Similarity assessment of charge variants for bevacizumab biosimilar formulations using imaged capillary isoelectric focusing

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ABSTRACT

The imaged capillary isoelectric focusing method was developed and validated for charge variant analysis of the bevacizumab innovator product and its biosimilar formulations ($N = 2$). The optimized method consists of: 4 M urea, 2.0 mM iminodiacetic acid, 0.35% m/v methylcellulose, 5.0 mM L-arginine, 4.0% v/v carrier ampholytes (narrow-range isoelectric point 5–8 and 8–10.5 with ratio of 1:3), 60 mM sodium hydroxide and 30 mM phosphoric acid as catholyte and anolyte solutions. The bevacizumab innovator and biosimilars formulations were observed with two acidic peaks followed by the main peak and two basic peaks. The validation of the developed method was done by performing intraday ($n = 6$) and interday ($n = 9$) precision studies with observed RSD < 2.0%. The limit of detection and limit of quantification of the developed method was found to be 0.016 and 0.048 mg mL$^{-1}$. The charge variant profiles observed for innovator and biosimilar formulations ($N = 2$) were then applied for statistical comparison using one-way ANOVA, Dunnett’s test. This study can serve as a reference to biosimilar manufacturers and startups on how to assess the charge variant profiles of bevacizumab during the manufacturing process.

GRAPHICAL ABSTRACT

KEYWORDS

Bevacizumab; biosimilars; charge variant analysis; imaged capillary isoelectric focusing; one way ANOVA

Introduction

In the last few decades, therapeutic monoclonal antibodies (mAbs) have revolutionized the treatment of autoimmune, oncologic, metabolic, and inflammatory diseases. Bevacizumab (BVZ) was first developed by Genentech and Hoffmann-La Roche as Avastin$^\text{®}$ (Avt). It is 93% human and 7% murine sequences recombinant humanized monoclonal IgG1 antibody with an approx. molecular weight of 149 kDa. It was first approved in the United States in 2004, for its use in the treatment of metastatic colon cancer in combination with standard chemotherapy.$^{[1]}$ Its mechanism of action involves selective binding to human vascular endothelial growth factor-A (VEGF-A) protein and thus inhibiting the VEGF-A binding to its receptors present on the surface of endothelial cells thereby suppressing proangiogenic signals. BVZ is a full-length IgG1κ isotype antibody composed of two identical light chains (214 amino acid residues) and two heavy chains (453 amino acid residues).

The development of therapeutic mAb biosimilar is a challenging task, as it requires an extensive orthogonal analytical approach to prove equivalence with the innovator product. The currently approved and marketed BVZ biosimilars in India and United States are listed below in Table 1. The charge heterogeneity is required to be monitored throughout the manufacturing process of biosimilars. It is a challenging task from an analytical point of view, due to the numerous unpredictable post-translational modifications that can occur during the characterization process.$^{[2]}$ These post-translational modifications occurring in the mAb molecule during the manufacturing and storage process like “deamidation of asparagine” or “oxidation of tryptophan” can result in the
formation of multiple variants with different charges on their surface. These variants are commonly termed acidic or basic when compared to the monomer peak. The variants with a lower isoelectric point (pI) are termed as acidic while variants with higher pI are termed as basic on being analyzed by isoelectric focusing (IEF) based techniques.[3]

That’s why these modifications are the answer to the question: why detailed protein charge profiles are required? The charge variants present in the structure of the mAb molecules influence the receptor binding, functional activity, shelf life, and thus overall patient safety.[2,4]

The analytical techniques that are used for charge variant analysis are ion-exchange chromatography coupled to tandem mass spectrometry (IX-MS) and IEF-based techniques.[2,5–8] The IEF-based technique like slab gel IEF and capillary isoelectric focusing (cIEF) is used for the separation of protein according to their pI during routine quality checks due to its simplicity in contrast to the IEX-MS technique which requires a complex setup.[9–11] The conventional cIEF has some disadvantages like undetected focusing step, focusing time solely based on current measurement (non-visual), and an increase in total run time due to the addition of the mobilization step. These shortcomings lead to the development of iCIEF technique.[12]

Some of the recently published BVZ research articles[13,14] include charge variant analysis by cIEF technique. The charge variant analysis study performed in these articles have a long run time. The total run time includes 15 min of focusing and 30 min of mobilization step. However, the method developed in the present article using iCIEF technique omits the mobilization step. This modification of iCIEF over cIEF reduces the total run time to 13 min.

Further modifications in iCIEF technique over cIEF involved monitoring the whole separation capillary during the focusing process, thus allowing visual control of the focusing step.[2,15] Once the focusing process is done the focused zones are then detected on-column using a detector (camera) that is continuously monitoring the separation column. Initially, in iCIEF, the separation capillary was directly connected to the catholyte and anolyte vial just like in cIEF. In later modified versions the separation capillary was connected to the inlet and outlet capillary using a hollow membrane which allows the transfer of hydroxyl ions and protons in the separation capillary.

Due to these advances and simplicity, the iCIEF technique is more preferred for the characterization, identification, purity determination, and quantitation of proteins over conventional cIEF.[1,16] In the present study, the iCIEF method was developed and validated for BVZ biosimilar formulations and innovator product. The pI value and area (%) of separated charge variants of Indian biosimilar formulations were compared with the innovator product, where the acidic, main, and basic species of the innovator product and biosimilar formulations were statistically assessed using one way ANOVA, post-hoc Dunnett’s test.

### Materials and methods

The present study was conducted on Maurice (ProteinSimple, San Jose, CA, USA) equipped with a UV and fluorescence detector. The cartridge containing fluorocarbon-coated capillary (PN PS-MC02-C) was obtained from ProteinSimple (effective length is 50 mm and the internal diameter is 0.1 mm). Samples are vacuum loaded with a typical separation time of 13–15 min depending on the molecule. Maurice works in a voltage range of 0–6.5 kV and also has a temperature control option that works in a range of 4–25 °C. The software used for data evaluation was “compass” for iCE 2.0.11.

BVZ innovator product “Avastin” (Avt), Biosimilar-1 (Bio-1), and Biosimilar-2 (Bio-2) formulations were purchased from the local market of Ahmedabad, Gujarat, India. The iCIEF method development kit (PN PS-MDK01-C) was procured from ProteinSimple. The system suitability kit (PN 046044), fluorescence calibration standard (PN 046025), and 1.0% m/v methylcellulose (MC) (PN 101876) were purchased from ProteinSimple.

The lyophilized pI markers (PN 046028) 3.38, 4.05, 5.85, 6.14, 7.05, 8.40, 9.99, and 10.17 were also purchased from ProteinSimple. The five types of ampholytes (PN PS-MDK01-A) including pharmalyte pH ranges 3–10, 2.5–5, 5–8, 8–10.5, and servalyte pH range 2–9 were also purchased from ProteinSimple.

L-arginine (purity ≥99%, PN 181003), urea (purity ≥99.5%, PN 108488), phosphoric acid (purity ≥85%, PN 543828), sodium hydroxide (purity ≥98%, PN 137020), iminodiacetic acid (purity ≥98%, PN 220000) and sodium chloride (purity ≥99.5%, PN 543832) were supplied by Merck Millipore (India). Milli-Q water used was made in-house.

### Selection of standard pI markers

The pI markers selected for checking the system suitability of the instrument were 3.38, 5.85, 6.14, 9.99, and 10.17. The selected pI markers were diluted 200 times into the final sample solution for internal pI calibration. The reported pI of BVZ is 8.3 thus the pI markers selected as standards were 6.14 and 9.50. The pI standards were mixed with each sample before injecting into the instrument.

### Master mix preparation

Master mix solution was prepared by mixing the following reagents in a centrifuge tube: 2.0 µl of pharmalyte (pH 5–8) and 6.0 µl of pharmalyte (pH 8–10.5), 56.0 µl of 0.35% m/v MC, 1.0 µl of standard pI markers 6.14 and 9.50, 80.0 µl of 4 M urea, 2.0 µl of cathodic stabilizer/blocking agent (5 mM L-arginine), 5.0 µl of anodic stabilizer/blocking agent (2 mM iminodiacetic acid), and 7.0 µl of water.

### Table 1. List of bevacizumab biosimilars approved in India and United States.

| Sr. No. | Biosimilar name     | Sr. No. | Biosimilar name     |
|---------|---------------------|---------|---------------------|
| 01      | Cizumab (India)     | 08      | Bevarest (India)    |
| 02      | VERSAVO (India)     | 09      | KRAVEBA (India)     |
| 03      | BevacRel (India)    | 10      | Bryxta (India)      |
| 04      | ADVAMAB (India)     | 11      | BEVATAS (India)     |
| 05      | AVASTIMAB (India)   | 12      | BEVAZZA (India)     |
| 06      | BEVICRA (India)     | 13      | ZIRABEV (US)        |
| 07      | Abeymmy (India)     | 14      | MVASI (US)          |
**Sample preparation**

All three Avt, Bio-1, and Bio-2 formulations were diluted up to 2.0 mg mL\(^{-1}\) with Milli-Q grade water. The 40.0 µl of the mAb solution is then mixed with 160.0 µl of the master mix solution to obtain the final concentration of 0.4 mg mL\(^{-1}\) which was then injected into iCIEF instrument. The study was performed in triplicate \((n = 3)\) where three batches of each formulation were injected into the instrument.

**Method development**

The fluorocarbon-coated capillary was cleaned daily before the start of the analysis by rinsing it with 0.5% m/v MC solution for 5 min and finally with water for 5 min. To decrease the precipitation, the cartridge is flushed with 4 M urea for 3 min and water for 5 min before commencement of analysis. 60 mM sodium hydroxide and 30 mM phosphoric acid solution were selected as the catholyte and anolyte solutions. The iCIEF separation process involves focusing with no mobilization step.\(^{[17]}\) System running was done for 1 min at 1.5 kV, then for 12 min at 3.0 kV in normal polarity, with a sample load duration of 55.0 s. The analysis was performed at the temperature of 10°C using a 280 nm filter.

**Statistical analysis**

GraphPad Prism software ver.9.2.0 was used to perform statistical analysis. The control and group samples were statistically compared using one-way ANOVA, post-hoc Dunnett’s test. The \(p\)-value was considered as the signifying parameter where \(p < 0.05\) represents a significant difference. The experiment was performed in triplicate.

**Results and discussion**

**The theory behind the working of iCIEF**

The iCIEF method was developed using BVZ biosimilar formulations. Method development requires optimization of various reagents and conditions used during analysis. MC was used in the method development process as a viscosity-enhancing as well as capillary coating agent that suppresses the residual electroosmotic flow (EOF). The total analysis time in iCIEF is short in comparison to cIEF given the absence of the mobilization step.\(^{[18]}\) It influences the longitudinal diffusion within the capillary and also reduces the analyte-wall interaction thus suppressing EOF. The migration time \((t_m)\) of the analytes is directly proportional to the concentration of the MC.

In the present study, 0.35% m/v was found to be the optimum concentration of the MC, generating good separation profiles with high resolution without clogging the capillary. Urea was used due to its solubility-enhancing property. The mAbs are highly sensitive to their surrounding environment and therefore get precipitated easily. Thus urea was added as an additive to prevent the precipitation of mAb molecules. The optimum urea concentration was found to be 4 M. Urea concentrations lower than 4 M show poorly defined separation profiles presumably due to poor solubility. The concentrations >4 M show substandard separation efficiency especially of basic variants possibly due to denaturation and unfolding of proteins. The amphotelyes are responsible for affecting the resolution and pH gradient of the charge variants inside the capillary.\(^{[19]}\)

The concentration of CAs selected was 4.0% v/v after referring to the iCIEF method development guide (Maurice, 046-296_RevD). From the trials, we understood that instead of using single wide range ampholytes, the use of two narrow range ampholytes gave better resolution. The mixture of two narrow range ampholytes 5–8 (25% v/v) and 8–10.5 (75% v/v) was used during detecting charge heterogeneity of BVZ. It is reported that as the sample temperature increases the viscosity of MC decreases which in turn causes a decrease in \(t_m\) and an increase in longitudinal diffusion leading to peak broadening.\(^{[20]}\) 10°C was selected as the ideal temperature as no peak broadening and splitting was observed.

The iCIEF profiles obtained for the Avt (Figure 1A), Bio-1 (Figure 1B), and Bio-2 (Figure 1C) formulations at a concentration of 0.4 mg mL\(^{-1}\) were observed to be similar. The acidic species consisting of two acidic peaks eluted first followed by the main peak and then by basic species consisting of two basic peaks.

**Determination of \(p_I\) using iCIEF**

The \(p_I\) is an important characteristic of mAbs which is defined as the pH at which the antibody contains zero electrical charges, and its value depends on the charged amino acids present in mAbs. The antibody carries a net positive charge if the pH of the surrounding environment is below the antibody’s \(p_I\), whereas the antibody will carry a net negative charge if the pH is above the \(p_I\).\(^{[21]}\) The standard markers with \(p_I\) 6.14 and 9.50 were used to measure the \(p_I\) of BVZ charge variants.

The \(p_I\) values of the biosimilar formulations and innovator products ranged from 7.4 to 8.2, and the primary peak regions of the acidic species, basic species, and main species were observed in the range of 7.4–7.7, 8.0–8.2, and 7.8–7.9, respectively. The calculated \(p_I\) values are shown in Table 2.

Although after applying Dunnett’s test, significant variations in the \(p_I\) value of acidic peak-2 \((p = 0.0262)\), main peak \((p = 0.0203)\), basic peak-1 \((p = 0.0019)\), and basic peak-2 \((p < 0.0001)\) were observed for biosimilar formulations and innovator product as seen in Figure 2. However, no significant variation was observed for the \(p_I\) value of acidic peak-1 \((p = 0.1126)\) between the biosimilar formulations and the innovator product.

As the \(p_I\) values in biosimilar formulations and innovator products don’t differ more than 0.4 units, the charge heterogeneity between them is considered comparable as per regulatory standards. These results demonstrate that the BVZ biosimilar formulations along with the innovator product have a similar \(p_I\) profile with a net positive charge on their surface which is essential for efficient fluid-phase
endocytosis (pinocytosis). It is reported from pharmacokinetic studies that the antibodies with a positive charge have a shorter half-life with high blood clearance and tissue retention.\textsuperscript{[22,23]}

**Charge variant analysis**

BVZ is a highly complex macromolecule in contrast to synthetically manufactured chemical drugs. It is heterogeneous in nature due to post-translational modifications that occur during the manufacturing stage like glycosylation and lysine clipping. It is also susceptible to several chemical modifications, such as oxidation or deamidation which can occur during the purification or storage stage.\textsuperscript{[24]} The statistical evaluation of the reported area (%) of acidic, main, and basic species as seen in Table 3 was done using one-way ANOVA. For multiple comparisons, statistical hypothesis testing Dunnett’s test was implemented.

The comparison of biosimilar formulations with innovator products for acidic variants as seen in Figure 3A shows a significant variation ($p = 0.0004$) with an observed $R^2$ of 0.9958. For the main peak as seen in Figure 3B, significant variation ($p = 0.0017$) was observed with an $R^2$ of 0.9884. Finally for basic variants as seen in Figure 3C, significant variation ($p = 0.0009$) was observed with an $R^2$ of 0.9925. After applying statistical tools and interpreting the results we summarized that, out of the two biosimilar formulations (Bio-1 and Bio-2), Bio-2 shows less significant variation and more similarity with the innovator product.

The possible contributors for significant variations observed for the acidic variants of BVZ biosimilars and innovators usually are either deamidation of asparagine (Asn) residue (N84), oxidation of methionine (Met) residue (M258), C-terminal lysine residue preservation or a high percentage of sialic acid present in the recombinant IgG1 antibody expressed in Chinese hamster ovary (CHO) cell line.\textsuperscript{[25,26]} The occurrence of deamidation takes place in

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**Figure 1. Electropherogram of innovator product: Avt (A), biosimilar formulations: Bio-1 (B) and Bio-2 (C).**

**Table 2.** $pI$ value of acidic, main and basic species in all three formulations ($n = 3$).

|          | Acidic peak-1 ± SD | Acidic peak-2 ± SD | Main peak ± SD | Basic peak-1 ± SD | Basic peak-2 ± SD |
|----------|--------------------|--------------------|----------------|-------------------|-------------------|
| Avt      | 7.472 ± 0.005      | 7.649 ± 0.005      | 7.883 ± 0.004  | 8.013 ± 0.002     | 8.075 ± 0.005     |
| Bio-1    | 7.549 ± 0.020      | 7.738 ± 0.030      | 7.908 ± 0.004  | 8.106 ± 0.005     | 8.268 ± 0.004     |
| Bio-2    | 7.523 ± 0.006      | 7.642 ± 0.007      | 7.872 ± 0.008  | 8.069 ± 0.006     | 8.241 ± 0.004     |
both variable and constant domains. However, the deamidation occurring in the complementary determining region (CDR) generates acidic species without fail.[3,27,28] Other modifications that can cause the generation of acidic species are trisulfide modification, thiosulfide modification, modification by maleuric acid, glycation, cysteinylation, reduction of disulfide bonds, presence of non-reduced species, non-classical disulfide linkages, presence of high mannose content and fragmentation.

The main peak was observed as the largest peak that elutes after the acidic peaks or before the basic peaks and serves as a reference peak for the detailed analysis of acidic and basic variants. The probable causes for significant variations observed for the main peak of biosimilars and innovators are modifications like; “glycosylation of the Asn residue in the constant heavy chain-2 (CH2) domain” or “heavy chain C-terminal lysine (Lys) removal” or “N-terminal glutamine (Gln) cyclization to Pyroglutamate (pGlu).”[29–31] The basic variants were observed as two peaks that were eluting after the main peak in the study.

It is reported that during biosimilar product development the incomplete removal of C-terminal lysine residues from either one or both heavy chains leads to the generation of basic variants. The other modifications responsible for the generation of basic variants are succinimide formation, amidation of proline (Pro), mutation of serine (Ser) to arginine (Arg), aglycosylated heavy chains, oxidation of methionine (Met), aggregation, fragmentation, isomerization of asparagine (Asn) to isoAspartic acid (isoAsp) and unformed disulfide bond.[3,26,30–35]

### Method validation

#### Precision

The developed iCIEF method was validated by performing a precision study using BVZ biosimilar as a model. Precision studies were performed to check the % deviations observed in the $pI$ values and area (%) of charge variants.[36] An intraday precision study was performed by injecting six replicate injections of 0.4 mg mL$^{-1}$ concentration in one day ($n = 6$). The interday precision study was done by injecting three injections of 0.4 mg mL$^{-1}$ concentration per day for three consecutive days ($n = 3$). The RSD (%) of not more than 2.0% was observed for the $pI$ values and area (%) values of acidic, main and basic species thus showing good precision as seen in Table 4.

However acidic species showed poor precision due to their low content. These variables in the precision study are mainly due to continuous degradation of the internal

#### Table 3. Area (%) of acidic, main and basic species in all three formulations ($n = 3$).

|          | Acidic peaks (%) ± SD | Main peak (%) ± SD | Basic peaks (%) ± SD |
|----------|-----------------------|--------------------|----------------------|
| Avt      | 36.0 ± 0.60           | 59.0 ± 0.50        | 4.9 ± 0.56           |
| Bio-1    | 23.4 ± 0.45           | 44.6 ± 0.66        | 32.0 ± 0.85          |
| Bio-2    | 28.5 ± 1.23           | 48.8 ± 1.30        | 22.6 ± 2.51          |

#### Figure 2. One way ANOVA test on observed $pI$ values of two acidic, one main and two basic peaks in Avt, Bio-1, and Bio-2 formulations.
coating of the capillary followed by increased adsorption of protein on the coating.\textsuperscript{[12,37,38]}

**Limit of detection (LOD) and limit of quantification (LOQ)**

The limit of detection (LOD) and limit of quantification (LOQ) of the developed iCIEF method were determined as per the ICH guideline\textsuperscript{[39]} using innovator product “Avt” as the standard reference. Six samples were prepared in the concentration range (0.16, 0.8, 0.4, 0.2, 0.1, and 0.05 mg/mL) near to the expected LOD and LOQ. Each sample was injected in triplicate (\(n = 3\)). Three calibration lines for concentration vs. peak area were plotted. The standard deviation (\(\sigma\)) of area for all the concentrations was calculated. The averaged slope (\(S\)) of the three lines and individual intercept points at concentration zero were calculated. Finally, the standard deviation (\(\sigma\)) and slope (\(S\)) values obtained were added to the equations: LOD = 3.3 * \(\sigma/S\) and LOQ = 10 * \(\sigma/S\). The LOD and LOQ of the developed iCIEF method were found to be 0.016 and 0.048 mg/mL, respectively.

Intraday precision at LOQ (0.048 mg/mL) of the method was performed by injecting six replicate injections of 0.05 mg/mL concentration in one day (\(n = 6\)). Similarly, interday precision at LOQ of the method was performed by injecting three injections of 0.05 mg/mL concentration per day for three consecutive days (\(n = 3\)). RSD (%) of not more than 2.0% was observed in both intraday and interday precision at LOQ of the method.

**Conclusion**

In the present study, we have developed a rapid and robust analytical method for the charge variant analysis of bevacizumab biosimilar formulations and innovator products using the iCIEF technique. The developed method is more rapid, simple, and precise than any of the earlier published cIEF methods.

Through similarity assessment, it was observed that the bevacizumab biosimilar formulations (\(N = 2\)) have a high % of basic variants compared to their innovator product. This observation is in agreement with a recently published article that also reported a high % of basic variants using the IEX-UV technique. The incomplete removal of C-terminal lysine residues during the manufacturing process is reported as the primary reason for the high % of basic variants. However, the excess of basic variants present in bevacizumab biosimilar formulations don’t affect their biological function and potency, as the modifications occurring on the terminal ends of the mAbs don’t participate in the receptor binding directly.

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Disclosure statement

The authors have declared no conflict of interest.

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Abbreviations

iCIEF imaged capillary isoelectric focusing
N number of formulations
MC methyl cellulose
CA carrier ampholytes
pI isoelectric point
n number of samples
ANOVA analysis of variance
mAb monoclonal antibody
BVZ bevacizumab
Avt avastin
Bio-1 biosimilar-1
Bio-2 biosimilar-2
IgG1 Immunoglobulin 1
VEGF-A vascular endothelial growth factor-A
IEF isoelectric focusing
IEX-MS ion exchange chromatography-mass spectroscopy
cIEF capillary isoelectric focusing
PN product number
kV kilovolt
P probability
EOF electroosmotic flow
tm migration time
R2 coefficient of determination
Met methionine
Asn asparagine
Lys lysine
Pro proline
Gln glutamine
pGlu pyroglutamate
Ser serine
Arg arginine
CH2 constant heavy chain-2
C-terminal carboxyl-terminal
N-terminal amino-terminal
CDR complementary determining region
CHO Chinese hamster ovary
LOD limit of detection
LOQ limit of quantification.