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RESEARCH ARTICLE

Development and Validation of a Stability Indicating RP-HPLC Method for the Simultaneous Estimation of Trastuzumab and Hyaluronidase-OYSK in Pharmaceutical Dosage Forms

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Abstract: The precise quantification of biologic combinations in pharmaceutical formulations necessitates robust and sensitive analytical techniques that can withstand the complexity of macromolecular structures. A simple, rapid, and reproducible Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Trastuzumab and Hyaluronidase-OYSK. Chromatographic separation was successfully achieved utilizing a Waters Alliance-e2695 system equipped with a Luna Phenyl-Hexyl column (250 x 4.6 mm, 5 µm). The isocratic mobile phase consisted of Acetonitrile and 0.1% Trifluoroacetic acid (TFA) in an equal ratio of 50:50 %v/v, pumped at a flow rate of 1.0 mL/min. Detection was monitored at 228 nm using a photodiode array detector at ambient temperature. The developed method demonstrated superior system suitability, with the number of theoretical plates exceeding 2000 and a tailing factor maintained below 2.0 for both analytes. Linearity was established across significant concentration ranges, and the correlation coefficient was found to be greater than 0.999. Precision was confirmed with a Relative Standard Deviation (RSD) of peak areas consistently below 2.0%. The method was extensively validated in accordance with International Council for Harmonisation (ICH) guidelines, covering parameters such as accuracy, precision, specificity, robustness, and sensitivity. Moreover, forced degradation studies under various stress conditions acidic, alkaline, thermal, oxidative, and photolytic revealed that the method effectively separates degradation products from the main analytes. These results indicate that the proposed RP-HPLC method is suitable, economical, and precise for the routine quality control and stability assessment of Trastuzumab and Hyaluronidase-OYSK in pharmaceutical dosage forms.

Keywords: RP-HPLC; Trastuzumab; Hyaluronidase-OYSK; Method Validation; Stability-Indicating Method.

1. Introduction

Breast cancer remains a significant global health burden, with HER2-positive cases representing a particularly aggressive subset of the disease [1]. The advent of targeted biological therapies has revolutionized treatment paradigms, with Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, serving as a cornerstone in the management of HER2-overexpressing metastatic breast and gastric cancers [2]. Trastuzumab functions by selectively binding to the extracellular domain of the human epidermal growth factor receptor 2 (HER2), effectively inhibiting the proliferation of tumor cells that overexpress this protein [3]. While initially administered via intravenous infusion, recent advancements have favored subcutaneous formulations to enhance patient compliance and reduce healthcare resource utilization [4].

The subcutaneous delivery of large macromolecules like monoclonal antibodies is often limited by the extracellular matrix of the subcutaneous tissue, primarily composed of hyaluronan, which acts as a barrier to fluid dispersion [5]. To overcome this physiological hurdle, formulations often incorporate Hyaluronidase-OYSK, a recombinant human endoglycosidase [6]. This enzyme acts by transiently depolymerizing hyaluronan, thereby increasing the permeability of the connective tissue and facilitating the rapid dispersion and systemic absorption of co-administered biologics [7]. The combination of Trastuzumab and Hyaluronidase-OYSK thus represents a critical evolution in oncology therapeutics, necessitating rigorous quality control measures [8]. Ensuring the safety and efficacy of such complex biopharmaceutical formulations requires high-precision analytical monitoring. Analytical chemistry provides the foundational tools for the qualitative and quantitative assessment of pharmaceutical substances, ensuring adherence to strict regulatory standards [9]. Among the various analytical methodologies available, High-Performance Liquid Chromatography

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(HPLC) is the most widely adopted technique in the pharmaceutical industry due to its superior resolution, sensitivity, and reproducibility [10]. However, the simultaneous analysis of proteins and enzymes poses unique challenges compared to small molecules, primarily due to issues related to conformational stability, adsorption to stationary phases, and the need for specific mobile phase additives to maintain peak symmetry [11].

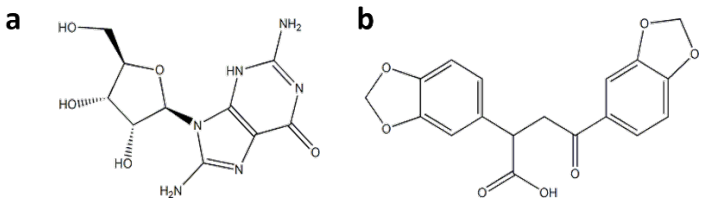

Figure 1. Structure of a. Trastuzumab and Hyaluronidase

Moreover, regulatory bodies such as the International Council for Harmonisation (ICH) mandate the development of stability-indicating assays [12]. These methods must possess the specificity to differentiate the active pharmaceutical ingredient (API) from its degradation products formed under various stress conditions, including hydrolysis, oxidation, and photolysis [13]. While existing literature provides methods for the individual analysis of Trastuzumab or Hyaluronidase [14, 15], there is a marked scarcity of research detailing a unified, stability-indicating RP-HPLC method for their simultaneous quantification in a single run [16]. The lack of a concurrent method increases the time and cost associated with routine quality control. Consequently, the present study aims to bridge this gap by developing and validating a simple, economical, and robust RP-HPLC method for the concurrent analysis of Trastuzumab and Hyaluronidase-OYSK, complying with the stringent validation protocols outlined in ICH Q2(R1) guidelines [17].

2. Materials and Methods

2.1. Chemicals and Reagents

The reliability of analytical data heavily depends on the purity of reagents used [18]. In this investigation, Acetonitrile of HPLC grade was employed as the organic modifier, while Trifluoroacetic acid (TFA) served as the ion-pairing agent. Milli-Q water, generated from an in-house purification system, was utilized for all aqueous preparations to minimize baseline noise and ghost peaks. Working standards of Trastuzumab and Hyaluronidase-OYSK were procured from reputable sources and stored under recommended conditions to prevent degradation prior to analysis. Commercial pharmaceutical formulations containing the target analytes were utilized for assay and recovery studies to demonstrate the method's applicability to marketed products.

2.2. Instrumentation and Chromatographic Conditions

Chromatographic analysis was executed on a Waters Alliance-e2695 HPLC system, a robust platform featuring a quaternary pump, an autosampler for precise injection, and a 2998 Photodiode Array (PDA) detector [19]. Data acquisition and signal processing were managed using Empower 2 software. The separation strategy employed a Luna Phenyl-Hexyl column (250 x 4.6 mm, 5 µm). This stationary phase was selected for its unique selectivity towards aromatic compounds and proteins, offering alternative interaction mechanisms (pi-pi interactions) compared to standard C18 columns [20].

Table 1. Optimized Chromatographic Conditions

Parameter	Condition
HPLC System	Waters Alliance-e2695 with PDA Detector (2998)
Stationary Phase (Column)	Luna Phenyl-Hexyl (250 x 4.6 mm, 5 µm)
Mobile Phase	Acetonitrile : 0.1% Trifluoroacetic acid (50:50 v/v)
Flow Rate	1.0 mL/min
Detection Wavelength	228 nm
Column Temperature	Ambient (25°C)
Injection Volume	10 µL
Run Time	6.0 minutes
Elution Mode	Isocratic

The mobile phase consisted of a mixture of Acetonitrile and 0.1% TFA in a 50:50 v/v ratio. The inclusion of TFA is critical in protein analysis as it suppresses the ionization of silanol groups on the column and ion-pairs with the basic residues of the proteins,

thereby improving peak shape and reducing tailing [21]. The mobile phase was filtered through a 0.45 µm membrane filter and degassed via ultrasonication. The flow rate was maintained at 1.0 mL/min, and the column temperature was controlled at ambient conditions (25°C). Detection was optimized at 228 nm, a wavelength that provides adequate sensitivity for the peptide bonds present in the analytes.

2.3. Preparation of Solutions

2.3.1. Preparation of Standard Solutions

A primary standard stock solution was prepared by accurately weighing 600 mg of Trastuzumab and 5 mg of Hyaluronidase-OYSK into a 10 mL clean, dry volumetric flask. The mixture was dissolved in the diluent (Acetonitrile) with the aid of ultrasonication to ensure complete solubilization and homogeneity. From this primary stock, a secondary dilution was performed by pipetting 0.5 mL of the solution into a 10 mL volumetric flask and diluting it to the mark with the diluent. Further working standard solutions were prepared to yield final concentrations of 6000 ppm (µg/mL) for Trastuzumab and 2.5 ppm (µg/mL) for Hyaluronidase-OYSK.

2.3.2. Preparation of Sample Solutions

For the assay of pharmaceutical formulations, an aliquot equivalent to the target dose was transferred into a 10 mL volumetric flask. The solution was diluted with the diluent, sonicated to ensure extraction and homogeneity, and filtered through a 0.45 µm syringe filter to remove any excipients or particulate matter that could foul the column [22]. Further dilutions were carried out to achieve the target concentrations within the established linearity range.

3. Results and Discussion

3.1. Method Optimization

The optimization process involved a systematic evaluation of chromatographic parameters to achieve acceptable resolution and peak symmetry.

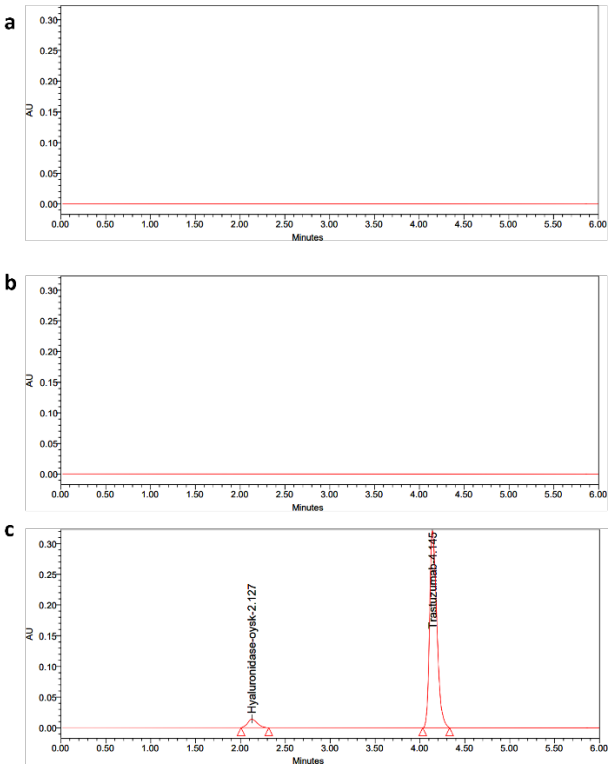

Figure 2. Chromatogram of a. Blank b. Placebo and c. Optimized Chromatogram

Initial trials utilizing methanol and phosphate buffers resulted in broad peaks and poor resolution, likely due to secondary interactions between the protein analytes and the stationary phase [23]. The transition to a Phenyl-Hexyl stationary phase, combined with a mobile phase of Acetonitrile and 0.1% TFA (50:50 v/v), provided sharp, well-defined peaks. The retention times were observed at approximately 2.127 minutes for Hyaluronidase-OYSK and 4.145 minutes for Trastuzumab. Chromatograms of the blank and placebo preparations showed no interfering peaks at these retention times, confirming the specificity of the method for the intended analytes.

3.2. Method Validation

The developed method underwent comprehensive validation in strict adherence to ICH Q2(R1) guidelines, evaluating parameters such as system suitability, linearity, precision, accuracy, robustness, and sensitivity [24].

3.2.1. System Suitability

System suitability testing is an integral part of liquid chromatographic methods, used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done [25]. Standard solutions were injected six times, and critical parameters were monitored. The results indicated that the theoretical plate count for both analytes exceeded 2000, signifying high column efficiency. The tailing factors were recorded at 0.85 for Hyaluronidase-OYSK and 1.06 for Trastuzumab, well within the acceptance limit of NMT 2.0, indicating symmetrical peak shapes. The resolution between the two peaks was 9.53, demonstrating excellent separation efficiency. The Relative Standard Deviation (% RSD) for the peak areas of six replicate injections was less than 2.0%, confirming the precision of the injection and flow systems.

Table 2. System Suitability Parameters

Parameter	Hyaluronidase-OYSK	Trastuzumab	Acceptance Criteria
Retention Time (min)	2.127	4.145	N/A
Theoretical Plates (N)	12375	17621	NLT 2000
Tailing Factor (T)	0.85	1.06	NMT 2.0
Resolution (Rs)	—	9.53	NLT 2.0
% RSD (n=6)	0.25	0.20	NMT 2.0%

NLT: Not Less Than; NMT: Not More Than; RSD: Relative Standard Deviation

3.2.2. Linearity

Linearity assesses the ability of the method (within a given range) to obtain test results that are directly proportional to the concentration of the analyte in the sample [26]. A series of standard solutions were prepared ranging from 25% to 150% of the target concentration. For Trastuzumab, the linearity range was 1500 to 9000 µg/mL, and for Hyaluronidase-OYSK, it was 0.63 to 3.75 µg/mL. The calibration curves, constructed by plotting peak area against concentration, yielded correlation coefficients (R^2) of 0.99988 for Hyaluronidase-OYSK and 0.99981 for Trastuzumab. These values indicate a robust linear relationship, justifying the use of single-point calibration for routine analysis [27]

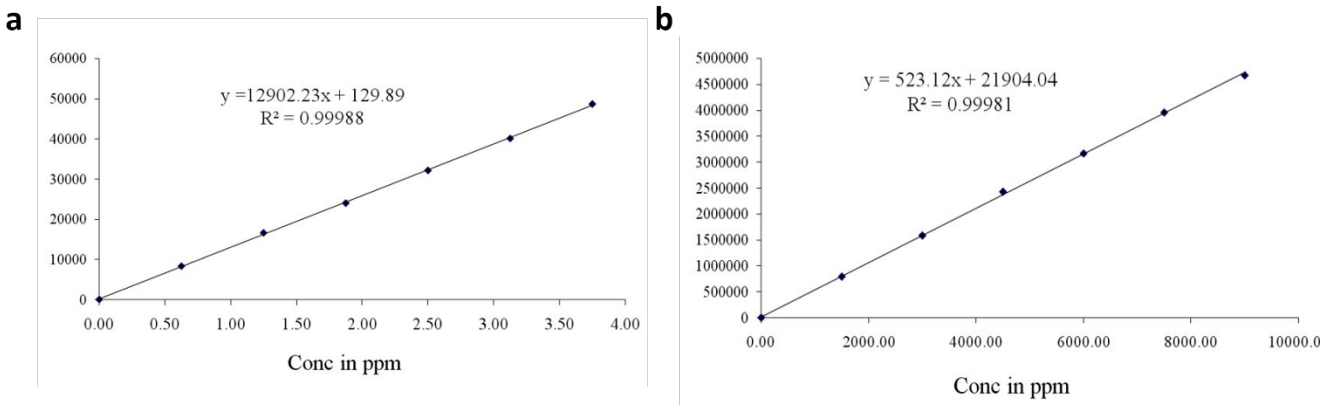

Figure 3. Linearity plots of Hyaluronidase-OYSK and Trastuzumab

Table 3. Linearity and Sensitivity (LOD & LOQ) Results

Parameter	Hyaluronidase-OYSK	Trastuzumab
Linearity Range (µg/mL)	0.63 – 3.75	1500 – 9000
Regression Equation	y = 12902x + 129.89	y = 523.12x + 21904
Correlation Coefficient (R^2)	0.99988	0.99981
Limit of Detection (LOD) (µg/mL)	0.075	0.600
Limit of Quantification (LOQ) (µg/mL)	0.250	1.800

3.2.3. Precision and Accuracy

Method precision was evaluated through repeatability studies involving six replicate injections of the standard solution. The % RSD values for Hyaluronidase-OYSK and Trastuzumab were 0.25% and 0.20%, respectively. These values are significantly lower than the acceptable limit of 2.0%, reflecting the high degree of agreement among individual test results [28]. Accuracy was determined using recovery studies at three concentration levels (50%, 100%, and 150%) by the standard addition method. The mean percentage recovery was found to be 100.3% for Hyaluronidase-OYSK and 99.9% for Trastuzumab. These high recovery values suggest that the method is accurate and that the excipients present in the formulation do not interfere with the quantification of the active drugs [29].

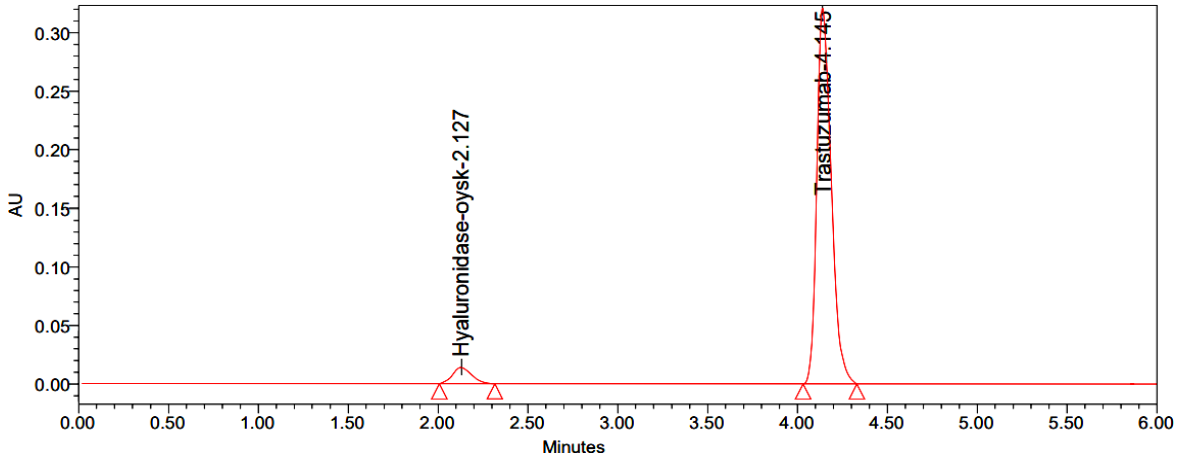

Figure 4. Precision of Chromatogram

Table 4. Accuracy (Recovery) Studies

Drug Name	Spike Level (%)	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean % Recovery
Hyaluronidase-OYSK	50%	0.0125	0.0125	100.3	100.3%
	100%	0.0250	0.0251	100.3	
	150%	0.0375	0.0376	100.2	
Trastuzumab	50%	30.00	29.89	99.6	99.9%
	100%	60.00	59.92	99.9	
	150%	90.00	90.24	100.3	

3.2.4. Robustness

Robustness measures the method's capacity to remain unaffected by small, deliberate variations in method parameters, providing an indication of its reliability during normal usage [30]. The method was challenged with variations in flow rate (± 0.1 mL/min) and organic phase composition (±5%). The analysis revealed that despite these perturbations, the system suitability parameters remained within acceptable limits. The % RSD for peak areas across all robustness conditions remained below 2.0%, confirming that the method is robust and suitable for routine analysis in different laboratory environments.

3.2.5. Sensitivity

The sensitivity of the method was quantified by determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) based on the signal-to-noise ratio method. The LOD is the lowest amount of analyte in a sample which can be detected but not

necessarily quantitated, while LOQ is the lowest amount that can be quantitatively determined with suitable precision and accuracy [31]. The LOD values were established at 0.60 µg/mL for Trastuzumab and 0.075 µg/mL for Hyaluronidase-OYSK. The LOQ values were found to be 1.80 µg/mL and 0.25 µg/mL, respectively. These low limits demonstrate the method's high sensitivity, making it suitable for detecting trace levels of the analytes.

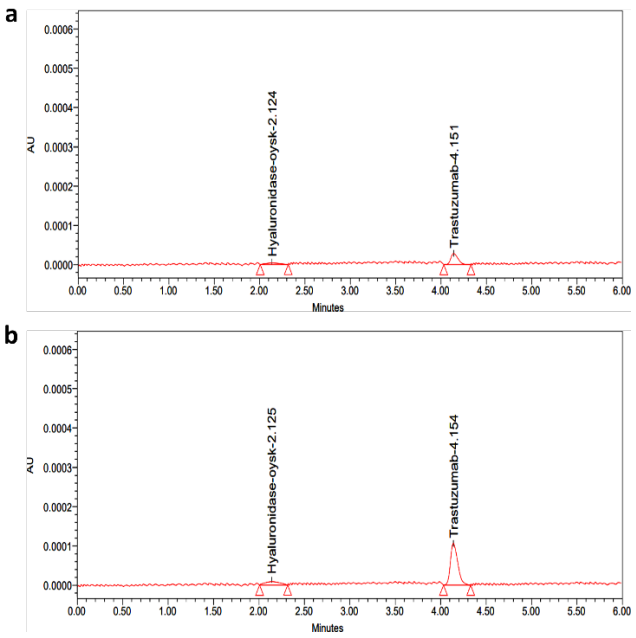

Figure 5. Chromatogram for LOD and LOQ

3.3. Stability and Degradation Studies

Forced degradation studies are essential for developing stability-indicating methods, providing insight into the degradation pathways and the stability of the molecule [32]. The drug samples were subjected to severe stress conditions:

- **Acid and Alkali Hydrolysis:** Exposure to 1N HCl and 1N NaOH at 60°C resulted in degradation percentages of approximately 9-11%. The chromatograms showed that the degradation products eluted at distinct retention times, well-resolved from the main analyte peaks.
- **Oxidative Degradation:** Samples treated with 3% hydrogen peroxide showed approximately 10-12% degradation. Oxidation is a common degradation pathway for proteins, often affecting methionine or cysteine residues [33]. The method successfully resolved these oxidative variants.
- **Thermal and Photolytic Degradation:** The samples exhibited relatively high stability under thermal (105°C) and photolytic stress, with degradation typically remaining below 5%.

Table 5. Results of Forced Degradation Studies

Stress Condition	Hyaluronidase-OYSK (% Degradation)	Trastuzumab (% Degradation)	Peak Purity Check
Acid Hydrolysis (1N HCl, 60°C)	9.3%	10.6%	Pass (Angle < Threshold)
Alkali Hydrolysis (1N NaOH, 60°C)	10.3%	11.2%	Pass (Angle < Threshold)
Oxidative (3% H_2O_2, 60°C)	10.9%	12.4%	Pass (Angle < Threshold)
Thermal (105°C)	2.2%	3.5%	Pass (Angle < Threshold)
Photolytic (UV Light)	3.5%	4.0%	Pass (Angle < Threshold)
Hydrolysis (Water, 60°C)	1.4%	1.7%	Pass (Angle < Threshold)
Reduction (10% $NaHSO_3$)	1.2%	2.7%	Pass (Angle < Threshold)

In all stress conditions, the purity angle was found to be less than the purity threshold for both analytes using the PDA detector. This peak purity analysis confirms that the analyte peaks are spectrally homogeneous and free from co-eluting degradation products [34].

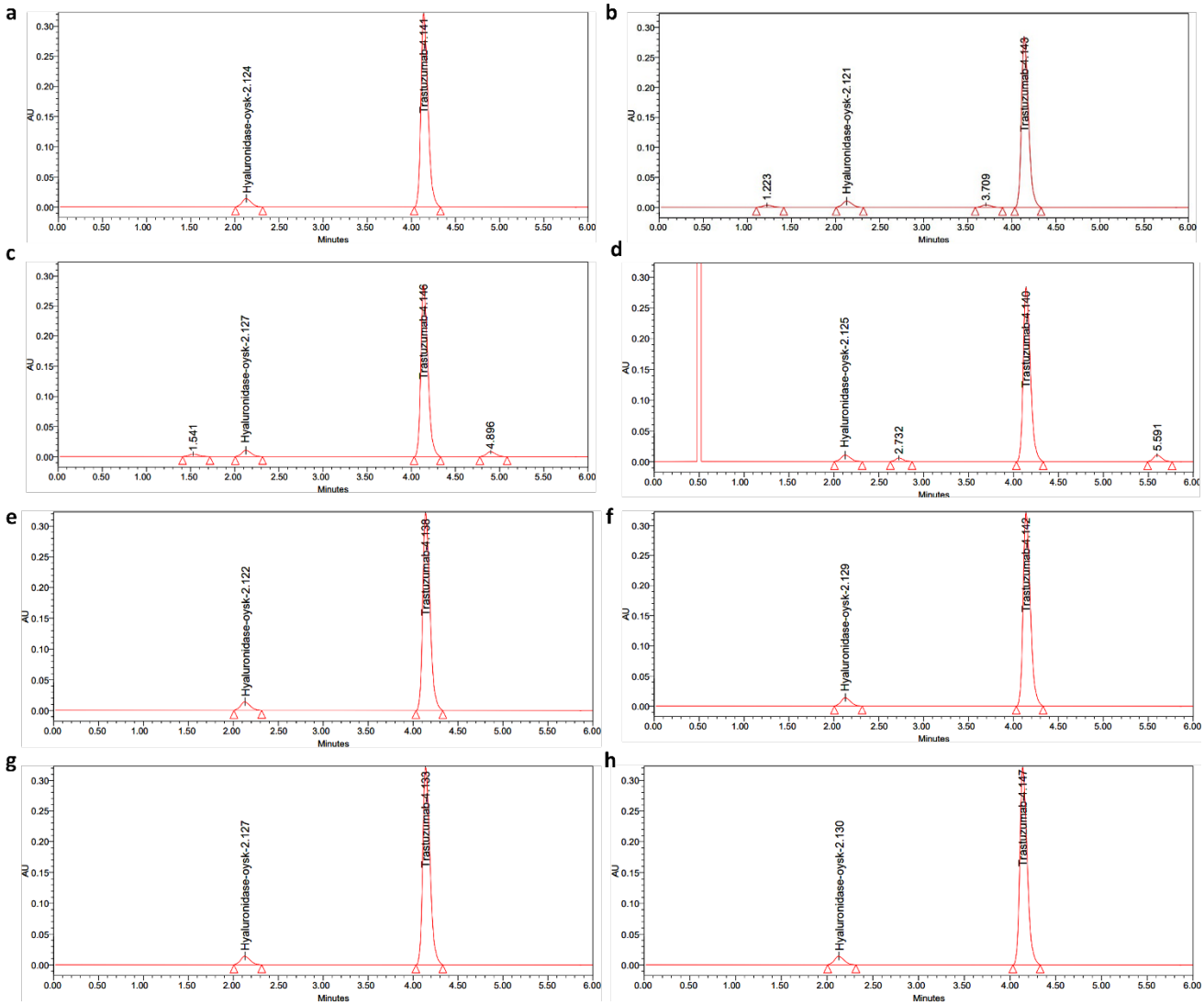

Figure 6. Force Degradation Studies of a. Control b. acid degradation c. alkali degradation d. peroxide degradation e. Thermal degradation f. hydrolysis degradation g. Photolytic degradation and h. Reduction degradation

4. Conclusion

The present study describes the successful development and validation of a simple, rapid, and sensitive RP-HPLC method for the simultaneous estimation of Trastuzumab and Hyaluronidase-OYSK. By utilizing a Phenyl-Hexyl stationary phase and simple mobile phase additives, the method overcomes common challenges associated with the chromatography of complex biologics. The validation data, demonstrating high linearity, precision, accuracy, and robustness, aligns with global regulatory standards. Moreover, the forced degradation studies unequivocally established the method's stability-indicating nature, as it effectively resolved the active drug peaks from a variety of stress-induced degradation products. Consequently, this method offers a reliable and economical solution for the routine quality control and stability testing of Trastuzumab and Hyaluronidase-OYSK combination therapies in the pharmaceutical industry.

References

[1] Hudis CA. Trastuzumab—mechanism of action and use in clinical practice. N Engl J Med. 2007;357(1):39-51.

[2] Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001;344(11):783-92.

[3] Nahta R, Esteva FJ. HER2-targeted therapy: lessons from trastuzumab and lapatinib. Clin Cancer Res. 2006;12(14):4307-14.

[4] Pivot X, Gligorov J, Müller V, Barrett-Lee P, Verma S, Knoop A, et al. Preference for subcutaneous or intravenous administration of trastuzumab in patients with HER2-positive early breast cancer (PrefHer): an open-label randomised study. Lancet Oncol. 2013;14(10):962-70.

[5] Frost GI. Recombinant human hyaluronidase (rHuPH20): an enabling platform for subcutaneous drug and fluid administration. Expert Opin Drug Deliv. 2007;4(4):427-40.

[6] Bookbinder LH, Hofer A, Haller MF, Zepeda ML, Keller GA, Lim JE, et al. A recombinant human enzyme for enhanced interstitial transport of therapeutics. J Control Release. 2006;114(2):230-41.

[7] Harb G, Leblond V, Quartier P, Mouton MC, Somani J. Recombinant human hyaluronidase-facilitated subcutaneous infusion of human immunoglobulins for primary immunodeficiency. J Allergy Clin Immunol. 2015;136(4):1073-81.

[8] Shpilberg O, Jackisch C. Subcutaneous administration of rituximab (MabThera) and trastuzumab (Herceptin) using hyaluronidase. Br J Cancer. 2013;109(6):1556-61.

[9] Skoog DA, West DM, Holler FJ, Crouch SR. Fundamentals of Analytical Chemistry. 9th ed. Belmont, CA: Brooks/Cole; 2014.

[10] Snyder LR, Kirkland JJ, Glajch JL. Practical HPLC Method Development. 2nd ed. New York: John Wiley & Sons; 1997.

[11] Fekete S, Guillarme D, Sandra P. Chromatographic, electrophoretic and mass spectrometric methods for the analytical characterization of protein biopharmaceuticals. Anal Chem. 2016;88(1):480-507.

[12] International Council for Harmonisation (ICH). Q1A(R2): Stability Testing of New Drug Substances and Products. Geneva: ICH; 2003.

[13] Blessy M, Patel RD, Prajapati PN, Agrawal YK. A review on forced degradation and stability indicating studies. J Pharm Anal. 2014;4(3):159-65.

[14] Budhraja RH, Shah MA, Suthar M, Ghante M. LC-MS/MS Validation Analysis of Trastuzumab Using dSIL Approach for Evaluating Pharmacokinetics. Molecules. 2016;21(11):1464.

[15] Katare KK, Mandapati U. Development and Validation of RP-HPLC Method for the Simultaneous Determination of Cisplatin, Capecitabine and Trastuzumab. Int J Pharm Qual Assur. 2022;13(1):76-81.

[16] Yarlagadda SR, Pavani Y, Mannam SR. Simultaneous Method Development and Validation of Trastuzumab and Hyaluronidase-OYSK and Its Pharmacokinetic studies with LC-MS/MS. J Pharm Sci Res. 2020;12(3):375-80.

[17] International Council for Harmonisation (ICH). Q2(R1): Validation of Analytical Procedures: Text and Methodology. Geneva: ICH; 2005.

[18] Prichard E, Barwick V. Quality Assurance in Analytical Chemistry. Chichester: John Wiley & Sons; 2007.

[19] Waters Corporation. Waters Alliance e2695 Separations Module Operator's Guide. Milford, MA: Waters Corporation; 2008.

[20] Croes K, Steffens A, Marchand DH, Snyder LR. Relevance of pi-pi interactions for retention in reversed-phase liquid chromatography. J Chromatogr A. 2005;1098(1-2):123-30.

[21] Mant CT, Hodges RS. High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation. Boca Raton: CRC Press; 1991.

[22] Kazakevich YV, LoBrutto R. HPLC for Pharmaceutical Scientists. Hoboken: Wiley-Interscience; 2007.

[23] Ahuja S, Rasmussen H. HPLC Method Development for Pharmaceuticals. Amsterdam: Elsevier; 2007.

[24] Food and Drug Administration (FDA). Guidance for Industry: Analytical Procedures and Methods Validation for Drugs and Biologics. Silver Spring, MD: FDA; 2015.

[25] Shabir GA. Validation of high-performance liquid chromatography methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. J Chromatogr A. 2003;987(1-2):57-66.

[26] Ermer J, Miller JH. Method Validation in Pharmaceutical Analysis: A Guide to Best Practice. Weinheim: Wiley-VCH; 2005.

[27] Araujo P. Key aspects of analytical method validation and linearity evaluation. J Chromatogr B. 2009;877(23):2224-34.

[28] Thompson M, Ellison SL, Wood R. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). Pure Appl Chem. 2002;74(5):835-55.

[29] Taverniers I, De Loose M, Van Bockstaele E. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. Trends Analyt Chem. 2004;23(8):535-52.

[30] Vander Heyden Y, Nijhuis A, Smeyers-Verbeke J, Massart DL. Guidance for robustness/ruggedness tests in method validation. J Pharm Biomed Anal. 2001;24(5-6):723-53.

[31] Shrivastava A, Gupta VB. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chron Young Sci. 2011;2(1):21-5.

[32] Bakshi M, Singh S. Development of validated stability-indicating assay methods—critical review. J Pharm Biomed Anal. 2002;28(6):1011-40.

[33] Torosantucci R, Schöneich C, Jiskoot W. Oxidation of therapeutic proteins and peptides: structural and biological consequences. Pharm Res. 2014;31(3):541-53.

[34] Forssén P, Fornstedt T. Peak purity assessment in liquid chromatography. J Sep Sci. 2014;37(19):2639-48.