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Analysis of Charge Variant Profiles in Antibody Conjugates

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***Abstract**: The aim of this study was to analyze the charge variant profiles of both unconjugated and conjugated antibodies. The first antibody exhibited the lowest alkalinity, with isoelectric point (pI) values ranging from 7.7 to 8.2. This was followed by the second antibody, which had pI values between 8.3 and 8.6. The third antibody was identified as the most alkaline, displaying pI values from 8.95 to 9.00. The first antibody was conjugated to a maytansine derivative via linkers, while the second and third antibodies were linked to tomaymycin. The conjugated antibodies demonstrated increased heterogeneity and more acidic characteristics compared to their unconjugated counterparts. Specifically, the first antibody conjugated with the maytansine derivative had a pI range of 7.0 to 8.1 (ΔpI: 1.1), the second antibody with tomaymycin ranged from 7.8 to 8.4 (ΔpI: 0.6), and the third antibody had pI values from 8.3 to 8.8 (ΔpI: 0.5). The charge variant profiles obtained through imaging capillary isoelectric focusing exhibited high repeatability both within and across days.*

Keywords: Antibody, Conjugates, Charge Variants, pI.

Abbreviations:
ADCs: Antibody-Drug Conjugates
FDA: Food and Drug Administration
cIEF: Chromatography and Capillary Isoelectric Focusing
icIEF: Imaged Capillary Isoelectric Focusing
ADCs: Antibody-Drug Conjugates
DAR: Drug-to-Antibody Ratio
RSD: Relative Standard Deviation

I. INTRODUCTION

Antibody-drug conjugates (ADCs) represent an up-and-coming class of targeted therapies for various diseases, particularly in oncology [1]. The development of ADCs gained momentum in 2000 when the U.S. Food and Drug Administration (FDA) approved Ozogamicin, marking the first instance of a drug conjugated to an antibody specifically for the treatment of acute myelogenous leukemia [2]. Since then, the field has expanded considerably, with eleven ADCs approved for various cancer treatments and over one hundred more currently undergoing clinical trials [3].

ADCs leverage the specificity of monoclonal antibodies(mAbs) to selectively target and eliminate cancer cells by binding to unique antigens present on the surface of these cells [4]. This targeted approach enhances the efficacy of the cytotoxic drugs and minimizes damage to healthy tissues [5]. The mechanism of action involves the covalent attachment of cytotoxic agents to mAbs through well-defined chemical bonds [6], allowing for the delivery of potent therapeutic agents directly to the malignant cells [7].

The ligands that link antibodies with drugs can be categorized into two main types: cleavable [8] and non-cleavable bonds [9].

Cleavable bonds are designed to release the cytotoxic agent in response to specific stimuli within the tumor microenvironment, such as enzymatic activity or alterations tin pH levels [10]. In contrast, non-cleavable bonds necessitate complete enzymatic degradation of the antibody before the release of the drug can occur [11]. The conjugation process typically involves binding the drug molecules to mAbs via thiol groups found in cysteine residues or amino groups in lysine residues [12], resulting in a complex and heterogeneous mixture of conjugates [13]. This heterogeneity can vary based on the drug-to-antibody ratio (DAR) and the specific sites of conjugation on the antibody [14].

Given the intricate nature of ADCs, precise characterization of their properties is essential to ensure batch-to-batch consistency and overall quality [15]. The assessment of charge heterogeneity is important as it serves as a critical quality control parameter [16]. Charge variants act as unique identifiers [17], akin to fingerprints, which help evaluate the consistency and stability of the ADC formulations [18].

To achieve this, several analytical techniques are employed, with ion exchange chromatography and capillary isoelectric focusing (cIEF) [19] being among the most widely used methods for monitoring the charge variant profiles of ADCs [20]. The separation principle in these techniques is based on differences in the charge variant isoelectric points (pI) [21]. Imaged capillary isoelectric focusing (icIEF) offers several advantages [22], including high sensitivity [23], reliability [24], and accuracy, making it an effective tool for determining [25] and quantifying charge variants [26]. Moreover, icIEF facilitates real-time monitoring of charge variant separation and enhances its utility in ADC characterization [27].

Maytansine derivatives and tomaymycin are potent cytotoxic agents commonly utilized for developing antibody-drug conjugates (ADCs) [28]. Maytansine derivatives inhibit tubulin polymerization [29], a crucial process in cancer cell division [30], thereby effectively disrupting mitotic spindle formation and leading to cell cycle arrest and eventual apoptosis [31]. This mechanism of action makes them particularly valuable in targeting rapidly proliferating tumor cells [32].

Tomaymycin, a bio

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antibiotic derived from the bacterium *Streptomyces achromogenes* [33], has demonstrated significant antitumor activity [34]. Its efficacy stems from its ability to interfere with critical cellular processes in cancer cells, contributing to its potential as a therapeutic agent in ADC formulations [35].

Characterizing the charge variant profiles of conjugated antibodies that utilize maytansine and tomaymycin derivatives is vital for several reasons [36]. First, it helps verify the consistency and efficiency of the conjugation process, ensuring that the intended drug-to-antibody ratio (DAR) is achieved [37]. Second, understanding the heterogeneity of these conjugates is essential for assessing their stability over time [38], which is crucial for maintaining therapeutic efficacy [39]. Lastly, this characterization allows for the evaluation of how different formulations impact the overall performance of the ADCs [40], including their pharmacokinetics, distribution, and efficiency in targeting tumor cells [41]. Consequently, thorough characterization is a key component for the successful development and clinical application of ADCs [42].

The primary objective of this study is to characterize the charge variant profiles of antibodies conjugated with maytansine and tomaymycin derivatives linked through cleavable bonds [43]. This characterization will be accomplished using the imaged capillary isoelectric focusing (icIEF) method optimized in prior research for its sensitivity and precision in differentiating charge variants [44]. By employing icIEF, the study aims to elucidate the heterogeneity of charge profiles that arise from the conjugation process [45], thereby providing insights into the stability and consistency of these antibody-drug conjugates [46]. Additionally, this research seeks to assess how variations in the conjugation strategy may influence the overall therapeutic efficacy and safety of the resulting antibodies [47]. Ultimately, the findings from this study will contribute to a deeper understanding of the physicochemical properties of ADCs, facilitating the optimization of their design and therapeutic applications.

II. MATERIALS AND METHODS

A.Materials

The study utilized several key materials, including the iCE280 chemical test kit and the ICE280 electrolyte solution group, both sourced from Convergent Bioscience. Methyl cellulose was used at 1% and 0.5%concentrations, while known standard isoelectric points (pI) of 6.61, 7.05, 8.18, and 9.5 were obtained from the same supplier. Pharmalyte solutions with pH ranges of 3-10 and 8-10.5 were acquired from GE Healthcare. Additional reagents such as urea, sucrose, histidine, and phosphoric acid were purchased from Sigma.

B. Naked and Conjugated Antibodies

In this investigation, three monoclonal antibodies, designated as Antibodies I, II, and III, were analyzed. The unmodified antibody solutions were prepared at a concentration of approximately 10 mg/mL in a phosphate buffer at pH 6.5. Following this, the antibodies underwent conjugation with cytotoxic molecules through the formation of cleavable linkages. Then, the resulting conjugated antibodies were formulated in a buffer solution containing 10 mM histidine, 10% sucrose, and N-methyl-2-pyrrolidone (NMP), maintaining a pH of 6.5 and a concentration of about 2 mg/mL.

C. Sample Preparation

For the preparation of both naked and conjugated antibody samples, the solutions were diluted to the required final concentration. This involved mixing a solution containing 0.35% methylcellulose with a 4% Pharmalyte mixture, combining solutions with pH ranges of 3-10 and 8-10.5 in a 1:1 ratio. Urea was added at a concentration of 2 M, along with standard materials corresponding to pI values of 6.61, 7.05, 8.18, and 9.5. After preparation, the samples were centrifuged at 6000 rpm for 3 minutes to eliminate any particulate matter. The clarified samples were then transferred to glass vials suitable for auto-injection and weighed again to ensure the removal of any air bubbles before being placed in the injector.

D. icIEF Device

The analyses of the prepared samples were conducted using the iCE280 system, which was equipped with an autoinjector from Convergent Bioscience [48]. The capillary column employed for the analysis had specific dimensions: a length of 50 mm, an inner diameter of 100 µm, and an outer diameter of 200 µm. During the analysis, a cathodic solution comprised of 100 mM NaOH and 0.1% methylcellulose was utilized, while the anodic solution contained 80 mM phosphoric acid and 0.1% methylcellulose. The protein concentration time was set to 7 or 10 minutes, with a potential difference of 3000 V applied [49]. Protein separation was monitored using a camera operating at a wavelength of 280 nm, allowing for the detection of the separated proteins [50].

III. RESULTS AND DISCUSSION

Charge heterogeneity is a significant factor contributing to the overall variability observed during the production of therapeutic monoclonal antibodies, whether they are in their native form or conjugated with drugs [51]. This heterogeneity can arise from various modifications that monoclonal antibodies undergo throughout the cell culture processes, purification, and formulation [52]. Therefore, characterizing these heterogeneous profiles, whether for unconjugated or drug-conjugated antibodies, is essential to ensure their purity and consistency across different production batches [53].

This study primarily aimed to characterize the heterogeneous profiles of unconjugated antibodies.

A. Heterogeneous Profile of Naked Antibodies

The charge variant profiles of three monoclonal antibodies were analyzed: the first and second antibodies specifically target the CD19 cell surface antigen, while the third antibody is engineered to recognize and bind to the EphA2 receptor. As illustrated in Figure 1, the first antibody exhibited a greater degree of charge heterogeneity and demonstrated a more acidic character compared to both the second and third antibodies.

This increased charge variability may influence

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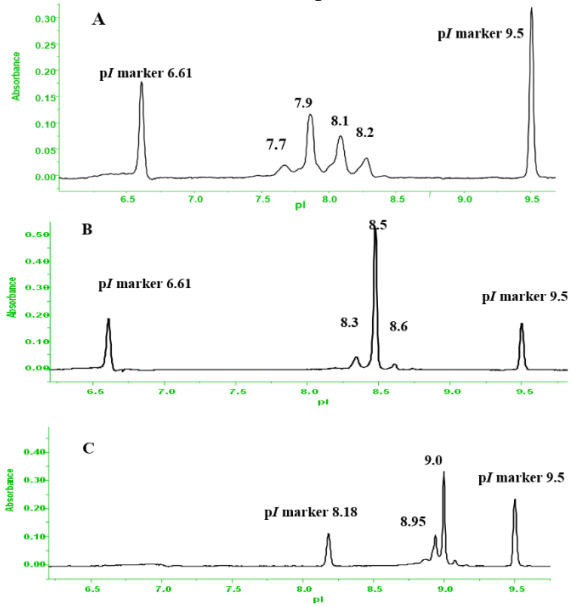
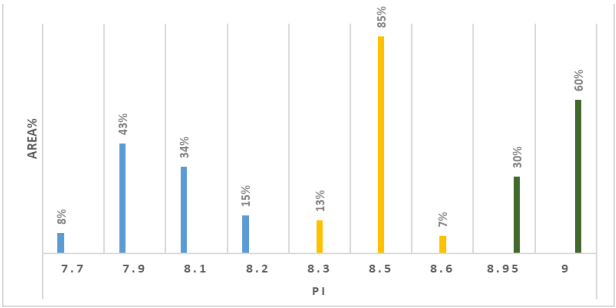
the antibody's biological activity, stability, and overall therapeutic efficacy, emphasizing the importance of thorough characterization in the development of effective monoclonal antibody therapies [54]. Further investigations into structural and functional implications of this charge heterogeneity are warranted to understand its impact on clinical outcomes.



[Fig.1: Graph of the heterogeneity of naked antibodies: (A) antibody I, (B) antibody II, (C) antibody III. Analytical conditions: The final concentration of the naked antibody in the sample is mg/mL0.2 after diluted in a solution containing 0.35% methylcellulose, 2% pharmalyte (3-10)/2% pharmalyte (8-10.5), M2 urea. Standard materials: pI 6.61, 8.18, 9.50]



[Fig.2: Percentage of Peak Area of Charge Variants of Naked Antibodies]

Figure 2 illustrates the percentage area of peaks corresponding to charge variants of the three unconjugated monoclonal antibodies analyzed. The first antibody exhibited four charge variants, with a differential isoelectric point (ΔpI) of 0.1. The observed pI values for these variants were 7.7 (8%), 7.9 (43%), 8.1 (43%), and 8.2 (15%). In contrast, the second antibody displayed three charge variants with a larger ΔpI of 0.3. The main peak for this antibody was observed at a pI of 8.50, constituting 85% of the total area, alongside two minor peaks at pI values of 8.3 (13%) and 8.6 (7%). The third antibody had two charge variants, characterized by a minimal ΔpI: 0.05, with pI values of 8.95 (30%) and 9.00 (60%).

The charge heterogeneity observed in these antibodies aligns with the known variability typically seen in therapeutic monoclonal antibodies [55]. Charge variants are generally categorized into primary types. They could be accompanied by secondary types that may be either more acidic or alkaline than the predominant form. For instance, the more acidic variant present in the second antibody (pI 8.3) can be attributed to the deamidations of one or more asparagine residues, resulting in a lower pI value than the main variant (pI 8.50). Conversely, the more alkaline charge variant (pI 8.6) likely arises from lysine residues (basic amino acids located at the carboxylic terminal end_.

Furthermore, studies assessing intraday and interday reproducibility of antibody heterogeneity using imaged capillary isoelectric focusing (icIEF) demonstrated excellent consistency. The relative standard deviation (RSD%) values for pI measurements were less than 0.4%, while the RSD% values for the peak area percentage for charge variants were below 4% (Table I). These results indicate a high level of reliability in the characterization of charge heterogeneity among the studied antibodies, reinforcing the importance of precise analytical techniques to develop and control therapeutic antibodies.

Table-I: Results of Repeatability of the Percentage of the Peaks of Charge Variants of Naked Antibodies

Recurrence Between Days				In-Day-Frequency				Fundamental Charge Variants
Area%		pI		Area%		pI		
RSD%*	Medium	RSD%*	Medium	RSD%*	Medium	RSD%*	Medium	
0.5	8%	0.2	7.7	0.5	8%	0.2	7.7	Antibody-1
1	43%	0.1	7.9	1	43%	0.1	7.9	
1.5	34%	0.2	8.1	1	34%	0.2	8.1	
1.9	15%	0.3	8.2	1.5	15%	0.3	8.2	Antibody-2
1	13%	0.2	8.3	1	13%	0.2	8.3	
3	85%	0.1	8.5	2	85%	0.1	8.5	
2	7%	0.1	8.6	1	7%	0.1	8.6	
3	30%	0.1	8.95	2	30%	0.1	9	Antibody-3
2.5	60%	0.1	9	2	60%	0.1	9	
*RSD: Relative Standard deviation								

B. Variants of Cleavable Conjugate Antibodies

The three monoclonal antibodies analyzed in this study were characterized using imaged capillary electrophoresis, focusing specifically on the amino groups of lysine residues. These antibodies were conjugated with

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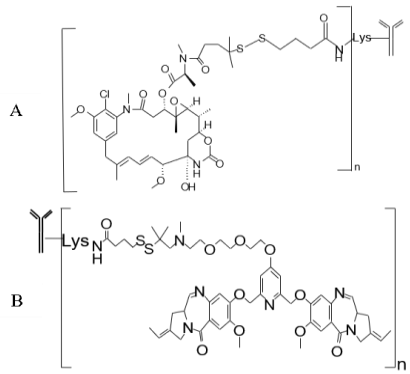
derivatives of maytansine (designated as Antibody I) and tomaymycin (designated as Antibodies II and III) through the formation of cleavable bonds, as illustrated in Figure 3.

The conjugation process involved the incorporation of ligands containing a disulfide bond, which plays a crucial role in the controlled release of the cytotoxic drug. Upon cellular uptake, this disulfide bond is cleaved, enabling the release of the toxic agent within the target cells [56]. This mechanism ensures that the therapeutic action is localized to the cancerous cells, thereby minimizing systemic toxicity and enhancing the overall efficacy of the treatment. Characterizing these cleavable conjugate antibodies is essential for understanding their stability, release kinetics, and therapeutic potential in the mixture of antibody-drug conjugates [57].



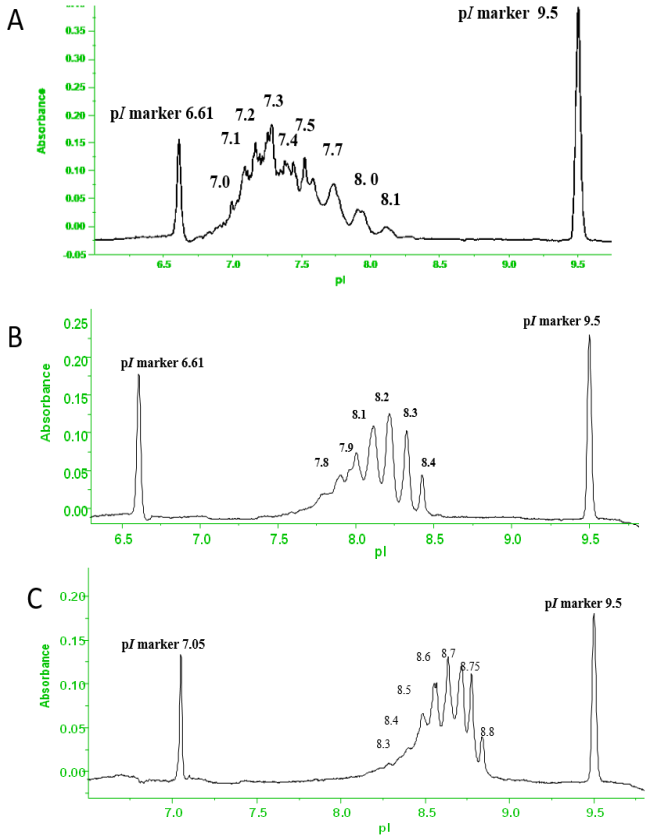
[Fig.3: Chemical Structure of Maytansine Derivatives (A) and Tomamycin (B) Molecules Conjugated with the Amino Group of Antibody Lysine Residues by Cleavable Bonds]

Figure 4 illustrates the charge heterogeneity observed in conjugated antibodies with cleavable derivatives of maytansine and tomaymycin. The analysis revealed that the conjugated antibodies exhibited a high heterogeneity compared to their unconjugated antibodies. In particular, these conjugated antibodies demonstrated an increase in acidic characteristics, which may be attributed to the modifications introduced during the conjugation process.

This enhanced heterogeneity and acidic nature can affect various pharmacokinetic and pharmacodynamic properties, including the stability, solubility, and efficacy of the antibody-drug conjugates. Understanding the implications of this charge variability is crucial for optimizing the therapeutic performance of these molecules, as it could influence their interactions with target cells and the overall therapeutic outcome. The findings underscore the importance of thorough characterization in the development of effective antibody-drug conjugates.

The isoelectric point (pI) values representing the charge heterogeneity of the first conjugated antibody ranged from 7.0 to 8.1, exhibiting a differential pI (ΔpI) of 1.1. For the second conjugated antibody, the pI values varied from 7.8 to 8.4, resulting in a ΔpI of 0.6. The third conjugated antibody displayed pI values from 8.3 to 8.8, with a ΔpI of 0.5. The observed increase in heterogeneity among the cleavable conjugate antibodies is reflected in the broader ΔpI ranges. This phenomenon can be attributed to the differing number of drug molecules associated with the amino groups of lysine residues within the antibodies.

Specifically, the first conjugated antibody exhibited more charge variants, totaling ten, in contrast to the second and third antibodies, which were conjugated with tomaymycin and displayed only seven charge variants. This disparity can be explained by the availability of lysine residues for conjugation; antibodies typically contain up to 80 lysine sites, providing numerous opportunities for modification. The variations in charge observed in the conjugated antibodies arise from the differing number of amine groups in lysine residues paired with the pharmaceutical agents.



[Fig.4: icIEF profile of naked conjugated antibodies: (A) first antibody conjugated with maytansine derivatives, (B) second antibody conjugated with Tomaymycin, and (C) third antibody conjugated with Tomaymycin. Analytical conditions: The final concentration of the conjugated antibody in the sample is mg\mL0.5 After diluting it in a solution containing 0.35% methylcellulose, 2% pharmalyte (3-10)/2% pharmalyte (8-10.5), M2 urea. Standard known materials6.61:pI, 7.05, 9.50]

The coupling of these amine groups with the drug molecules results in a reduction of pI values, with this decrease becoming more pronounced as the number of bonded amino groups increases. Consequently, this leads to a composite profile of charge heterogeneity characterized by pI values that are more acidic than those of the unconjugated antibodies.

Figure 5 further elucidates the percentage areas of the peaks corresponding to the cleavable conjugate antibodies. Notably, the percentage of charge variants observed in the naked antibodies was markedly lower. These findings underscore the successful conjugation of the studied antibodies, highlighting the effectiveness of the conjugation process in

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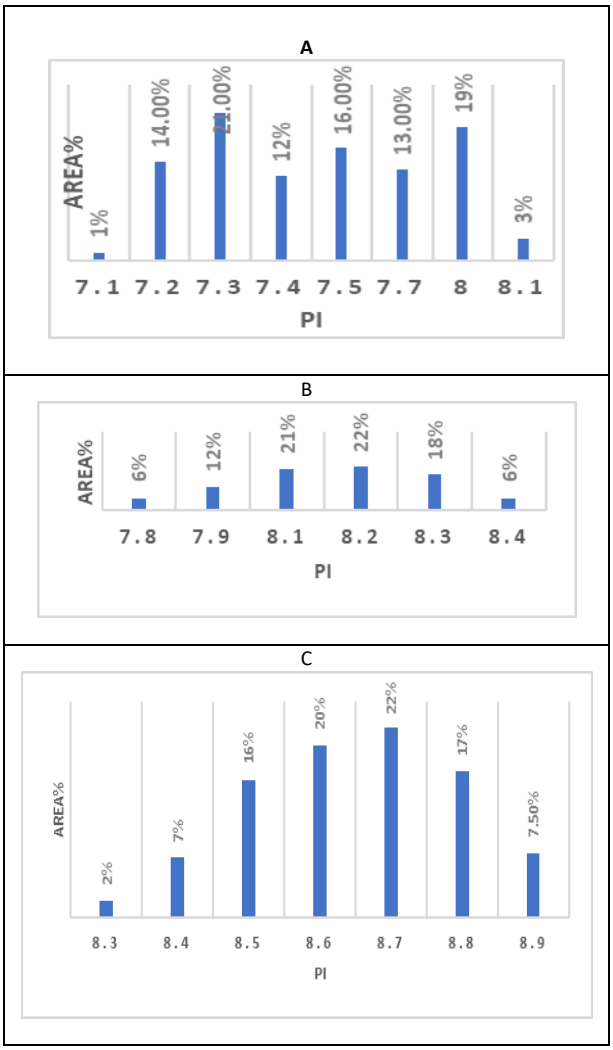
altering their charge properties.



[Fig.5: Percentage area of peaks of cleavable conjugate antibodies: (A) the first antibody conjugated with maytansine derivatives, (B) the second antibody conjugated with Tomaymycin, and (C) the third antibody paired with Tomaymycin. The analytical conditions are given in Fig.4]

Table-II: Results of Frequency for Percentage Peaks and pI for Charge Variants of Antibodies Conjugated with Drugs

(n=6) between days				(n=6) within the day				Fundamental charge variants
Area%		pI		Area%		pI		
RSD %	medium	Monitor %	medium	RDS %	medium	RSD%	medium	
0.5	6%	0.2	7.80	1.5	14%	0.2	7.20	
1	12%	0.1	7.90	1	21%	0.2	7.30	
1.5	21%	0.2	8.10	1	12%	0.2	7.40	Antibody-1 - maytansine
1.9	22%	0.3	8.20	1.5	16%	0.3	7.50	
1	18%	1.5	8.30	0.5	13%	0.1	7.70	
1.5	6%	0.5	8.40	1	19%	0.1	8.00	
1	12%	0.1	7.90	1	12%	0.1	7.90	Antibody-2 Tomamycin
1.5	21%	0.2	8.10	1	21%	0.2	8.10	
1.9	22%	0.3	8.20	1.5	22%	0.3	8.20	
2	18%	0.1	8.30	1.5	18%	0.2	8.30	
2	16%	0.2	8.50	0.5	16%	0.2	8.50	
1	20%	0.3	8.60	1	20%	0.3	8.60	Antibody-3 -Tomamycin
1.5	22%	0.1	8.70	1.5	22%	0.2	8.70	
1	17%	0.2	8.80	0.5	17%	0.2	8.80	

The characterization of charge heterogeneity in the studied conjugated antibodies was conducted using imaged capillary electrophoresis, and the results demonstrated high precision and reproducibility. The analysis revealed that the relative standard deviation (% RSD) values for the pI of the charge variants remained below 0.3%, indicating a reliable and consistent measurement over both intraday and interday assessments. Furthermore, the % RSD values for the area percentages of the detected peaks were less than 2%, highlighting the robustness of the technique in quantifying the charge heterogeneity of these antibodies. These findings, summarized in Table II, underscore the effectiveness of imaged capillary electrophoresis as a valuable tool for the precise characterization of antibody properties, which is essential for ensuring the quality and efficacy of therapeutic monoclonal antibodies.

IV. CONCLUSION

Capillary electrophoresis was employed to assess the heterogeneity profiles of three unconjugated monoclonal antibodies that were conjugated with derivatives of maytansine and tomaymycin through the formation of cleavable bonds. A comparative analysis of the charge heterogeneity among these unconjugated antibodies revealed that the third antibody exhibited more acidic properties and high heterogeneity than the first and second antibodies, which displayed a relatively more uniform charge distribution.

A marked increase in heterogeneity was observed for the conjugated antibodies, characterized by a broader range of pH values associated with the charge variants compared to their unconjugated counterparts. Additionally, the conjugated antibodies exhibited enhanced acidic characteristics, as indicated by lower pI values of the charge heterogeneity, which further differentiated them from the unconjugated antibodies.

The stability and reproducibility of the measurements were confirmed, as the pI values and the percentage area of the peaks corresponding to charge variations demonstrated excellent consistency both within individual days and across multiple days of analysis [58]. These findings highlight the effectiveness of capillary electrophoresis in accurately characterizing the charge heterogeneity of antibody-drug conjugates [59], which is critical for evaluating their therapeutic potential and ensuring quality control in biopharmaceutical development [60].

DECLARATION STATEMENT

I must verify the accuracy of the following information as the article's author.

- **Conflicts of Interest/ Competing Interests:** Based on my understanding, this article has no conflicts of interest.
- **Funding Support:** This article has not been funded by any organizations or agencies. This independence ensures that the research is conducted with objectivity and without any external influence.
- **Ethical Approval and Consent to Participate:** The content of this article does not necessitate

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ethical approval or consent to participate with supporting documentation.

- **Data Access Statement and Material Availability:** The adequate resources of this article are publicly accessible.
- **Authors Contributions:** The authorship of this article is contributed solely.

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