycle. In this context, this study aimed to develop and evaluate PREVER, a framework based on the Safe and Sustainable by Design (SSbD) concept to support impurity qualification and specification setting. The applicability of PREVER was assessed using a 600 mg praziquantel tablets. To achieve this, PREVER integrated a literature review, forced degradation studies, *in silico* toxicological, and long-term stability studies to identify, qualify, and establish specifications for impurities. The literature identified six major potential degradation products (DPs): praziquanamine, cyclohexane carboxylic acid (CHA), mono- and dihydroxypraziquantel, and impurities with theoretical masses of 330.19434 (DP-1) and 310.16813 (DP-2). Forced degradation studies identified four of these degradation products (praziquanamine, CHA, DP-1, and DP-2). Comparison between the predicted and observed degradation profiles enabled the identification of relevant impurities and the establishment of provisional specifications based on *in silico* toxicological predictions: 0.5% for DP-1, 0.3% for DP-2, and 0.2% for praziquanamine, CHA, and monohydroxypraziquantel. Subsequently, long-term stability studies were conducted to evaluate the actual degradation profile of the product and refine the proposed specifications. Although several degradation products were initially identified and qualified based on the predicted degradation profile, no impurities were detected throughout the long-term stability study. Based on these findings, a conservative specification of 0.2% for any individual impurity and total impurities was established. Collectively, these results demonstrate that PREVER provides a proactive and scientifically justified approach for impurity qualification and specification setting. By integrating predictive, experimental, and stability data, PREVER supports science-based decision-making aligned with SSbD principles and regulatory expectations.

1. Introduction

Schistosomiasis constitutes a significant helminthic disease, affecting an estimated 250 million individuals, predominantly in low- and middle-income countries across the African continent. The cornerstone of public health interventions aimed at controlling the disease is preventive chemotherapy, which involves the large-scale empirical administration of praziquantel (PZQ) to school-aged children residing in endemic regions. PZQ a derivative of pyrazinoisoquinoline, demonstrates robust anthelmintic efficacy. This active pharmaceutical ingredient (API) is widely utilized in the treatment of schistosomiasis and cysticercosis and is further indicated for various trematode and cestode infections[1–3].

Farmanguinhos, a state-owned Brazilian pharmaceutical company, has successfully developed and registered 600-mg praziquantel tablets. This is an important milestone, as approximately 72,000 individuals were diagnosed with schistosomiasis in Brazil between 2002 and 2022, highlighting the need for effective treatments[4].

The development of these drug products (DPs) complies with regulatory frameworks that increasingly emphasize robust safety investigations during the early stages of development. A significant advancement in this field is the Safe and Sustainable by Design (SSbD) approach, initially introduced by the U.S. Food and Drug Administration (FDA) in 2016 and more recently refined by the European Commission. This approach seeks to minimize potential health risks and hazards by considering the entire life cycle of a product, thereby preventing unnecessary setbacks. The European contribution further provides a structured framework for the voluntary implementation of SSbD, with a strong emphasis on integrating New Approach Methodologies (NAMs). These include data derived from *in vitro* assays, as well as *in silico* models and tools—

such as Quantitative Structure–Activity Relationship (QSAR) models—to support and guide innovation towards the development of safer, non-hazardous products[5,6].

The stability of medicines and pharmaceutical ingredients can be affected by various stress conditions, including oxygen, heat, and light. The context of drug production and storage is susceptible to the emergence of impurities and degradation products. Even in small quantities, their presence can affect the efficacy and safety of pharmaceutical products; consequently, drug purity has always been associated with quality. This investigation is highly important and must be carried out from the beginning and throughout all stages involved in the handling and production of pharmaceutical products[7].

Stress testing, or forced degradation studies, plays a pivotal role in evaluating the intrinsic stability of drug substances (DS) and drug products (DP). When properly designed, these studies not only support the development of stability-indicating methods but also help elucidate degradation pathways and guide formulation, packaging, and shelf-life strategies. Increasingly, in silico tools are being applied to predict chemical degradation and toxicological endpoints. By integrating large datasets, these platforms simulate degradation behavior, assess potential safety risks, and enhance the efficiency of experimental design, ultimately supporting impurity risk assessment. In particular, Quantitative Structure–Activity Relationship (QSAR) models provide a valuable alternative to conventional toxicological testing, aligning with regulatory guidance while reducing reliance on animal studies and resource-intensive approaches. The combined application of forced degradation studies and in silico predictions strengthens science-based decision-making throughout pharmaceutical development[8–12].

Our group has made significant progress in demonstrating the pathway for building quality into stability-indicating analytical methods[13–15]. However, this task

remains challenging, particularly for DP lacking compendial method descriptions and established specifications for related substances[16]. In this context, we propose an innovative framework, the PREVER strategy, which integrates forced degradation studies, long-term stability data, and *in silico* predictions to support the establishment of appropriate specifications for related substances in PZQ 600 mg tablets.

2. Material and methods

2.1. *Chemicals and reagents*

All chemicals and reagents are at least of analytical grade. Purified water was used (Merck Milipore, Germany). Trifluoroacetic acid (TFA) Methanol and Acetonitrile (ACN) HPLC-grade were purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Neon (Rio de Janeiro, Brazil), sodium hydroxide was purchased from Synth (Rio de Janeiro, Brazil) and hydrogen peroxide was purchased from Scharlau (Barcelona, Spain). Praziquantel reference standard was purchased from Brazilian Pharmacopeia and 600 mg Praziquantel tablets were developed at Farmanguinhos (Rio de Janeiro, Brazil).

2.2.*2.2 Methodology*

For the determination of impurity specifications based on the safety profile of degradation products, the PREVER strategy was applied. Developed at Farmanguinhos, PREVER is a proactive process management approach designed to support decision-making during the early stages of pharmaceutical development. As illustrated in Figure 1**,** the acronym PREVER comprises six sequential steps: Prediction, Reproduction, Evaluation, Verification, Establishment, and Review.

Initially, the degradation profile of pharmaceutical interest was predicted using literature review (Prediction), followed by the experimental reproduction of degradation pathways under stress conditions (Reproduction). Subsequently, the related substances identified experimentally were subjected to an i*n silico* toxicological and safety assessment (Evaluation). The predicted degradation behavior was then confirmed through long-term stability studies of the drug product (Verification). Based on the actual degradation data obtained throughout the product lifecycle, impurity acceptance criteria and specifications were defined (Establishment) and subsequently reassessed according to new regulatory requirements and updated scientific knowledge (Review)**.**

[Figure 1]

2.3.*Part I: Prediction of potential pharmaceutically relevant degradation profile*

2.3.1.*Literature review*

A comprehensive search of scientific literature was conducted to identify studies on forced degradation involving the drug praziquantel and/or drug products containing praziquantel. This search was carried out across multiple databases, including ScienceDirect, the Coordination for the Improvement of Higher Education Personnel (CAPES) Journal Portal, PubMed, and Google Scholar. The following keywords were used: praziquantel, indicative stability method, forced degradation, and stability study. Searches were performed in both Portuguese and English.

A thorough review of the retrieved studies was then conducted to highlight key aspects, including the conditions under which degradation occurred, the percentage of degradation products detected, and the analytical parameters described in the respective methodologies.

2.4.*Part II: Experimental assessment of potential pharmaceutically relevant degradation profile*

2.4.1.*Stock solution preparation of PZQ tablets*

A stock solution of PZQ at a concentration of 2 mg/mL was prepared. 20 tablets were ground to a fine powder. Approximately 120 mg of the powder (100 mg of API) was transferred to a volumetric flask and 50 mL of diluent was added. The solution was sonicated for 10 min or until complete dissolution of the API was achieved. After sonication, the volume was completed with diluent to obtain a final concentration of 2 mg/mL of PZQ. The diluent, consisting of 50% v/v methanol in water, was used for the preparation of all sample solutions.

2.4.2.*Control samples preparation of PZQ tablets*

Non-degraded (control) samples were prepared from the stock solution and diluted volumetrically to final concentrations of 250 µg/mL for the analysis of related substances, 50 µg/mL for PZQ content determination, and 0.5 µg/mL for impurity quantification. Following dilution, all samples were filtered through a 0.45 µm hydrophilic membrane filter.

2.4.3.*Stress Testing of PZQ tablets and Endpoints determination*

The selection of the endpoints in the present study was based on the current Brazilian regulatory framework applicable to pharmaceutical products, complemented by evidence from the scientific literature and results from a prior *in silico* study[17–19]. Stress testing was performed under acidic and basic hydrolysis, as well as oxidative, metallic, thermal, and photolytic conditions. The objective was to achieve a degradation level of approximately 10% compared to non-degraded samples.

For the solution-phase stress studies of PZQ tablets, a 1 mg/mL solution of PZQ was subjected to stress conditions by mixing the stock solution with the stressor solution in a 1:1 (v/v) ratio. Acidic hydrolysis was performed using 0.1 M aqueous hydrochloric acid at 70 °C for 3 hours, while basic hydrolysis was conducted with 0.05 M aqueous sodium hydroxide at room temperature for 2 hours. Oxidative degradation was induced using a 3% (v/v) aqueous hydrogen peroxide solution at 70 °C for 10 hours. For the metallic stress condition, a 0.1 M aqueous copper (II) acetate solution was employed at room temperature for 3 hours. After the degradation period, the samples were diluted to final concentrations of 250 and 50 µg/mL for HPLC analysis.

Dry and wet thermal degradation studies were conducted by exposing solid-state PZQ tablets to 70 °C under two distinct conditions: dry heat and high humidity. For the high humidity condition, the samples were placed in open flasks inside a water vapor-saturated environment. In both cases, the tablets were maintained in an oven for 96 hours.

Photodegradation studies were performed in a photostability chamber (Ethik Technology, São Paulo, Brazil), under an overall illumination of 2.4 million lux-hours and a total ultraviolet energy of 2000 Wh/m^2. The solid-state drug was exposed to light inside an inert, transparent glass bottle. A light-protected sample, maintained under identical conditions, served as the control. After the exposure period, the solid samples were reconstituted in the diluent and further diluted to 250 and 50 µg/mL for HPLC analysis.

2.4.4.*Mass Balance and confidence interval*

To evaluate the extent of degradation and ensure the accuracy of quantification, a mass balance assessment was performed. The mass balance was determined using the absolute mass balance (AMB) calculation (Equation 1). The percentage of PZQ

degradation in the drug product was compared with a control sample containing approximately 50 µL/mL of PZQ. The percentage of each degradation product was calculated relative to the area of a standard solution with a concentration of approximately 0.5 µL/mL of PZQ [18,19]. Where: remaining drug is the percentage of API quantified after stress, formed degradation products correspond to the sum of degradation products generated during stress, initial drug assay is the API content before degradation, and impurities prior to degradation correspond to the initial impurity levels in the unstressed sample.

$$AMB = \frac{\%remaining\ drug + \%formed\ degradation\ products}{\%initial\ drug\ assay + \%impurities\ prior\ to\ degradation}\ x\ 100 \qquad \text{Equation 1}$$

To assess the impact of analytical variability on the mass balance calculations, the intermediate precision values of the method, for assay and related substances analyses respectively, were used as reference. The response variance was calculated according to Equation 2 [19]. Where: (1) R = % Absolute mass balance (AMB); (2) a = % Mass balance (MB); (3) b = % Initial content of the sample; (4) σ = Standard deviation

$$\frac{(\sigma_R)^2}{R} = \left[\left(\frac{\sigma_a}{a}\right)^2 + \left(\frac{\sigma_b}{b}\right)^2\right] x\ 100 \qquad \text{Equation 2}$$

The confidence interval value was determined as twice the standard deviation obtained from the experimental variability value, which corresponds approximately to a 95% confidence interval under the assumption of a normal distribution of the data (ANVISA, 2025).

2.4.5. *Stability-indicating method validation*

2.4.5.1. Chromatographic conditions

The samples were evaluated by HPLC, using a Shimadzu HPLC system (Shimadzu, Japan) equipped with a LC 20 AT pump, degassing unit DGU-20A5, autosampler SIL-20AC, column oven CTO-20A, and a Diode Array Detector (DAD) SPD-M20A with a thermostatic flow cell. Shimadzu LC Solution software was used for processing chromatographic data. The analysis was performed using a binary mobile phase. An ACE CN (250 ×4.6 mm, 5 µm) column was used. Mobile phase A was an aqueous solution of TFA 0.025 % and ACN (75:25 v/v) while mobile phase B was ACN. The analyses were performed under isocratic mode, for 25 min. The flow rate was set to 1.5 mL/min and the injection volume was 25 µL (retention time of PZQ: 6.3 min). The absorbance at wavelength 210 nm was monitored. The stability indicative method was previously developed and validated in accordance with ICH requirements.

2.4.5.2. Validation Parameters

The specificity of the method was determined by comparing the chromatograms obtained by injecting the diluent (blank), PZQ standard, forced degradation samples, as well as the placebo and PZQ formulation samples. PZQ peak purity in forced degradation samples was evaluated using a photodiode array detector between 200 – 400 nm.

The linearity of the analytical method was evaluated by constructing three independent calibration curves, each prepared in triplicate. For the assessment of related substances, the concentration range was 0.05 to 0.6 µg/mL, comprising six concentration levels per curve. For content determination, the range was 0.04 to 0.06 mg/mL, with five concentration levels per curve. Calibration plots were obtained by plotting peak area versus analyte concentration. Linearity was statistically assessed by linear regression

analysis using the least squares method and further evaluated by analysis of variance (ANOVA). The homoscedasticity of the method was also examined using the Cochran C-test.

Precision was evaluated through repeatability (intra-day) and intermediate precision (inter-day). Repeatability was assessed by analyzing six individual control samples containing 250 μg/mL of PZQ on the same day. Intermediate precision was determined by repeating the procedure on two consecutive days using two different analysts. For the related substances assessment, control samples (250 μg/mL) were spiked with Related Substance A and Related Substance B at a concentration of 5 μg/mL each. The variability of the results was expressed as the relative standard deviation (RSD).

The accuracy of the method was evaluated through nine determinations across the linear range, using three concentration levels—low, medium, and high—with three replicates per level. The study employed the standard addition (recovery) approach, in which known amounts of the analyte standard were spiked into the sample matrix. For content determination, the spiking levels corresponded to 80%, 100%, and 120% of the nominal sample concentration (50 μg/mL). For related substances, spiking levels were 0.05%, 0.20%, and 0.24% relative to the sample concentration (0.5 μg/mL). Results were expressed as the percentage of praziquantel (PZQ) recovered, and the relative standard deviation (RSD) was calculated at each level to evaluate precision.

The robustness of the analytical method was assessed by introducing deliberate and controlled variations to the chromatographic parameters, and the method's efficiency under each modified condition was evaluated based on the recovery of PZQ. These included adjusting the column temperature to 28°C and 32°C for the related substances method, and to 27°C and 33°C for the PZQ content; varying the flow rate to 1.4 mL/min and 1.6 mL/min; altering the mobile phase composition to acetonitrile:0.025% TFA at

ratios of 24:76 v/v and 26:74 v/v; and employing chromatographic columns from different manufacturing batches and with varying usage histories. Samples were prepared from the stock solution (*item 2.3.1*) and volumetrically diluted in three 20 mL volumetric flasks containing solvent at the working concentration to obtain 50 µg/mL for PZQ content determination, and in three 20 mL volumetric flasks containing placebo at the working concentration to obtain 0.5 µg/mL for impurity quantification. Following dilution, all samples were passed through a 0.45 µm hydrophilic membrane filter prior to chromatographic injection.

2.4.6.*LC-HRMS Studies by ESI-Orbitrap*

Chromatographic separation was performed on an ACE ULTRACORE 2.5 SuperC18 column (50 × 2.1 mm, 2.5 µm; Advanced Chromatography Technologies, Aberdeen, UK) using a Thermo Scientific Vanquish UHPLC system equipped with an LC TriPlus autosampler. Mobile phase A consisted of water containing 0.1% formic acid and 5 mM ammonium formate, and mobile phase B was acetonitrile with 0.1% formic acid. Chromatographic elution was performed at a flow rate of 0.300 mL/min and a column temperature of 40 °C. The gradient program was as follows: 0–0.5 min, 5% B; 0.5–1.0 min, 5–10% B; 1.0–2.0 min, 10–25% B; 2.0–10.0 min, 25–90% B; 10.0–14.0 min, 90–100% B; 14.0–16.0 min, 100% B; 16.0–16.1 min, 100–5% B; 16.1–20.0 min, 5% B (re-equilibration). Total run time was 20 min.

High-resolution mass spectrometric data were acquired on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a heated electrospray ionization source (HESI). Mass calibration was performed daily using the manufacturer's calibration solution prior to sample analysis, with lock masses set at m/z 391.2843 and 445.1200 in positive mode. The capillary temperature was set to 380 °C, the spray voltage was 3.90 kV, the S-lens RF level was

80.0, and the auxiliary gas heater temperature was 380 °C. Source gas parameters were a sheath gas flow rate of 60, an auxiliary gas flow rate of 20, and a sweep gas flow rate of 0 (arbitrary units).

Data were acquired in a data-dependent acquisition (DDA) mode using a Full MS/dd-MS^2 (Top10) experiment over a total runtime of 20 min. Full scan MS data were collected in positive ionization mode over a scan range of m/z 200–800 at a resolving power of 70,000 FWHM (at m/z 200), an AGC target of 1×10^6, a maximum injection time of 50 ms, and 10 microscans. Data-dependent MS^2 fragmentation (dd-MS^2) was performed at a resolving power of 17,500 FWHM, an AGC target of 1×10^5, and a maximum injection time of 20 ms, with a loop count of 10, MSX count of 1, TopN of 10, and an isolation window of 2.0 m/z. Fragmentation was performed using stepped normalized collision energy (NCE) of 30 eV. Data-dependent settings included a minimum AGC target of 8.0×10^3, an intensity threshold of 4.0×10^5, a dynamic exclusion window of 30.0 s, and isotope exclusion enabled. All spectra were acquired in centroid mode. Data acquisition and processing were performed using Thermo Fisher Scientific TraceFinder 4.0 software.The molecular formulas obtained were in good agreement with the theoretical values expected for the analyzed compounds, presenting mass deviations within ±5 ppm.

2.5.*Part III: In silico qualification of potential related substances*

2.5.1.*In silico toxicological profile of PZQ impurities*

The in vitro Ames mutagenicity of the PZQ impurities — praziquanamine, PZQ impurity (theoretical mass = 330.19434), cyclohexanecarboxylic acid, PZQ impurity (theoretical mass = 310.16813), and monohydroxypraziquantel — was predicted using

the QSAR Toolbox version 4.8 (Database version 4.8), freely accessible through the OECD platform (https://qsartoolbox.org/download/).

The Ames test evaluates the potential of a compound to induce point mutations in the DNA of Salmonella typhimurium. It employs several bacterial strains (TA1537, TA1535, TA100, TA98, and TA97), each optimized to detect specific classes of mutagenic agents. The standard version of the assay identifies only direct-acting mutagens — substances capable of interacting with DNA without the need for metabolic activation[20].

Similarly, the systemic toxicity of the same PZQ impurities was predicted using the QSAR Toolbox to support the derivation of acceptable exposure limits. The Acceptable Level (AL) was determined based on the predicted No Observed Effect Level (NOEL) obtained from repeated-dose toxicity studies in Rattus norvegicus (oral, gavage administration). The AL was calculated according to the following equation[21]:

$$AL\left(\frac{\mu g}{d}\right) = \frac{PoD\left(\frac{mg}{kg \cdot d}\right) \times 50\ kg \times 1000}{AF1 \times AF2 \times AF3 \times AF4 \times AF5 \times AF6 \times AF7} \qquad \text{Equation 3}$$

where:

AL (µg/d) – Acceptable Level of the impurity, expressed in micrograms per day.

PoD (mg/kg/d) – Point of Departure, derived from toxicological studies (NOEL or NOAEL).

50 kg – Default human body weight assumed for calculation purposes.

1000 – Conversion factor from milligrams to micrograms.

AF1–AF7 – Adjustment (assessment) factors accounting for interspecies differences, intraspecies variability, exposure duration, dose–response extrapolation, severity of effect, data completeness, and other uncertainties.

Both the Ames mutagenicity and systemic toxicity predictions were performed within the QSAR Toolbox using the *read-across* approach. In each case, five structurally

and physiochemically similar analogues were selected, under the assumption that compounds belonging to the same chemical category exhibit comparable toxicological behavior. Structural similarity, *log P* values, and mechanistic relevance were the main criteria applied for analogue selection[22].

2.6.*Part IV: Evaluation of real degradation profile and specification definitions*

2.6.1.*Stability studies of PZQ tablets*

The stability study was conducted following ICH guidelines for climatic zone IVb. Tablets were stored in a stability chamber at 30 °C ± 2 °C and 75% ± 5% relative humidity (RH) for 24 months. After this period, the tablets were finely powdered for sample preparation. Twenty tablets were accurately weighed and ground using a mortar and pestle. Precisely 120 mg of powder, equivalent to 100 mg of praziquantel, were weighed in triplicate for assay and in duplicate for related substances (SR) analysis and transferred to 100 mL volumetric flasks. Approximately 80 mL of diluent were added, followed by 5 minutes of sonication with occasional agitation. The volume was completed with diluent and homogenized. For assay, a 5.0 mL aliquot was transferred to a 100 mL volumetric flask, diluted to volume to obtain a final concentration of 0.05 mg/mL. For SR, the aliquot was diluted in a 20 mL volumetric flask to a final concentration of 0.25 mg/mL. All solutions were filtered through a 0.45 µm polytetrafluoroethylene (PTFE) membrane prior to HPLC analysis, discarding the first 5 mL of filtrate[23]

2.5.1.*Specification definitions*

The drug product specification was established using the PREVER approach, which integrates the systematic assessment of impurity safety with real degradation behavior throughout pharmaceutical development. This methodology involved the prediction of degradation products, the experimental reproduction and identification of impurities, the in silico toxicological evaluation of the related substances, and the verification of the actual degradation profile under long-term stability conditions. Based on these data, impurity acceptance criteria and specifications were established and supported by a science-based strategy of the potential toxicity of the detected impurities.[24].

3. Results and Discussion

3.1.*Part I: Prediction of potential pharmaceutically relevant degradation profile*

3.1.1.*Literature review*

Four studies reporting forced degradation of PZQ were identified in the literature. According to published data, PZQ remains stable in its original or solid state under dry/wet thermal and photolytic conditions, showing major degradation only under acidic and alkaline hydrolysis in solution, and minor degradation under oxidative and neutral conditions. Figure 2 presents the known degradation products of PZQ reported in the literature.

[Figure 2]

In the study by Kumar and Chalannavar (2022), PZQ degraded predominantly under acidic and alkaline hydrolysis. Praziquanamine (theorical mass = 202.11061) was detected only under acidic hydrolysis, formed by cleavage of the cyclohexanecarbonyl group from PZQ through amide hydrolysis. This reaction yields both praziquanamine and cyclohexanecarboxylic acid (theorical mass = 128.08373 — not detected in the study) (Figure 2). In addition, a known PZQ impurity with theorical mass of 330.19434 was formed under both acidic and alkaline hydrolysis conditions and was structurally characterized. The formation of this impurity from PZQ under acidic and basic degradation conditions is explained by hydrolysis of the amide functional group in the piperazine ring (Figure 2). The authors also reported the presence of another impurity formed under alkaline hydrolysis; however, it was suggested that this product was a secondary by product of the mass of 330 impurity, which, under elevated temperature, may undergo a transamidation reaction—where the amine portion of an amide is replaced by another amine—resulting in a new amide, thus classifying it as a secondary impurity.

Furthermore, slight degradation was observed under neutral (1.2%) and peroxide oxidation (0.3%) conditions, forming an unknown product referred to as DP-4 with theorical mass 310.16813 [25]

Špehar et al. (2021) reported a degradation profile similar to that described above, in which praziquanamine and the known impurity (theorical mass = 330.19434) were observed after acidic and alkaline hydrolysis, respectively (Figure 2). Under peroxide oxidation, two known products were predominantly detected: di-hydroxypraziquantel (theorical mass = 344.17361) and mono-hydroxypraziquantel (theorical mass = 328.17869), along with two unknown impurities (theorical mass = 147 and 216). The first two are classified as oxidative metabolites of PZQ (Figure 2). In addition, the authors conducted an accelerated stability study (40 °C/75% RH) by storing solid-state PZQ in a climatic chamber for three months. At the end of the study, only PZQ-related substance B was detected, with a drug loss of just 2.18%, confirming the product's stability under wet thermal degradation [26].

Havlíková et al. (2016) also detected praziquanamine after acidic hydrolysis of PZQ. In the same study, the authors reported an additional degradation product formed under acidic hydrolysis conditions. This compound was assigned the molecular formula $C_{19}H_{24}N_2O_4$, with a theoretical exact mass of 344.17361, and was detected as the protonated ion $[M+H]^+$ at m/z 345.2 in positive ion full-scan MS. Under alkaline hydrolysis, only the known PZQ impurity with a theoretical exact mass of 330.19434 was observed (Figure 2) [27]. Hashem et al. (2017) also identified degradation products qualitatively under solution-phase conditions (acidic/alkaline hydrolysis and peroxide oxidation) [28].

Overall, all cited studies demonstrated a similar PZQ degradation profile, with major degradation occurring under acidic and alkaline hydrolysis, producing

praziquanamine and the known impurity (theorical mass = 330.19434). Under peroxide oxidation, products primarily related to the oxidative metabolism of PZQ were formed (Figure 2).

3.2. *Part II: Experimental assessment of potential pharmaceutically relevant degradation profile*

3.2.1. *Stress Testing of PZQ tablets and Endpoints determination*

The rationale for selecting the endpoints under forced degradation conditions was established through an expert scientific judgment to anticipate potential degradation pathways of PZQ. The resulting insights were employed to refine the experimental design of the forced degradation study and to perform a rational assessment of the API's reactivity with excipients, including the potential formation of excipient-related impurities [12]. Thus, based on the scientific investigation of the probable degradation pathways of PZQ and its experimentally determined degradation profile. The following parameters were defined: (1) concentration of the degrading agent, (2) specific reaction temperature, and (3) reaction duration.

The results of the forced degradation studies are summarized in Table 2. The absolute mass balance values were considered acceptable, remaining within the confidence interval calculated from the standard deviation data of intermediate precision. A total of four degradation products (DPs) were detected above the reporting threshold (0.05%) after the degradation study, including praziquanamine, which has been previously reported as a degradation product of PZQ. In all forced degradation studies, the developed method demonstrated selectivity for the impurities listed in the specification, for those not listed in the specification, and for the API. PZQ eluted at a

retention time (RT) of 6.3 minutes, while praziquanamine, DP-1, DP-2 and DP-3, eluted at RRTs of 0.41, 0.55, 0.73 and 0.81 minutes, respectively.

[Table 1]

In this study, PZQ was initially evaluated under acidic and alkaline hydrolysis, oxidative degradation (peroxide and metal-catalyzed conditions), and thermal degradation. A subsequent photodegradation study was also performed. Mild degradation conditions were first assessed in accordance with literature and WHO guidelines [17,29]. PZQ in the drug product remained stable under thermal and photolytic stress, as well as oxidation by metal ions. In contrast, significant API degradation (approximately 10%) was observed only under acidic and alkaline hydrolysis, as well as peroxide-induced oxidation. The chromatograms obtained from the DAD detector are presented in Figure 3, respectively.

[Figure 3]

Under acidic hydrolysis stress, praziquanamine and DP-1 were detected, with 89.64% of PZQ degraded after 3 h of heating. In addition, a low-intensity signal at a retention time of 4.327 min was observed at a relative level of 0.2%. This signal may indicate the formation of a minor secondary degradation product under acidic conditions in the presence of methanol. Under alkaline hydrolysis, only DP-1 was detected, with 89.51% of PZQ degraded at room temperature after 2 h. In addition, three significant degradation products (DP-2 and DP-3), including praziquanamine, were detected following oxidative degradation with peroxide, with 90,99% of PZQ degraded after 10 h of heating. Absolute mass balance values of 92%, 104%, and 91% were achieved for acidic hydrolysis, alkaline hydrolysis, and oxidative degradation, respectively. The variation in mass balance can be justified and adjusted based on the expected

experimental variability, as determined by the RSD obtained during the intermediate precision assessment in the analytical method validation for assay and related substances (RS) of PZQ. The RSD values for assay and RS were 3.0% and 3.6%, respectively, both within the specification limits. Accordingly, a confidence interval of 84–100%, 95–113% and 83–100% was calculated for acidic hydrolysis, alkaline hydrolysis, and oxidative degradation, respectively, reaching 100% in all cases, as recommended by the Brazilian guideline [19].

It is noteworthy that the formation of praziquanamine occurs concomitantly with the formation of cyclohexanecarboxylic acid in a stoichiometric proportion. However, this compound was not detected experimentally—unlike praziquanamine—likely due to the loss of its chromophore group. Consequently, the impurity deficit observed in the mass balance for acidic and oxidative degradation may be related to the low detectability of cyclohexanecarboxylic acid. Considering the stoichiometric relationship between praziquanamine and cyclohexanecarboxylic acid formation, it is inferred that both are generated in equivalent amounts. Furthermore, since praziquanamine is not formed during the long-term stability study of the drug product, it is reasonable to conclude that cyclohexanecarboxylic acid is also absent from the actual degradation profile of PZQ.

Moreover, under oxidative forced degradation conditions, three degradation products of PZQ were experimentally detected, including praziquanamine, as also reported in scientific literature [26].

3.2.2.*Stability-indicating method validation*

3.2.2.1.Chromatography conditions

The objective of a selective method was to separate all impurities from PZQ. The chromatographic method for the determination of PZQ and its impurities employs

reversed-phase systems with an *end-capped* C18 column as the stationary phase and an acidic binary mobile phase consisting of ACN as the organic solvent and an aqueous solution of TFA. Screening protocols for the development of an advanced chromatographic method in this study were based on these previously described approaches [27,28].

Here, samples were evaluated using DAD detectors. In addition, acidic mobile phases were tested with TFA, in different concentrations and binary mixtures of TFA and ACN in different proportions. A binary mobile phase with aqueous solution of TFA 0.025 % and ACN (75:25 v/v) (mobile phase A), in combination with a C18 column, showed superior separation efficiency in terms of resolution, sensitivity, and retention. ACN (mobile phase B) was selected as the organic modifier due to its lower viscosity and reduced baseline noise compared to methanol. Selective separation was achieved under isocratic elution mode. A detection wavelength of 210 nm was chosen, allowing better visualization of the degradation products present in the samples. PZQ eluted with a retention time (RT) of approximately 6.3 minutes.

The chromatographic signal of PZQ exhibited a purity value of 1.000, confirming the absence of co-eluting peaks. Furthermore, the chromatographic data obtained was considered satisfactory for all detected compounds, with resolutions greater than 1.5, theoretical plates exceeding 2000, and peak asymmetry between 0.8 and 1.2, as recommended by the USP [30].

3.2.2.2.Validation Parameters

The proposed method was validated in accordance with ICH guideline Q2(R2)[31] and RDC 166/2017[32] for selectivity, linearity, precision, accuracy, and robustness. The analytical procedure validation results for PZQ and its impurities are presented in Table 3.

548 [Table 2]

549 The method demonstrated selectivity, linearity in the range of 0.04–0.06 mg/mL
550 for PZQ assay and 0.05–0.6 μg/mL for impurities (R > 0.99), as well as satisfactory
551 precision and accuracy.

552 Repeatability and intermediate precision of PZQ at a concentration of 250 μg/mL
553 were evaluated (Table 3). Repeatability was assessed on the same day, while intermediate
554 precision was assessed over three separate days. The maximum %RSD values were below
555 2.0% and 3.7% for the PZQ assay, and below 6% and 11% for impurities, for repeatability
556 and intermediate precision, respectively, indicating that the developed method exhibited
557 high precision. The method also proved to be accurate and robust, with good PZQ
558 recovery at the tested concentrations, even when deliberate variations were introduced
559 into the analytical procedure. The %RSD remained below 3.7% for PZQ assay and below
560 7.3% for related substances (Table 3).

561

562 3.2.3.*LC-HRMS Studies by ESI-Orbitrap*

563 LC–HRMS analysis using ESI–Orbitrap-MS/MS was performed to characterize
564 and support the structural confirmation of the degradation products (DPs) of PZQ. The
565 instrumental parameters were optimized to enhance the responses of the protonated
566 molecular ions $[M+H]^+$ of the analytes under investigation. The monoisotopic masses of
567 the expected DPs were previously calculated based on their elemental compositions.
568 Initially, PZQ was analyzed to establish its mass spectral behavior and to provide a basis
569 for comparison with the DPs formed under stress conditions[33]. The proposed elemental
570 compositions for PZQ and its DPs showed mass errors ranging from −1.48 to 4.75 ppm
571 (Table 4), with all values within the commonly accepted ±5 ppm tolerance for HRMS-
572 based assignments.

573 [Table 3]

The reconstructed ion chromatograms (RIC) obtained by ESI-Orbitrap-MS/MS for the PZQ, are shown in Figure 4. The ESI-Orbitrap-MS/MS spectrum of PZQ showed the protonated molecular ion $[M+H]^+$ at m/z 313.19037, corresponding to the elemental composition $C_{19}H_{25}N_2O_2^+$, with a mass error of −2.19003 ppm (Figure 4A). The fragmentation profile was characterized by the formation of an abundant product ion at m/z 203.11781, assigned to $C_{12}H_{15}ON_2^+$, which represented the base peak of the spectrum and was consistent with cleavage of the cyclohexylcarbonyl moiety from the PZQ structure. This pathway is also supported by the formation of the complementary cyclohexanecarboxylic acid-related fragment, as represented in the proposed fragmentation scheme (Figure 4B). Another relevant fragment was observed at m/z 256.16940, assigned to $C_{17}H_{22}ON^+$, suggesting cleavage of the lactam/amide portion of the molecule while retaining the cyclohexylcarbonyl-containing fragment. According to the proposed fragmentation pathway, this ion may subsequently undergo further dissociation to generate the ion at m/z 174.09132, assigned to $C_{11}H_{12}ON^+$. Additional fragmentation of this isoquinoline-containing ion may lead to the formation of the product ion at m/z 146.09627, assigned to $C_{10}H_{12}N^+$, probably through loss of a carbonyl-containing neutral fragment. A lower-mass ion at m/z 83.08640, assigned to $C_6H_{11}^+$, was also observed, supporting the contribution of the cyclohexyl moiety to the fragmentation pathway. Overall, the MS/MS spectrum of PZQ was dominated by cleavage of the cyclohexylcarbonyl group and subsequent fragmentation of the pyrazinoisoquinoline nucleus, as summarized in the proposed fragmentation scheme shown in Figure 4B.

[Figure 4]

The ESI-Orbitrap-MS/MS spectra of the PZQ-related impurities showed diagnostic fragments consistent with the proposed structures and with the main fragmentation pathways observed for the drug substance. Overall, the dissociation profiles indicated partial preservation of the isoquinoline/pyrazinoisoquinoline core of PZQ, as well as cleavages associated with the cyclohexylcarbonyl group and the amide or lactam moieties of the molecule. In addition, the degradation products identified in this study are in agreement with findings previously reported in scientific literature[25–27]. The fragmentation spectra of the DPs are shown in Figure 5, whereas the proposed fragmentation pathway is presented in Figure 6.

Praziquanamine, detected under acidic hydrolysis and oxidative degradation with peroxide, showed a protonated ion at m/z 203.11768, corresponding to the elemental composition $C_{12}H_{15}ON_2^+$. This ion was also observed as the base peak in the MS/MS spectrum of PZQ, indicating that praziquanamine represents the heterocyclic portion formed after cleavage of the cyclohexylcarbonyl moiety from PZQ. Subsequent fragmentation generated ions at m/z 174.09123 and 146.09627, assigned to $C_{11}H_{12}ON^+$ and $C_{10}H_{12}N^+$, respectively. These fragments are consistent with successive neutral losses from the pyrazinoisoquinoline core, mainly involving the carbonyl/lactam portion. The presence of these ions reinforces that praziquanamine retains the characteristic heterocyclic core of PZQ but lacks the cyclohexylcarbonyl chain (Figure 5 and Figure 6).

DP-1, detected under acidic and basic hydrolysis conditions, showed a protonated ion at m/z 331.20093, compatible with the elemental composition $C_{19}H_{27}O_3N_2^+$. The proposed structure suggests opening of the pyrazine/lactam ring of PZQ, with formation of a carboxylic acid group and retention of the cyclohexylcarbonyl moiety. In the MS/MS spectrum, fragments at m/z 285.19537 and 256.16925 were observed, which may be related to cleavages of the open-chain structure and neutral losses involving the

carboxylic acid function and/or the amide portion. The ion at m/z 256.16925 is particularly relevant, since it was also observed in the fragmentation of PZQ, suggesting a common dissociation pathway with retention of the cyclohexyl-containing fragment. In addition, the ions at m/z 175.12299 and 146.09631 indicate subsequent fragmentation of the isoquinoline region, confirming the preservation of this structural core in DP-1 (Figure 5 and Figure 6).

Additionally, under acidic conditions in the presence of methanol, a signal at 4.327 min was observed, compatible with a possible secondary degradation product (Figure 3). This compound exhibited a protonated ion $[M+H]^+$ at m/z 345.21727, consistent with the proposed methyl ester derivative. The proposed structure suggests formation of the corresponding methyl ester, possibly through acid-catalyzed esterification of the carboxylic acid moiety. This assignment is supported by the expected mass increase of 14.01565 Da relative to DP-1, corresponding to the conversion of the carboxylic acid group into a methyl ester, from the theoretical exact mass of 330.19434 Da for DP-1 to 344.20999 Da for the proposed methyl ester derivative (Figure 6). Acid-catalyzed esterification of carboxylic acids in alcoholic media is a well-established reaction, and methanolic HCl has been described as an effective reagent for the formation of methyl esters from free carboxylic acids [34]. In the proposed fragmentation pathway, this secondary degradation product may generate the diagnostic ion at m/z 203.11789, associated with the pyrazinoisoquinoline-related core, followed by further fragmentation to m/z 146.09643. In parallel, the ion at m/z 256.16959 may arise from an alternative cleavage pathway involving retention of the cyclohexyl-containing fragment.

DP-2, detected under oxidative degradation with peroxide, showed a protonated ion at m/z 311.17657, assigned to the elemental composition $C_{19}H_{23}O_2N_2^+$. Compared with protonated PZQ, observed at m/z 313.19037, this difference of approximately 2 Da

suggests a product with a lower degree of hydrogenation, compatible with the formation of an additional unsaturation or a dehydrogenation process relative to the original PZQ structure. The MS/MS spectrum showed fragments at m/z 283.18045, 201.10223, and 173.10738. The fragment at m/z 283.18045 may be associated with the loss of a small neutral molecule from the precursor ion, whereas the ions at m/z 201.10223 and 173.10738 indicate fragmentation involving the heterocyclic core. The presence of a fragment close to the ion at m/z 203, observed for PZQ, suggests that DP-2 preserves the pyrazinoisoquinoline substructure, although modified by an additional unsaturation (Figure 5 and Figure 6).

DP-3, also detected under oxidative degradation with peroxide, showed a protonated ion at m/z 329.18567, corresponding to the elemental composition $C_{19}H_{25}O_3N_2^+$. This composition is consistent with the addition of one oxygen atom relative to protonated PZQ, supporting the assignment of DP-3 as a monohydroxylated PZQ derivative. A diagnostic product ion was detected at m/z 219.11269, assigned to $C_{12}H_{15}O_2N_2^+$. This ion is particularly important because it can be interpreted as the oxygenated analogue of the characteristic PZQ fragment at m/z 203.11781 ($C_{12}H_{15}ON_2^+$), generated after cleavage of the cyclohexylcarbonyl moiety. The mass difference of approximately 15.995 Da between these two fragments indicates retention of the additional oxygen atom in the pyrazinoisoquinoline-related portion of the molecule, supporting hydroxylation of the heterocyclic core rather than the cyclohexyl group. The ion at m/z 219.11269 may undergo subsequent fragmentation to generate the base peak at m/z 191.11781, assigned to $C_{11}H_{15}ON_2^+$, probably through the loss of CO. This sequential fragmentation is consistent with dissociation of the oxygenated heterocyclic core. The additional product ion at m/z 134.09656 ($C_9H_{12}N^+$) may be attributed to further cleavage and rearrangement of the isoquinoline-containing portion of the molecule.

Overall, the formation of the ion at m/z 219.11269 represents the strongest evidence for the proposed DP-3 structure, since it preserves the elemental features of the pyrazinoisoquinoline fragment while incorporating the additional oxygen atom. Therefore, the observed fragmentation behavior supports the tentative assignment of DP-3 as a monohydroxylated degradation product of PZQ, with the hydroxyl group located in the heterocyclic core (Figure 5 and Figure 6).

[Figure 5]

[Figure 6]

Taken together, the fragmentation data indicates that the identified impurities arise from structural modifications of PZQ involving cleavage of the cyclohexylcarbonyl group, opening of the heterocyclic ring, dehydrogenation, oxidation, or formation of a secondary product under acidic methanolic conditions. The presence of common ions, especially at m/z 203, 256, 174/175, and 146, reveal shared fragmentation pathways and confirm the preservation of characteristic PZQ substructures. Thus, the MS/MS spectra support the proposed structures of the degradation products and demonstrates their direct relationship with the pyrazinoisoquinoline scaffold of praziquantel.

3.3.*Part III: In silico qualification of potential related substances*

3.3.1.*In silico toxicological profile of PZQ impurities*

According to the Brazilian Health Regulatory Agency, in 2023 the impurity section of the Common Technical Document (CTD), a regulatory dossier, was the second most frequently questioned. Major objections were raised when deficiencies that could be addressed were identified, as well as in cases involving other noncritical issues requiring clarification. In some instances, the section was refused when regulatory

699 requirements were not met. This underscores the importance of establishing impurity
700 specifications based on evaluation data that demonstrates the biological safety of an
701 individual impurity or an impurity profile at the specified level(s)[35]. At the same time,
702 regulatory authorities are increasingly adopting *in silico* technologies to improve
703 efficiency, cost-effectiveness, and ethical aspects of safety assessments. In genotoxicity
704 testing, these model are recognized by the ICH M7 guideline for predicting bacterial
705 mutagenicity and have demonstrated accuracy comparable to laboratory studies. When
706 sufficiently reliable, in silico methods can replace or complement experimental testing,
707 strengthening regulatory evaluations through a weight-of-evidence approach[36].

708 The target compound was characterized in the OECD QSAR Toolbox using
709 structural, mechanistic, and metabolic profilers to support prediction of the endpoint
710 "Human Health Hazards → Genetic Toxicity → in vitro → Bacterial Reverse Mutation
711 Assay (Ames Test) → Gene mutation → *Salmonella typhimurium* → With and Without
712 metabolic activation." Analogues with similar structural or mechanistic features were
713 identified and grouped into a chemical category, and experimental data were collected
714 from the Bacterial Mutagenicity ISSSTY, Toxicity Japan MHLW, Genotoxicity Pesticides
715 EFSA, ECHA REACH (Test Materials), and Genotoxicity OASIS databases. Category
716 consistency was evaluated to ensure similar mutagenic behavior among analogues, and
717 when coherence was observed, the mutagenicity outcome was extrapolated to the target
718 compound through the read-across approach[37–39].

719 The results of all five impurities investigated are presented in Figure 7 and Figure
720 8. The plot depicts the correlation between the experimental outcomes of chemical
721 analogues (y-axis) and a selected molecular descriptor (x-axis), with logP serving as the
722 default parameter. The red dot represents the predicted value for targets compounds,
723 obtained from the relationship between the descriptor and the brown dots, which

correspond to the analogues included in the read-across analysis. The blue dots indicate experimental data from analogues that were excluded from the analysis due to insufficient structural similarity or limited relevance. The prediction was generated using data from five analogue compounds. According to the prediction, all five impurities, namely praziquanamine (A), DP-1 (theoretical mass = 330.19434, B), cyclohexanecarboxylic acid (C), DP-2 (theoretical mass = 310.16813, D), and monohydroxypraziquantel (E), showed negative results in the Ames test for gene mutation, both with and without metabolic activation.

The determination of interim impurity specifications during the early development phase is essential for defining decision criteria that support long-term stability studies and guide the overall product development strategy. In the present study, the NOEL-based read-across assessment (Figure 7) was applied to estimate the toxicological reference values for the identified impurities: praziquanamine (161 mg/kg/day), DP-1(theoretical mass = 330.19434; 352 mg/kg/day), cyclohexanecarboxylic acid (121 mg/kg/day), DP-2(theoretical mass = 310.16813, 196 mg/kg/day) and monohydroxypraziquantel (118 mg/kg/day).

Based on the predicted NOEL values, acceptable limits (AL) for each impurity were derived using the assessment factors (AF1–AF7) recommended by the ICH Q3D(R2) framework. AF1, accounting for interspecies extrapolation, was set at 5, corresponding to data from *Rattus norvegicus*. AF2, a factor of 10, was applied to address interindividual human variability. AF3 was set at 2, rather than 10, considering the short-term administration of praziquantel and the limited treatment duration. This adjustment is consistent with the use of short-term toxicity studies (up to one month) from structural analogues employed in the read-across approach [21,40].

AF4 and AF5, which address severe toxicity, were both set to 1, as the NOEL was considered a conservative and sufficiently protective point of departure. AF6, which compensates for differences in the route of exposure, was set at 2, considering the oral administration and the reported oral bioavailability of praziquantel of up to 80.[41] Finally, a conservative AF7 factor of 5 was applied, considering that several analogues used in the prediction exhibited structural similarity below 50%[21].

Based on these parameters and the previously described equation, the acceptable limits (AL) and interim impurity specifications were determined as follows: praziquanamine (8,050 µg/day; 0.2%), DP-1(theoretical mass = 330.19434; 17,600 µg/day; 0.5%), cyclohexanecarboxylic acid (6,050 µg/day; 0.2%), DP-2 (theoretical mass = 310.16813, 9,800 µg/day; 0.3%), and monohydroxypraziquantel (5,900 µg/day; 0.2%). These values were calculated considering a maximum daily dose of 3500 mg for praziquantel[23].

Overall, the results indicate that the proposed specifications were derived using a conservative approach for most impurities, as the calculated limits remained abovethe conventional qualification threshold of 0.15% established for praziquantel-related substances. This outcome supports the toxicological acceptability of the impurities and provides a sound basis for defining interim specifications during the early stages of product development[23].

[Figure 7]

[Figure 8]

3.4.*Part IV: Evaluation of real degradation profile and specification definitions*

772 3.4.1.*Stability studies of PZQ tablets and specification definitions*

773 The stability study was conducted in accordance with the ICH guidelines for
774 climatic zone IVb. Tablets were stored in a stability chamber at 30 °C ± 2 °C and 75% ±
775 5% relative humidity (RH) for 24 months. At the end of the study, the samples were
776 evaluated using the validated stability-indicating method, ensuring adequate separation
777 and chromatographic performance for all degradation products detected in the predictive
778 profile. The results of the actual impurity profile of PZQ are shown in Figure 9.

779 [Figure 9]

780

781 Figure 9 demonstrates the absence of individual chromatographic signals above
782 0.05%, corresponding to the reporting threshold, which supports the selected degradation
783 endpoints and confirms the stability of PZQ under the proposed storage conditions.
784 Furthermore, praziquanamine, a relevant impurity previously identified in theoretical and
785 forced degradation studies, was not detected at the end of the stability study. Considering
786 the expected stoichiometric relationship between praziquanamine and
787 cyclohexanecarboxylic acid formation, these findings also suggest that
788 cyclohexanecarboxylic acid was not generated at detectable levels under the evaluated
789 conditions.

790 Overall, the results indicate that all five impurities identified during the forced
791 degradation studies — praziquanamine, DP-1(theoretical mass = 330.19434),
792 cyclohexanecarboxylic acid, DP-2(theoretical mass = 310.16813), and
793 monohydroxypraziquantel — were absent under long-term stability conditions.
794 According to the PREVER strategy, shelf-life impurity specifications should be
795 established based on the real degradation profile obtained during stability studies rather
796 than exclusively on forced degradation data. Therefore, a specification limit of 0.2% for
797 any individual impurity and total impurities was established using a conservative risk-

based approach, considering the lowest toxicological threshold predicted for the most critical impurities identified during forced degradation studies[42].

4. Conclusion

The establishment of specifications for impurity control remains a complex challenge, particularly during the early stages of the pharmaceutical product lifecycle, when limited information regarding product stability is available. In this context, the present study aimed to present the PREVER strategy as a guideline for a proactive approach to specification setting, based on the results obtained for the pharmaceutical product containing 600 mg praziquantel tablets developed by Farmanguinhos, in support of the product registration dossier. The results demonstrated that the pharmaceutically relevant degradation profile predicted in silico was consistent with the potential degradation profile established through forced degradation studies. Consequently, interim specifications were established for the five relevant impurities based on in silico toxicity predictions, namely: praziquanamine (0.2%), DP-1(theoretical mass = 330.19434; 0.5%), cyclohexanecarboxylic acid (0.2%), DP-2 (theoretical mass = 310.16813 (0.3%), and monohydroxypraziquantel (0.2%). However, the results obtained from the real degradation profile during long-term stability studies indicated that no impurities were detected over 24 months. Therefore, a conservative specification of 0.2% for any individual impurity and total impurities was established. Our findings indicate that the PREVER approach is aligned with the principles of Safe and Sustainable by Design (SSbD) and can provide a safe and well-defined pathway for impurity qualification processes and specification setting in pharmaceutical products.

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Determination of Impurity Specification

STEP 1:
P
PREDICTION

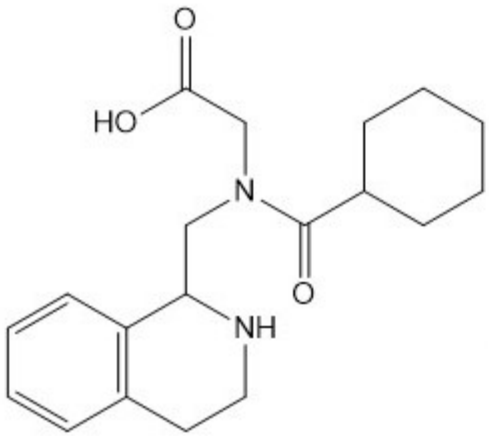
STEP 2:
R
REPRODUCTION

STEP 3:
E
EVALUATION

STEP 4:
V
VERIFICATION

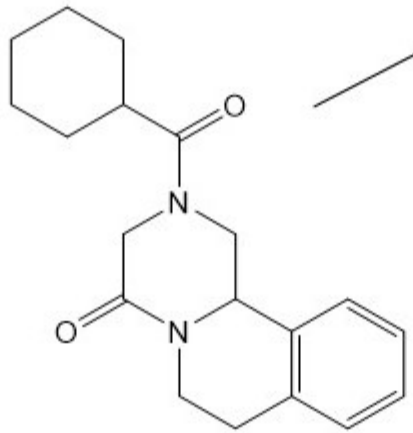
STEP 5:
E
ESTABLISHMENT

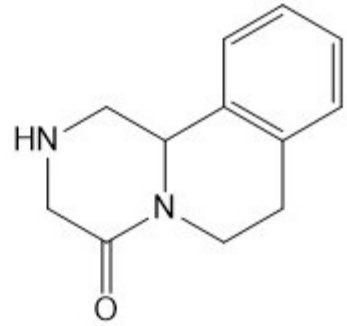
STEP 6:
R
REVIEW



Theorical mass: 330.19434
Acid and basic hydrolysis

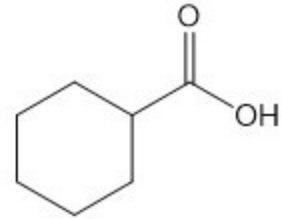
Praziquantel
Theorical mass: 312.18378



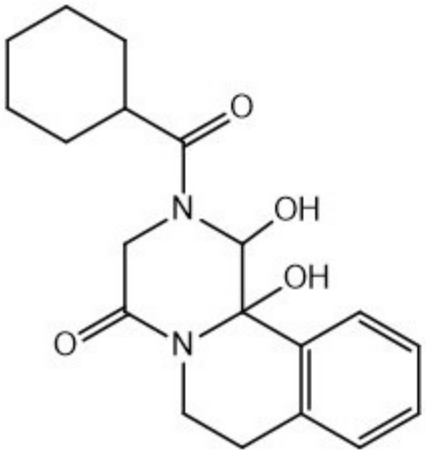


Praziquanamine
Theorical mass: 202.11061
Acid and oxidative degradation

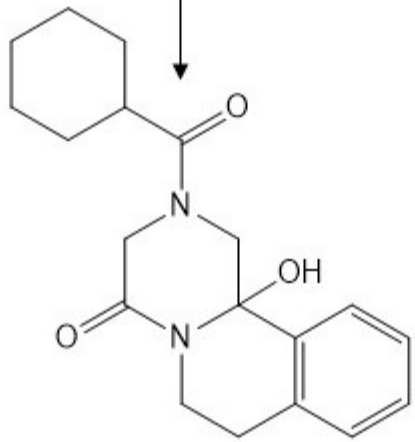
+



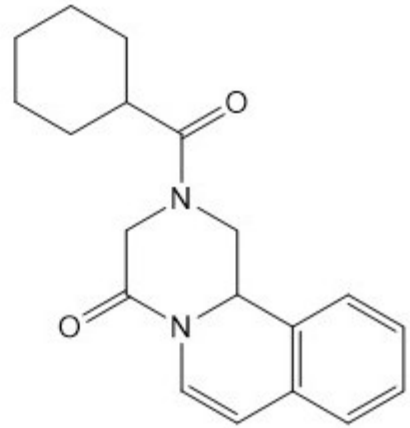
Cyclohexanecarboxylic acid
Theorical mass: 128.08373
Acid and oxidative degradation



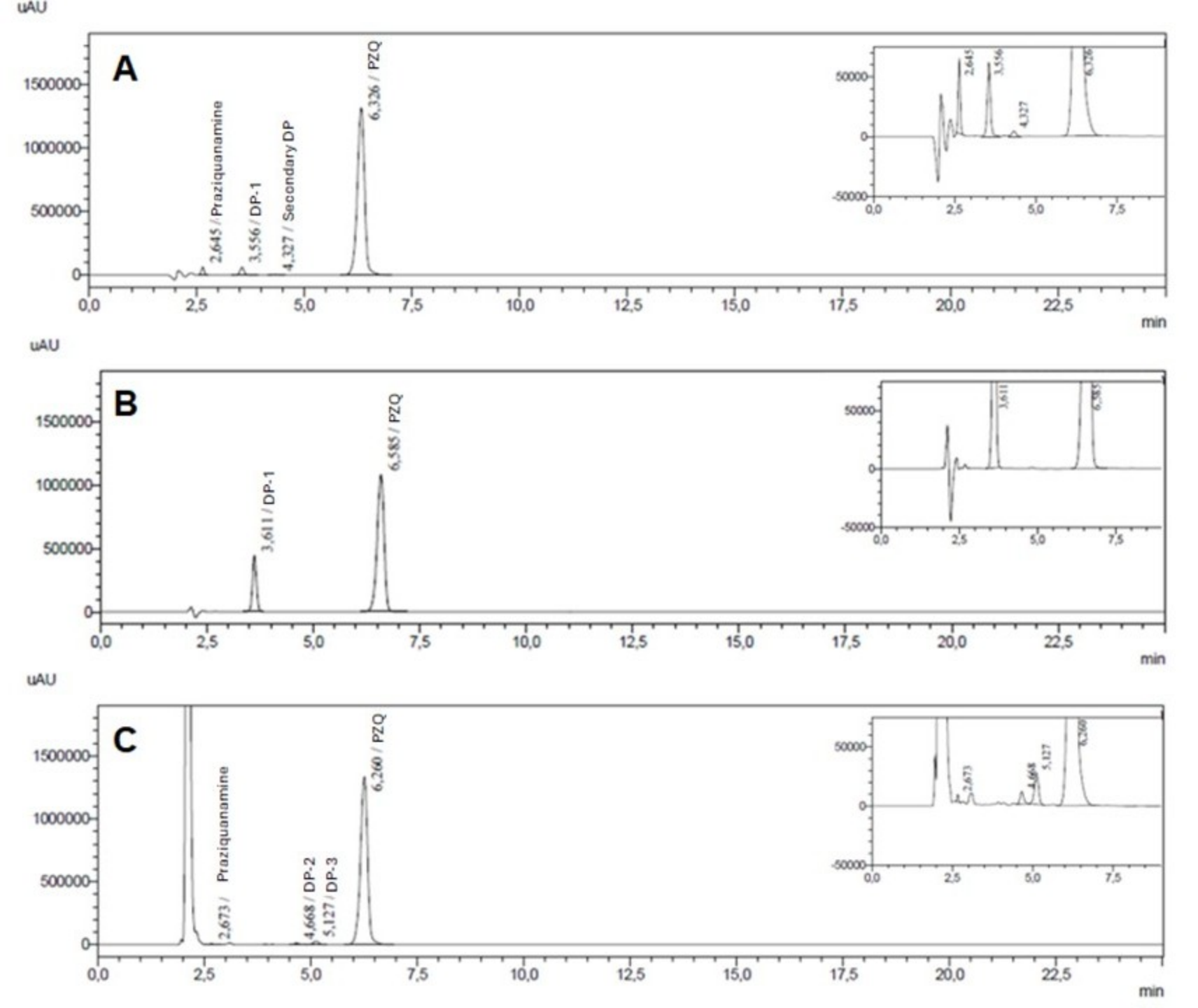
Di-hydroxypraziquantel
Theorical mass: 344.17361
Peroxide oxidation

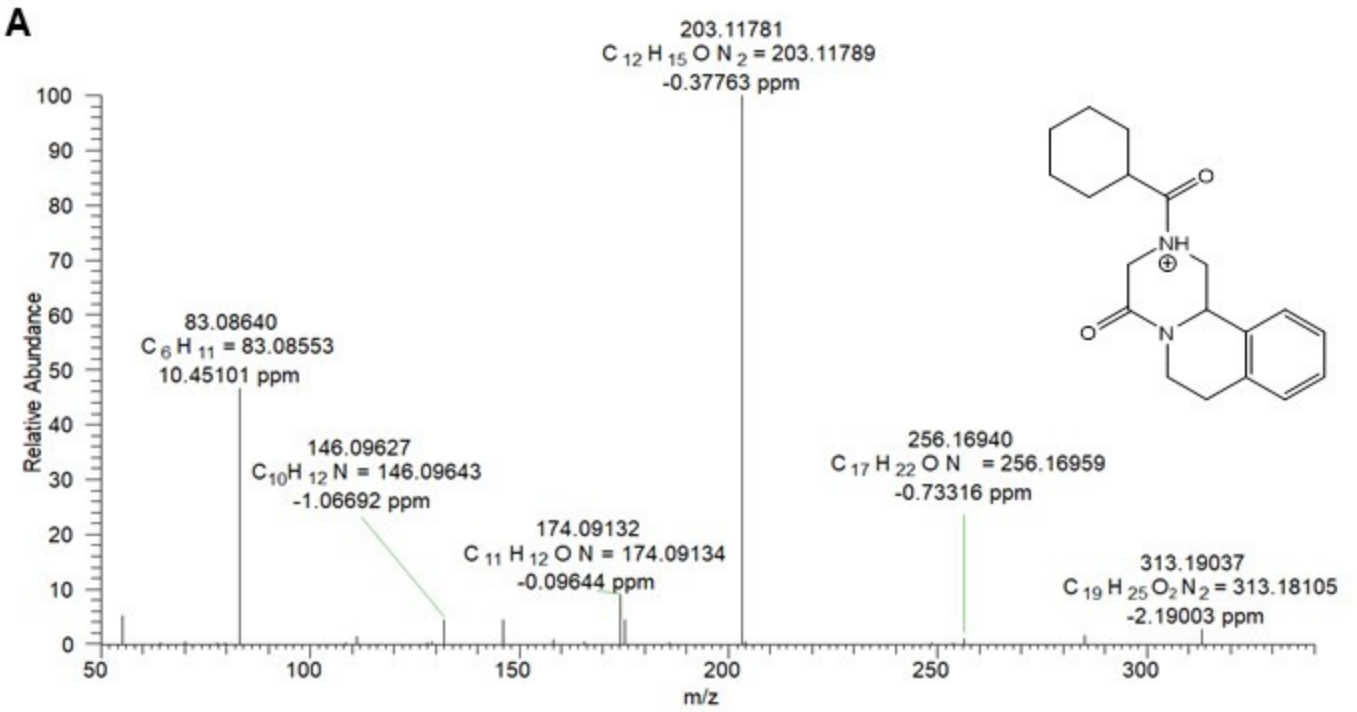


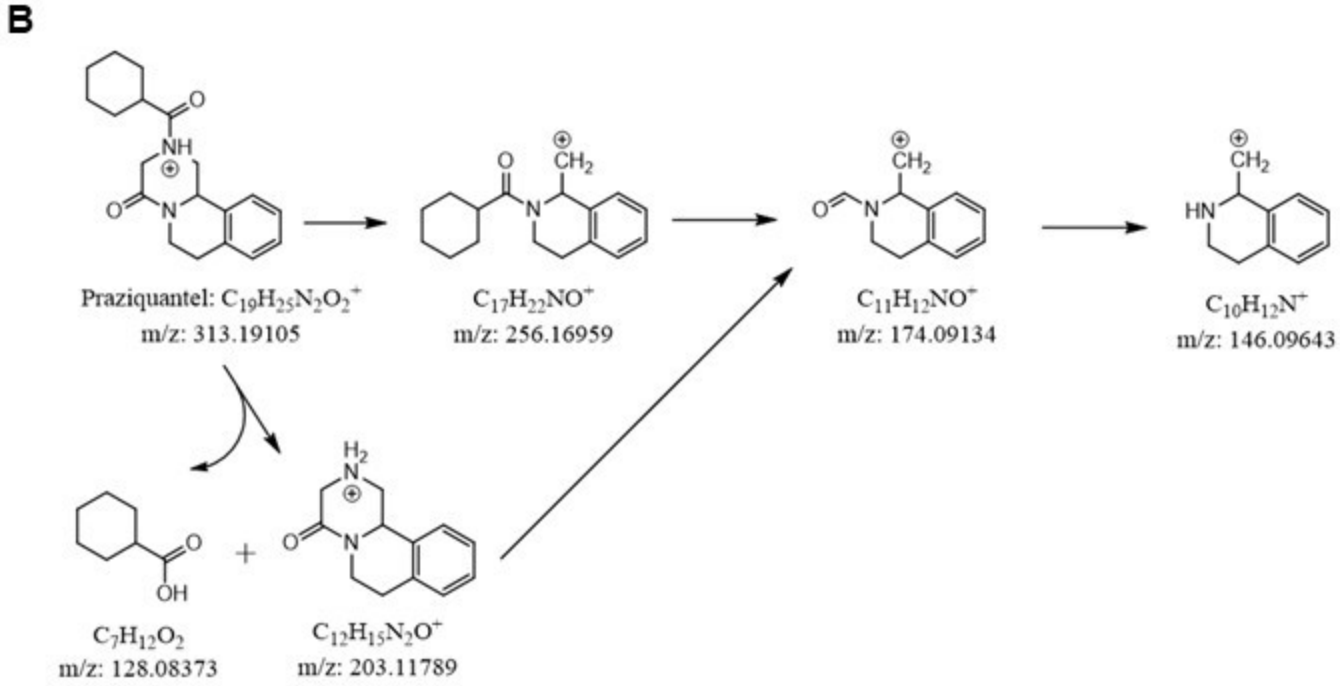
Mono-hydroxypraziquantel
Theorical mass: 328.17869
Peroxide oxidation

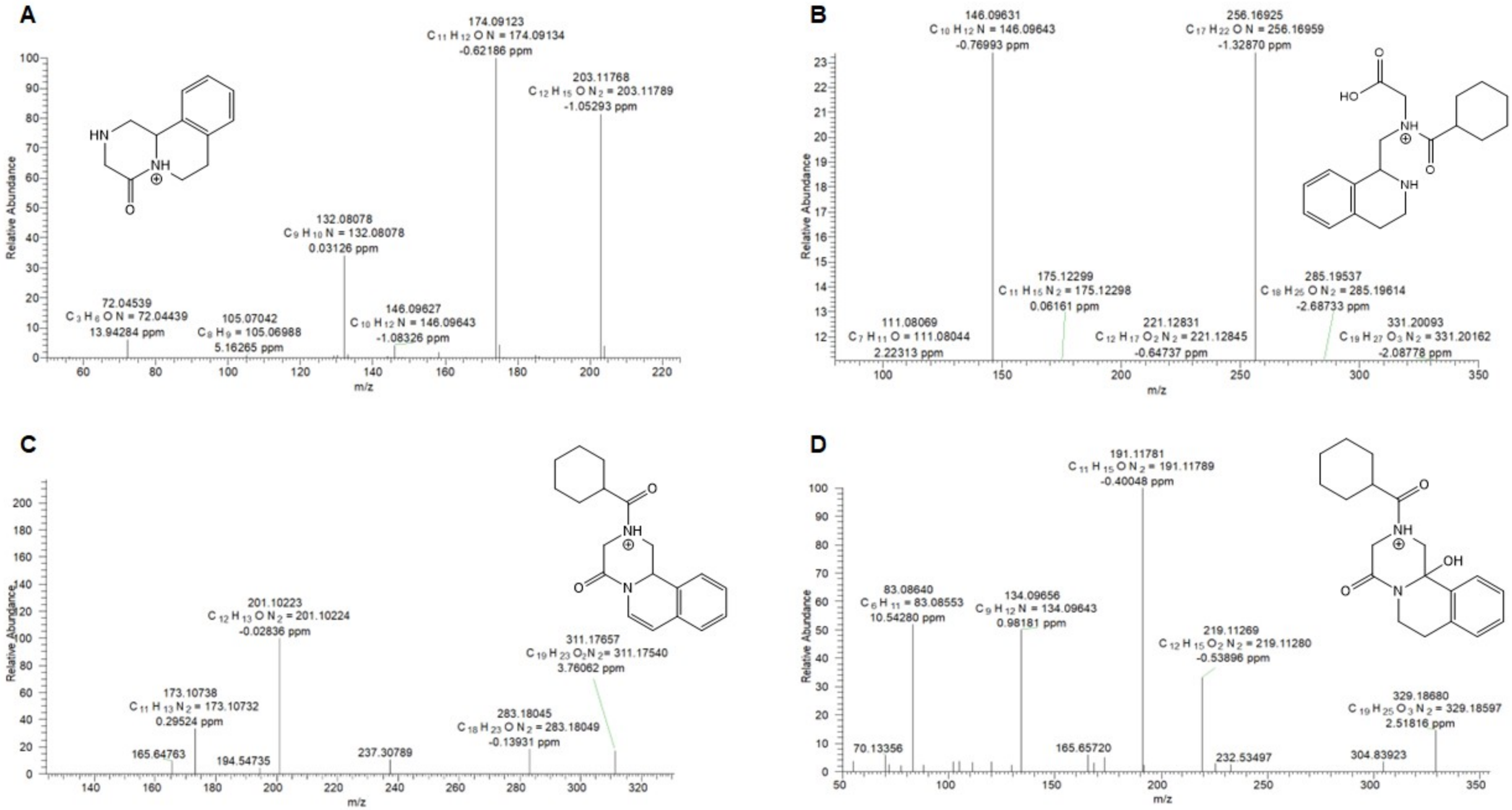


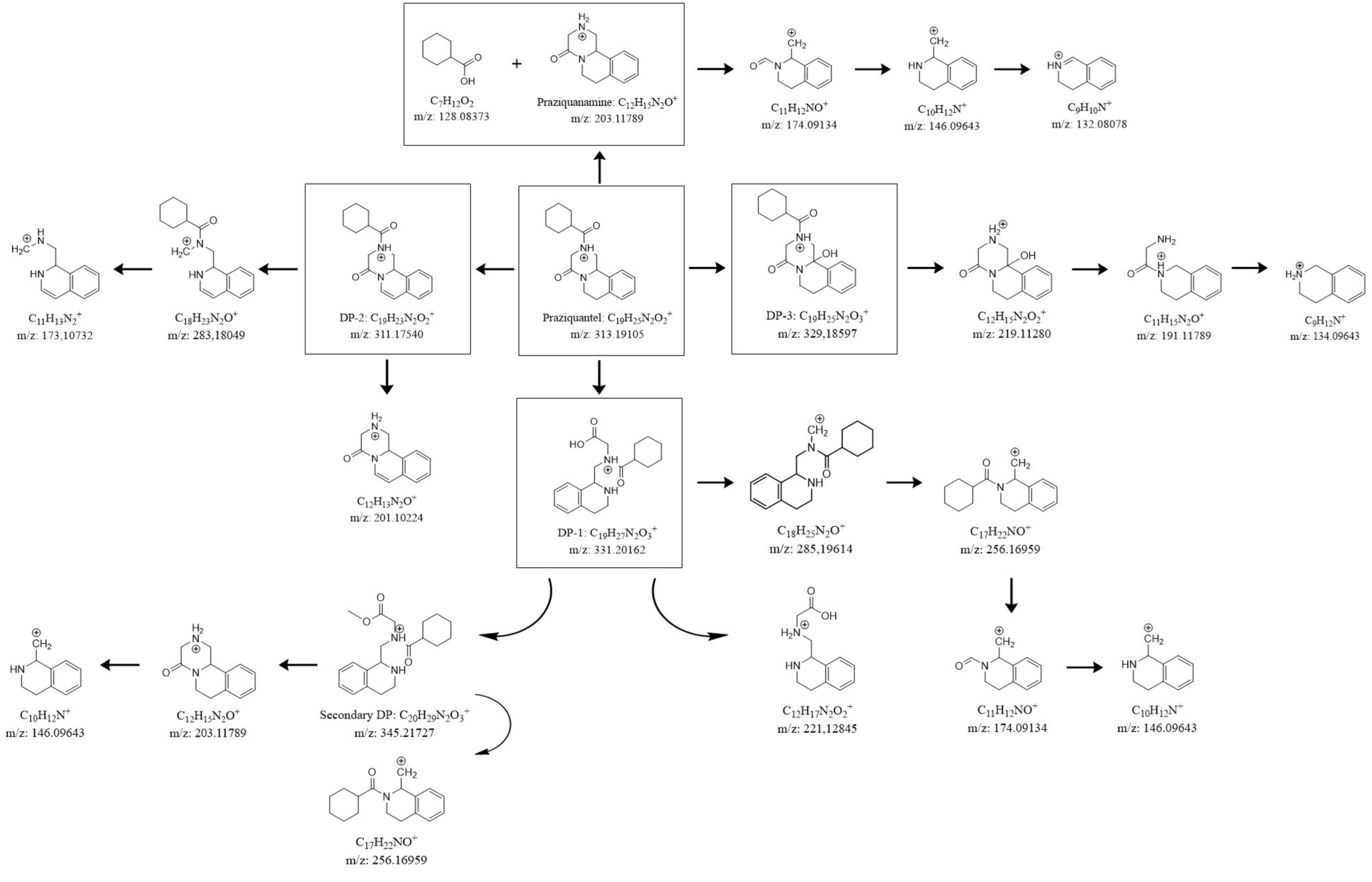
Theorical mass: 310.16813
Peroxide oxidation

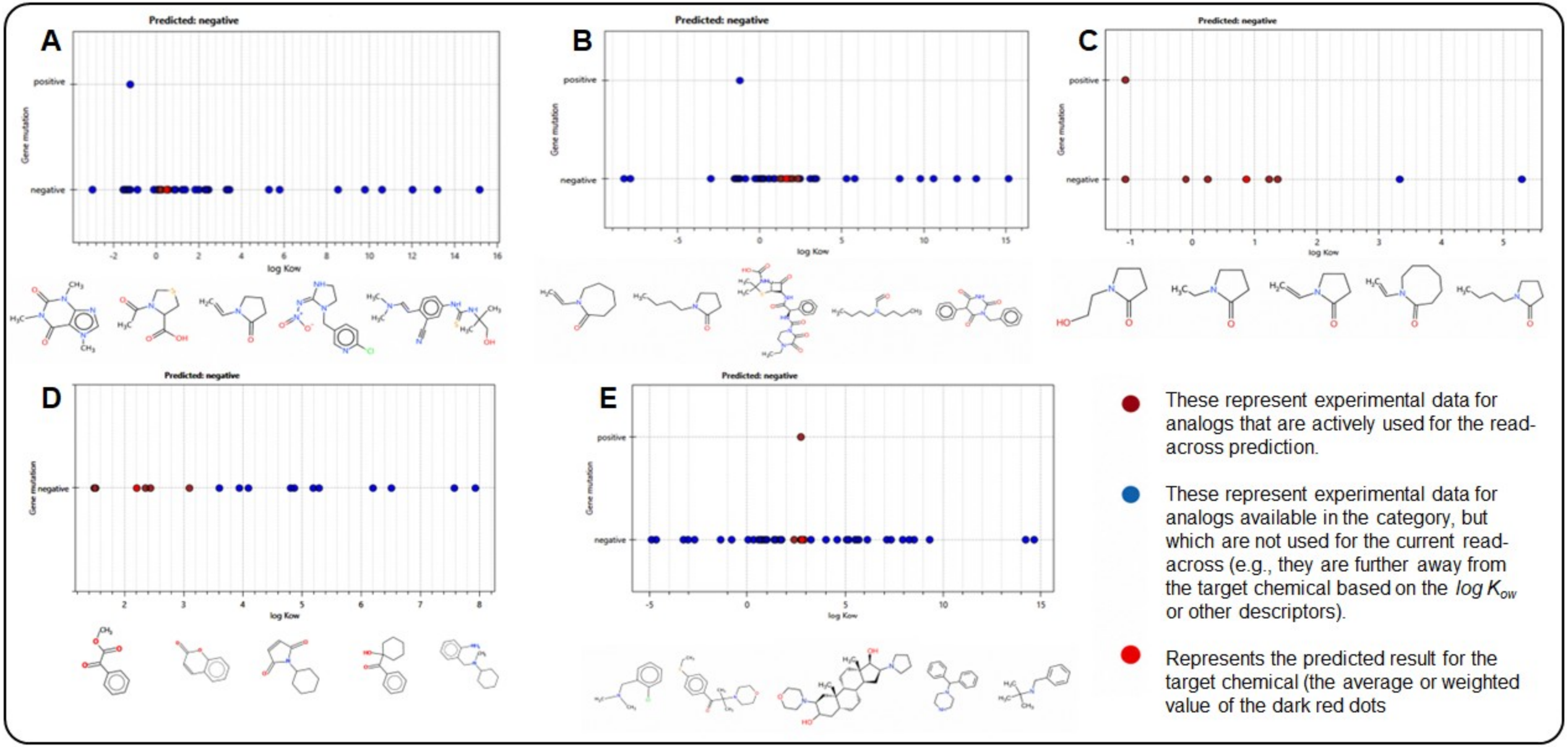

A
uAU
1500000
1000000
500000
0
2,645 / Praziquanamine
3,556 / DP-1
4,327 / Secondary DP
6,326 / PZQ
0,0
2,5
5,0
7,5
10,0
12,5
15,0
17,5
20,0
22,5
min
50000
-50000
B
3,611 / DP-1
6,585 / PZQ
C
2,673 /
Praziquanamine
4,668 / DP-2
5,127 / DP-3
6,260 / PZQ

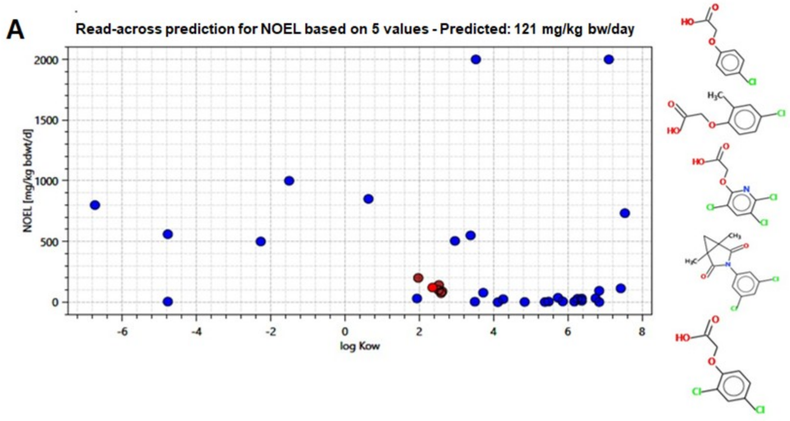

A
203.11781
C 12 H 15 O N 2 = 203.11789
-0.37763 ppm
83.08640
C 6 H 11 = 83.08553
10.45101 ppm
146.09627
C10H 12 N = 146.09643
-1.06692 ppm
174.09132
C 11 H 12 O N = 174.09134
-0.09644 ppm
256.16940
C 17 H 22 O N = 256.16959
-0.73316 ppm
313.19037
C 19 H 25 O2 N 2 = 313.18105
-2.19003 ppm
Relative Abundance
m/z

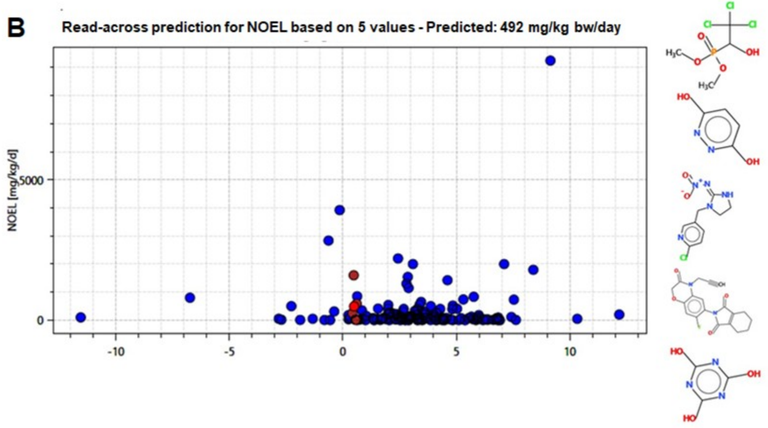

B
Praziquantel: C19H25N2O2+
m/z: 313.19105
C17H22NO+
m/z: 256.16959
C11H12NO+
m/z: 174.09134
C10H12N+
m/z: 146.09643
C7H12O2
m/z: 128.08373
C12H15N2O+
m/z: 203.11789

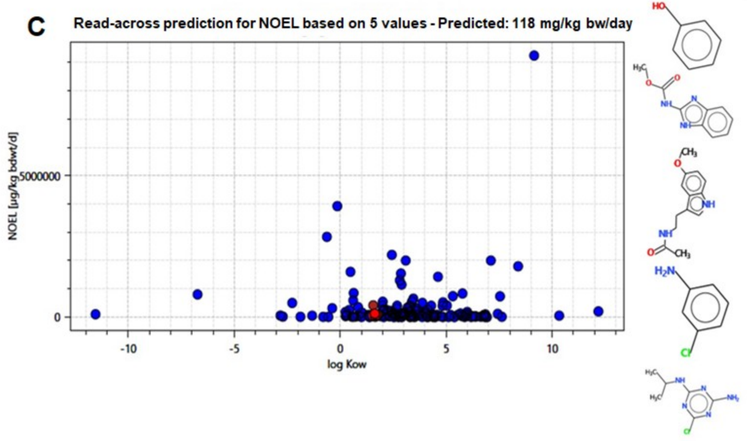

A
174.09123
C11 H12 O N = 174.09134
-0.62186 ppm
203.11768
C12 H15 O N2 = 203.11789
-1.05293 ppm
132.08078
C9 H10 N = 132.08078
0.03126 ppm
72.04539
C3 H6 O N = 72.04439
13.94284 ppm
105.07042
C8 H9 = 105.06988
5.16265 ppm
146.09627
C10 H12 N = 146.09643
-1.08326 ppm
Relative Abundance
m/z
B
146.09631
C10 H12 N = 146.09643
-0.76993 ppm
256.16925
C17 H22 O N = 256.16959
-1.32870 ppm
175.12299
C11 H15 N2 = 175.12298
0.06161 ppm
285.19537
C18 H25 O N2 = 285.19614
-2.68733 ppm
111.08069
C7 H11 O = 111.08044
2.22313 ppm
221.12831
C12 H17 O2 N2 = 221.12845
-0.64737 ppm
331.20093
C19 H27 O3 N2 = 331.20162
-2.08778 ppm
Relative Abundance
m/z
C
201.10223
C12 H13 O N2 = 201.10224
-0.02836 ppm
311.17657
C19 H23 O2 N2 = 311.17540
3.76062 ppm
173.10738
C11 H13 N2 = 173.10732
0.29524 ppm
283.18045
C18 H23 O N2 = 283.18049
-0.13931 ppm
165.64763
194.54735
237.30789
Relative Abundance
m/z
D
191.11781
C11 H15 O N2 = 191.11789
-0.40048 ppm
83.08640
C6 H11 = 83.08553
10.54280 ppm
134.09656
C9 H12 N = 134.09643
0.98181 ppm
219.11269
C12 H15 O2 N2 = 219.11280
-0.53896 ppm
329.18680
C19 H25 O3 N2 = 329.18597
2.51816 ppm
70.13356
165.65720
232.53497
304.83923
Relative Abundance
m/z

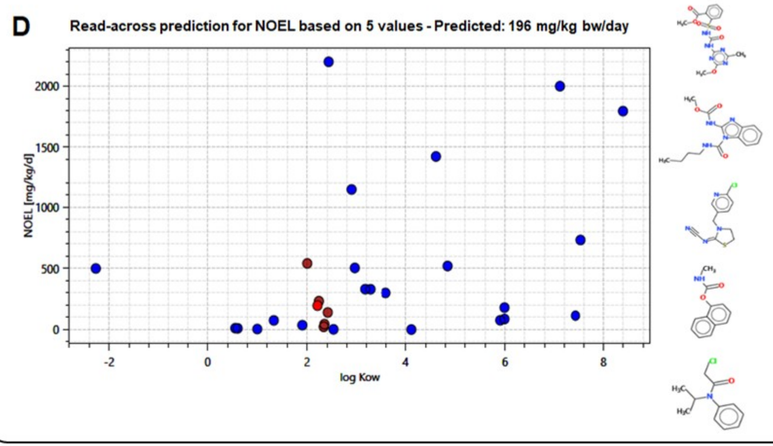

$C_7H_{12}O_2$
m/z: 128.08373
Praziquanamine: $C_{12}H_{15}N_2O^+$
m/z: 203.11789
$C_{11}H_{12}NO^+$
m/z: 174.09134
$C_{10}H_{12}N^+$
m/z: 146.09643
$C_9H_{10}N^+$
m/z: 132.08078
$C_{11}H_{13}N_2^+$
m/z: 173,10732
$C_{18}H_{23}N_2O^+$
m/z: 283,18049
DP-2: $C_{19}H_{23}N_2O_2^+$
m/z: 311.17540
Praziquantel: $C_{19}H_{25}N_2O_2^+$
m/z: 313.19105
DP-3: $C_{19}H_{25}N_2O_3^+$
m/z: 329,18597
$C_{12}H_{15}N_2O_2^+$
m/z: 219.11280
$C_{11}H_{15}N_2O^+$
m/z: 191.11789
$C_9H_{12}N^+$
m/z: 134.09643
$C_{12}H_{13}N_2O^+$
m/z: 201.10224
DP-1: $C_{19}H_{27}N_2O_3^+$
m/z: 331.20162
$C_{18}H_{25}N_2O^+$
m/z: 285,19614
$C_{17}H_{22}NO^+$
m/z: 256.16959
$C_{10}H_{12}N^+$
m/z: 146.09643
$C_{12}H_{15}N_2O^+$
m/z: 203.11789
Secondary DP: $C_{20}H_{29}N_2O_3^+$
m/z: 345.21727
$C_{12}H_{17}N_2O_2^+$
m/z: 221,12845
$C_{11}H_{12}NO^+$
m/z: 174.09134
$C_{10}H_{12}N^+$
m/z: 146.09643
$C_{17}H_{22}NO^+$
m/z: 256.16959

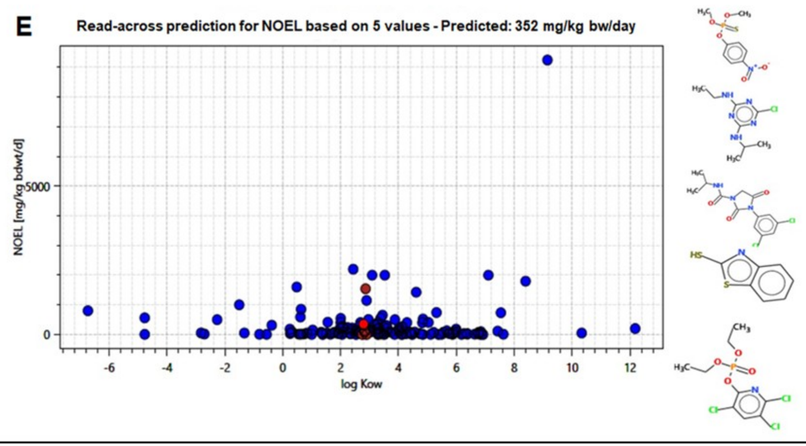

A
Predicted: negative
positive
negative
Gene mutation
log Kow
B
Predicted: negative
positive
negative
Gene mutation
log Kow
C
Predicted: negative
positive
negative
Gene mutation
log Kow
D
Predicted: negative
negative
Gene mutation
log Kow
E
Predicted: negative
positive
negative
Gene mutation
log Kow
These represent experimental data for analogs that are actively used for the read-across prediction.
These represent experimental data for analogs available in the category, but which are not used for the current read-across (e.g., they are further away from the target chemical based on the log K_{OW} or other descriptors).
Represents the predicted result for the target chemical (the average or weighted value of the dark red dots











- These represent experimental data for analogs that are actively used for the read-across prediction.
- These represent experimental data for analogs available in the category, but which are not used for the current read-across (e.g., they are further away from the target chemical based on the *log* K_{OW} or other descriptors).
- Represents the predicted result for the target chemical (the average or weighted value of the dark red dots

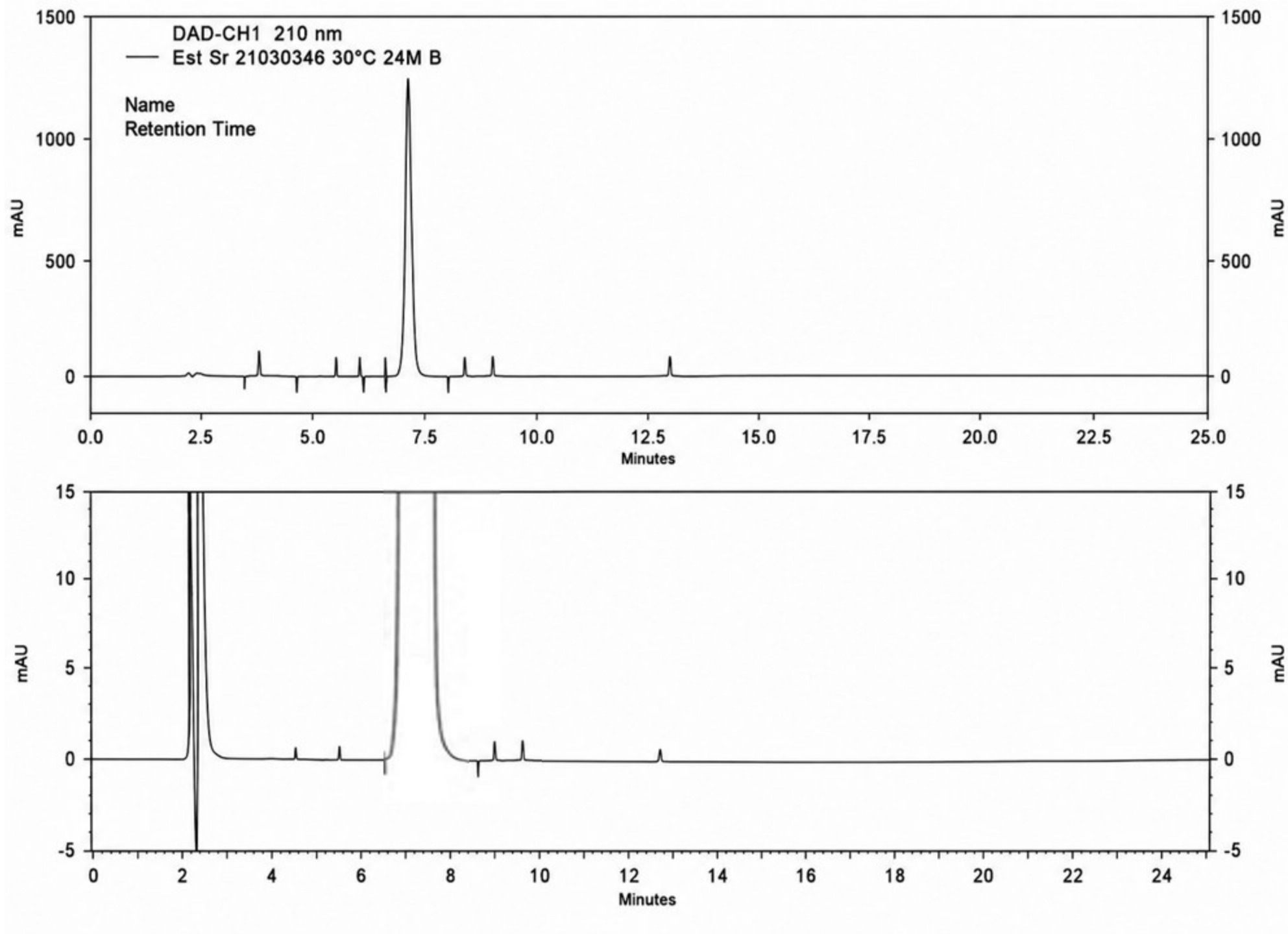

DAD-CH1 210 nm
Est Sr 21030346 30°C 24M B
Name
Retention Time
mAU
Minutes
1500
1000
500
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
15
10
5
0
-5
2
4
6
8
12
14
16
18
20
22
24

Figure 1 illustrates the steps of the PREVER strategy, and a detailed description is presented in the following sections.

Figure 2. PZQ-related impurities described in scientific literature

Figure 3. Representative chromatogram of praziquantel 600 mg tablets at a concentration of 0.25 mg/mL after degradation by **(A)** acidic hydrolysis, **(B)** alkaline hydrolysis, and **(C)** oxidative degradation.

Figure 4. ESI-Orbitrap-MS/MS analysis of praziquantel (PZQ). **(A)** Product ion spectrum of the protonated molecular ion $[M+H]^+$ at m/z 313.19037, showing the main diagnostic. **(B)** Proposed fragmentation pathway for PZQ.

Figure 5. ESI-Orbitrap-MS/MS analysis of PZQ-related impurities. **(A)** Product ion spectrum of Praziquanamine with protonated molecular ion $[M+H]^+$ at m/z 203.11768; **(B)** Product ion spectrum of DP-1 with protonated molecular ion $[M+H]^+$ at m/z 331.20093 (DP-1); **(C)** Product ion spectrum of DP-2 with protonated molecular ion $[M+H]^+$ at m/z 311.17657 and **(D)** Product ion spectrum of DP-3 with protonated molecular ion $[M+H]^+$ at m/z 329.18567.

Figure 6. Proposed fragmentation pathways for the main product ions observed in the product ion spectra of the protonated molecules $[M+H]^+$ of PZQ, Praziquanamina, DP-1, DP-2 and DP-3, including their theoretical masses.

Figure 7. Read-across Ames test prediction for impurities praziquanamine (A), DP-1 (theorical mass = 330.19434 – B), cyclohexanecarboxylic acid (C), DP-2 (theorical mass = 310.16813 - D), mono-hydroxypraziquantel (E). Ames test based on 5 values. The molecular structure represents the analogs set of chemicals selected for prediction. ***Legend***: Red dot - predicted results for the target chemical. Brown dots - experimental results available for the analogs that are used for the read-across. Blue dots - experimental results available for the analogues but not used for read-across

Figure 8. Read-across NOEL prediction for impurities praziquanamine (A), DP-1 (theorical mass = 330.19434 – B), cyclohexanecarboxylic acid (C), DP-2 (theorical mass = 310.16813 - D), mono-hydroxypraziquantel (E). NOEL prediction for impurities based on 5 values. The molecular structure represents the analogs set of chemicals selected for prediction. ***Legend****:* Red dot - predicted results for the target chemical. Brown dots - experimental results available for the analogs that are used for the read-across. Blue dots - experimental results available for the analogues but not used for read-across

Figure 9. Representative chromatographic profile of the PZQ tablet sample after 24 months under long-term stability conditions for climatic zone IVb. Tablets were stored at

30 °C ± 2 °C and 75% ± 5% RH, in accordance with ICH stability guidelines. Chromatograms were obtained by HPLC-DAD at 210 nm. The upper chromatogram shows the full-scale profile, while the lower chromatogram shows an expanded view to improve visualization of low-intensity degradation-related peaks.

Table 1. Results obtained after the forced degradation study of PZQ under acidic and alkaline hydrolysis and oxidation conditions.

Compounds	RRT	Degradation conditions/ Degradation percentage (%)		
		Acid	Basic	Oxidative
PZQ	na	89.64	89.51	90.99
Praziquanamine	0.41	1.56	nd	0.13
DP-1	0.55	2.35	16.24	nd
DP-2	0.73	nd	nd	0.50
DP-3	0.81	nd	nd	1.34
Drug degradation (%)		10.3	10	9.0
Total impurities (%)		4.1	16.24	1.96
Absolute mass balance (%)		92	104	91
Standard deviation		4.09	4.39	4.07
Confidence interval (%)		84 - 100	95 – 113	83 – 100

nd = not detected; na = not applicable

Table 2. Summary of validation results

Parameter	Assay	Related impurities
Selectivity	Absence of interference	Absence of interference
Linearity	R = 0.999, Random pattern of residues	R = 0.999, Random pattern of residues
Repeatability	Pooled RSD 0.5%	Pooled RSD 3.1%
Intermediate precision	RSD 3%	RSD 3.6%
Accuracy	101% - 102%	90% - 98%
Robustness	RSD < 3.7%	RSD < 7.3%

Table 3. Elemental composition, theoretical mass $[M+H]^+$, precursor ion [M+H]+, product ions (using high resolution MS/MS experiments) and elemental composition (experimental) of praziquantel and its degradation products.

	Precursor ion [M+H]+ (*m/z*)	**Precursor ion and product ion [M+H]+ (*m/z*)**			
		Experimental m/z	**Theorical m/z**	**Error (ppm)**	**Elemental composition**
PZQ	**313.19037**	**313.19038**	**313.18971**	**2.12240**	**$C_{19}H_{25}O_2N_2$**
		256.16940	256.16959	-0.73316	$C_{17}H_{22}ON$
		203.11781	203.11789	-0.37763	$C_{12}H_{15}ON_2$
		174.09132	174.09134	-0.09644	$C_{11}H_{12}ON$
		146.09627	146.09643	-1.06692	$C_{10}H_{12}N$
Praziquanamine	**203.11768**	**203.11768**	**203.11768**	**-1.05293**	**$C_{12}H_{15}ON_2$**
		174.09123	174.09134	-0.62186	$C_{11}H_{12}ON$
		146.09627	146.09643	-1.08326	$C_{10}H_{12}N$
		132.08078	132.08078	0.03126	$C_9H_{10}N$
DP-1	**331.20093**	**331.20093**	**331.20093**	**1.96613**	**$C_{19}H_{27}O_3N_2$**
		285.19537	285.19614	-2.68733	$C_{19}H_{27}O_3N_2$
		256.16925	256.16959	-1.32870	$C_{17}H_{22}ON$
		221.12831	221.12845	-0.64737	$C_{12}H_{17}O_2N_2$
		175.12299	175.12298	0.06161	$C_{11}H_{15}N_2$
		146.09631	146.09643	-0.76993	$C_{10}H_{12}N$
DP-2	**311.17657**	**311.17657**	**311.17540**	**3.76062**	**$C_{19}H_{23}O_2N_2$**
		283.18045	283.18049	-0.13931	$C_{18}H_{23}ON_2$
		201.10223	201.10224	-0.02836	$C_{12}H_{13}ON_2$
		173.10738	173.10732	0.29524	$C_{11}H_{13}N_2$
DP-3	**329.18567**	**329.18567**	**329.18597**	**-0.91196**	**$C_{19}H_{25}O_3N_2$**
		219.11269	219.11280	-0.53896	$C_{12}H_{15}O_2N_2$
		191.11781	191.11789	-0.40048	$C_{11}H_{15}ON_2$
		134.09656	134.09643	0.98181	$C_9H_{12}N$

All experimental molecular formula were obtained with <5 ppm mass error.