ORIGINAL ARTICLE

Design and Verification of A opposed Polarity Separation Technique for Zenocutuzumab in Large-quantity Drug Substance and Delivery System Forms

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Abstract

Background: Zenocutuzumab is a novel therapeutic monoclonal antibody requiring precise and reliable investigative techniques for routine monitoring, quality, and stability evaluation. The present investigation is directed to progress and confirm the perceptive a rapid, sensitive, and robust opposing polarity chromatographic separation method for the quantitative assessment of Zenocutuzumab in majority medicine substance and pharmaceutical therapeutic preparations. **Materials and Methods:** Effective separation of components was obtained by employing are versed-phase an reversed-phase C18 chromatographic column analytical column (150 mm × 4.6 mm, 3.5 µm particle diameter) served as the separation medium under isocratic conditions. The eluent consisted of KH_2PO_4 Buffer solution titrated to pH 2.5 with orthophosphoric acid) and ethanol in the volumetric ratio of 70:30 volume-to-volume. A constant flow rate of 1.0 mL/min was maintained throughout, and analysis was performed at ambient temperature. The analytical procedure was validated in accordance with the requirements of International Council for Harmonization (ICH) guidelines. Stability-indicating stress studies were conducted. The sample was subjected to acidic, basic, and oxidative environments, reductive, photolytic, hydrolytic, and thermal stress conditions. **Results:** Zenocutuzumab showed a linear response spanning the concentration range of 25–150 µg/mL. The detection and quantification limits were evaluated to be 0.6 µg/mL and 2.0 µg/mL, respectively. The total run time was <5 min. Precision and accuracy studies yielded results within acceptable limits. Validation parameters confirmed the method's specificity, linearity, robustness, and reproducibility. The method successfully quantified Zenocutuzumab in both bulk and formulated samples and effectively separated degradation products during stability studies. **Conclusion:** The established reversed-phase high-performance liquid chromatography method is easy to perform, quick, and precise, and reliable for the quantitative determination of Zenocutuzumab. Its compliance with ICH validation criteria and ability to assess stability under various stress conditions make it suitable for standard quality control and stability monitoring testing of Zenocutuzumab in drug formulation systems.

Key words: Development, high-performance liquid chromatography, validation, zenocutuzumab

INTRODUCTION

Zenocutuzumab, marketed as *Bizengri,* is a humanized monoclonal antibody (mAb)[1-4] utilized in the intervention of Non-Small Cell Pulmonary Carcinoma (NSCLC),[2,3] and pancreatic cancer.[5,6] It is a low-fucose, full-length humanized immunoglobulin G1 that functions as a bivalently engaging mAb.[7] The receptors human epidermal growth factor receptor 2 and 3 (HER2 and HER3), belonging to the HER2 receptor family (types 2 and 3) using therapies that interfere with the function of these two receptors to treat diseases like cancer. The most frequently reported adverse effects include diarrhea[8,9] musculoskeletal

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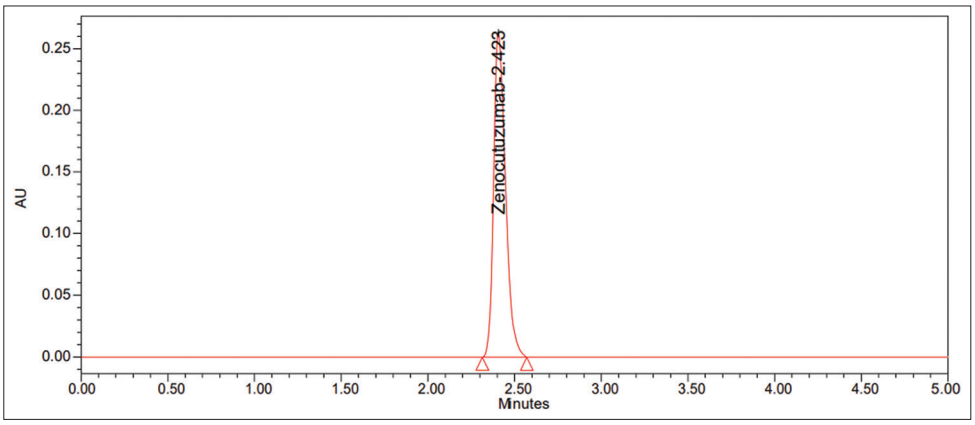

Figure 1: Chromatographic profile for system suitability-I

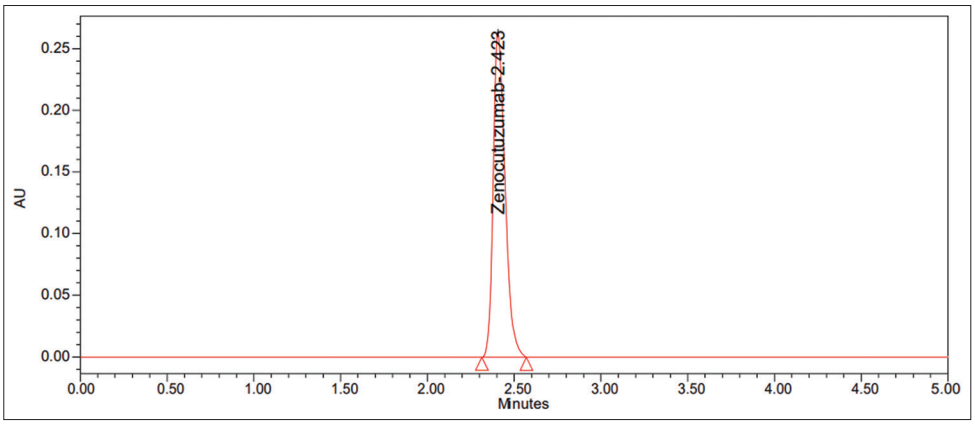

Figure 2: System suitability evaluation chromatogram – 2

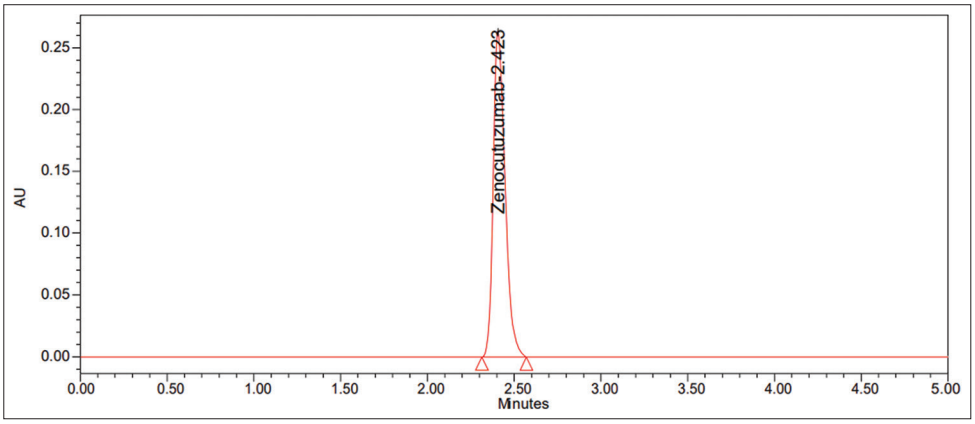

Figure 3: Chromatogram for system suitability analysis (3)

pain,[10,11] fatigue,[12,13] gastrointestinal discomfort, parenteral therapy reactions, respiratory distress, erythema, bowel irregularity,[14,15] expulsion of gastric contents, mesenteric discomfort, and tissue swelling.[16] Severe or life-threatening lab test deviations that occur frequently among patients in a study or treatment setting, observed during therapy, consist of elevated gamma-glutamyl transferase levels, reduced hemoglobin concentration, hyponatremia, and thrombocytopenia.[17,18] Zenocutuzumab is specifically indicated for post-pubertal study population with sophisticated surgically untreatable or disseminated NSCLC or pancreatogenic endocrine carcinoma characterized by growth factor *NRG1* gene fusion, who have experienced disorder evolution following earlier pharmacologic therapy.

MATERIALS AND METHODS

Experimental chemicals and materials

Ethanol, KH_2PO_4, and spectroscopy-grade water were procured from Merck Incorporated, Worli, an urban

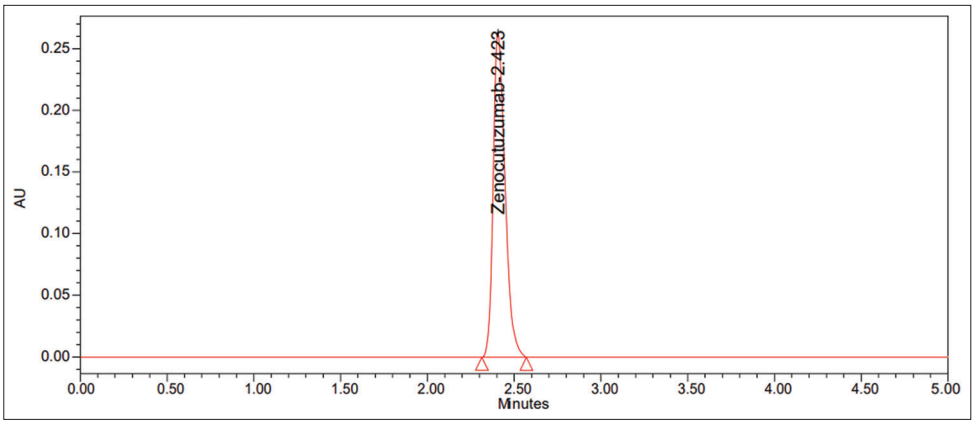

Figure 4: System suitability test chromatogram (IV)

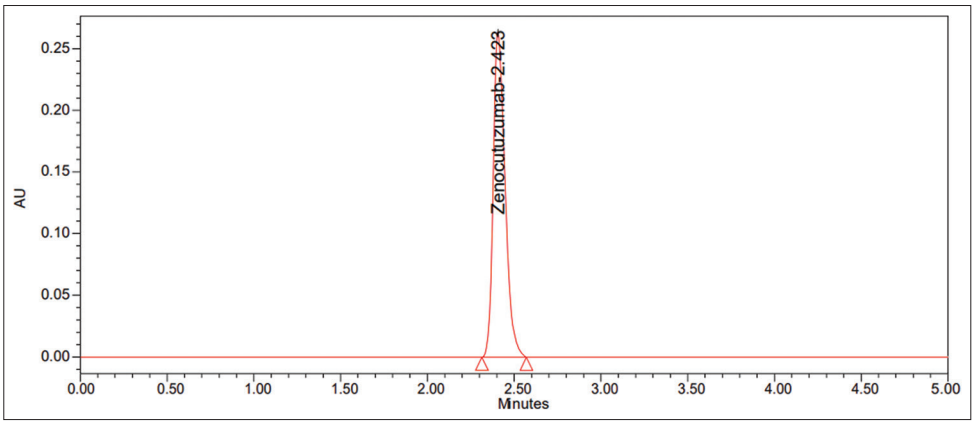

Figure 5: System suitability chromatogram 5

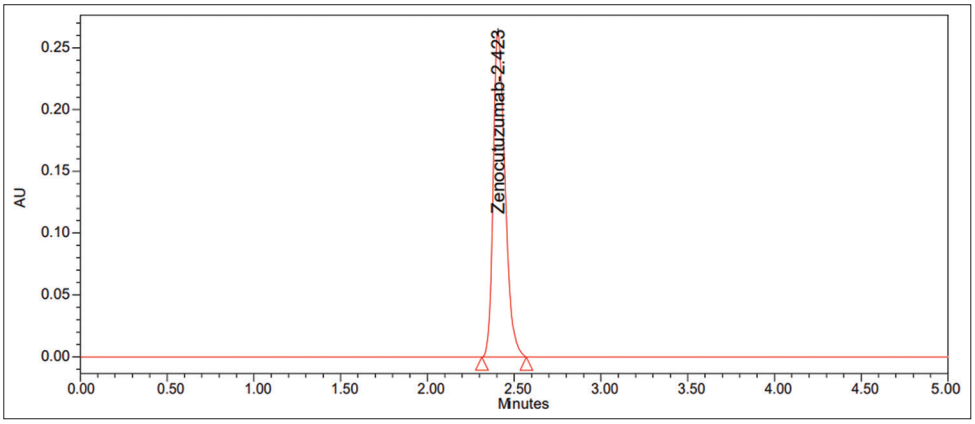

Figure 6: Chromatogram representing system suitability, part 6

precinct of Mumbai. The Zenocutuzumab Active medicinal ingredient used as the reference compound was procured from Zydus Cadila Healthcare Ltd. Ahmedabad, India**.**

Experimental scientific instrumentation

A liquid chromatographic system (Waters Alliance unit) equipped with a multi-solvent gradient pump and a multi-wavelength ultraviolet detector (model 2998) was implemented for the quantification. Measurement acquisition and processing were carried out executing analysis through Empower software, version 2.

Chromatography methodology

Separation by chromatography was carried out in Elution was performed using a constant mobile-phase composition at

Table 1: Optimized separation conditions for the analysis using RP-HPLC of zenocutuzumab

Parameter	Conditions
Stationary phase	An octadecyl silica (C18) Waters X-Terra column with dimensions 150×4.6 mm and 3.5 µm particle size
Eluent composition	KH_2PO_4 pH adjusted to 2.5 using OPA and ethanol in a 70:30 v/v proportion
Loading injection volume	A 10 µL injection volume
Pump elution rate of flow	A column flow adjusted to 1.0 mL/min
Column chamber measured temperature	25°C (controlled temperature)
Monitoring spectral wavelength	271 nm
Total analysis time	5 min
Analyte elution time zenocutuzumab	2.423 min

OPA: Orthophosphoric acid, RP-HPLC: Reversed-phase high-performance liquid chromatography

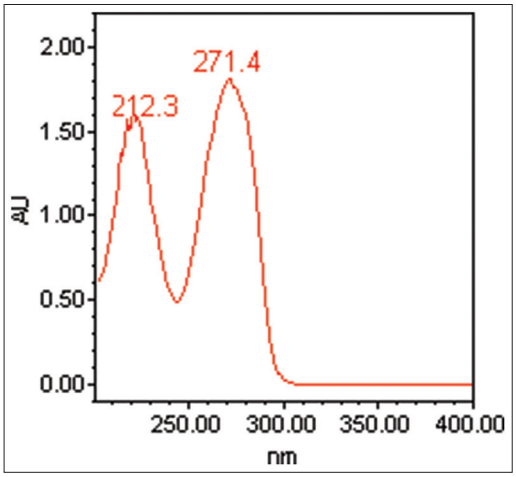

Figure 7: PDA spectra of Zenocutuzumab

ambient thermostatic condition, using a reversed-phase C18 column (Waters X-Terra, 150 × 4.6 mm, 3.5 µm). A 30:70 volume-to-volume (v/v) mixture constituted the eluting phase of ethyl hydroxide and monopotassium phosphate ion (PO_4^{3-}) KH_2PO_4 ionic strength–maintaining solution (pH maintained at 2.5, adjusted with orthophosphoric acid [OPA]), flowed through the system at 1.0 mL/min. Absorbance was measured at 271 nm on a diode array detection system, with 10 µL of sample injected. The separation was achieved, and the analysis was completed within 5 min.

Development of pH-stabilizing aqueous solution

1.36 g of KH_2PO_4 was combined with 1 L of ultra-pure HPLC water to form a solution, and the pH was standardized to 2.5 using OPA. The solution was purified using a 0.45 µm membrane filtration unit to remove insoluble particles before injection.

Preparation of mobile phase

Combine ethanol with KH_2PO_4, a solution composed of OPA and pH 2.5 buffer in 30:70 (v/v) proportion, and pass the mixture subjected to 0.45 µm filtration.

Diluent

As a carrier solvent, the flowing phase is utilized.

Both calibration and quality control samples were systematically prepared

Weighed out precisely 10 mg quantity of Zenocutuzumab working certified reference substance and poured into a 10 mL calibrated volumetric flask. Dispense approximately 7 mL of diluent, followed by sonication for a period of 30 min to attain complete solubility. Make up the measured volume with the diluent. Further, aliquot 1 mL of the solution and then diluted to a total volume of 10 mL with the diluting medium.

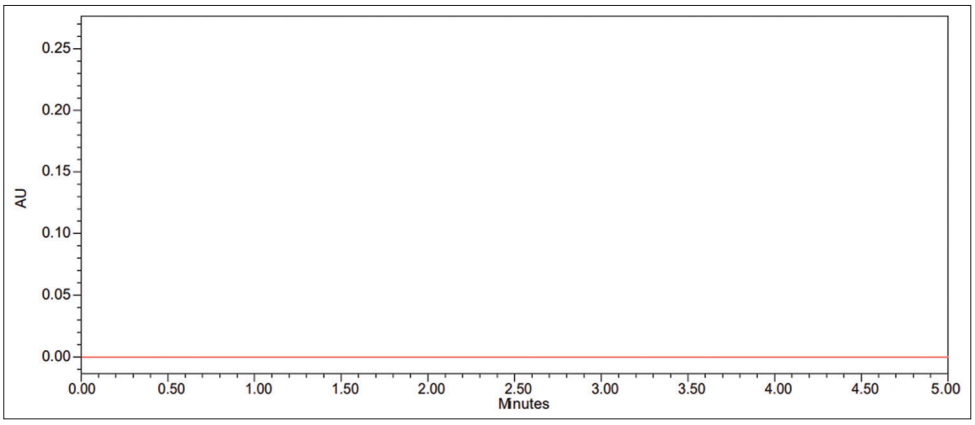

Figure 8: Chromatographic output representation of the baseline sample

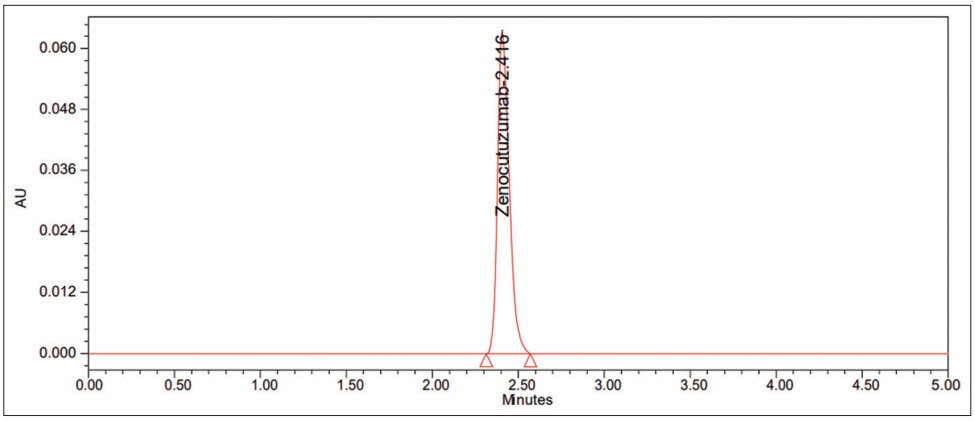

Figure 9: Calibration plots of Zenocutuzumab

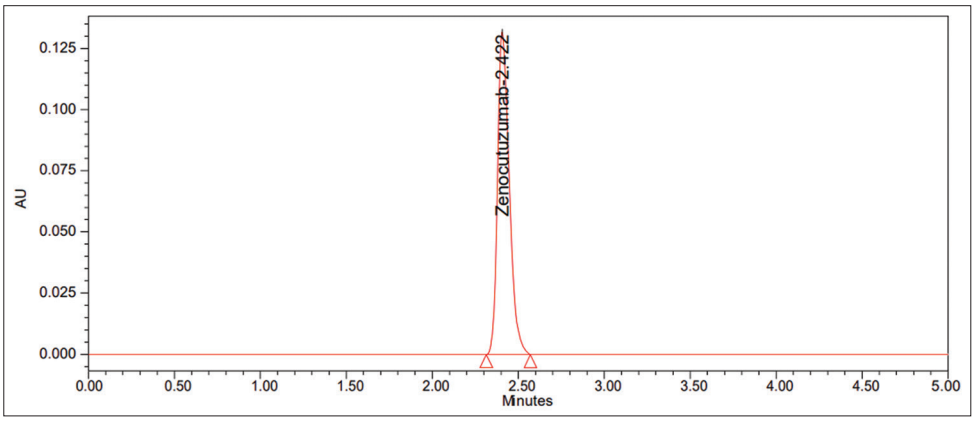

Figure 10: Chromatogram of linearity-25%

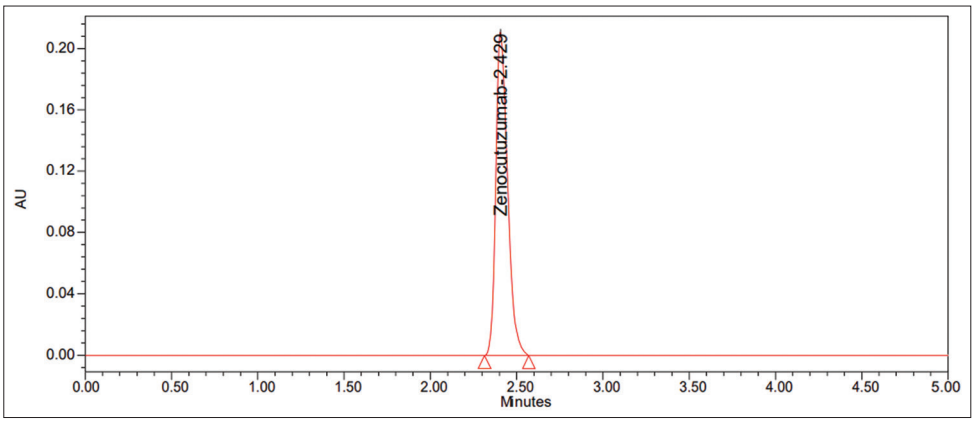

Figure 11: Chromatogram for 50% linearity level

Table 2: Analytical system performance evaluation

Quantitative measure	Zenocutuzumab
Column performance parameter	8426
Peak symmetry factor	0.98
Elution time	2.423 min

Method for sample solution preparation

Pipette 0.5 mL of the Zenocutuzumab, the analytical aliquot of the sample, was transferred into a 10 mL measuring calibrated flask and added 7 mL of analytical-grade solvent. Ultrasonic agitation the mixture until quantitatively

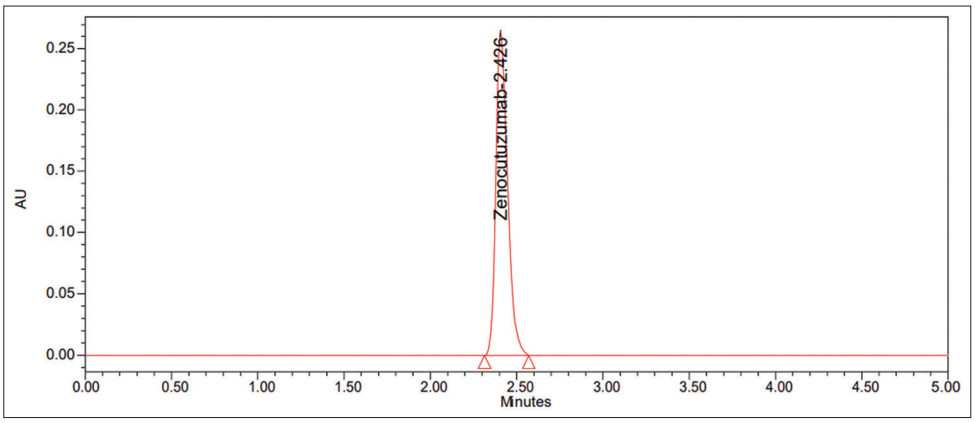

Figure 12: Chromatogram showing linearity at 75% concentration

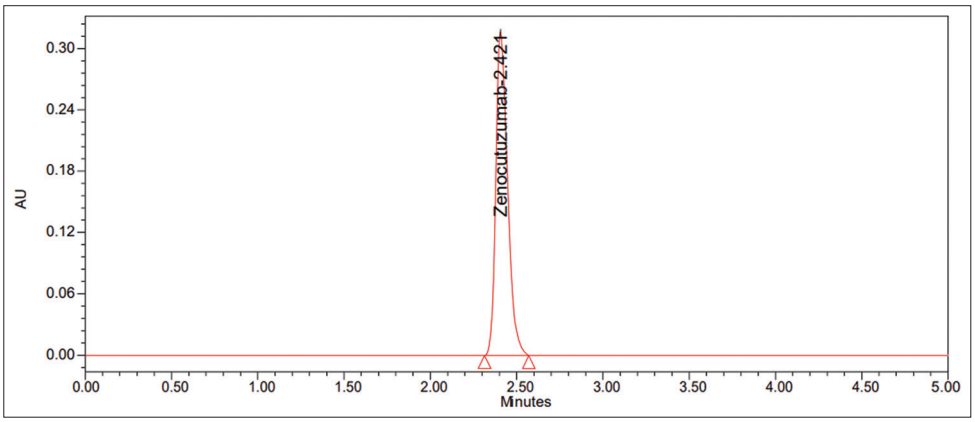

Figure 13: Linearity chromatogram at 100% level

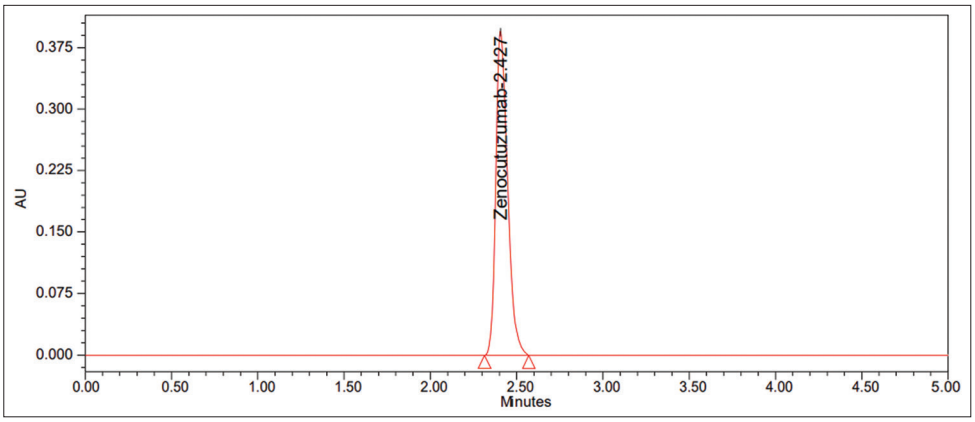

Figure 14: Chromatographic profile for 125% linearity

completely dissolved and then adjusted to a total volume with diluent. An analytical portion of 1 mL of the stock solution was formulated, added to 10 mL Precision flask, and adjusted to reach 10 mL using diluent. Finally, filter the solution using a 0.45 µm pore size filter porous membrane nylon-based material dispensing injector syringe. All parameters were assessed and validated based on International Council for Harmonization (ICH) guidelines as well as literature references.

DISCUSSION

The research undertaken in this study aimed to optimize a time-efficient, precise, and straightforward hydrophobic phase chromatography separation analytical experimental procedure for the assay and appraisal of Zenocutuzumab in both substance drug and therapeutic formulations. Various mobile phase combinations were evaluated to optimize the

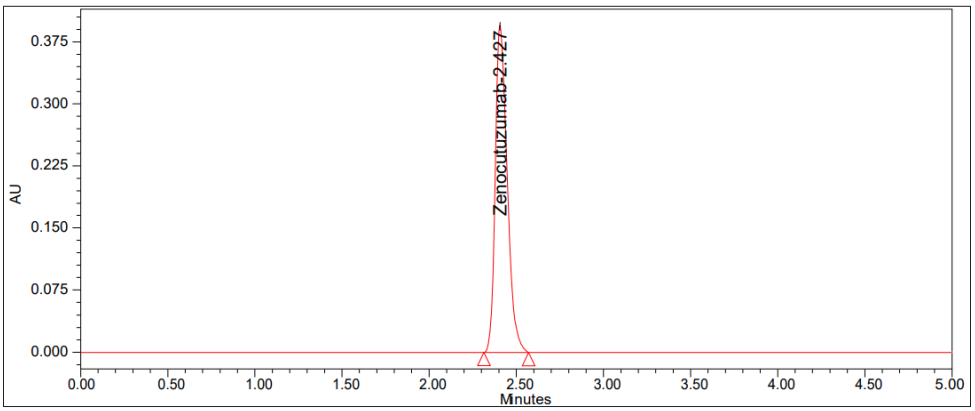

Figure 15: Linearity assessment chromatogram (150%)

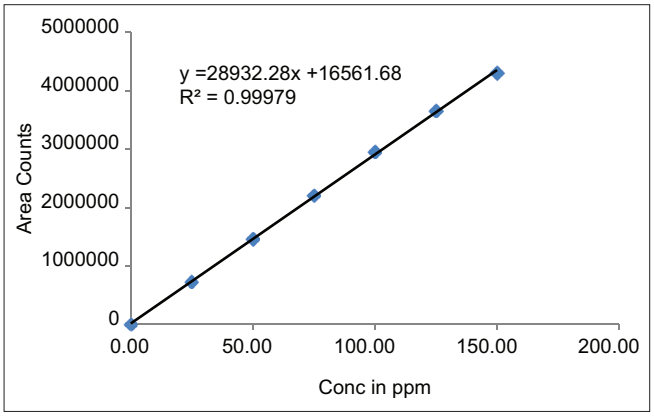

Figure 16: Calibration plots of zenocutuzumab

Table 3: Evaluation results of method linearity

Serial number	Zenocutuzumab	
	Concentration of zenocutuzumab (micrograms per milliliter)	Measured area
1	25.00	728094
2	50.00	1460231
3	75.00	2207819
4	100.00	2951643
5	125.00	3655402
6	150.00	4302189
Correlation coefficient		0.99979
Slope		28932.28
Intercept		16561.68

chromatographic conditions for Zenocutuzumab analysis. The final optimized mobile phase consisted of KH_2PO_4 buffered to pH 2.5 with OPA and ethanol in a 70:30 v/v proportion. Different wavelengths were tested to achieve sufficient sensitivity for detecting low concentrations of Zenocutuzumab, and a wavelength of 271 nm was ultimately selected, providing strong drug absorbance. The method employed delivered with a flow rate maintained at 1 mL/min, with Zenocutuzumab exhibiting a retention time of 2.423 min. The implemented approach was assessed for accuracy and precision under ICH regulatory guidelines, with all parameters meeting the specified limits. Observations were completed with a total elution time of 5.0 min.

Table 4: Method reliability under different conditions

S.No.	Conc. (µg/mL)	Zenocutuzumab peak area	% Assay
1.	100	2945715	100.7
2.	100	2965404	101.4
3.	100	2944531	100.6
4.	100	2926410	100.0
5.	100	2913467	99.6
6.	100	2912121	99.5
Average		2934608	
Standard deviation		20928.637	
% RSD			0.71

% RSD: Percentage relative standard deviation

Table 5: Outcomes of method reproducibility (ruggedness)

S. No.	Conc. (µg/mL)	Zenocutuzumab peak area	% Assay
1.	100	2946187	100.7
2.		2912303	99.5
3.		2968172	101.4
4.		2957994	101.1
5.		2931620	100.2
6.		2938776	100.4
% RSD			0.67

% RSD: Percentage relative standard deviation

Future directions

Insufficient impurity and degradation profiling

Current literature provides limited insight into the separation of closely related impurities, charge variants, and

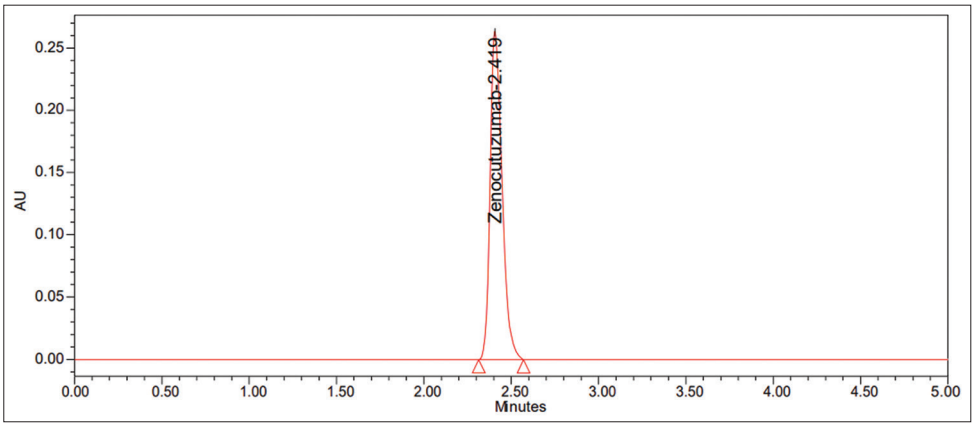

Figure 17: Chromatogram of method precision

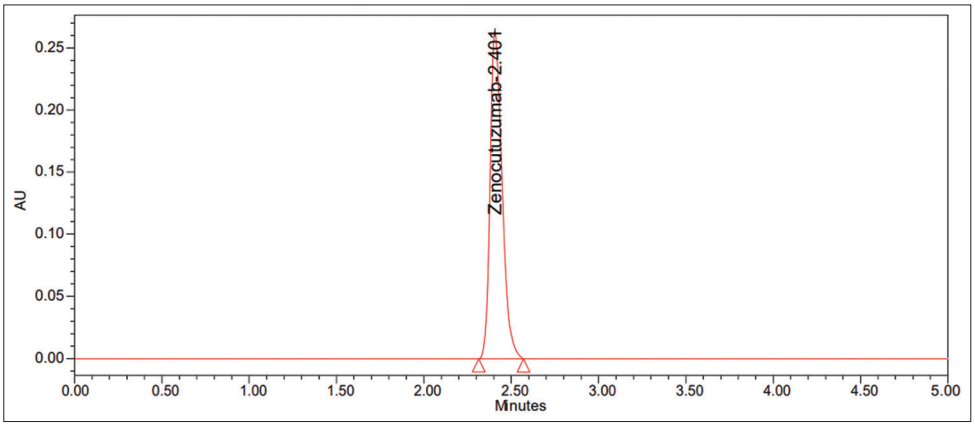

Figure 18: Chromatogram of intermediate precision

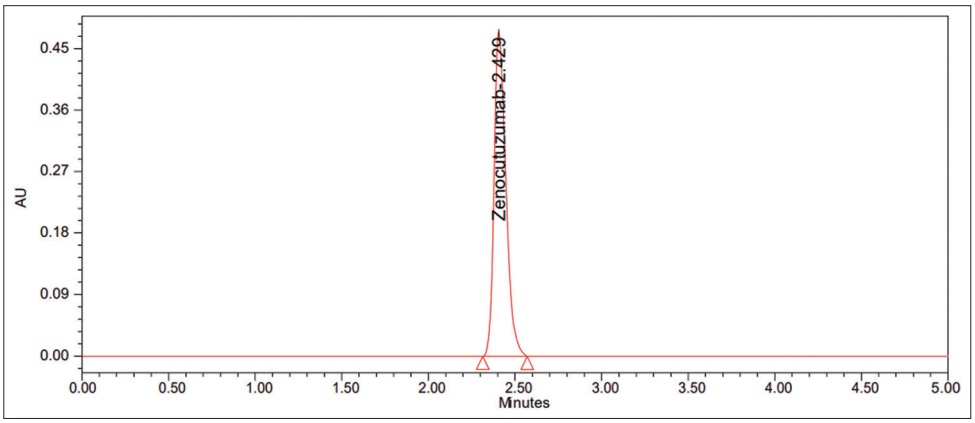

Figure 19: Chromatogram for accuracy at 80% level

degradation products of Zenocutuzumab using reversed-phase techniques.

Compatibility with advanced chromatographic techniques

The method can be further optimized and transferred to high-resolution platforms such as ultra-high performance liquid chromatography or liquid chromatography-mass spectrometry to enable enhanced sensitivity, reduced run time, and detailed impurity and variant profiling of Zenocutuzumab.

Evaluation across diverse delivery systems

Further studies should investigate the method's applicability to emerging delivery systems, including lipid-based carriers, polymeric systems, and sustained-release formulations, to ensure consistent performance across different formulation matrices.

Automation and high-throughput analysis

Future research may explore automation and method scalability for routine quality control testing, particularly

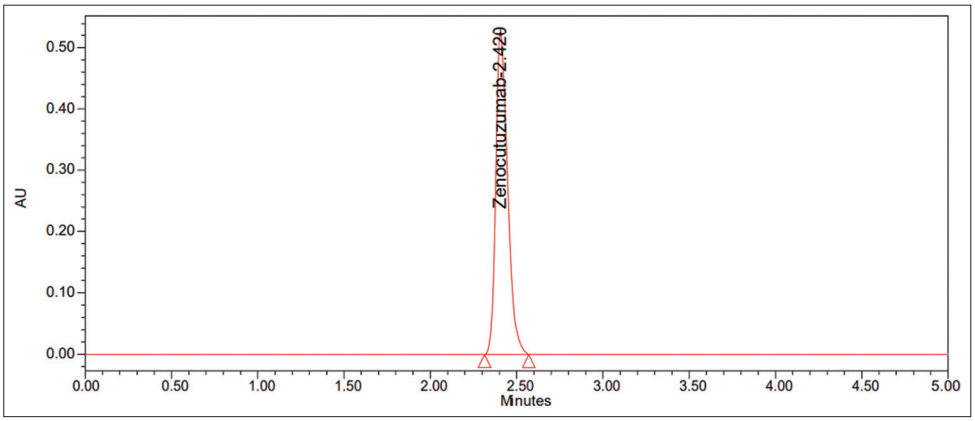

Figure 20: Chromatogram showing 100% accuracy level

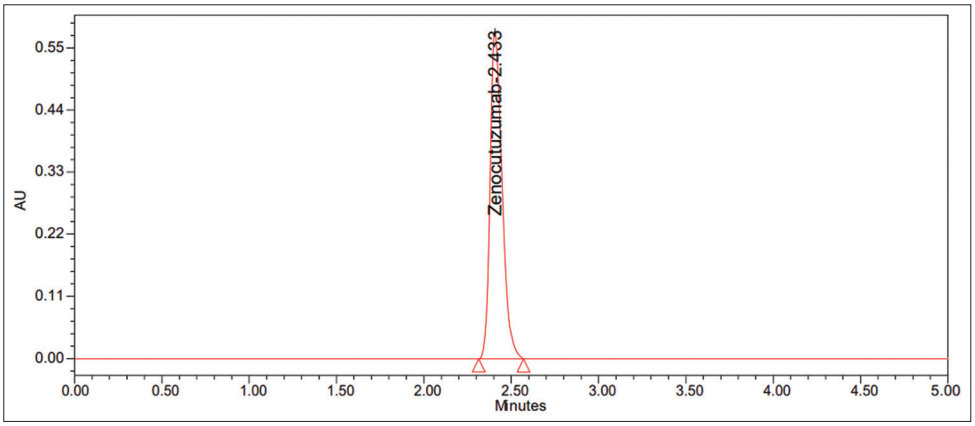

Figure 21: Accuracy study chromatogram (120%)

Table 6: Accuracy evaluation of the analytical method

Analytical exactness	Sample peak area	Concentration of zenocutuzumab	Amount recovered	% Extraction efficiency % recovery±SD, %RSD (*n*=3)
80	5276431	18.00	18.03	99.8±0.42, 0.420
	5254984	18.00	17.96	
	5230376	18.00	17.88	
100	5858471	20.00	20.02	100.2±0.10, 0.100
	5870163	20.00	20.06	
	5861727	20.00	20.04	
120	6451245	22.00	22.05	100.3±0.55, 0.550
	6495670	22.00	22.2	
	6425812	22.00	21.96	

% RSD: Percentage relative standard deviation

Table 7: Results of the accuracy of zenocutuzumab

S. No.	Zenocutuzumab area	% Assay
1.	2955412	100.8
2.	2943780	

for large-batch manufacturing and real-time process monitoring.

Regulatory lifecycle management

Additional work can address method adaptability under ICH Q12 guidelines, supporting post-approval changes and continuous process verification for commercial manufacturing.

System suitability

System suitability was established by administering a standard solution through injection of Zenocutuzumab at

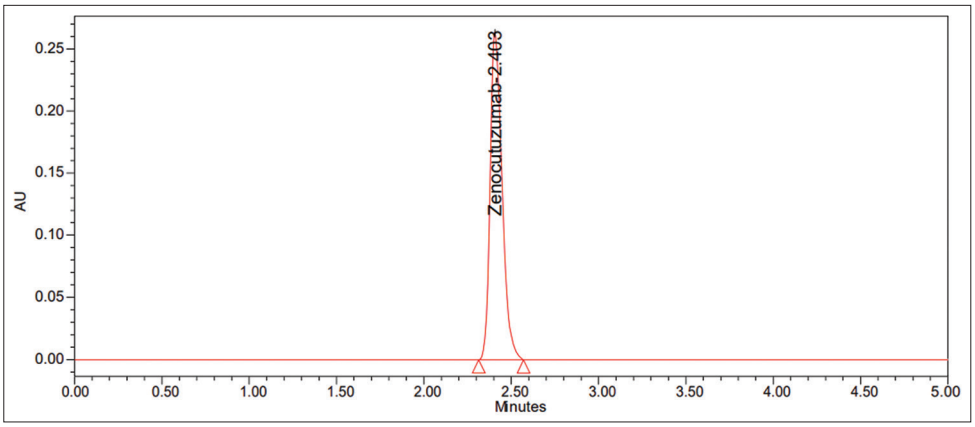

Figure 22: Assay chromatogram 1

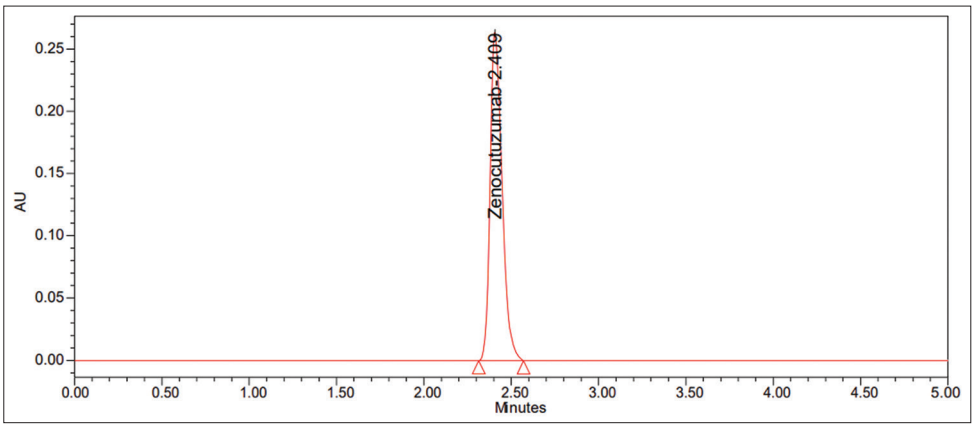

Figure 23: Chromatographic profile of assay – 2

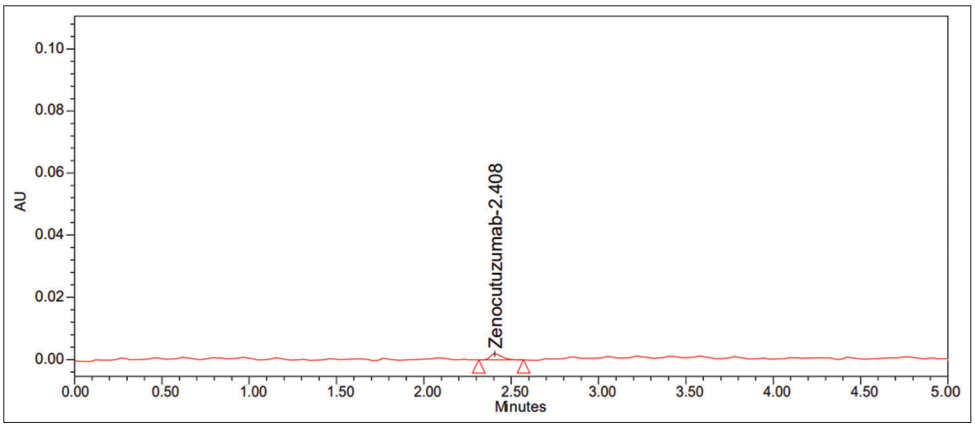

Figure 24: Chromatogram for limit of detection

Table 8: Evaluation of detection and quantification thresholds

Zenocutuzumab			
Detection limit		**Quantification limit**	
Measured amount	Analytical signal-to-noise ratio	Measured amount	Signal relative to noise
0.6 µg/mL	3	2.0 µg/mL	10

Table 9: Results of method consistency under variations

Condition	Coefficient of variation (%) zenocutuzumab
Pump rate (1.1 mL/min)	0.150
Pump rate (0.9 mL/min)	0.360
Solvent phase (33:67)	0.25
Solvent phase (27:73)	0.32

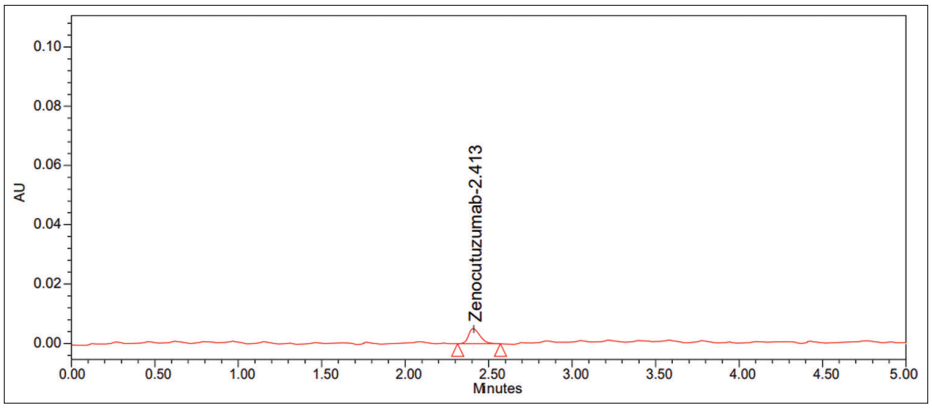

Figure 25: Chromatogram showing the limit of quantification

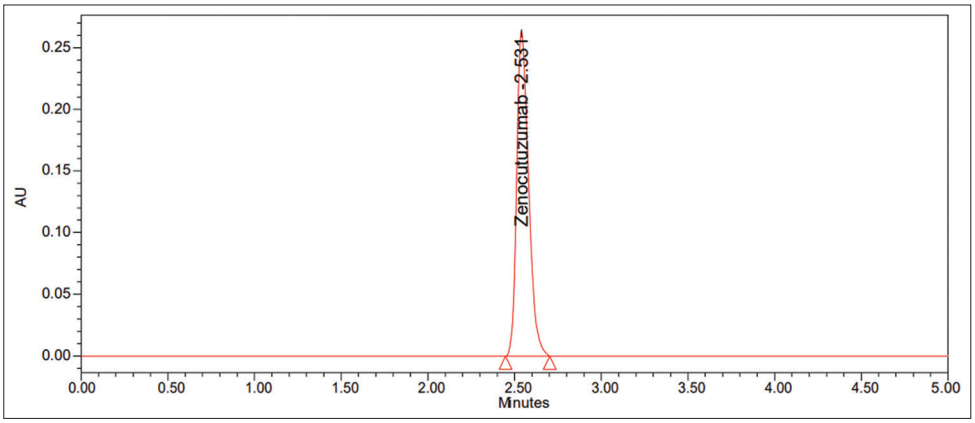

Figure 26: Chromatogram at lower flow rate (0.9 mL/min)

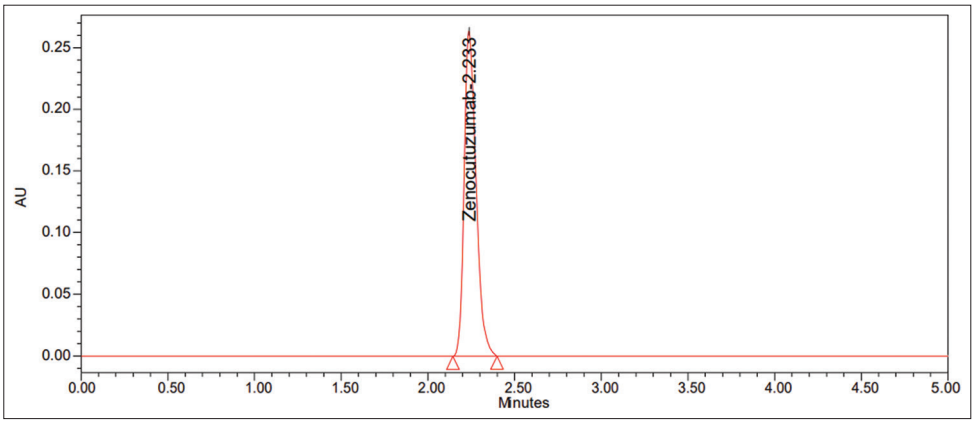

Figure 27: Chromatographic profile at 1.1 mL/min (high flow rate)

Table 10: Data from stability-indicating degradation studies

Forced degradation variable	Zenocutuzumab % deg
Control degradation	0
Acidic degradation	12.3
Basic degradation	10.4
Oxidative degradation	12.7
Reductive degradation	4.5
Heat-induced degradation	10.9
Light-induced degradation	3.1
Hydrolytic stress	1.5

100 µg/mL with six replicate determinations. The obtained results demonstrate that all system suitability parameters satisfied the acceptance criteria.

Analytical selectivity

"No signal from the blank affected Peak retention period of Zenocutuzumab."

Analytical linearity

The method's linearity was observed by constructing a calibration plot using chromatographic peak area

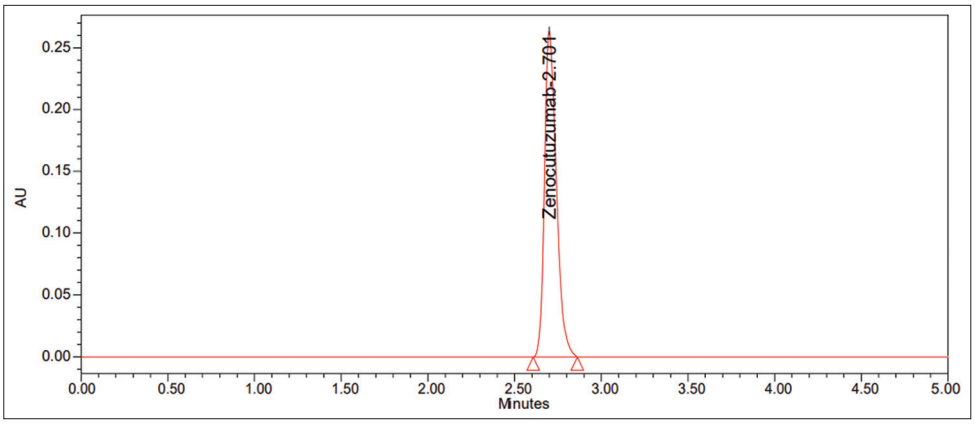

Figure 28: Chromatogram with lower organic phase composition (27:73)

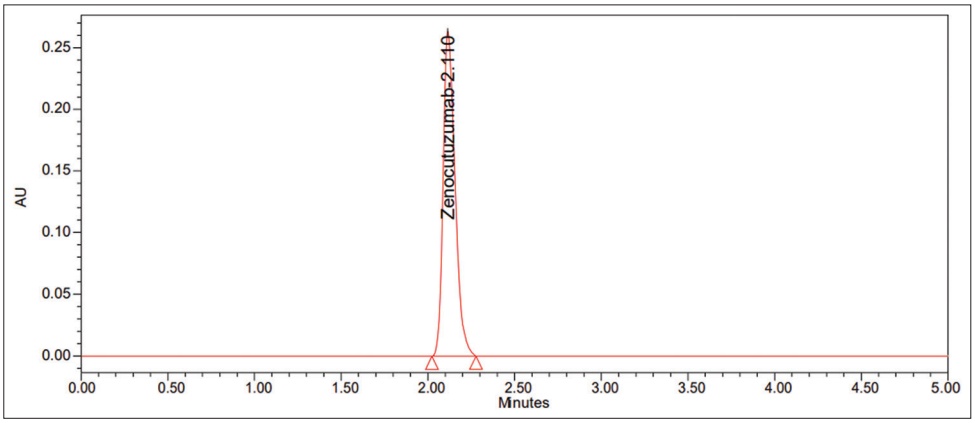

Figure 29: Chromatogram obtained using higher organic content (33:67)

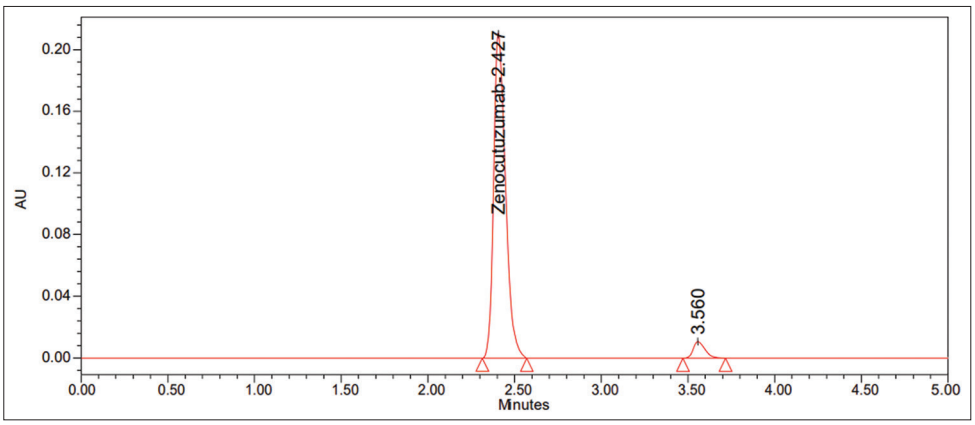

Figure 30: Acid degradation chromatogram

response against relevant concentrations. The data curve showed a straight-line relationship within 25–150 µg/mL of Zenocutuzumab. The resulting regression equation was Y = 28932.28X + 16561.68, with R^2 = 0.99979.

Repeatability

The reproducibility applied technique evaluated for short-term (repeatability) and intermediate precision variability. Within-day precision assessments were performed by analyzing six replicates of the Zenocutuzumab immediately prepared sample solution identical experimental conditions. Reproducibility under varied conditions was assessed within the same experimental laboratory by conducting the experiment was performed by various analysts on different instruments. The optimized method demonstrated high consistency, as Percentage relative standard deviation (% RSD) values were found to be below 2%. In addition, satisfactory the obtained drug recoveries were achieved at all spiked concentration levels, confirming the accuracy of the testing procedure.

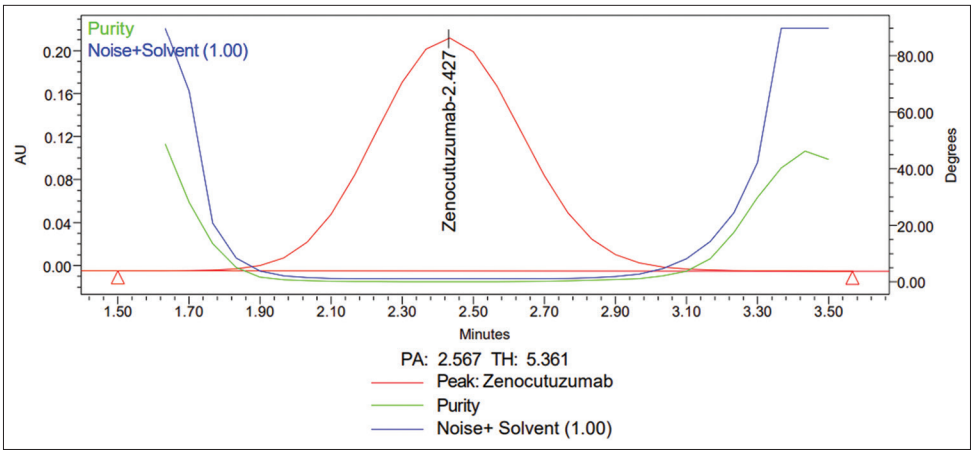

Figure 31: Purity profile of Zenocutuzumab

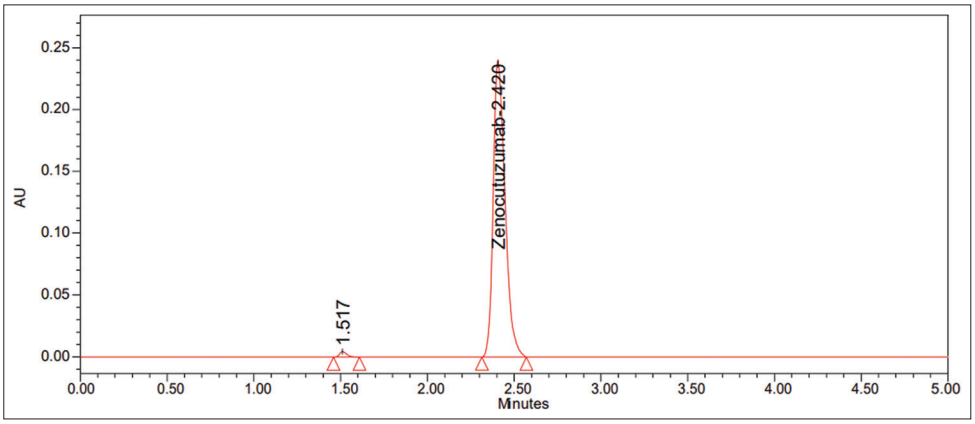

Figure 32: Chromatogram for alkali stress degradation

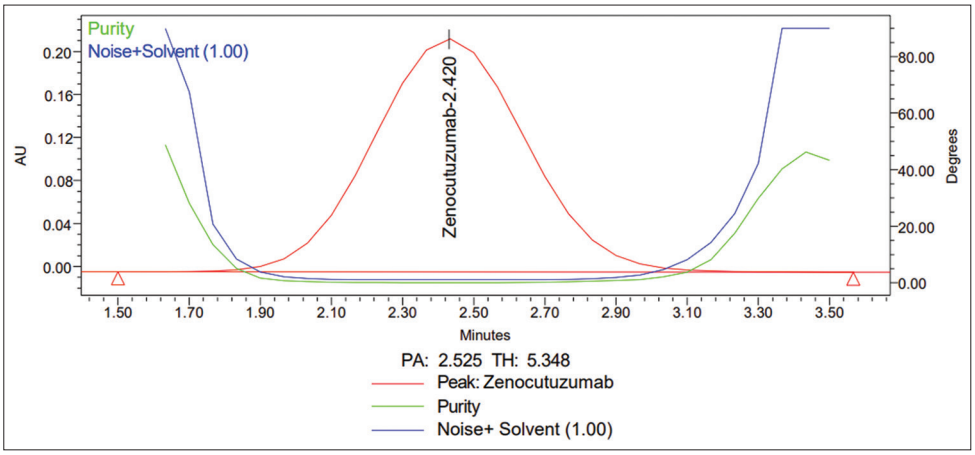

Figure 33: Chromatographic purity plot of Zenocutuzumab

Reproducibility

Intermediate precision represents the within-lab variability, such as results obtained on separate days by different laboratory analysts, or using various instruments in the same laboratory.

Acceptance criteria

The % RSD of the areas obtained from six sample solutions should be not more than 2%.

Closeness to true value

The trueness of the method performance was characterized through quantitative recovery evaluation across three levels (80%, 100%, and 120%). Sample solutions of Zenocutuzumab were prepared at concentrations of 180, 200, and 220 micrograms per milliliter. Each spiked solution was assayed in three independent runs following the established test procedure. Accuracy was confirmed with recoveries

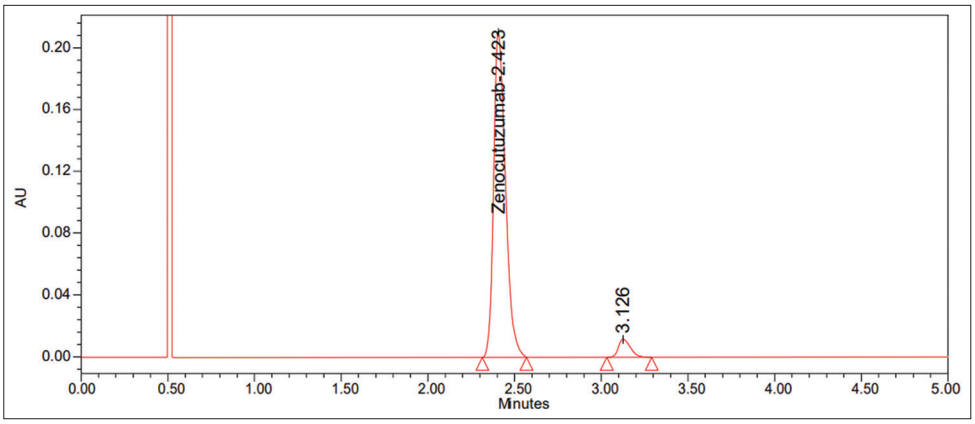

Figure 34: Chromatogram of oxidative (peroxide) degradation

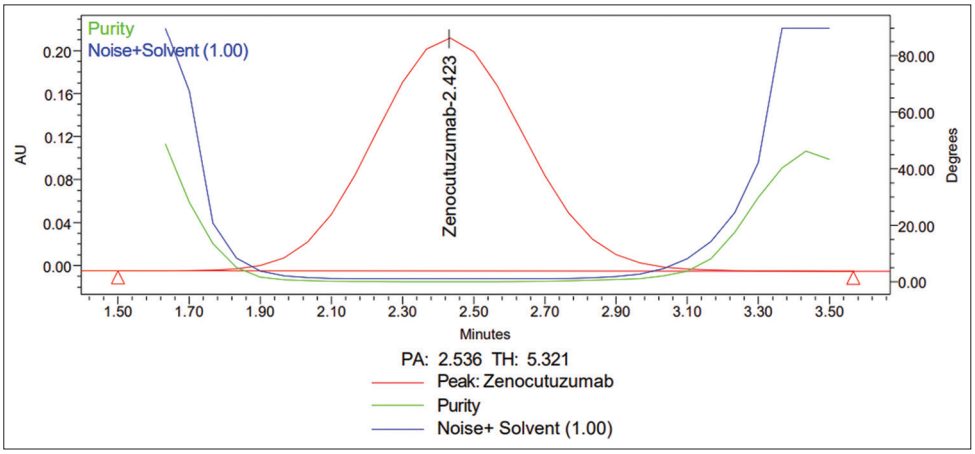

Figure 35: Zenocutuzumab purity chromatogram plot

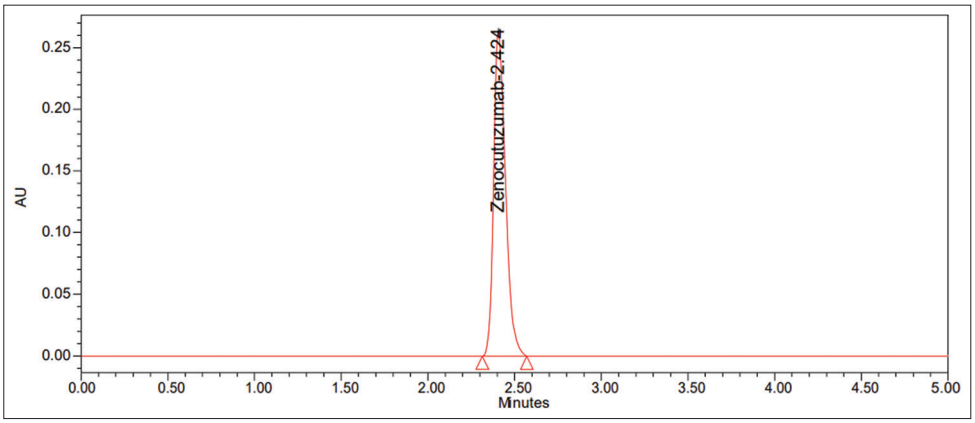

Figure 36: Chromatogram showing reduction-induced degradation

near 100%, and precision was demonstrated by RSD values <2%%. Accuracy (% recovery), central tendency (mean), and repeatability (RSD) were determined. Recovery evaluations confirmed the method's suitability and reproducibility within the validated range.

Assay

The assay of the drug substance was determined by HPLC by comparing the sample's peak area with that of the reference material. Assay by HPLC showed that the drug content was within 98–102% of the labeled claim. Two different assay sample solutions results are shown in above the table.

Minimum detectable concentration and minimum quantifiable concentration

The limit of detection (LOD) for Zenocutuzumab was found adjusted to 0.6 microgram per milliliter with peak signal

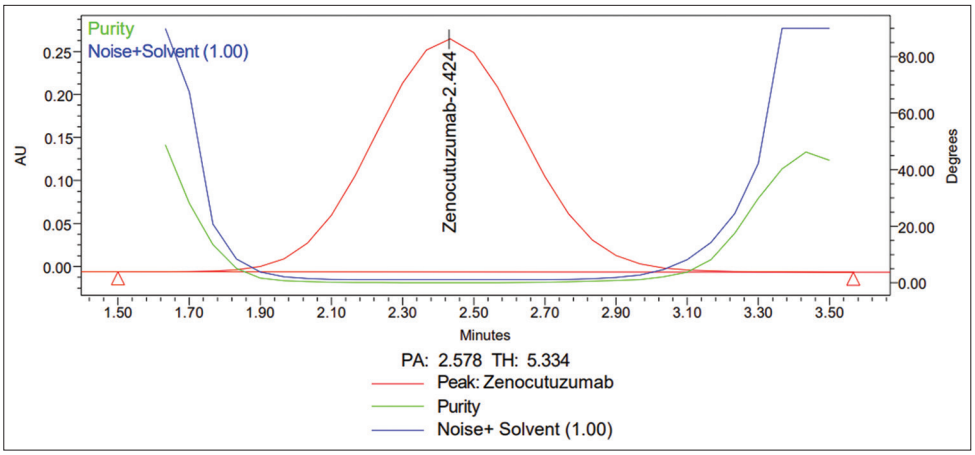

Figure 37: Purity assessment plot of Zenocutuzumab

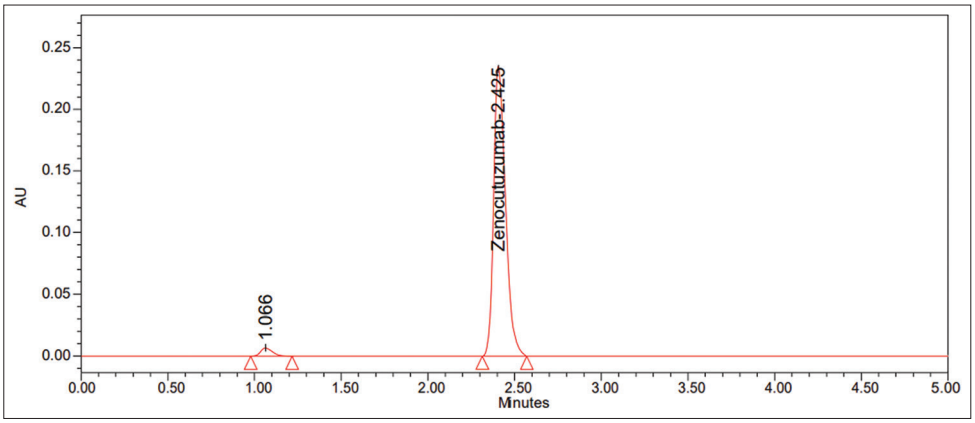

Figure 38: Thermal degradation study chromatogram

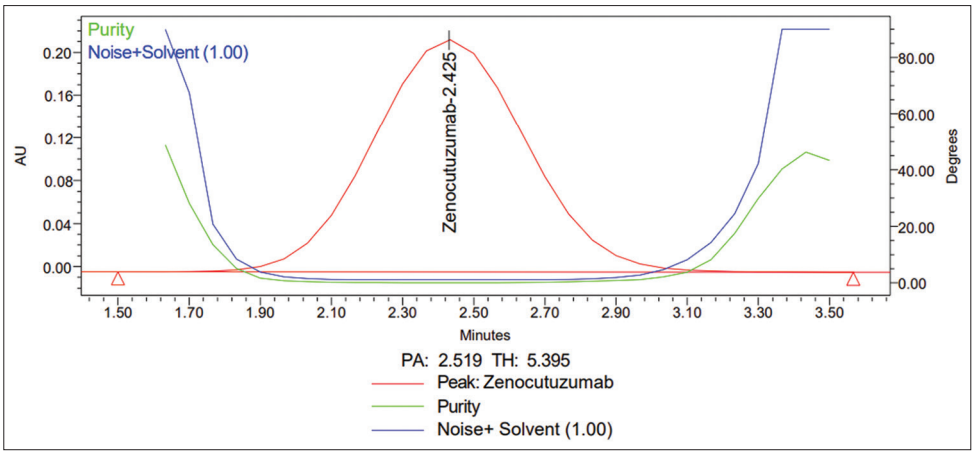

Figure 39: Plot showing purity of Zenocutuzumab

relative to noise ratio at a 3:1 ratio. The limit of quantification (LOQ) was determined as 2.0 µg/mL, corresponding to the minimum quantifiable signal corresponding to S/N = 10. Validation of the analytical method was carried out according to ICH Q2 (R1) guidelines.

$$LOD = (3.3 \times \sigma)/s$$

$$LOQ = (10 \times \sigma)/s$$

Method reliability under variable conditions

The reliability of the analytical procedure based on chromatography was evaluated by altering the solvent delivery rate and the composition of the mobile phase. The RSD values were within acceptable limits.

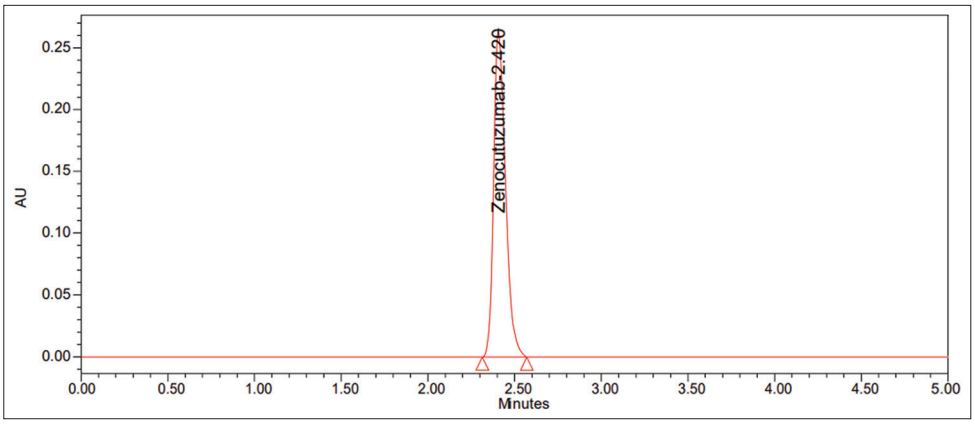

Figure 40: Photodegradation study chromatogram

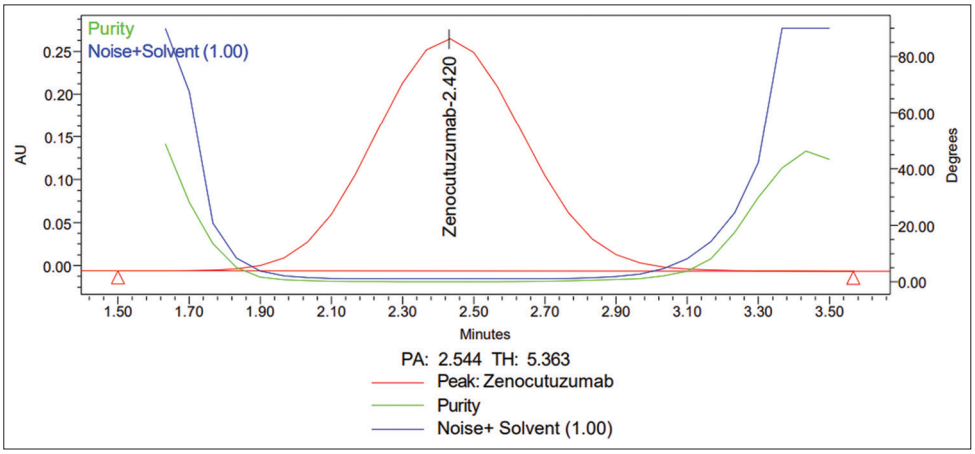

Figure 41: Purity evaluation plot for Zenocutuzumab

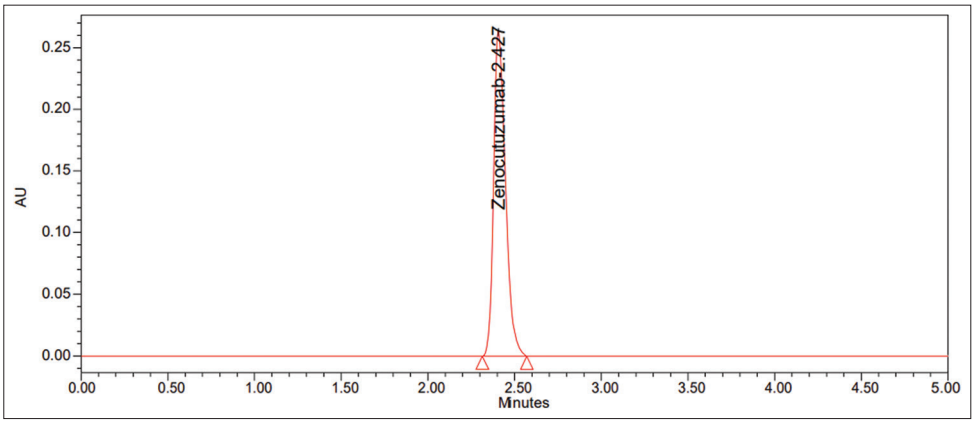

Figure 42: Chromatogram of hydrolytic degradation

Stability testing under forced conditions

The developed chromatographic procedure can be applied for drug release kinetics and stability evaluation assessments to enable method performance assessments and may be regarded as stability-monitoring a technique**.** Stress-induced decomposition experiments were performed in accordance with ICH guidelines, including degradation under acidic, alkaline, oxidative, reductive, heat-mediated, light-induced, and water-mediated stress conditions. Examination of the chromatograms demonstrating that the compounds maintained stability under applied stress, although degradation products were detected.

CONCLUSION

In the present work, a new, rapid, cost-effective, sensitive, and readily accessible HPLC method was developed for the quantification of Zenocutuzumab in both bulk drug and

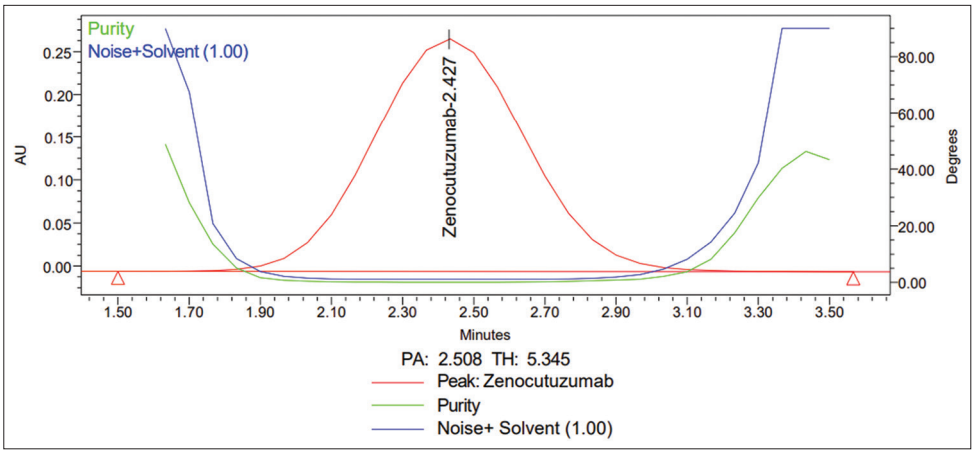

Figure 43: Purity evaluation plot for Zenocutuzumab

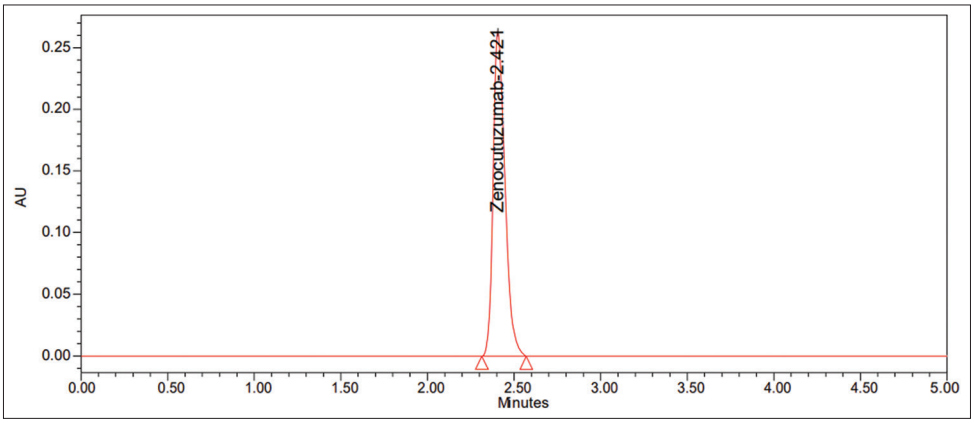

Figure 44: Chromatographic profile of control degradation

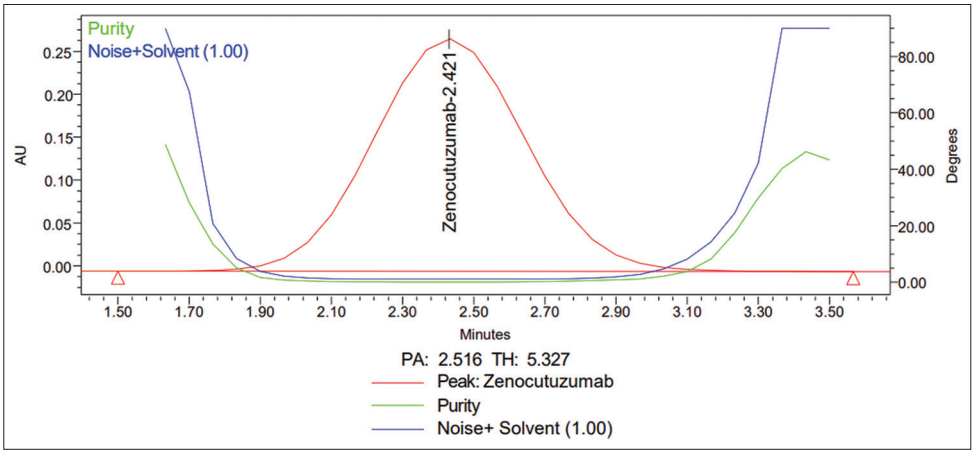

Figure 45: Purity determination profile of Zenocutuzumab

marketed pharmaceutical formulations. A notable advantage of the proposed method is that no previously established HPLC procedures have been reported for this analyte. The method is characterized by a short analysis time, economical operation, simplicity, high sensitivity, reliability, and good reproducibility, making it suitable for routine analysis of a large number of samples.

The method was validated for various parameters, including linearity, accuracy, specificity, robustness, and precision, and all results were found to be within acceptable limits. The %RSD values for all validation studies were below 2%, confirming the reliability of the method and the consistency of the results obtained.

Therefore, the developed method can be effectively applied in quality control laboratories for routine analysis of Zenocutuzumab in bulk and marketed dosage forms without the need for any preliminary separation steps.

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AUTHOR'S CONTRIBUTIONS

All authors participated in designing the study, conducting experiments, analyzing data, and preparing the manuscript, and approved the final version.

SUMMARY

An optimized, fast, and sensitive HPLC procedure was implemented for the analysis of the quantification of Zenocutuzumab in both bulk drug and marketed formulations. A key utility of this technique approach is that no prior high-performance liquid chromatography method has been reported for this analyte. The procedure achieves expedited run times, affordability, ease of use, detection capability, method robustness, and repeatability**,** which are particularly valuable when analyzing a substantial number of samples. The analytical method was comprehensively validated, and all assessed parameters for linearity, accuracy, specificity, robustness, and precision conformed to set acceptance standards. The RSD values for all parameters were below 2%**,** confirming the method's reliability and the consistency of the results obtained. Therefore, this optimized HPLC technique can be conveniently used for standard testing and quality evaluation of Zenocutuzumab in medicinal compound formulations without requiring any initial chromatographic separation.

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