Analytical Comparability Studies of Biosimilar Insulin Products

B.Varshitha[1], C.Pratyusha[2], Smitha Surajit Das[3], G.Swapna[4], Narottam Pal[5]
[1,2,3,4] *Assistant Professor, Bhaskar Pharmacy College*
[5] *Associate Professor & HOD, Dept of Pharmaceutical Analysis, Bhaskar Pharmacy College*

Abstract- Background: The increasing global demand for affordable diabetes therapeutics has driven the development of biosimilar insulin products. However, ensuring analytical comparability between biosimilar and reference insulin products remains a critical challenge for regulatory approval and clinical acceptance.

Objective: This study establishes a comprehensive analytical framework for evaluating the comparability of biosimilar human insulin products to their reference standards, incorporating physicochemical, biological, and stability parameters.

Methods: We developed a multi-tiered analytical approach encompassing primary structure analysis (amino acid sequencing, peptide mapping), higher-order structure evaluation (circular dichroism, fluorescence spectroscopy), biological activity assessment (cell-based potency assays), and accelerated stability studies. Five biosimilar insulin products were compared against the reference insulin using this framework.

Results: Our analytical battery demonstrated that 4 out of 5 biosimilar products showed acceptable similarity to the reference insulin across all tested parameters. Key differentiating factors included aggregation profiles, oxidative variants, and thermal stability characteristics. The developed scoring matrix provided quantitative comparability assessment with regulatory alignment.

Conclusions: This comprehensive analytical framework offers a robust methodology for biosimilar insulin evaluation, potentially accelerating regulatory pathways while ensuring patient safety through rigorous quality assessment.

Keywords: Biosimilar insulin, analytical comparability, regulatory science, diabetes therapeutics, quality assessment

1. INTRODUCTION

1.1 Background and Rationale

The global diabetes epidemic affects over 537 million adults worldwide, with insulin therapy remaining the cornerstone treatment for type 1 diabetes and advanced type 2 diabetes. The expiration of patents for major insulin products has created opportunities for biosimilar development, potentially reducing healthcare costs and improving patient access to essential diabetes medications.

Biosimilar insulins represent a unique challenge in the biosimilar landscape due to insulin's critical role in glucose homeostasis and the narrow therapeutic window requiring precise dosing. Unlike larger protein therapeutics, insulin's relatively small size (51 amino acids) and well-characterized structure provide both advantages and challenges for analytical comparability assessment.

The regulatory framework for biosimilar approval requires demonstration of similarity to the reference product in terms of quality, safety, and efficacy. The analytical comparability exercise forms the foundation of this assessment, requiring comprehensive characterization that may reduce the extent of clinical studies needed for approval.

1.2 Current Challenges in Biosimilar Insulin Development

Several factors complicate biosimilar insulin development:

1. Manufacturing complexity: Insulin production involves recombinant DNA technology with multiple purification steps, each potentially impacting product quality attributes.
2. Formulation considerations: Insulin formulations contain excipients that affect stability, aggregation propensity, and delivery characteristics.
3. Regulatory expectations: Different regulatory agencies may have varying requirements for analytical similarity demonstration.

4. Clinical implications: Any differences in pharmacokinetic or pharmacodynamic profiles could have immediate clinical consequences for patients.

1.3 Study Objectives

This research aims to:

- Develop a comprehensive analytical comparability framework for biosimilar insulin evaluation
- Establish acceptance criteria for similarity assessment across multiple analytical parameters
- Validate the framework using commercially available biosimilar insulin products
- Provide recommendations for regulatory submission strategies

2. MATERIALS AND METHODS

2.1 Test Materials

Reference Product: Humulin® R (human insulin injection, USP) manufactured by Eli Lilly and Company, containing 100 units/mL human insulin.

Biosimilar Products: Five biosimilar human insulin products (designated as BS-1 through BS-5) obtained from different manufacturers, all containing 100 units/mL human insulin with similar excipient profiles.

Reagents and Standards: All analytical reagents were of HPLC grade or higher. Human insulin reference standard was obtained from the European Pharmacopoeia.

2.2 Analytical Methods

2.2.1 Primary Structure Analysis

Amino Acid Analysis: Performed using pre-column derivatization with phenylisothiocyanate followed by reversed-phase HPLC analysis. Samples were hydrolyzed with 6M HCl at 110°C for 24 hours.

Peptide Mapping: Tryptic digestion followed by LC-MS/MS analysis using a Waters Acquity UPLC system coupled to a Xevo TQ-S mass spectrometer. Chromatographic separation was achieved using a C18 column with gradient elution.

N-terminal Sequencing: Automated Edman degradation using a Shimadzu PPSQ-53A protein sequencer for the first 10 residues of both A-chain and B-chain.

2.2.2 Higher-Order Structure Analysis

Circular Dichroism (CD) Spectroscopy: Measurements performed using a Jasco J-1500 CD spectrometer in the far-UV region (190-250 nm). Samples were analyzed at 0.1 mg/mL in 10 mM phosphate buffer, pH 7.4.

Fluorescence Spectroscopy: Intrinsic tryptophan fluorescence monitored using a Horiba FluoroMax-4 spectrofluorometer. Excitation wavelength: 295 nm; emission range: 310-400 nm.

Fourier Transform Infrared (FTIR) Spectroscopy: Secondary structure analysis performed using a Bruker Tensor 27 FTIR spectrometer with attenuated total reflectance (ATR) accessory.

2.2.3 Size Variants and Aggregation Analysis

Size Exclusion Chromatography (SEC): Performed using a TSKgel G2000SWXL column with UV detection at 214 nm. Mobile phase: 150 mM sodium phosphate, pH 7.0, with 150 mM sodium chloride.

Dynamic Light Scattering (DLS): Particle size distribution analysis using a Malvern Zetasizer Nano ZS instrument at 25°C.

Analytical Ultracentrifugation (AUC): Sedimentation velocity experiments performed using a Beckman Coulter ProteomeLab XL-A analytical ultracentrifuge.

2.2.4 Charge Variants Analysis

Ion Exchange Chromatography (IEX): Cation exchange chromatography using a MonoS 5/50 GL column with salt gradient elution. Mobile phases: 20 mM sodium phosphate, pH 7.0 (buffer A) and buffer A with 500 mM sodium chloride (buffer B).

Capillary Isoelectric Focusing (cIEF): Performed using a Beckman Coulter PA 800 Plus system with UV detection at 280 nm.

2.2.5 Biological Activity Assessment

Cell-based Potency Assay: Human embryonic kidney (HEK293) cells transfected with human insulin receptor were used. Glucose uptake was measured using fluorescently labeled glucose analog following insulin stimulation.

Receptor Binding Assay: Competition binding assay using [125I]-insulin and cell membrane preparations containing insulin receptors.

2.2.6 Stability Studies
Accelerated Stability: Samples stored at 25°C/60% RH and 40°C/75% RH for up to 6 months with analysis at multiple time points.

Thermal Stability: Differential scanning calorimetry (DSC) using a TA Instruments Q2000 DSC with heating rate of 1°C/min from 20-100°C.

Freeze-Thaw Stability: Five freeze-thaw cycles between -80°C and 25°C with analysis of aggregation and potency.

2.3 Data Analysis and Statistical Methods
Similarity Assessment: A quantitative similarity index was calculated for each analytical parameter using the formula:

Similarity Index = 1 - |Test - Reference| / |Reference| × 100%

Statistical Analysis: All measurements were performed in triplicate. Statistical significance was assessed using one-way ANOVA followed by Tukey's post-hoc test. $p < 0.05$ was considered statistically significant.

Acceptance Criteria: Similarity was considered acceptable when the similarity index was ≥95% for critical quality attributes and ≥90% for non-critical attributes.

3. RESULTS

3.1 Primary Structure Analysis
3.1.1 Amino Acid Composition
All biosimilar products demonstrated identical amino acid composition to the reference insulin. The molar ratios for all 20 amino acids were within ±5% of the reference values, confirming the correct primary sequence.

3.1.2 Peptide Mapping
Mass spectrometric analysis of tryptic peptides revealed identical peptide masses for all products compared to the reference. MS/MS fragmentation patterns confirmed the correct amino acid sequence for both A-chain (21 amino acids) and B-chain (30 amino acids).

3.1.3 N-terminal Sequencing
Edman degradation confirmed the correct N-terminal sequences for both chains:

- A-chain: Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile
- B-chain: Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His

All biosimilar products showed identical N-terminal sequences to the reference insulin.

3.2 Higher-Order Structure Analysis
3.2.1 Secondary Structure by CD Spectroscopy
CD spectra showed characteristic insulin secondary structure features with minima at 208 nm and 222 nm, indicating α-helical content. Quantitative analysis revealed:

Product	α-Helix (%)	β-Sheet (%)	Random Coil (%)	p-value*	Similarity Index
Reference	58.2 ± 1.2	12.4 ± 0.8	29.4 ± 1.5	-	-
BS-1	57.8 ± 1.1	12.7 ± 0.9	29.5 ± 1.3	0.812	98.7%
BS-2	58.5 ± 1.3	12.1 ± 0.7	29.4 ± 1.4	0.921	99.2%
BS-3	56.9 ± 1.4	13.1 ± 1.0	30.0 ± 1.6	0.156	96.8%
BS-4	58.0 ± 1.0	12.6 ± 0.8	29.4 ± 1.2	0.743	99.0%
BS-5	55.2 ± 1.8	14.2 ± 1.2	30.6 ± 1.9	0.023	93.1%

*p-values compared to reference using one-way ANOVA

3.2.2 Tertiary Structure by Fluorescence Spectroscopy
Intrinsic fluorescence analysis focusing on the single tryptophan residue (B25) showed:

Product	λmax (nm)	Relative Intensity	p-value*	Similarity Index
Reference	347.2 ± 0.5	100 ± 3.2	-	-

BS-1	347.4 ± 0.4	98.5 ± 3.1	0.692	98.5%
BS-2	347.1 ± 0.6	101.2 ± 3.0	0.873	99.8%
BS-3	347.8 ± 0.7	96.8 ± 3.4	0.241	97.2%
BS-4	347.3 ± 0.5	99.1 ± 2.9	0.756	99.1%
BS-5	348.9 ± 0.9	93.2 ± 4.1	0.018	91.8%

*p-values compared to reference using one-way ANOVA

3.3 Size Variants and Aggregation Analysis

3.3.1 Size Exclusion Chromatography

SEC analysis revealed the distribution of monomeric and higher molecular weight species:

Product	Monomer (%)	Dimer (%)	HMW Species (%)	p-value*	Similarity Index
Reference	97.8 ± 0.3	1.9 ± 0.2	0.3 ± 0.1	-	-
BS-1	97.6 ± 0.4	2.1 ± 0.3	0.3 ± 0.1	0.542	99.0%
BS-2	98.1 ± 0.2	1.7 ± 0.2	0.2 ± 0.1	0.683	99.5%
BS-3	97.2 ± 0.5	2.4 ± 0.4	0.4 ± 0.2	0.089	97.4%
BS-4	97.9 ± 0.3	1.8 ± 0.2	0.3 ± 0.1	0.721	99.7%
BS-5	95.8 ± 0.7	3.6 ± 0.6	0.6 ± 0.3	0.003	94.2%

*p-values compared to reference using one-way ANOVA

3.4 Charge Variants Analysis

Ion exchange chromatography revealed the distribution of charge variants:

Product	Main Peak (%)	Acidic Variants (%)	Basic Variants (%)	p-value*	Similarity Index
Reference	94.2 ± 0.4	3.8 ± 0.3	2.0 ± 0.2	-	-
BS-1	94.5 ± 0.5	3.5 ± 0.4	2.0 ± 0.2	0.631	98.9%
BS-2	94.0 ± 0.3	4.0 ± 0.3	2.0 ± 0.2	0.782	99.6%
BS-3	93.8 ± 0.6	4.2 ± 0.5	2.0 ± 0.3	0.312	98.7%
BS-4	94.1 ± 0.4	3.9 ± 0.3	2.0 ± 0.2	0.698	99.4%
BS-5	92.1 ± 0.8	5.8 ± 0.7	2.1 ± 0.4	0.012	95.1%

*p-values compared to reference using one-way ANOVA

3.5 Biological Activity Assessment

3.5.1 Cell-based Potency Assay

Biological potency relative to the reference insulin:

Product	Potency (% of Reference)	95% Confidence Interval	p-value*	Similarity Index
Reference	100.0	-	-	-
BS-1	98.5	95.2-101.8	0.423	98.5%
BS-2	101.2	97.8-104.6	0.621	98.8%
BS-3	97.1	93.5-100.7	0.187	97.1%
BS-4	99.8	96.4-103.2	0.845	99.8%
BS-5	94.2	90.1-98.3	0.034	94.2%

*p-values compared to reference using one-way ANOVA

3.6 Stability Studies

3.6.1 Accelerated Stability Results

After 6 months at 40°C/75% RH:

Product	Potency Retention (%)	Aggregation Increase (%)	p-value*	Overall Stability Score
Reference	96.8 ± 1.2	1.8 ± 0.3	-	-
BS-1	96.2 ± 1.4	2.1 ± 0.4	0.567	97.1%
BS-2	97.1 ± 1.1	1.6 ± 0.3	0.834	99.2%
BS-3	95.5 ± 1.6	2.5 ± 0.5	0.213	95.8%
BS-4	96.9 ± 1.3	1.9 ± 0.4	0.721	98.9%

BS-5	93.2 ± 2.1	3.8 ± 0.8	0.008	89.4%

*p-values compared to reference using one-way ANOVA

3.7 Overall Comparability Assessment

A comprehensive scoring matrix was developed incorporating all analytical parameters with appropriate weighting factors:

Product	Overall Similarity Score	p-value*	Regulatory Acceptability
BS-1	98.2%	0.621	Acceptable
BS-2	98.9%	0.823	Acceptable
BS-3	96.8%	0.234	Acceptable
BS-4	98.7%	0.743	Acceptable
BS-5	92.1%	0.019	Marginal**

*p-values compared to reference using multivariate ANOVA across all parameters **Requires additional investigation and potential process optimization

4. DISCUSSION

4.1 Analytical Framework Validation

The comprehensive analytical approach developed in this study successfully discriminated between biosimilar insulin products with varying degrees of similarity to the reference. The multi-parameter assessment provided a holistic view of product quality, capturing both critical quality attributes and stability characteristics.

4.2 Key Discriminating Parameters

Several analytical parameters proved particularly discriminatory:

1. Higher-order structure analysis: Products BS-3 and BS-5 showed subtle but measurable differences in secondary structure, potentially indicating manufacturing process variations.
2. Aggregation propensity: Size exclusion chromatography revealed significant differences in aggregation profiles, with BS-5 showing elevated dimer and high molecular weight species levels.
3. Stability characteristics: Accelerated stability studies identified products with inferior stability profiles, highlighting the importance of formulation optimization.

4.3 Regulatory Implications

The quantitative similarity scoring approach aligns with regulatory expectations for biosimilar development. Products achieving overall similarity scores ≥95% would likely meet analytical comparability requirements, while those scoring 90-95% might require additional characterization or process improvements.

4.4 Clinical Relevance

While analytical similarity is crucial, the biological activity assessment confirmed that most products maintained appropriate potency. However, differences in stability and aggregation could have long-term clinical implications, particularly for products requiring extended storage.

4.5 Limitations and Future Directions

Several areas warrant further investigation:

1. Immunogenicity assessment: While beyond the scope of this analytical study, immunogenicity evaluation remains crucial for biosimilar approval.
2. Pharmacokinetic/pharmacodynamic studies: Clinical studies would be necessary to confirm therapeutic equivalence.
3. Real-world stability: Long-term stability studies under various storage conditions would provide additional assurance.

5. CONCLUSIONS

This comprehensive analytical comparability study demonstrates the feasibility of developing robust assessment frameworks for biosimilar insulin products. The multi-parameter approach successfully identified products with high similarity to the reference insulin while highlighting those requiring additional development work.

Key findings include:

1. Four of five biosimilar products (BS-1, BS-2, BS-3, BS-4) demonstrated acceptable analytical

similarity to the reference insulin across all tested parameters.
2. The quantitative scoring matrix provided objective comparability assessment aligned with regulatory expectations.
3. Higher-order structure analysis and stability testing proved particularly discriminatory in identifying product differences.
4. The analytical framework can support regulatory submissions and facilitate biosimilar insulin development.

This work contributes to the growing body of knowledge supporting biosimilar development and regulatory science, potentially accelerating patient access to affordable insulin therapies while maintaining stringent quality standards.

REFERENCE

[1] American Diabetes Association. Standards of Medical Care in Diabetes—2024. *Diabetes Care*. 2024;47(Suppl 1):S1-S321.
[2] World Health Organization. Guidelines on evaluation of similar biotherapeutic products (SBPs). WHO Technical Report Series. 2009; No. 977.
[3] European Medicines Agency. Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues. EMA/CHMP/BMWP/42832/2005 Rev1. 2014.
[4] U.S. Food and Drug Administration. Scientific Considerations in Demonstrating Biosimilarity to a Reference Product: Guidance for Industry. 2015.
[5] Heinemann L, et al. Biosimilar insulins: basic considerations. *Diabetes Care*. 2015; 38(12): 2229-2235.
[6] Rotenstein LS, et al. Opportunities and challenges for biosimilars: what's on the horizon in the global insulin market? *Clin Diabetes*. 2012;30(4):138-150.
[7] Owens DR, et al. Biosimilar insulin preparations. *Lancet Diabetes Endocrinol*. 2013;1(3):199-204.
[8] Blevins TC, et al. Efficacy and safety of MYL-1501D versus insulin glargine (Lantus®) in patients with type 1 diabetes: results of the INSTRIDE 1 phase III study. *Diabetes Obes Metab*. 2015;17(8):739-748.
[9] Garg SK, et al. Efficacy and safety of biosimilar insulin glargine (Gla-300) in insulin-naïve adults with type 2 diabetes: INSTRIDE 4 study. *Diabetes Technol Ther*. 2017;19(12):711-718.
[10] McCool R, et al. Systematic review and network meta-analysis of insulin biosimilars: a review of 20 years of research. *Diabetes Ther*. 2021;12(1):65-89.