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Analyzing Charge Variant Profiles of Monoclonal Antibodies Conjugated to Cytotoxic Agents

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Abstract: ***This study investigates the charge variant profiles of monoclonal antibodies (mAbs) conjugated to cytotoxic agents, focusing on their implications for therapeutic efficacy and safety. Antibody-drug conjugates (ADCs) are a promising class of targeted therapies, particularly in oncology. The research centers on three specific mAbs: mAB1 and mAB2, targeting EphA2, and mAB3, targeting CD19. Using imaged capillary isoelectric focusing (icIEF), we characterized the charge variants of these mAbs in both their unconjugated state and after conjugation to maytansine and tomaymycin derivatives using non-cleavable linkers. Our findings indicate that mAB1 and mAB2 exhibit greater charge heterogeneity compared to mAB3, with distinct isoelectric points (pI) reflecting their structural diversity. Specifically, mAB1 displayed two charge variants with pI values of 9.00 and 8.95, while mAB3 showed a predominant variant at pI 8.50. Conjugation increased charge heterogeneity and acidity across the ADCs, particularly evident in mAB1 conjugates, which demonstrated a broader pI range. The icIEF method proved effective, showing high repeatability for intra-day and inter-day analyses. These results highlight the critical role of charge variant characterization in ensuring the quality and consistency of ADCs. They underscore how variations in charge profiles can influence mAb pharmacokinetics and therapeutic outcomes, offering insights for developing more effective and safer antibody-drug conjugates in clinical applications.***

Keywords: Monoclonal Antibodies, Conjugates, icIEF, Charge Variant Profile.

Abbreviations:
ADCs: Antibody-Drug Conjugates
FDA: Food and Drug Administration
AML: Acute Myeloid Leukemia
pI: Isoelectric Point
icIEF: Imaged Capillary Isoelectric Focusing
NMP: N-methyl-2-pyrrolidone
ID: Inner Diameter
OD: Outer Diameter
CCD: Charge-Coupled Device
mAbs: Monoclonal Antibodies
RSD: Relative Standard Deviation

I. INTRODUCTION

Antibody-drug conjugates (ADCs) represent a significant advancement in targeted therapeutic strategies, particularly in oncology [1], demonstrating substantial efficacy against various malignancies [2]. A pivotal moment in ADC development occurred in 2000 with the U.S. Food and Drug Administration (FDA) approval of ozogamicin [3], establishing ADCs as effective therapeutic agents for acute myeloid leukemia (AML) [4]. Since this milestone, the landscape of ADCs has rapidly expanded [5], with 14 ADCs currently approved for various cancer types and over 100 additional candidates in various stages of clinical trials [6].

ADCs harness the specificity of monoclonal antibodies (mAbs) to selectively bind to antigens expressed on tumor cell surfaces [7]. This targeted approach allows for precise delivery of potent cytotoxic agents designed to induce apoptosis in cancer cells [8]. These cytotoxic drugs are covalently linked to mAbs using specialized linkers [9], categorized as cleavable or non-cleavable [10]. Cleavable linkers release their cytotoxic payload in response to stimuli within the tumor microenvironment [11], such as enzymatic cleavage or pH changes [12]. Non-cleavable linkers require complete mAb degradation for drug release [13], offering advantages such as extended plasma half-life and reduced off-target toxicity [14], thereby enhancing the therapeutic index of ADCs [15].

The conjugation process primarily targets amino acid residues, notably cysteine (Cys) and lysine (Lys) [16], resulting in a heterogeneous mixture of ADC variants characterized by varying drug-to-antibody ratios (DAR) and conjugation sites [17]. This inherent complexity necessitates meticulous ADC characterization to ensure consistency and quality across batches [18]. Charge variant analysis is crucial in this regard, serving as a key marker for evaluating ADC formulation stability and uniformity [19].

Established analytical techniques such as ion exchange chromatography and capillary isoelectric focusing (cIEF) are routinely used to analyze charge variant profiles by separating proteins based on their isoelectric points (pI) [20]. Imaged cIEF (icIEF) is particularly notable for its reliability, sensitivity, and high resolution in determining and quantifying protein pI [21], enabling real-time monitoring of charge variant separation and offering insights into ADC stability and heterogeneity [22].

In ADC formulations, maytansine derivatives and tomaymycin are commonly employed as potent cytotoxic agents [23]. Maytansine derivatives inhibit tubulin polymerization within cancer cells, disrupting mitotic processes [24]. Tomaymycin, derived from Streptomyces achromogenes, exhibits significant antitumor activity through its mechanism of action [25].

This study aims to characterize the charge variant profiles of maytansine derivatives and tomaymycin when conjugated to mAbs using non-cleavable linkers [26]. Advanced icIEF methodology, as developed in previous studies [27], will be employed to enhance

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understanding of the stability and therapeutic potential of these ADC formulations [28].

II. MATERIALS AND METHODS

A. Materials

The ICE280 chemical test kit and ICE280 electrolyte solution kit were purchased from Convergent Bioscience [29]. Methylcellulose was obtained at concentrations of 1% and 0.5% [30]. Isoelectric point (pI) markers at values of 6.61, 7.05, 8.18, and 9.5 were also sourced from Convergent Bioscience [31]. Pharmalyte solutions covering pH ranges of 3–10 and 8–10.5 were acquired from GE Healthcare [32]. Additional reagents, including urea, sucrose, histidine, and phosphoric acid, were procured from Sigma [33].

B. Monoclonal Antibodies and Antibody-Drug Conjugates

This study focused on three monoclonal antibodies: mAB1 and mAB2, targeting the EphA2 receptor, and mAB3 [34], targeting the CD19 antigen [35]. Initially, unconjugated solutions of these antibodies were prepared at a concentration of approximately 10 mg/mL in a phosphate buffer at pH 6.5 [36]. Subsequently, mAB1 was conjugated to maytansinoid compounds, while mAB2 and mAB3 were conjugated to tomaymycin derivatives [37]. The conjugation process employed a non-cleavable linker to ensure stability [38]. The resulting antibody-drug conjugates (ADCs) were suspended in a formulation buffer containing 10 mM histidine, 10% sucrose [39], and N-methyl-2-pyrrolidone (NMP) [40], adjusted to a pH of 6.5 [41], achieving an approximate concentration of 2 mg/mL [42].

C. Sample Preparation

To prepare samples of monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs), [43] solutions were diluted to achieve the desired final concentrations [44]. Dilution was carried out in a solution comprising 0.35% methylcellulose [45], a mixture of 4% pharmalytes (combining equal volumes of pH ranges 3–10 and 8–10.5) [46], and 2 M urea [47]. Isoelectric point (pI) markers with values of 6.61, 7.05, 8.18, and 9.5 were added to aid in subsequent analysis [48].

Following dilution [49], the samples underwent centrifugation at 6000 rpm for 3 minutes to remove any particulate matter [50]. The clarified supernatant was transferred to glass autosampler vials, and a second centrifugation step was performed to eliminate any remaining air bubbles [51]. Subsequently, the prepared samples were loaded into the autosampler carousel for further analysis [52].

D. icIEF Instrumentation

The icIEF (imaged capillary isoelectric focusing) method was conducted using an iCE280 instrument coupled with a PrinCE autosampler [53], both supplied by Convergent Bioscience [54]. The analysis utilized a capillary column measuring 50 mm in length, with an inner diameter (ID) of 100 µm and an outer diameter (OD) of 200 µm [55]. This transparent capillary column was housed in a glass cartridge, and its inner surface was coated with a fluorocarbon material to minimize electroosmotic flow [56].

For the analysis of antibody-drug conjugates (ADCs) and monoclonal antibodies (mAbs) [57], a cathodic solution containing 100 mM NaOH and 0.1% methylcellulose was used, along with an anodic solution comprising 80 mM H_3PO_4 and 0.1% methylcellulose [58]. The protein focusing phase was set for either 7 minutes for unconjugated mAbs or 10 minutes for ADCs, with a voltage of 3000 V applied during this period [59]. Detection of the focused proteins was performed using a charge-coupled device (CCD) camera set to a wavelength of 280 nm, enabling precise measurement of the isoelectric focusing profiles of the samples [60].

III. RESULTS AND DISCUSSION

Charge variants contribute significantly to the heterogeneity observed in the production of therapeutic monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs). This variability stems from modifications occurring throughout cell culture, purification, and formulation processes. Precise characterization of the charge variant profile is crucial for verifying the purity of therapeutic mAbs and ADCs, and for ensuring consistency across production batches. The main aim of this study was to analyze the charge variant profiles of unconjugated mAbs.

A. Charge Variant Profile of Unconjugated mAbs

This study comprehensively characterized the charge variant profiles of three monoclonal antibodies (mAbs): mAB1 and mAB2, targeting the CD19 cell surface antigen, and mAB3, designed to bind specifically to the EphA2 receptor. The imaged capillary isoelectric focusing (icIEF) technique was employed to assess the charge heterogeneity of these mAbs, with results depicted in Figure 1. Notably, mAB1 and mAB2 exhibited greater charge heterogeneity and a more basic charge profile compared to mAB3.

Typically, the charge profiles of mAbs include major and minor species categorized as either more acidic or more basic. Figure 2 illustrates the percentage area occupied by various charge variants for the three mAbs studied. For mAB1, two distinct charge variants were identified with isoelectric point (pI) values of 9.00 (64%) and 8.95 (36%), resulting in a ΔpI of 0.1. Conversely, mAB2 displayed three charge variants (ΔpI: 0.3), predominantly at pI values of 9.00 (60%) and 8.95 (30%). These observations are consistent with prior research on charge variant profiles in therapeutic mAbs. The presence of a more acidic variant at pI 8.95 in both mAB1 and mAB2 compared to the major variant (pI 9.00) may be attributed to deamidation processes affecting one or more asparagine (Asn) residues, causing a shift in pI.

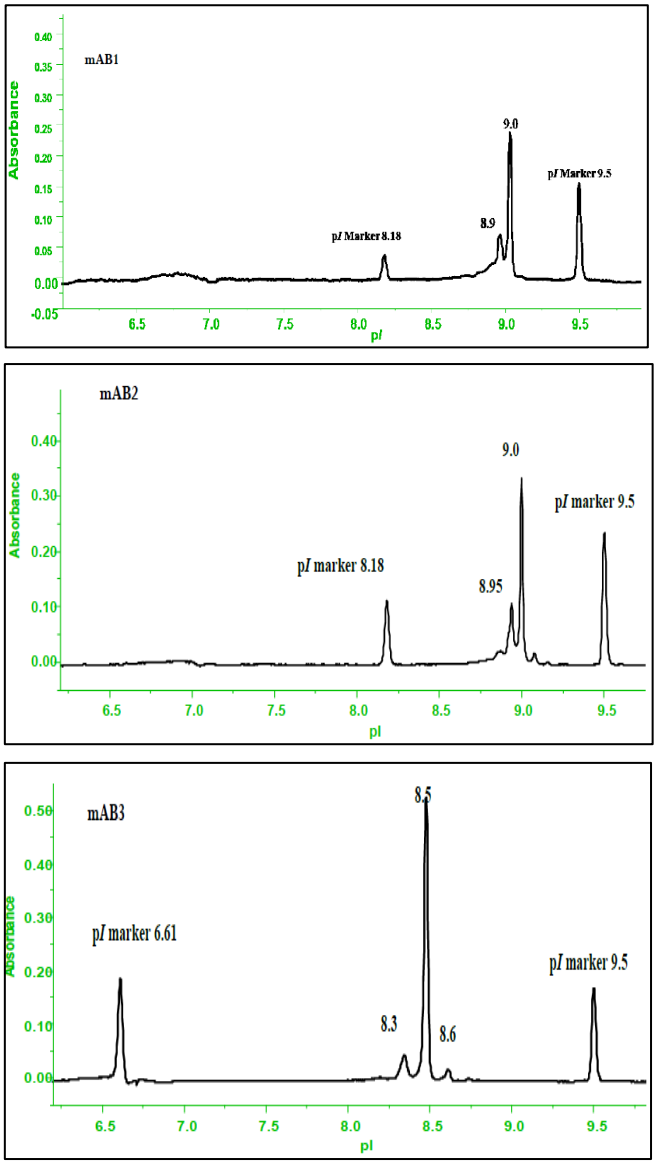
For mAB3, three charge variants were identified (ΔpI: 0.3), with a major variant at pI 8.5 (85%), and minor peaks at pI values of 8.3 (13%) and 8.6 (7%). The presence of a more acidic variant (pI 8.3) relative to the main species (pI 8.5) may also be linked to deamidation processes, while the more basic variant (pI 8.6) could be influenced by the

presence of a C-terminal lysine, consistent with previous studies suggesting its role in forming basic species.

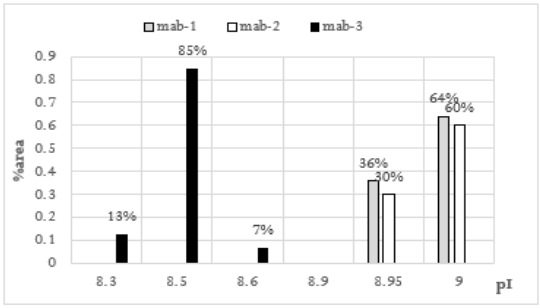
The icIEF profiles of the mAbs demonstrated strong intra- and inter-day repeatability, with relative standard deviation (RSD) values below 0.26% for pI measurements and below 8% for the percentage area occupied by charge variants. Detailed findings are summarized in Table I, underscoring the robustness and reliability of icIEF for characterizing charge variants in therapeutic mAbs.

Table I. Statistical Results of Intra- day and Inter-day Repeatability of icIEF Profile of mABs

Major charge species	Intra- day repeatability (n=6)				Inter- day repeatability (n=6, 3 day)			
	*p*I		Area%		*p*I		Area%	
	Mean	RSD%	Mean	RSD%	Mean	RSD%	Mean	RSD%
mAB1	8.95	0.2	34%	3	8.95	0.25	35%	4
	9.00	0.25	64%	3	9.00	0.20	65%	4
mAB2	8.95	0.15	30%	5	8.95	0.16	31%	6
	9.00	0.17	60%	5	9.00	0.18	60%	6
mAB3	8.5	0.2	85%	6	8.5	0.1	86%	7



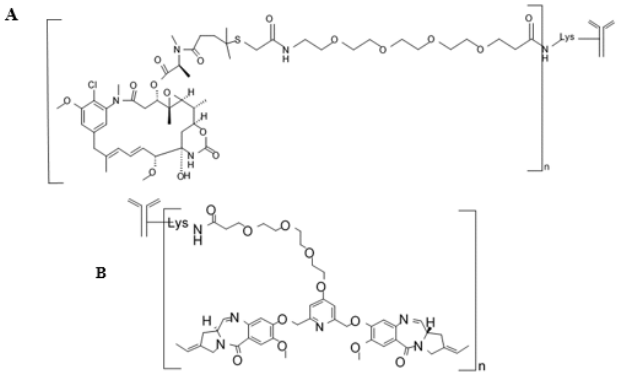
[Fig.1: Illustratation of the Analysis Conducted Using icIEF to Assess the Charge Variant Profile of Unconjugated mABs. The Final Concentration of the Unconjugated mABs in the Sample Matrix was 0.2 mg/mL, and They Were Diluted in a Solution of 0.35% Methyl Cellulose. Other Conditions Were Mentioned in the Materials and Methods]



[Fig.2: Representation of the Area Percentage of Charge Variants Observed in the Studied Unconjugated Antibodies]

B. Charge Variant Profile of Non-Cleavable ADCs

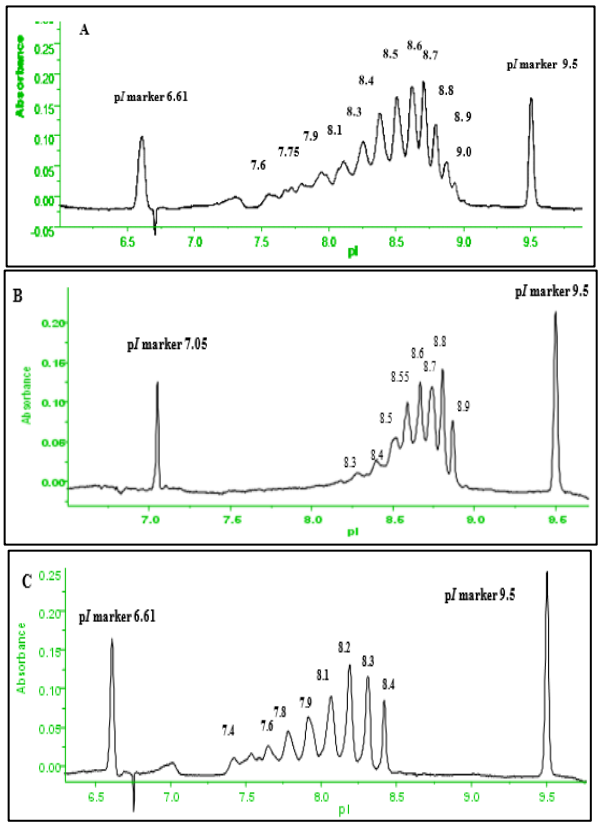
The monoclonal antibodies (mAbs) were characterized and subsequently conjugated utilizing the amino groups located on lysine residues. Specifically, mAB1 was coupled with maytansine derivatives, whereas mAB2 and mAB3 were conjugated with tomaymycin. This conjugation process employed a cleavable linker that was designed to release the cytotoxic agent under specific conditions. Figure 3 provides a schematic depiction of the conjugation strategy.



[Fig.3: Showcases the Structure of Non-Cleavable Maytansinoid ADCs (DM4-NHAc-(PEG)4) in Panel (A) and non-Cleavable Tomaymycin ADCs (Tomaymycin-Pyridine-(PEG)4) in Panel (B)]

Figure 4 illustrates the charge variant profiles of antibodies conjugated to non-cleavable linkers incorporating maytansine derivatives and

tomaymycin. Our analysis indicated that these conjugated antibodies exhibited increased levels of heterogeneity and acidity compared to their unconjugated counterparts. This heightened charge heterogeneity likely stems from structural modifications introduced during conjugation, affecting the overall charge distribution and isoelectric points of the antibodies. Moreover, the presence of non-cleavable linkers may enhance the stability of the conjugates, thereby influencing their physicochemical properties and charge variant profiles.



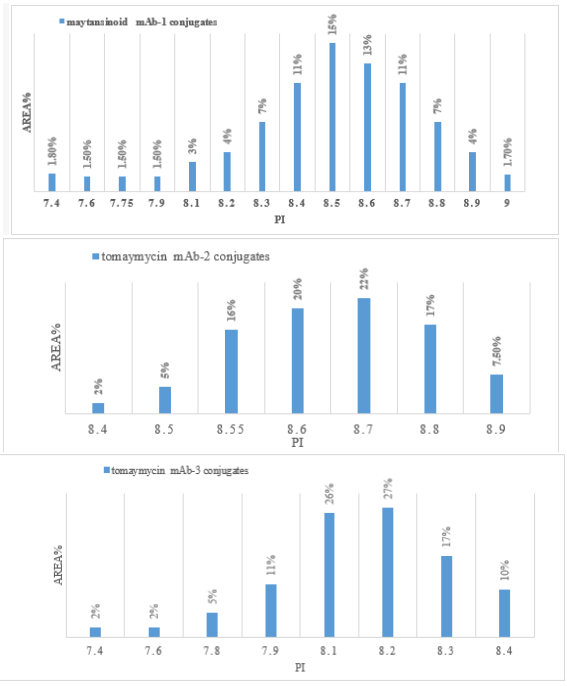
[Fig.4: Illustration of the Analysis Conducted using icIEF for the Evaluation of (a) the Non-Cleavable Maytansinoid mAB1 Conjugate and (b, c) the non-Cleavable Tomaymycin mAB2 Conjugate. The Experiments were Conducted Under Specific Conditions, Including a Final Concentration of 0.5 mg/mL in 0.35% Methyl Cellulose. Other Conditions were Mentioned in Materials and Methods]

Figure 5 provides a comprehensive analysis of the percentage area occupied by charge variants in non-cleavable antibody-drug conjugates (ADCs). The isoelectric point (pI) ranges for the non-cleavable maytansinoid conjugate mAB1 were determined to be between 7.4 and 8.9, resulting in a ΔpI of 1.4. In contrast, the non-cleavable tomaymycin conjugates, mAB2 and mAB3, exhibited pI ranges of 8.2 to 8.9 (ΔpI: 0.7) and 7.4 to 8.4 (ΔpI: 1.0), respectively.

The observed increase in charge heterogeneity among the non-cleavable conjugates, indicated by broader ΔpI values and relatively lower pI values of isoforms, can be attributed to the number of cytotoxic drugs linked to the free amino groups of lysine (Lys) residues within the antibodies. Specifically, the non-cleavable maytansinoid conjugates of mAB1 showed a higher number of distinct charge variants (14) compared to tomaymycin conjugates, which exhibited 8 charge variants for mAB2 and 9 for mAB3. This difference arises from variations in the availability of lysine residues for conjugation in each mAb, as antibodies can contain up to 80 lysine residues.

The charge variants in the ADCs originate from varying numbers of amine groups on lysine residues conjugated to the linker-drug complex. This modification leads to reduced pI values as the quantity of modified amino groups increases, contributing to the observed acidic profile in the ADC species. These findings align with previous studies documenting similar observations in mAb conjugates.

It is notable that the percentages of charge isoforms corresponding to unconjugated antibodies were relatively low, indicating the successful conjugation process for the mAbs. This suggests that the majority of the antibody population underwent effective modification, achieving the desired characteristics of the ADCs.



[Fig.5: % Area of Major Charge Variant of Non-Cleavable Conjugated Antibody]

The isoelectric focusing (icIEF) profile of the antibody-drug conjugates (ADCs) exhibited exceptional reproducibility, both within a single day and across multiple days. This was quantified by the low relative standard deviation (RSD%) values observed. Specifically, the RSD% for the isoelectric point (pI) values was less than 0.26%, indicating precise measurement of the pI of the charge variants present in the ADCs. Furthermore, the RSD% for the area percentage attributed to the various charge variants remained below 7%, demonstrating consistent and reliable quantification of the different charged species within the ADC formulations. Detailed data on the repeatability of these measurements is provided in Table II.

Table II. Statistical Results of Intra- Day and Inter-day Repeatability of icIEF Profile of ADCs

Major charge species	Intra- day Repeatability (n=6)				Inter- day Repeatability (n=6, 3 day)			
	*p*I		Area%		*p*I		Area%	
	Mean	RSD%	Mean	RSD%	Mean	RSD%	Mean	RSD%
Maytansinoid mAB1 conjugates	8.3	0.2	7%	3	8.3	0.25	7%	3
	8.4	0.25	11%%	3	8.4	0.20	10.5%	2
	8.5	0.1	15%	2	8.5	0.25	15.5%	5
	8.6	0.1	13%	5	8.6	0.1	13.1%	4
	8.7	0.2	11%	4	8.7	0.25	11.2%	3
	8.8	0.2	7%	3	8.8	0.1	6,8%	2
Tomaymycin mAB2 conjugates	8.5	0.2	5%	6	8.5	0.27	5%	6
	8.55	0.15	16%	2	8.55	0.17	16.1%	2
	8.6	0.17	20%	2	8.6	0.18	29.9%	2
	8.7	0.25	22%	3	8.7	0.29	21.95%	3
	8.8	0.1	17%	4	8.8	0.15	17.01%	4
Tomaymycin mAB3 conjugates	7.8	0.15	5%	5	7.8	0.15	5.1%	4
	7.9	0.17	11%	5	7.9	0.18	11.2%	3
	8.1	0.25	26%	5	8.1	0.28	25.6%	5
	8.3	0.1	17%	5	8.3	0.15	17%	4
	8.4	0.1	10%	3	8.4	0.16	10%	3

IV. CONCLUSION

In this study, isoelectric focusing (icIEF) was utilized to examine the charge variant profiles of three monoclonal antibodies (mAbs) and their corresponding non-cleavable conjugates. Comparative analysis among the unconjugated mAbs revealed that mAB3 displayed a more acidic and homogeneous charge profile, with its principal charge variant having an isoelectric point (pI) of 8.50. In contrast, mAB1 and mAB2 showed two distinct charge variants, with pIs of 9.00 and 8.95, respectively.

During the conjugation process, mAB1 was conjugated to a maytansine derivative using a non-cleavable linker, while both mAB2 and mAB3 were conjugated to tomaymycin molecules. The resulting non-cleavable conjugated antibodies exhibited increased heterogeneity and acidity compared to their unconjugated forms.

These findings underscore the efficacy of icIEF as a robust analytical tool for assessing the charge profiles of antibody-drug conjugates (ADCs). Furthermore, the pI and area percentage values for the charge variants of both unconjugated mAbs and ADCs demonstrated high repeatability, affirming the reliability of these measurements across multiple days and within a single day.

DECLARATION STATEMENT

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

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- **Authors Contributions:** The authorship of this article is contributed solely.

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