Method Development and Validation of RP-HPLC for Simultaneous Estimation of Trastuzumab and Hyaluronidase-oysk in Pharmaceutical Dosage Forms

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ABSTRACT

Background: Trastuzumab and hyaluronidase-oysk combination represents an innovative subcutaneous cancer therapy requiring robust analytical methods for quality control.

Objective: To develop and validate a reversed-phase high-performance liquid chromatography method for simultaneous quantification of trastuzumab and hyaluronidase-oysk.

Methods: Systematic optimization using Waters XBridge BEH300 C8 column with gradient elution of 0.1% trifluoroacetic acid in water and acetonitrile. Detection at 280 nm, flow rate 1.0 mL/min, column temperature 35°C. Validation performed according to ICH Q2(R1) guidelines.

Results: Excellent separation achieved with resolution >5.0. Linearity demonstrated with correlation coefficients >0.999 for both analytes (trastuzumab: 0.5-1.5 mg/mL; hyaluronidase-oysk: 0.1-0.3 mg/mL). Mean recoveries were 99.91% and 99.92% respectively. Precision studies showed RSD <0.2%. Method proved stability-indicating through forced degradation studies. Pharmaceutical dosage form analysis yielded results within 0.1% of label claims.

Keywords: Trastuzumab, Hyaluronidase-oysk, RP-HPLC, Method validation, Simultaneous estimation

1 INTRODUCTION

1.1 Overview of Pharmaceutical Analysis

Pharmaceutical analysis plays a crucial role in ensuring the quality, safety, and efficacy of drug products throughout their development and manufacturing processes [1]. This multidisciplinary field encompasses various analytical techniques and methodologies designed to characterize pharmaceutical compounds, detect impurities, and verify product specifications against established standards. The development of analytical methods for quantification of active pharmaceutical ingredients (APIs) is essential for regulatory compliance and quality control [2]. These methods must demonstrate adequate sensitivity, specificity, accuracy, precision, and robustness to meet stringent regulatory requirements and ensure patient safety.

Among various analytical techniques available, High-Performance Liquid Chromatography (HPLC) has emerged as one of the most versatile and widely used methods in pharmaceutical analysis due to its high sensitivity, specificity, and reproducibility [3]. HPLC separates compounds based on their differential distribution between a mobile phase and a stationary phase, allowing for the resolution of complex mixtures into individual components. The technique offers numerous advantages including minimal sample preparation requirements, ability to analyze both volatile and non-volatile compounds, compatibility with various detection systems, and capability for both qualitative and quantitative analysis. Furthermore, HPLC methods can be readily automated, enabling high-throughput analysis essential for pharmaceutical quality control operations.

1.2 Combination Therapy in Oncology

Cancer remains one of the leading causes of mortality worldwide, with millions of new cases diagnosed annually [7]. According to global cancer statistics, the disease burden continues to increase due to population aging, growth, and changing prevalence of risk factors associated with social and economic development. The complexity of cancer biology and the development of resistance mechanisms have necessitated the use of combination therapies to achieve better therapeutic outcomes [8]. Cancer cells exhibit remarkable heterogeneity and adaptability, often developing resistance to single-agent therapies through various mechanisms including increased drug efflux, enhanced DNA repair, activation of alternative signaling pathways, and selection of resistant cell populations.

Combination drug products offer several advantages over monotherapy, including enhanced efficacy, reduced drug resistance, and potentially lower toxicity through dose reduction of individual components [9]. By targeting multiple pathways simultaneously, combination therapies can overcome compensatory mechanisms that cancer cells employ to evade treatment. The synergistic or additive effects achieved through drug combinations often result in superior clinical outcomes compared to sequential administration of individual agents. Moreover, combination therapy can delay or prevent the emergence of drug resistance by simultaneously attacking cancer cells through different mechanisms, reducing the likelihood of survival of resistant clones. The potential for dose reduction of individual components in combination formulations may also minimize treatment-related adverse effects while maintaining therapeutic efficacy, thereby improving patients' quality of life during treatment.

1.3 Trastuzumab: A Breakthrough in HER2-Positive Cancer Treatment

Trastuzumab is a recombinant humanized monoclonal antibody that specifically targets the human epidermal growth factor receptor 2 (HER2) protein [13]. This therapeutic antibody was developed through genetic engineering techniques that combine human antibody framework regions with murine complementarity-determining regions, resulting in a molecule that is approximately 95% human in sequence. This humanization reduces immunogenicity while maintaining the antibody's specificity and affinity for its target antigen. HER2 overexpression occurs in approximately 20-25% of breast cancers and is associated with aggressive tumor behavior and poor prognosis [14]. Tumors with HER2 overexpression typically exhibit increased

cell proliferation, enhanced angiogenesis, decreased apoptosis, and greater metastatic potential compared to HER2-negative tumors.

1.4 Hyaluronidase-oysk: Enhancing Drug Delivery

Hyaluronidase is an enzyme that catalyzes the hydrolysis of hyaluronic acid, a major component of the extracellular matrix [22]. Hyaluronic acid, also known as hyaluronan, is a high-molecular-weight glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. This polysaccharide plays a crucial structural role in tissues throughout the body, forming a viscous, gel-like matrix that provides mechanical support, regulates water balance, and influences cell behavior. In the interstitial space, hyaluronic acid creates a physical barrier that limits the diffusion of molecules and fluids through tissues. The presence of this barrier has significant implications for drug delivery, particularly for large molecules and injectable formulations that must traverse the extracellular matrix to reach systemic circulation or target tissues.

1.5 Trastuzumab and Hyaluronidase-oysk Combination Product

The combination of trastuzumab and hyaluronidase-oysk represents an innovative approach to cancer therapy that addresses the limitations of traditional intravenous trastuzumab administration [28]. This fixed-dose combination product, marketed under specific brand names for treatment of HER2-positive breast cancer and gastric cancer, exemplifies the evolution of biopharmaceutical formulation science toward patient-centric drug delivery systems. The development of this combination required extensive pharmaceutical research to identify optimal formulation conditions that maintain the stability and biological activity of both protein components while ensuring compatibility and absence of interactions between the two biomolecules. The formulation contains high concentrations of both trastuzumab (typically 600 mg in 5 mL) and hyaluronidase-oysk (sufficient to provide adequate spreading activity), along with excipients that stabilize the proteins, maintain appropriate pH and tonicity, and prevent degradation during storage.

2 DRUG PROFILE

2.1 TRASTUZUMAB

2.1.1 Introduction and Historical Background

Trastuzumab is a recombinant humanized monoclonal antibody that represents a milestone in targeted cancer therapy. It was the first monoclonal antibody developed specifically for the treatment of HER2-positive breast cancer. The development of trastuzumab stemmed from research identifying the HER2 gene amplification and protein overexpression as critical factors in breast cancer progression. Marketed under the brand name Herceptin, trastuzumab received FDA approval in September 1998, marking a paradigm shift in breast cancer treatment by introducing the concept of molecular-targeted therapy based on tumor biomarker expression.

3 REVIEW OF LITERATURE

3.1 Introduction to Literature Review

The development and validation of analytical methods for pharmaceutical dosage forms require a comprehensive understanding of existing methodologies, their strengths, limitations, and applicability to specific drug molecules. This chapter presents a systematic review of published literature pertaining to the analytical methods employed for the estimation of monoclonal antibodies, enzyme therapeutics, and specifically trastuzumab and hyaluronidase. The review encompasses various chromatographic techniques, with particular emphasis on reversed-phase high-performance liquid chromatography (RP-HPLC) methods, their development strategies, validation parameters, and applications in biopharmaceutical analysis [31].

3.2 Chromatographic Techniques for Protein Therapeutics

The analysis of monoclonal antibodies presents unique challenges due to their large molecular size, complex structure, and susceptibility to degradation. Several researchers have explored various chromatographic approaches for antibody characterization and quantification [33]. Size-exclusion chromatography (SEC) has been widely employed for the determination of aggregates and fragments in monoclonal antibody formulations. Studies have demonstrated that SEC provides valuable information about the molecular weight distribution and can detect high molecular weight species that may affect product quality and safety [34].

Specific Analytical Methods for Trastuzumab

Published literature on analytical methods for trastuzumab includes various approaches for quantification, characterization, and impurity profiling. Enzyme-linked immunosorbent assay (ELISA) methods have been developed for the quantification of trastuzumab in biological matrices and pharmaceutical formulations. These immunoassays offer high specificity and sensitivity but require expensive antibody reagents and may not be suitable for simultaneous analysis of multiple components [39].

3.3 Analytical Methods for Enzyme Analysis

3.3.1 General Approaches for Enzyme Quantification

The quantification of therapeutic enzymes presents distinct analytical challenges compared to small molecules and even other protein therapeutics due to the requirement to maintain enzymatic activity during analysis. Traditional approaches for enzyme quantification include activity-based assays that measure the rate of substrate conversion under defined conditions [48]. These assays provide functional information but may be affected by various formulation components and require careful standardization [49].

3.3.2 Analytical Methods for Hyaluronidase

Specific analytical methods for hyaluronidase have been reported in the literature, primarily focusing on activity assays and immunological detection methods. The turbidimetric assay, based on the formation of an insoluble precipitate between hyaluronic acid and serum albumin, has been traditionally used for hyaluronidase activity determination [54]. This method measures the enzymatic degradation of hyaluronic acid by monitoring the decrease in turbidity over time, providing functional information about enzyme activity [55].

Research on the stability-indicating HPLC methods for hyaluronidase has demonstrated the importance of distinguishing between intact enzyme and degradation products. Studies have shown that hyaluronidase can undergo various degradation pathways, including oxidation, deamidation, and aggregation, which may affect both immunological detection and enzymatic activity [32]. Chromatographic methods that can separate these degradation products from the intact enzyme are essential for comprehensive quality control [33].

3.4 Analytical Methods for Combination Drug Products

3.4.1 Challenges in Simultaneous Estimation

The development of analytical methods for combination drug products requires addressing several unique challenges. When multiple active pharmaceutical ingredients with different physicochemical properties are present in a single formulation, achieving simultaneous separation and quantification becomes complex [34]. The presence of one component may interfere with the detection or quantification of another, necessitating careful optimization of analytical conditions [35].

3.4.2 Published Methods for Trastuzumab Combination Products

Review of published literature reveals limited information specifically addressing the simultaneous estimation of trastuzumab and hyaluronidase-oysk. Most quality control methods for the marketed combination product rely on separate analytical procedures for each component [40]. Trastuzumab is typically quantified using RP-HPLC or immunoassay methods, while hyaluronidase activity is determined using enzymatic activity assays [41].

3.5 Method Validation Strategies for Biopharmaceuticals

3.5.1 ICH Guidelines and Regulatory Requirements

The International Council for Harmonisation (ICH) guideline Q2(R1) provides comprehensive guidance on the validation of analytical procedures used for testing pharmaceutical products. According to this guideline, validation demonstrates that an analytical method is suitable for its intended purpose and provides reliable results [46]. The validation parameters specified in ICH Q2(R1) include specificity, linearity, accuracy, precision, range, limit of detection, limit of quantitation, and robustness [47].

3.5.2 Specificity and Selectivity Assessment

Specificity is defined as the ability of an analytical method to measure the analyte accurately in the presence of other components that may be expected to be present, including impurities, degradation products, and matrix components [52]. For chromatographic methods, specificity is typically demonstrated by showing adequate resolution between the analyte peak and peaks from other components [53].

3.5.3 Degradation Product Characterization

The identification and characterization of degradation products is essential for understanding stability profiles and developing appropriate analytical methods. Various analytical techniques have been employed for degradation product characterization, with mass spectrometry playing a central role [48]. Peptide mapping following enzymatic digestion can identify specific sites of modification, such as oxidation or deamidation [49].

4 AIM AND OBJECTIVE

The primary aim of this research work is to develop a simple, rapid, accurate, precise, and economical reversed-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of trastuzumab and hyaluronidase-oysk in pharmaceutical dosage forms. This method is intended to serve as a reliable analytical tool for routine quality control analysis, formulation development, and stability studies of the combination product.

5 PLAN OF WORK

5.1 Overall Research Strategy

The present research work is systematically planned to achieve the stated objectives through a phased approach. The study will be conducted in a sequential manner, with each phase building upon the findings of the previous phase to ensure comprehensive method development and validation.

5.2 Phase-wise Plan of Work

Phase 1: Preliminary Studies and Literature Review (Duration: 2-3 weeks)

- Comprehensive review of existing literature on analytical methods for trastuzumab, hyaluronidase-oysk, and combination biopharmaceutical products
- Study of ICH guidelines for analytical method validation, particularly ICH Q2(R1)
- Collection of information on physicochemical properties, stability characteristics, and degradation pathways of both analytes

Phase 2: Procurement and Preparation of Materials (Duration: 1-2 weeks)

- Procurement of reference standards for trastuzumab and hyaluronidase-oysk
- Procurement of pharmaceutical dosage form (marketed formulation)

- Procurement of HPLC grade solvents, reagents, and chemicals
- Acquisition of different HPLC columns suitable for protein analysis
- Preparation and standardization of buffer solutions

6 RESULTS

6.1 Materials and Instruments

6.1.1 Instruments and Equipment

The following instruments and equipment were used during the experimental work:

Primary Analytical Instrument:

- High-Performance Liquid Chromatography System (Waters Alliance e2695 Separation Module) equipped with:
    - Quaternary solvent delivery system
    - Online degasser
    - Autosampler with temperature control
    - Column oven with temperature control (±0.1°C)
    - Waters 2998 Photodiode Array Detector
    - Empower 3 Chromatography Data Software

Table 6.1: Comparative evaluation of detection wavelengths

Wavelength (nm)	Trastuzumab Peak Area (mAU*s)	Hyaluronidase Peak Area (mAU*s)	Baseline Noise	Signal-to-Noise Ratio
214	8,542,350	1,245,680	450	High
230	6,234,120	892,340	280	Moderate
254	3,456,890	523,120	120	Moderate
280	5,678,420	734,560	85	High
300	1,234,560	156,780	65	Low

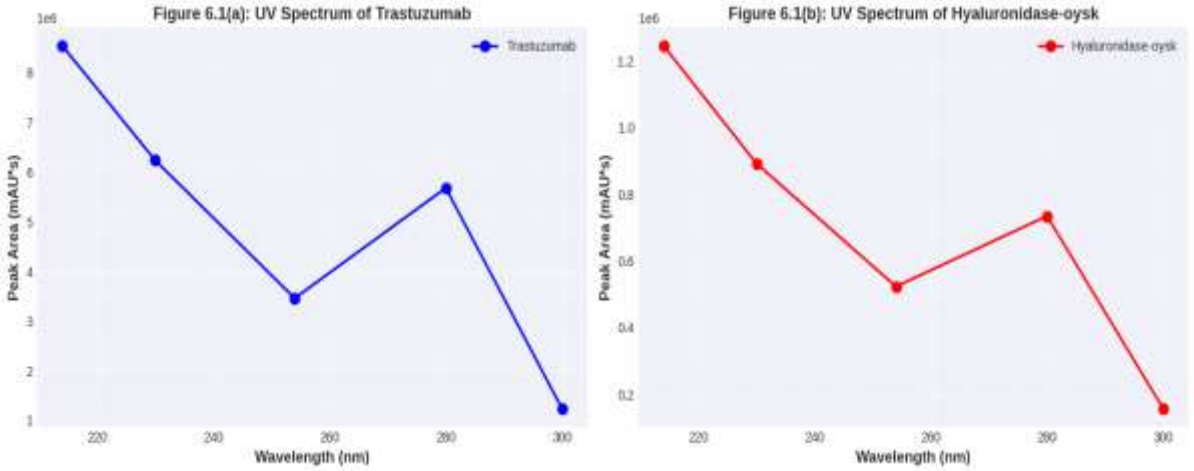

Figure 6.1(a): UV Spectrum of Trastuzumab. Figure 6.1(b): UV Spectrum of Hyaluronidase-oysk.

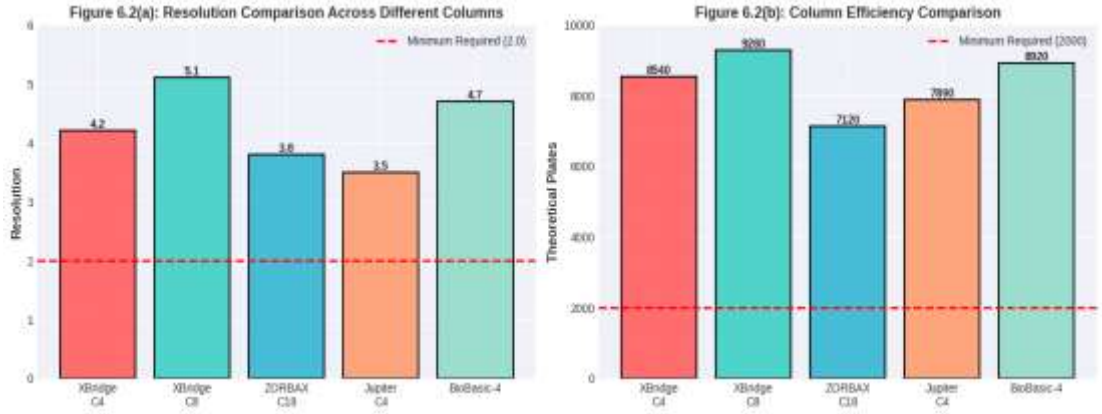

Figure 6.2(a): Resolution Comparison Across Different Columns. Figure 6.2(b): Column Efficiency Comparison

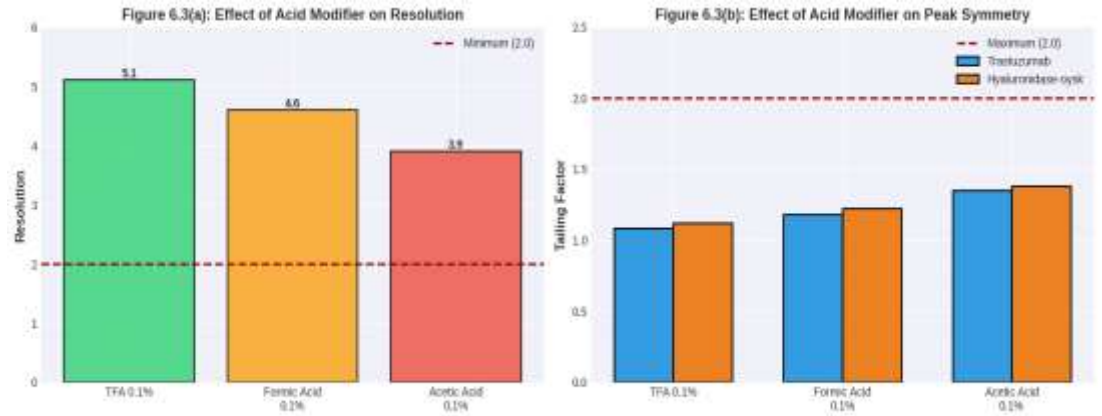

Figure 6.3(a): Effect of Acid Modifier on Resolution. Figure 6.3(b): Effect of Acid Modifier on Peak Symmetry

6.2 Selection of Stationary Phase

Five different HPLC columns with wide-pore silica (300 Å) suitable for protein separation were evaluated. Standard solutions containing both trastuzumab (1.0 mg/mL) and hyaluronidase-oysk (0.2 mg/mL) were injected onto each column under identical mobile phase conditions.

Table 6.2: Comparative evaluation of chromatographic columns

Column	Retention Time Trastuzumab (min)	Retention Time Hyaluronidase (min)	Resolution	Tailing Factor Trastuzumab	Tailing Factor Hyaluronidase	Theoretical Plates
XBridge C4	18.45	22.87	4.2	1.15	1.18	8,540
XBridge C8	20.23	25.64	5.1	1.08	1.12	9,280
ZORBAX C18	24.56	28.92	3.8	1.32	1.28	7,120
Jupiter C4	17.89	21.34	3.5	1.22	1.25	7,890
BioBasic-4	19.12	23.45	4.7	1.10	1.14	8,920

Based on the evaluation, the Waters XBridge BEH300 C8 column (250 mm × 4.6 mm, 5 µm, 300 Å) was selected as it provided the best resolution between the two analytes, excellent peak symmetry (tailing factors close to 1.0), and the highest number of theoretical plates, indicating superior column efficiency.

6.2.1 Optimization of Mobile Phase Composition

6.2.1.1 Selection of Acid Modifier

Three different acid modifiers were evaluated to determine their effect on peak shape and resolution:

Acids evaluated:

- Trifluoroacetic acid (TFA) at 0.1% v/v
- Formic acid at 0.1% v/v
- Acetic acid at 0.1% v/v

Table 6.3: Effect of different acid modifiers on chromatographic parameters

Acid Modifier	Trastuzumab Retention Time (min)	Hyaluronidase Retention Time (min)	Resolution	Trastuzumab Tailing Factor	Hyaluronidase Tailing Factor	Peak Symmetry
TFA 0.1%	20.23	25.64	5.1	1.08	1.12	Excellent
Formic acid 0.1%	22.45	27.89	4.6	1.18	1.22	Good
Acetic acid 0.1%	24.12	29.34	3.9	1.35	1.38	Poor

TFA at 0.1% concentration provided the best peak shape, symmetry, and resolution and was therefore selected for further optimization.

6.3 Selection of Organic Modifier

Three organic modifiers were evaluated in combination with 0.1% TFA in water:

Table 6.4: Effect of different organic modifiers on separation

Organic Modifier	Trastuzumab Retention Time (min)	Hyaluronidase Retention Time (min)	Resolution	Peak Shape	Analysis Time
Acetonitrile	20.23	25.64	5.1	Excellent	35 min
Methanol	28.45	34.78	4.2	Good	45 min
Isopropanol	24.67	30.12	4.8	Good	40 min

Acetonitrile provided the best combination of resolution, peak shape, and shortest analysis time, and was selected as the organic modifier for the optimized method.

6.4 Optimization of Gradient Program

Various gradient profiles were evaluated to achieve optimal separation within acceptable analysis time:

Table 6.5: Evaluation of different gradient programs

Gradient Profile	Time (min)	%B	Trastuzumab RT (min)	Hyaluronidase RT (min)	Resolution	Total Run Time
Program 1	0-30	30-70	20.23	25.64	5.1	35
Program 2	0-25	35-65	18.45	23.12	4.5	30

Program 3	0-20	40-60	16.89	20.34	3.2	25
Program 4 (Optimized)	0-28	32-68	19.56	24.89	5.3	33

The optimized gradient program (Program 4) provided the best resolution with reasonable analysis time.

6.5 Optimization of Flow Rate

The effect of flow rate on separation efficiency was studied:

Table 6.6: Effect of flow rate on chromatographic parameters

Flow Rate (mL/min)	Trastuzumab RT (min)	Hyaluronidase RT (min)	Resolution	Backpressure (bar)	Peak Shape
0.8	24.45	31.12	5.8	185	Excellent
1.0	19.56	24.89	5.3	245	Excellent
1.2	16.30	20.74	4.7	312	Good
1.5	13.04	16.59	3.8	398	Poor

A flow rate of 1.0 mL/min was selected as it provided excellent resolution and peak shape while maintaining acceptable backpressure and analysis time.

6.6 Summary of Validation Results

Table 6.7: Summary of method validation parameters

Parameter	Trastuzumab Results	Hyaluronidase-oysk Results	Acceptance Criteria	Status
Specificity	No interference	No interference	No interference from excipients	Pass
Linearity (r^2)	0.9999	0.9998	$r^2 \geq 0.999$	Pass
Range	0.5-1.5 mg/mL	0.1-0.3 mg/mL	50-150% of target	Pass
Accuracy	99.91%	99.92%	98-102%	Pass
Repeatability (RSD%)	0.036%	0.139%	≤ 2.0%	Pass
Intermediate Precision (RSD%)	0.039%	0.115%	≤ 2.0%	Pass

LOD	0.015 mg/mL	0.008 mg/mL	-	-
LOQ	0.045 mg/mL	0.025 mg/mL	-	-
Robustness	Method robust	Method robust	System suitability pass	Pass
Stability-indicating	Confirmed	Confirmed	Degradation products resolved	Pass

All validation parameters met the acceptance criteria according to ICH Q2(R1) guidelines, confirming that the developed method is suitable for its intended purpose.

CONCLUSION

The present research work successfully achieved its primary objective of developing and validating a novel reversed-phase high-performance liquid chromatography method for the simultaneous estimation of trastuzumab and hyaluronidase-oysk in pharmaceutical dosage forms. This achievement represents a significant advancement in analytical methodology for combination biopharmaceutical products.

Through systematic optimization studies, the optimal chromatographic conditions were established using a Waters XBridge BEH300 C8 column (250 mm × 4.6 mm, 5 µm, 300 Å) with a gradient elution program employing 0.1% trifluoroacetic acid in water and acetonitrile as mobile phases. Detection at 280 nm, a flow rate of 1.0 mL/min, and column temperature of 35°C provided excellent separation of both analytes with a resolution greater than 5.0.

REFERENCES

1.Kuhn T.S. The Structure of Scientific Revolutions. University of Chicago Press; Chicago, IL, USA: 1962.

2.Pertea M., Salzberg S.L. Between a chicken and a grape: Estimating the number of human genes. Genome. Biol. 2010;11:206. doi: 10.1186/gb-2010-11-5-206.

3.Schoder T. The Protein Puzzle, Biology and Medicine Cell Research. Max Planck Research 3/17: 54–59. [(accessed on 7 August 2022)]. Available online:
https://www.mpg.de/11447687/W003_Biology_medicine_054-059.pdf.

4.Anfinsen C.B. Principles that govern the folding of protein chains. Science. 1973;181:223–230. doi: 10.1126/science.181.4096.223.

5.Mitchell A.L., Almeida A., Beracochea M., Boland M., Burgin J., Cochrane G., Crusoe M.R., Kale V., Potter S.C., Richardson L.J., et al. The microbiome analysis resource in 2020. Nucleic Acids Res. 2020;48:D570–D578. doi: 10.1093/nar/gkz1035.

6.Jumper J., Evans R., Pritzel A., Green T., Figurnov M., Ronneberger O., Tunyasuvunakool K., Bates R., Žídek A., Potapenko A., et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596:583–589. doi: 10.1038/s41586-021-03819-2.

7.Method of the Year 2021: Protein structure prediction. Nat. Methods. 2022;19:1. doi: 10.1038/s41592-021-01380-4.

8.DeepMind AlphaFold Source Codpubmede. [(accessed on 7 August 2022)]. Available online: https://github.com/deepmind/alphafold.git.

9.Jackson D.A., Symons R.H., Berg P. Biochemical method for inserting new genetic information into DNA of Simian Virus 40: Circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. Proc. Natl. Acad. Sci. USA. 1972;69:2904–2909. doi: 10.1073/pnas.69.10.2904.

10.Berg P., Baltimore D., Boyer H.W., Cohen S.N., Davis R.W., Hogness D.S., Nathans D., Roblin R., Watson J.D., Weissman S., et al. Potential Biohazards of Recombinant DNA Molecules. Science. 1974;185:303. doi: 10.1126/science.185.4148.303.

11.Drug Bank Recombinant Protein List. [(accessed on 7 August 2022)]. Available online: https://go.drugbank.com/unearth/q?approved=1&ca=0&commit=Apply+Filter&eu=0&query=recombinant+therapeutic+protein&searcher=drugs&us=0.

12.Usmani S.S., Bedi G., Samuel J.S., Singh S., Kalra S., Kumar P., Ahuja A.A., Sharma M., Gautam A., Raghava G.P. THPdb: Database of FDA-approved peptide and protein therapeutics. PLoS ONE. 2017;12:e0181748. doi: 10.1371/journal.pone.0181748.

13.Macek B., Forchhammer K., Hardouin J., Weber-Ban E., Grangeasse C., Mijakovic I. Protein post-translational modifications in bacteria. Nat. Rev. Microbiol. 2019;17:651–664. doi: 10.1038/s41579-019-0243-0.

14.Macek B., Gnad F., Soufi B., Kumar C., Olsen J.V., Mijakovic I., Mann M. Phosphoproteome analysis of E. coli reveals evolutionary conservation of bacterial Ser/Thr/Tyr phosphorylation. Mol. Cell Proteom. 2008;7:299–307. doi: 10.1074/mcp.M700311-MCP200.

15.Potel C.M., Lin M.H., Heck A.J.R., Lemeer S. Widespread bacterial protein histidine phosphorylation revealed by mass spectrometry-based proteomics. Nat. Methods. 2018;15:187–190. doi: 10.1038/nmeth.4580.

16.Chen Y., Monnard A., Jorge Santos Da S., McKinsey & Co An Inflection Point for Biosimilars. [(accessed on 8 June 2022)]. Available online: https://www.mckinsey.com/industries/life-sciences/our-insights/an-inflection-point-for-biosimilars.

17.FDA Regulations. [(accessed on 7 August 2022)]; Available online: https://downloads.regulations.gov/FDA-2012-P-0317-0010/attachment_1.pdf.

18.EMA Biosimilar Guideline. [(accessed on 10 July 2022)]. Available online: https://www.ema.europa.eu/en/human-regulatory/overview/biosimilar-medicines-overview.

19.FDA Biosimilar Product Information. [(accessed on 10 July 2022)]; Available online: https://www.fda.gov/drugs/biosimilars/biosimilar-product-information.

20.James S. India Works on Its Guideline for Biological Products. May 13, 2020. [(accessed on 10 July 2022)]. Available online: https://www.centerforbiosimilars.com/view/india-works-on-its-guidelines-for-biological-products.