RESEARCH ARTICLE

A UPLC Method for Simultaneous Estimation of Trastuzumab and Hyaluronidase in Bulk and Pharmaceutical Dosage Form

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ABSTRACT

Objective: The proposed research aimed at establishing a quick, accurate, and exact ultra-performance liquid chromatography (UPLC) methodology for contemporaneously analyzing trastuzumab and hyaluronidas in bulk and pharmaceutical formulation, with a focus on the stability-indicating properties of the assay.

Method: Using a Water X-Bridge C18 column (50 mm x 4.6 mm x 2.5 µ) and a mobile phase of ACN: Buffer (pH 2.5) [50% v/v], pushed at 0.5 mL/min, compounds were separated chromatographically. An ultraviolet (UV) detector fixed at 260 nm was employed to identify the isolated compounds.

Results: The findings showed that 1.3529 and 2.404 minutes were optimal for separating Trastuzumab and Hyaluronidase, correspondingly. The current procedure has been verified in accordance with ICH standards Q2 R1, and stability-indicating tests have been performed in accordance with ICH standards Q1A R2. Both the intra- and inter-day precisions were determined to be satisfactory. The suggested approach showed linearity between 30 to 180 µg/mL of trastuzumab and between 12.50-75µg/mL of hyaluronidase. It was determined that the limit of detection (LoD) and limit of quantitation (LoQ) for trastuzumab were 0.36 and 1.2 µg/mL, correspondingly, whereas those for hyaluronidase were 0.15 and 0.5 µg/mL. The approach was shown to have a recovery rate around 98.6 to 99.7%.

Conclusion: The suggested approach successfully separated the chemical compounds from their by-products. Therefore, trastuzumab and hyaluronidase routine evaluation and stability-indicating tests were both effectively implemented using the approach.

Keywords: UPLC, Validation, Trastuzumab, ICH guidelines Hyaluronidase, Forced degradation studies

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INTRODUCTION

When combined with additional cancer therapies, the injectable formulation of trastuzumab (TST) and hyaluronidase (HYA) can be administered for managing HER2-overexpressing, node-positive or negative (ER/PR-negative or having one high-risk characteristic) breast cancer.[1,2] For patients with HER2-positive cancers, the FDA authorized the drug trastuzumab in October 2017, especially for breast, gastric and gastroesophageal malignancies. Its molecular formula is $C_{6448}H_{9948}N_{1720}O_{2012}S_{442}$ oligosaccharide reset ($C_{47}H_{61}ClN_4O_{13}S$).[3,4]

Some malignancies that overproduce a molecule known as the HER2 protein may be stopped in their tracks by using the innovative treatment formulation of trastuzumab and hyaluronidase-oysk.[5,6] Individuals with malignancies that have been demonstrated to overexpress HER2 should be the only ones to get this treatment.[7,8]

According to the research done, no ultra-performance liquid chromatography (UPLC) technique for concurrent quantification of trastuzumab and hyaluronidase in bulk and pharmaceutical formulations has been published in the literature. Trastuzumab and hyaluronidase-oysk concentrations, both singly and in mixtures, have been determined by spectroscopic high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS), according to a small number of published studies. Trastuzumab evaluation in combination with various medications has only been documented through a limited number of spectroscopic approaches.[9-11] Only a couple of HPLC techniques for determining hyaluronidase-oysk alone

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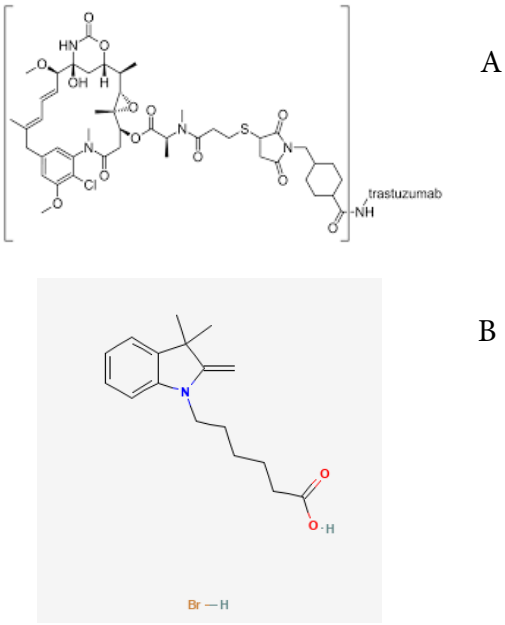


Figure 1: Structures of (A) Trastuzumab (B) Hyaluronidase

or in tandem with other medications have been revealed.[12,13] Figure 1 show the molecular makeup of trastuzumab and hyaluronidase-oysk, respectively.

MATERIALS AND METHODS

Chemicals

Supriya Labs in Mumbai generously provided us with a free sample of trastuzumab and hyaluronidase-oysk. Water (HPLC grade, Milli Q or comparable) and other solvents from Merck Specialties Pvt. Ltd., in Mumbai, India.

Instrumentation

Chromatographic condition

For this experiment, researchers employed a photodiode array detector (model 2998) and an Agilent model:1290 Infinity equipped with 2.0 data handling system for monitoring purposes.

Preparation of standard solution

Carefully transfer 200 mg of trastuzumab and 50 mg of hyaluronidase-oysk to a flask, then add 70 mL of dilutant followed by sonication to disperse the mixture. In addition, an examination in the UV area revealed that the highest absorption occurred at 260 nm after being diluted from 5 to 50 mL using a dilutant.

Preparation of sample solution

About 1-mL of sample (equal to 120 mg of trastuzumab + Hyaluronidase-oysk 50 mg) is put in a flask, and then 70 mL of dilutant are added, followed by sonication to dissolve the dilution. And then, water up the concentration from 5 to 50 mL.
Average Weight: 1-mL
Label Claim: Trastuzumab 120 mg + Hyaluronidase-oysk 2000 units (Eq. to 50 mg)

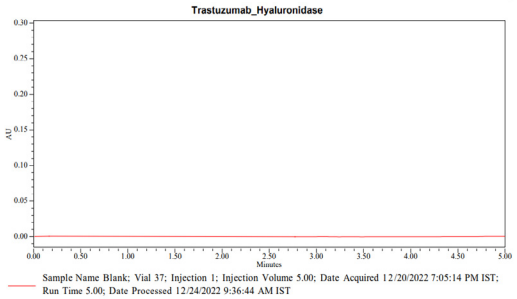


Figure 2: Blank chromatogram

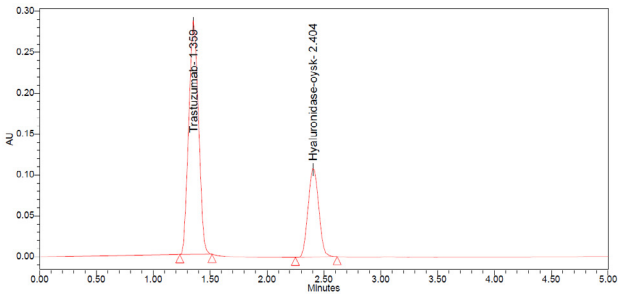


Figure 3: Standard sample chromatogram

Method Development

An in-depth analysis of chromatographic settings, including column type and temperature, mobile phase, and flowing velocity, is essential for optimizing chromatographic methodology, achieving symmetrical peak design, and enhancing resolution. The mobile phase was optimized by trying out numerous mixtures of appropriate solvents, and in the end, ACN: H2O (pH 3.5) (50:50 v/v) was chosen as the optimal mobile phase at 0.2 mL/min. Table 1 displays the optimum chromatographic parameters. Figures 2 and 3 displayed the blank and optimized chromatogram, respectively.

Method Validation

System suitableness, linearity, accuracy, robustness, precision, and selectivity are only few of the factors that were considered while validating the suggested technique in accordance with ICH Q2R1 recommendations.[14-16]

Linearity

According to ICH, linearity is the extent to which the method of analysis yields test findings that scale linearly with the quantity of analyte in the given sample.[17,18] The spectrum of an analyte concentrations, defined as the difference between the highest and lowest concentrations, is an indicator of the analytical method's accuracy, precision, as well as linearity. In order to establish linearity, small portions were prepared and analyzed in threefold throughout an amount range of 30 to 180 µg/mL for trastuzumab and 12.5 to 75.0 µg/mL for hyaluronidase-oysk. By comparing the calibration graph to the linear regression formula, a correlation coefficient was calculated.[19,20] Figures 4 and 5 displayed the linearity results.

Table 1: Optimized findings of trastuzumab and hyaluronidase-oysk

S. No	*Components*	*Optimized conditions*
1	Column	Water X-Bridge C18, 50 x 4.6 mm, 2.5 µ
2	Wavelength	260 nm
3	Injection volume	5 µL
4	Column temperature	Ambient
5	Flow rate	0.5 mL/min
6	Run time	5 minutes

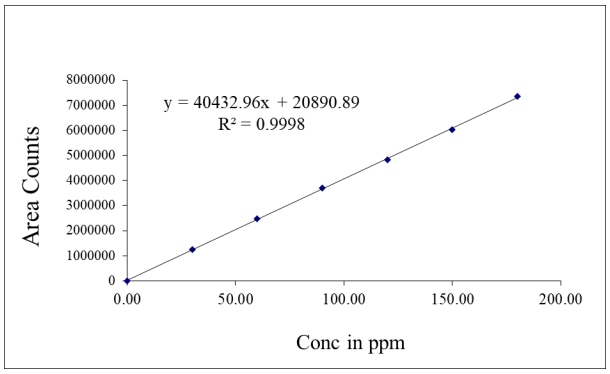


Figure 4: Calibration curve of trastuzumab

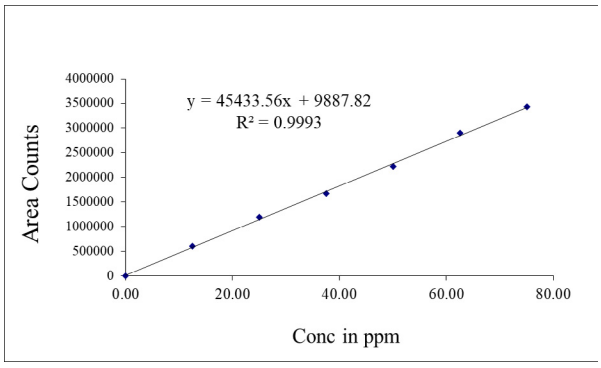


Figure 5: Calibration curve of hyaluronidase-oysk

Precision

Analysis precision was calculated using both intra- and inter-day standards.[21,22] The RSD is a measure of accuracy.

Accuracy

Accuracy is measured by how much data can be recovered.[23] Pre-examined samples are assessed at three concentrations before being spiked with established quantities of standard trastuzumab and hyaluronidase-oysk.

LoD and LoQ

According to ICH, the minimum detectable concentration of an analyte (although it is not always its quantitative value) is known as the limit of detection.[24,25] The minimal detectable concentration of a component in a sample using an appropriate analytical technique is called the LoQ.

The LoD and LoQ were calculated through data from a calibration graph.

Forced degradation studies

Stability-indicating characteristic of the suggested technique was assessed using forced degradation studies (FDS). Experiments were performed under a variety of conditions, including acid hydrolysis (0.5N HCl/60°C/1h), alkaline hydrolysis (0.5N NaOH/60°C/1h), oxidative (10% H_2O_2/60°C/1h), hydrolytic (water/60°C/1h), photolytic (UV energy-254 nm/3 days/dark control), and thermal degradation (105°C/75%RH/24 hours).[26,27] Trastuzumab and hyaluronidase-oysk9 were both tested for their stability by subjecting them to FDS under different stress settings.[28-30]

RESULTS

Linearity

Both trastuzumab (y = 40432.96x + 20890.89) and hyaluronidase-oysk (y = 45433056x + 9887.82), when tested across the dosage ranges of 30 to 180 and 12.50 to 75.00 µg/mL, correspondingly, were found to have linear concentration-response relationships. Table 2 displays the linearity information for trastuzumab and hyaluronidase-oysk.

LoD and LoQ

Trastuzumab's LoD and LoQ were determined to be 0.36 and 1.2 µg/mL, respectively, whereas hyaluronidase-oysk's were 0.15 and 0.5 µg/mL.

Precision

Intra- and inter-day precision findings for trastuzumab and hyaluronidase-oysk, respectively, reveal a %RSD of 0.66 and 0.98, respectively, demonstrating the approach's accuracy. Tables 3 and 4, respectively highlight the outcomes of accuracy.

Accuracy

The reliability of the procedure was tested by analyzing recoveries after 50, 100, and 150% spiking. Using %RSD, we calculated that the mean recovery rates for trastuzumab were 100.4, 100.3, and 99.8, while those for hyaluronidase-oysk were 99.3, 98.4, and 99.6. The results were 0.75, 0.510, 0.21 for trastuzumab and 0.960, 0.370, 0.570 for hyaluronidase-oysk. Tables 4 and 5 show the results of the accuracy values.

Table 2: Linear regression data for calibration curves (n = 6)

Parameters	*Trastuzumab*	*Hyaluronidase*
Linearity	30–180	12.5–75.0
$r^{2 \pm}$ SD	0.99986 ± 0.0001	0.99936 ± 0.0001
Slope ± SD	40432.96 ±	45433.56 ± 37.8
Intercept ± SD	20890.89 ± 1444.9	9887.82 ± 972.2

Table 3: Findings for the precision of (n = 6)

Drug	*Conc. (µg)*	*Mean ± SD*	*%RSD Mean ± SD*	*%RSD*
Trastuzumab	50	100.66	0.666	0.66
Hyaluronidase-oysk	120	99.9	0.975	0.98

Table 4: Accuracy for trastuzumab (n = 3)

Drug	*Level of injections*	*Mean*	*SD*	*% RSD*	*%recovery*
Trastuzumab	50	100.4	0.75	0.750	100.4
	100	100.3	0.51	0.510	100.3
	150	98.8	0.20	0.200	98.8

Table 5: Accuracy for hyaluronidase (n = 3)

Drug	*Level of injections*	*Mean*	*SD*	*% RSD*	*%recovery*
Hyaluronidase	50	99.3	0.96	0.960	99.3
	100	98.4	0.370	0.370	98.4
	150	99.6	0.57	0.570	99.6

Table 6: Robustness of trastuzumab (n = 6)

Parameter	*Mean ± SD*	*%RSD*
Flow rate (-)	99 ± 0.874	0.88
Flow rate (+)	98.7 ± 0.361	0.37
ORG minus	98.9 ± 0.987	1
ORG plus	995949.2 ± 9458.3	0.9

Table 7: Robustness of hyaluronidase-oysk (n = 6)

Parameter	*Mean ± SD*	*%RSD*
Flow rate (-)	99.4 ± 0.6	0.6
Flow rate (+)	99 ± 0.709	0.72
ORG minus	99.8 ± 0.926	0.93
ORG plus	100 ± 0.153	0.715

Robustness

In terms of robustness, there is not a significant variance in peak area or resolution comparing trastuzumab and hyaluronidase-oysk after making minor and intentional modifications to mobile phase proportion, column temperatures, and rate of flow. Information on robustness may be found in Tables 6 and 7.

Assay

With %RSD of 0.361 for trastuzumab and 0.899 for hyaluronidase-oysk, the analysis of the drug product showed that there was not any peak interference by degradants, additives, or impurities, at retention duration of TST and HYA (Figure 6).

Forced Degradation Studies

Sample solutions were subjected to a variety of stresses in order to conduct accelerated degradation investigations. Trastuzumab and hyaluronidase-oysk were found to degrade under acidic, alkaline, peroxide, and heat environments, according to the results of degradation experiments. Chromatograms depicting degradation as a function of pH, acidity, peroxide concentration, temperature, and light intensity are presented in Figures 7, 8, 9, 10, and 11. Figures 12 and 13 displays that neither trastuzumab nor hyaluronidase-oysk showed any degradation peaks under hydrolytic conditions. Table 8 is a summary of the findings we collected on degradation.

DISCUSSION

In order to effectively resolve trastuzumab and hyaluronidase-oysk, it was decided to optimize the mobile phase. Symmetric peak and effective peak resolution in chromatography were achieved by adjusting the mobile phase's pH and amount of organic matter. Retention durations of 1.359 and 2.404 minutes were observed after elution of trastuzumab and hyaluronidase-oysk, respectively. In order to accurately measure both trastuzumab and hyaluronidase-oysk at the same time, an approach was developed with an optimal wavelength of 260 nm. Trastuzumab and hyaluronidase-oysk were successfully separated by chromatography using a mobile phase of ACN and buffer (pH 2.5) [50:50%, v/v] and a

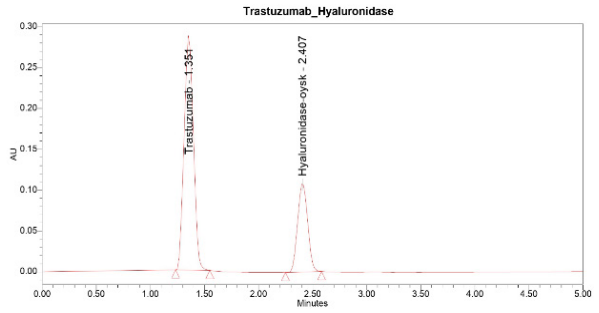
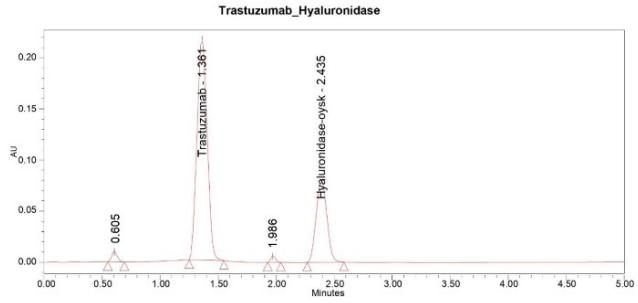
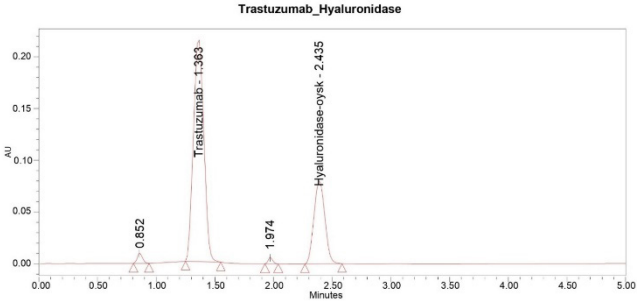


Figure 6: Chromatogram of marketed formulation sample



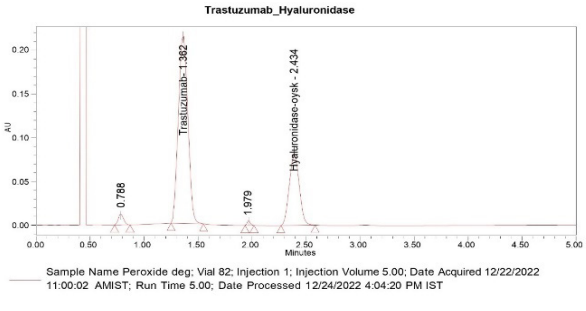
Observation: It may be neutralized by heating 1-mL of HCl (1N) for 30 minutes at 60°C, then adding 1-mL of NaOH (1N) after it has cooled. Drug degradation caused by

Figure 7: Chromatogram of acid degradation



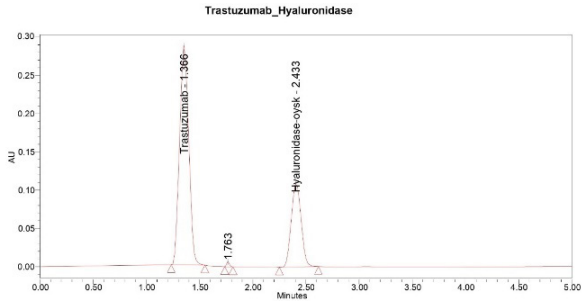
Observation: It may be neutralized by heating 1-mL of NaOH (1N) for 30 minutes at 60°C, then adding 1-mL of HCl (1N) after it has cooled. Drug degradation caused by

Figure 8: Chromatogram of alkali degradation



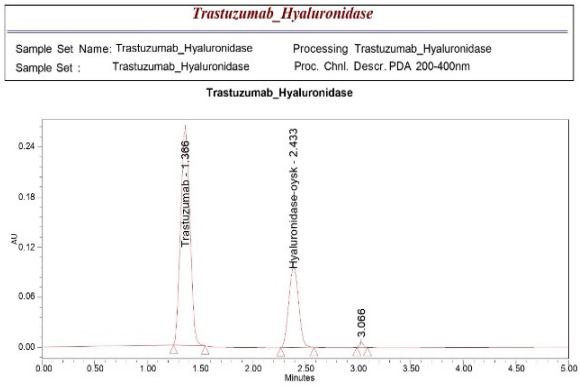
Observation: Put 1-mL of a 30% H_2O_2 solution in a water bath at 60°C for 30 minutes. Drug degradation caused by

Figure 9: Chromatogram of peroxide degradation



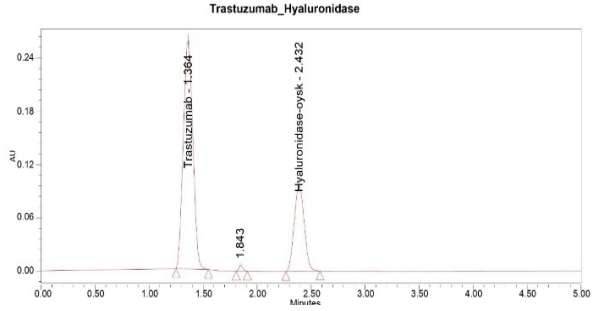
Observation: A 30-minute incubation at 60°C is done for a 1-mL solution of 30% sodium bisulfate.

Figure 10: Chromatogram of reduction degradation



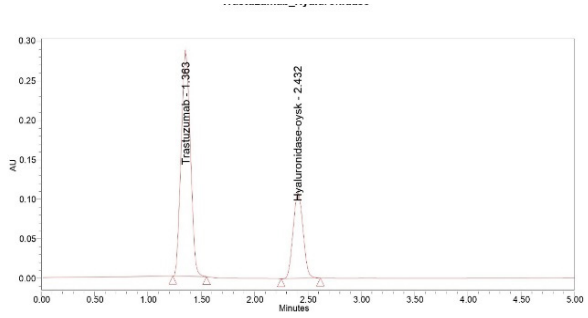
Five mL of the sample was heated in a 105°C hot air oven for three days. Next, a VF holding 10 mL is filled with the 0.5 mL sample.

Figure 11: Chromatogram of thermal degradation (105°C for 72 hours)



Observation: Using a photostability chamber, we observed the effects of exposing 5 mL of the compound to 1.2 million lx h and 200 W h/m^2 of light.

Figure 12: Chromatogram of photodegradation (1.2 millxh and 200Wh/m2 light)



Observation: In a 10 mL volumetric flask (VF), place 1-mL of the Sample stock solution. Fill the remaining 9 mL with HPLC water and warm it using a water bath set to 60°C for 30 minutes.

Figure 13: Chromatogram of hydrolysis degradation (heat on water bath 60°C for 30 minutes)

Table 8: Summary of degradation data

Stress condition	*%Degradation*			
	Trastuzumab	*Hyaluronidase-oysk*	*Peak 1*	*Peak 2*
Acidic	1.361	2.435	0.605	1.986
Base	1.363	2.435	0.852	1.974
Oxidative	1.362	2.434	0.788	1.979
Reduction	1.366	2.433	1.763	…..
Thermal	1.366	2.433	3.066	…..
Photolytic	1.364	2.432	1.843	…..
Hydrolytic	1.363	2.432	…	…..

Water X-Bridge C18, 50 mm 4.6 mm 2.5 µ column running at 0.5 mL/min. When looking at the linearity range, statistics having a correlation of 0.999 indicates a very excellent correlation. For intermediate precision intervals, the relative standard deviation (RSD) of TST and HYA, respectively, was 0.98 and 0.66, indicating the reproducibility of the analytical approach. The suggested analytical approach has a low LoD, which indicates that it is also extremely sensitive. This indicates that the suggested approach has sample accuracy in accordance with ICH requirements, as 99.8 and 99.1% of the spiking trastuzumab and hyaluronidase-oysk, respectively, were recovered. The suggested approach is quite robust, as shown by the fact that even small, intentional modifications to the method's settings have no effect on the findings of SST. It was found that neither hydrolytic nor photo-degradation occurred during the FDS. According to ICH recommendations, forcible degradation of under twenty percent is considered permissible. Trastuzumab and hyaluronidase-oysk showed

degradation of under 20% using the suggested approach, which indicates stability.

CONCLUSION

The current UPLC technique used for the measurement of bulk and dosage forms adopts great sensitivity and reliability. The present procedure was validated using the ICH Q2R1 recommendations. When compared to other approaches, the suggested approach stood out with regard to validation criteria and stability-indicating investigations. Findings for LoQ, LoD, and accuracy were all within the permitted limits during validation, demonstrating that the instrument can provide reliable findings down to very low concentrations. Peaks in degradation may be identified in the research.

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