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ORIGINAL ARTICLE

Robust Analytical Method Development and Validation for HER2 Antibody and Enzymatic Adjuvant in Clinical Preparations

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ABSTRACT
Trastuzumab and Hyaluronidase-OYSK are both integral to cancer treatment. This study focused on the development of a reverse-phase high-performance liquid chromatography technique that has been validated for simultaneous assay of Trastuzumab and Hyaluronidase-OYSK using Waters Alliance-e2695HPLC with Agilent Eclipse XDB (250x 4.6mm, 5µ) column with UV detection at 228 nm, and the run time is 8 minutes. The mobile phase containing Acetonitrile: Ammonium formate pH-3.0/OPA in the ratio of 30:70% v/v with a flow rate of 1.0 ml/min. The retention times for Trastuzumab 2.092 min and Hyaluronidase-OYSK 3.469 min were determined. The method exhibited a concentration range of 1500–9000 µg/ml for Trastuzumab and 0.63–3.75 µg/ml for Hyaluronidase-OYSK. The Limit of Detection and Limit of Quantification were determined to be 0.6µg/ml and 1.8 µg/ml for Trastuzumab, 0.07 µg/ml and 0.25 µg/ml for Hyaluronidase-OYSK, respectively. The %recovery of Trastuzumab was 99.95% and Hyaluronidase-oysk was 100% respectively. The method that was developed showed a high level of accuracy, precision, specificity and robustness, as evidenced by the relative standard deviation (RSD) being less than 2% as per ICH guidelines. Hence, successfully this method can be used for routine analysis and stability study.
Keywords: Trastuzumab, Hyaluronidase-OYSK, HPLC, Stability indicating

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INTRODUCTION

The advancement of targeted cancer therapies has significantly improved clinical outcomes for patients with various malignancies. Among these, Trastuzumab, a humanized monoclonal antibody targeting the human epidermal growth factor receptor 2 (HER2), has revolutionized the treatment landscape for HER2-positive breast and gastric cancers. By specifically binding to HER2 receptors, Trastuzumab inhibits tumor cell proliferation and mediates antibody-dependent cellular cytotoxicity, thereby enhancing therapeutic efficacy and patient survival rates [1-8]. Despite its clinical success, the therapeutic efficacy of Trastuzumab can be further optimized through strategic combination with adjunctive agents. Hyaluronidase, an enzyme that catalyzes the degradation of hyaluronic acid in the extracellular matrix, has emerged as a promising adjunct in enhancing drug delivery and distribution within tumor tissues [9]. Specifically, the formulation of hyaluronidase-oysk (a recombinant form of hyaluronidase) in combination with Trastuzumab is posited to improve the intratumoral penetration and bioavailability of the antibody, thereby potentially overcoming resistance mechanisms and enhancing antitumor activity [10]. The successful co-administration of Trastuzumab and hyaluronidase-

oysk necessitates rigorous analytical methodologies to ensure the stability, compatibility, and accurate quantification of both agents within the formulation. High-Performance Liquid Chromatography (HPLC) is a pivotal analytical technique widely employed in the pharmaceutical industry for the separation, identification, and quantification of complex biological molecules. HPLC offers high resolution, sensitivity, and reproducibility, making it an indispensable tool for the characterization of biopharmaceutical combinations[11]. Previous studies have demonstrated the application of HPLC in the analysis of monoclonal antibodies and enzyme-based therapies individually. For instance, HPLC methods have been successfully developed for the quantification and purity assessment of Trastuzumab, utilizing techniques such as size-exclusion chromatography (SEC) and reversed-phase HPLC (RP-HPLC)[12]. Similarly, analytical methods for hyaluronidase have leveraged HPLC for enzymatic activity assays and purity profiling [13]. A literature survey reported that, few methods are available for simultaneous estimation of Trastuzumab and hyaluronidase-oysk and a few articles reported spectrophotometric techniques for estimation of Trastuzumab alone and with other drugs such as, LC-MS/MS, and RP-HPLC [14-17].

However, the simultaneous analysis of Trastuzumab and hyaluronidase-OYSK within a combined formulation presents unique analytical challenges, including potential interactions between the two proteins, differences in their physicochemical properties, and the need for method validation to ensure specificity and accuracy. The development of a robust and validated HPLC method tailored for this combination is essential for quality control, regulatory compliance, and the successful translation of this combination therapy from bench to bedside. This study aims to create a simple, precise, accurate, relatively sensitive, and fast RP-HPLC technique for estimating Trastuzumab and hyaluronidase-oyskin. The developed method was validated as per ICH guidelines[18,19] and can be applied successfully to quality control determinations.

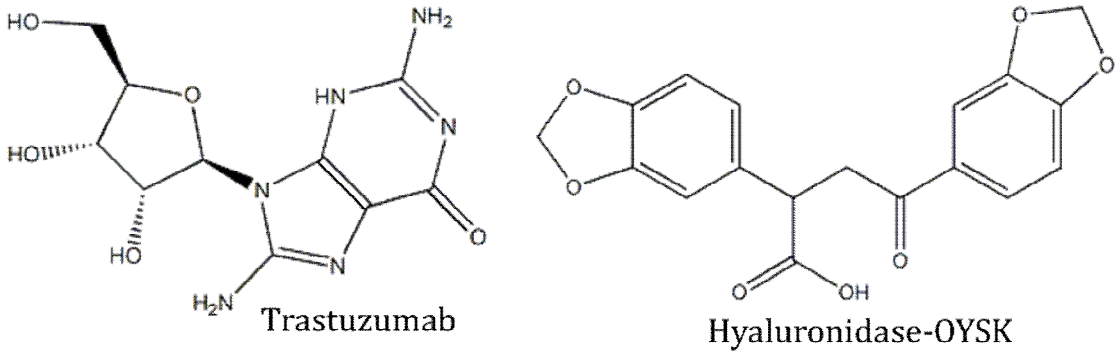

Figure 1: Structure of Trastuzumab and hyaluronidase-OYSK

MATERIAL AND METHODS

Chemicals and Reagents

Trastuzumab and hyaluronidase-oyskin pure drugs were received from Pharma life research lab, Hyderabad. Trastuzumab, and hyaluronidase-oyskin injections (Herceptin Hylecta) were obtained from the local market. Acetonitrile HPLC grade was received from Rankem, Water (Milli Q) was produced in-house, and Ammonium Formate, orthophosphoric acid, and Formic Acid of HPLC grade were obtained from analytical reagents.

Preparation of standard solution

Accurately weigh and transfer 600 mg of Trastuzumab and 5 mg of Hyaluronidase-oysk working standard into a 10 ml clean, dry volumetric flask add Diluent and sonicate to dissolve it entirely and make volume up to the mark with the same solvent and again pipette 0.5ml of the above Hyaluronidase-Oysk solution into 10ml volumetric flask and dilute up to the mark with diluent (Stock solution). Further, pipette 1 ml of the above stock solutions into a 10 ml volumetric flask and dilute with diluent up to the mark. (6000ppm of Trastuzumab, 2.5ppm of Hyaluronidase-oysk)

Preparation of Sample Solution

Transfer 0.5ml of Trastuzumab and Hyaluronidase-oysk sample into a 10mL clean, dry volumetric flask, add diluent, and sonicate it up to 30 mins to dissolve, and centrifuge for 30min. Dissolve it entirely and make the volume rise to the mark with the same solvent. Then, it is filtered through a 0.45-micron Injection filter. (6000ppm of Trastuzumab, 2.5ppm of Hyaluronidase-oysk).

Method Validation

Specificity/Selectivity

The method's ability to distinguish and quantify the analyte in the presence of other components like impurities, degradation products, or matrix components. To ensure no interference at the analyte retention time, analyze blank samples, spiked samples with known analyte concentrations, and samples containing potential interferences.

Linearity

The method's ability to elicit results directly proportional to the concentration of the analyte within a given range. Prepare calibration curves by analyzing samples of known concentrations (typically 5–7 levels) and plot the response vs. concentration. Calculate the correlation coefficient (R^2), which should be ≥ 0.99 for most applications.

Accuracy

The closeness of the test results to the actual value. Analyze known concentrations of the analyte and compare the measured value with the actual value. Express results as a percentage recovery, typically within 98-102% for pharmaceutical assays.

Precision

Perform replicate analyses (typically six) on homogeneous samples and calculate %RSD (Relative Standard Deviation), with the acceptable limit generally being ≤ 2%.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The lowest amount of analytes can be detected but not necessarily quantified. The lowest amount of analyte can be quantitatively determined with acceptable precision and accuracy. Based on the signal-to-noise ratio, 3:1 for LOD and 10:1 for LOQ is typically sufficient. LOD and LOQ can also be estimated from the response's calibration curve slope and standard deviation.

Robustness

The method's capacity to remain unaffected by minor but deliberate variations in method parameters (e.g., changes in pH, temperature, flow rate). Intentionally vary method parameters and assess if the changes affect the results significantly. The method should maintain acceptable performance under varying conditions**.**

Degradation Studies:

Degradation studies for Trastuzumab and Hyaluronidase-OYSK were conducted under various stress conditions to assess their stability. Oxidative degradation involved adding 1 ml of 20% hydrogen peroxide to 1 ml of stock solution, heating at 60°C for 30 minutes and analyzing by HPLC after dilution to 6000 µg/ml and 2.5 µg/ml. Acid degradation was performed by refluxing 1 ml of stock solution with 1 ml of 2N hydrochloric acid at 60°C for 30 minutes, followed by HPLC analysis at the same concentrations. Similarly, alkali degradation was conducted by refluxing the stock solution with 2N sodium hydroxide at 60°C for 30 minutes. Dry heat degradation was tested by placing the drug at 105°C for 6 hours, and Photostability was assessed by exposing the drug solution to UV light for seven days or 200 Watt hours/m^2. In neutral degradation studies, the drug was refluxed in water for 6 hours at 60°C. Reduction degradation was executed by treating the sample with 1ml of 10% Sodium bisulfate at 60 °C for 1 hour. For each condition, the resulting solution was diluted to 6000 µg/ml and 2.5 µg/ml, and 10 µl was injected into the HPLC system to record chromatograms and eluate sample stability.

RESULTS AND DISCUSSION

The chromatographic analysis was performed using an Agilent Eclipse XDB (250x4.6 mm, 5 µ) with a mobile phase consisting of acetonitrile (ACN) and ammonium formate pH-3/OPA in a ratio of 30:70. The flow rate was set at 1 ml/min, and the detector wavelength was 228 nm. The column temperature was maintained at 30°C, and an injection volume of 10 µL was used. The total run time for each analysis was 8.0 minutes. Both peaks have good resolution, tailing factor, Theoretical plate count, and resolution. The total runtime for each validation parameter was set to 8 minutes.

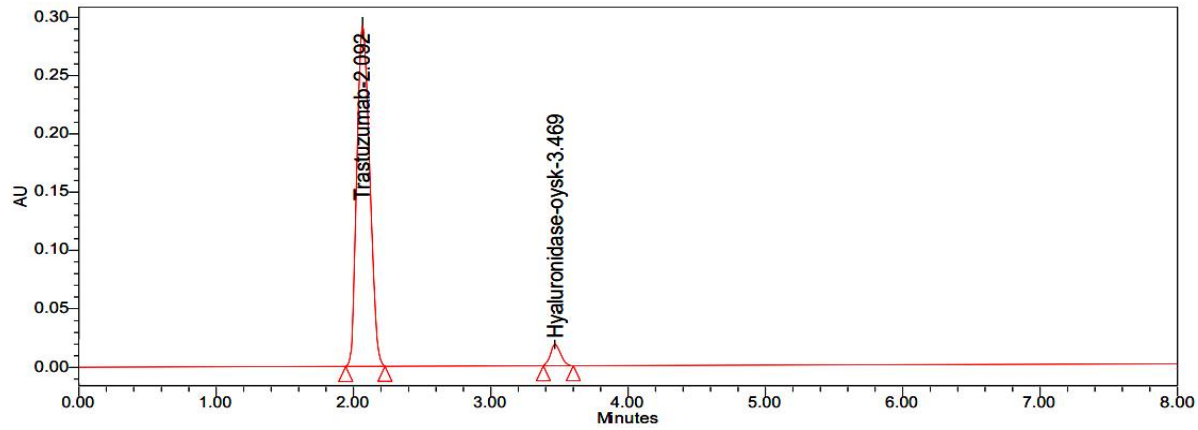

Figure 2: Optimized Chromatogram

With the optimized chromatographic conditions, **t**he Trastuzumab peak was observed at 2.092 min with peak area 3854216, tailing factor 1.05 Hyaluronidase-oysk peaks was observed at 3.469min, with peak area 99653, tailing factor 1.03 and resolution 8.35.

Method Validation

The following parameters were studied to validate the HPLC method for the determination of Trastuzumab and Hyaluronidase-OYSK as per the protocol and demonstrate that the method is appropriate for its intended use. All the validation parameters were carried out according to ICH.

Specificity

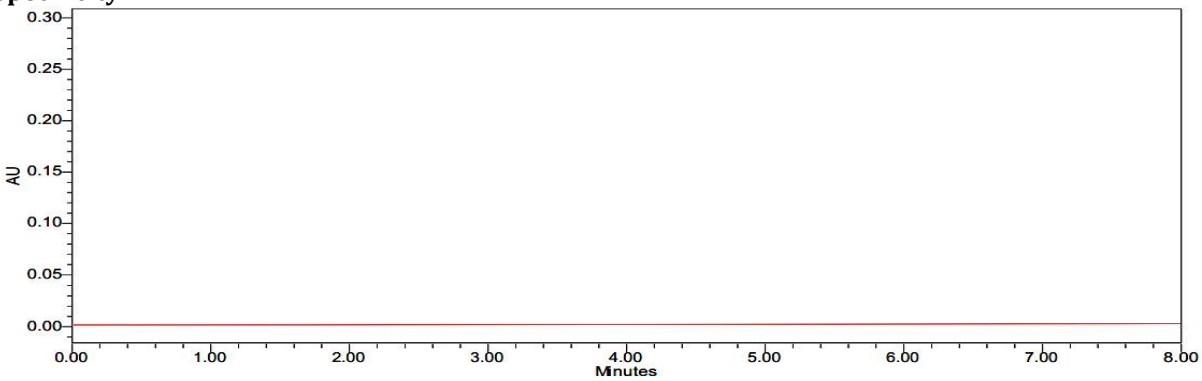

Figure 3: Chromatogram of blank

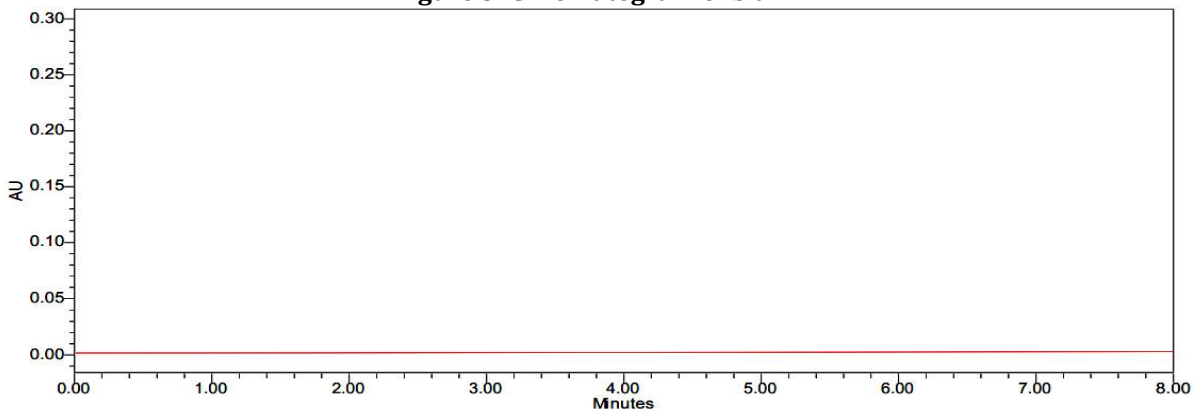

Figure 4: Chromatogram of placebo

Linearity

Six linear concentrations of Trastuzumab (1500–9000 µg/ml) and Hyaluronidase-oyskin (0.63-3.75µg/ml) were injected and duplicated. Average areas were mentioned in table No. 1, and the linearity equations obtained for Trastuzumab was y = 657.35x +18080.18, and for Hyaluronidase-oyskin, was y =39647.54x +116.43. The correlation coefficient obtained was 0.999 for the two drugs.

Table 1. Linearity for Trastuzumab & Hyaluronidase-oysk

S.No.	Trastuzumab		Hyaluronidase-oysk	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	1500.00	1084369	0.63	24757
2	3000.00	1954782	1.25	49682
3	4500.00	3012467	1.88	74235
4	6000.00	3864271	2.50	99524
5	7500.00	4883659	3.13	125469
6	9000.00	6033548	3.75	147522
Regression equation	y = 657.35x +18080.18		y =39647.54x +116.43	
Slope	657.35		39647.54	
Intercept	18080.18		116.43	
R^2	0.99940		0.99989	

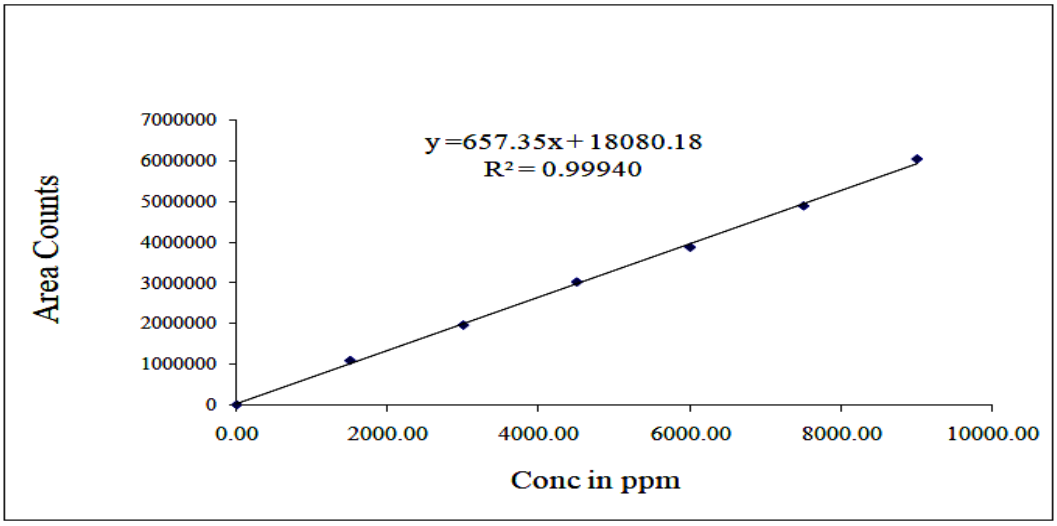

Figure 5: Calibration curve of Trastuzumab

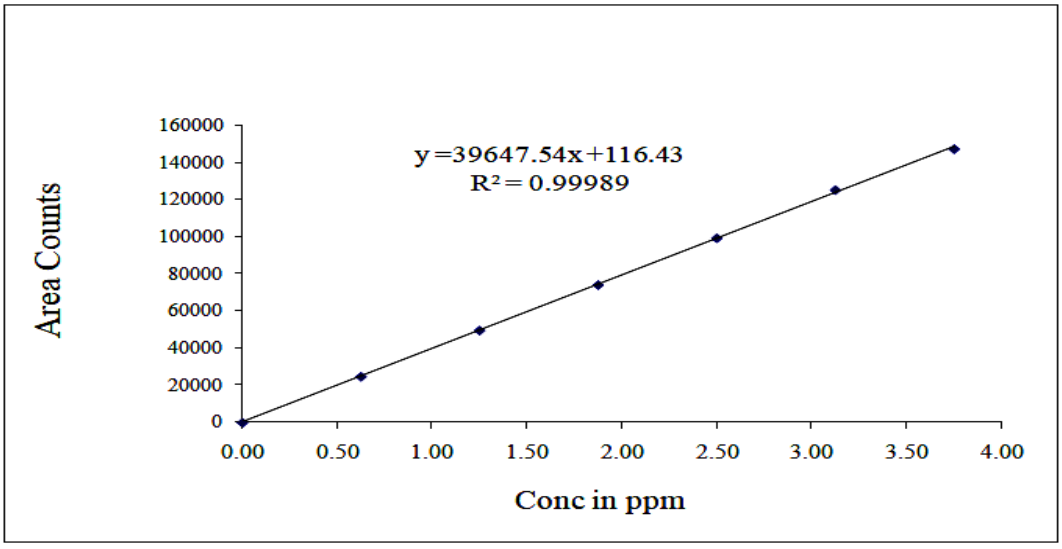

Figure 6: Calibration curve of Hyaluronidase-oysk

Precision

Method Precision

The %RSD (Relative Standard Deviation) for Trastuzumab & Hyaluronidase-oysk was calculated using six replicate injections. The mean area for Trastuzumab was 3875955 with a standard deviation (S.D) of 14040, resulting in a %RSD of 0.36%. Similarly, the mean area for Hyaluronidase-oysk was 99730, with a

standard deviation of 186.17, giving a %RSD of 0.19%. These low %RSD values indicate high precision and reproducibility of the method for both compounds.

Intermediate precision (Day_ Day Precision)

Intermediate precision of Trastuzumab & Hyaluronidase-oysk based on six replicate injections. The mean area for Trastuzumab on day-1 and day-2 was 3862205, 3861724 with a standard deviation (S.D) of 21178.7, 15459.7 resulting in a %RSD of 0.55%, 0.4% respectively. Hyaluronidase-oysk mean area on day-1 and day-2 was 99571, 99549 with a standard deviation of 269.8, 336.4 yielding a %RSD of 0.27%, 0.34% respectively. These results indicate good intermediate precision for both analytes, demonstrating the method's consistency when tested under different conditions or over different days.

Accuracy

Three levels of Accuracy samples were prepared by the standard addition method. Triplicate injections were given for each level of accuracy, and the mean Recovery was 100.04% and 100% for Trastuzumab & Hyaluronidase-oysk, respectively.

Sensitivity

The Limit of Detection (LOD) for Trastuzumab was 0.6 µg/ml, and the Limit of Quantitation (LOQ) was 1.8 µg/ml. Hyaluronidase-oysk LOD was 0.075 µg/ml, and the LOQ was 0.25 µg/ml. These values indicate the method's ability to detect and quantify deficient concentrations of both analytes with high sensitivity.

Robustness

The method's robustness for Trastuzumab & Hyaluronidase-oysk was evaluated under various conditions by altering the flow rate, mobile phase composition, and temperature. When the flow rate was decreased to 0.9 ml/min, the %RSD for Trastuzumab was 0.21%, and for Hyaluronidase-oysk, was 0.15%. When the flow rate was increased to 1.1 ml/min, the %RSD values were 0.4% and 0.75%, respectively. For mobile phase composition changes, a ratio of 27A:73B resulted in %RSD values of 0.25% for Trastuzumab and 0.21% for Hyaluronidase-oysk, while a 33A:67B ratio yielded %RSDs of 0.35% and 0.63%. Lastly, temperature variations at 27°C showed %RSDs of 0.6% and 1.0%, indicating that the method is robust under these conditions.

Table 2. Robustness data for Trastuzumab & Hyaluronidase-oysk

S.No.	Condition	%RSD of Trastuzumab	%RSD of Hyaluronidase-oysk
1	Flow rate (-) 0.9ml/min	0.21	0.15
2	Flow rate (+) 1.1ml/min	0.40	0.75
3	Mobile phase (-) 27A:73B	0.25	0.21
4	Mobile phase (+) 33A:67B	0.35	0.63

Assay

Herceptin Hylecta, bearing the label claim Trastuzumab and Hyaluronidase-oysk. An assay was performed with the above formulation. The average % Assay for Trastuzumab and Hyaluronidase-oysk obtained was 99.8% and 100%, respectively.

Degradation Studies

The method's specificity was demonstrated through forced degradation studies conducted on the sample using acid, alkaline, oxidative, reductive, and thermal degradation. The sample was exposed to these conditions, and the main peak was studied for peak purity, thus indicating that the method effectively separated the degradation products from the pure active ingredient. Regulatory guidance in ICH Q2A, Q2B, Q3B, and FDA 21 CFR section 211 requires developing and validating stability-indicating potency assays.

Table 3. Forced Degradation results for Trastuzumab and Hyaluronidase-oysk

Degradation	Area	% Assay	% Deg	Area	% Assay	% Deg
Control	3871216	100	0	99632	100	0
Acid	3454213	89.2	10.8	87596	87.9	12.1
Alkali	3352232	86.6	13.4	88696	89.0	11.0
Peroxide	3312784	85.6	14.4	86220	86.5	13.5
Reduction	3834218	99.0	1.0	96584	96.9	3.1
Thermal	3765041	97.2	2.8	95989	96.3	3.7
Photolytic	3698359	95.5	4.5	98795	99.1	0.9
Hydrolysis	3742106	96.6	3.4	98632	98.9	1.1

CONCLUSION

The study concluded that the HPLC method developed and validated for the simultaneous quantification of Trastuzumab and Hyaluronidase-oysk in drug products proved to be simple, precise, accurate, and

sensitive. The method demonstrated excellent separation of both analytes with well-defined retention times and consistent results across different levels of accuracy. Validation parameters, including % recovery, LOD, and LOQ, confirmed the method's reliability and robustness for routine analysis. This method can be effectively applied for quality control and routine analysis of Trastuzumab and Hyaluronidase-oysk in pharmaceutical formulations.

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