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Monitoring the charge variant profile of antibody-tomaymycin conjugates by icIEF method

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ABSTRACT

This study aimed to evaluate the charge variant profile of antibody-tomaymycin conjugates using an imaged capillary isoelectric focusing method (icIEF). Two unconjugated antibodies were examined, along with two conjugation methods involving cleavable and non-cleavable linkers. The cleavable linker contained one amine group. mAb-1 demonstrated greater homogeneity with a single main peak at a p*I* of 8.5, whereas mAb-2 exhibited two main peaks at p*I* values of 8.95 and 9.00. Conjugation with tomaymycin molecules resulted in an increased charge heterogeneity of both cleavable and non-cleavable conjugates. The non-cleavable conjugates displayed a higher number of additional acidic variants (p*I* range: 7.4 to 8.4 for mAb-1 and 8.2 to 8.9 for mAb-2) compared to the cleavable conjugates (p*I* range: 7.8 to 8.4 for mAb-1 and 8.4 to 8.9 for mAb-2). The linker nature had a lesser influence on the charge heterogeneity of the antibody-tomaymycin conjugates compared to the nature of the unconjugated antibody.

Keywords: antibody, charge isoforms, conjugates, linker

INTRODUCTION

Monoclonal antibodies (mAbs) constitute a very important therapeutic class developed to treat various diseases, including cancer[1]. Conjugation of antibod-

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ies with chemotherapeutic drugs has been extensively studied to improve their antitumor activity[2-4]. To date, the FDA has approved twelve antibody drug conjugates (ADCs) for marketing[5].

ADCs are composed of cytotoxic drugs covalently attached to monoclonal antibodies via a chemical linker. The conjugation process of drug molecules often involves cysteine (Cys) and lysine (Lys) residues on the monoclonal antibodies[6].

The choice of chemical linkers plays a critical role in determining the efficacy of investigated ADCs[5-9]. The linkers must ensure the chemical stability of the ADC in the bloodstream and facilitate the rapid release of cytotoxic agents to target cells. ADC linkers in ADCs can be classified into two groups: cleavable and non-cleavable linkers[5-8]. Over 80% of approved ADCs employ cleavable linkers[9].

Cleavable linkers efficiently release the active agents from ADCs within targeted cells through chemical mechanisms such as pH changes and redox reactions (hydrazone or disulfide bonds) or enzymatic activity (peptide-based or phosphate-based linkers)[7-9]. Non-cleavable linkers (thioether and maleimidocaproyl) are chemically and enzymatically stable[7-9].

The physicochemical properties of ADCs significantly influence their therapeutic effectiveness, emphasizing the importance of evaluating ADC homogeneity. Homogeneous drug conjugation is crucial for producing effective anticancer ADCs[6,10]. Therefore, assessing the charge heterogeneity of ADCs using relevant methods is essential[11-14].

Monitoring the charge variants forms of mAbs or ADCs provides valuable information on protein stability, batch to batch purity, degradation pathways, and more. Various chromatographic and electrophoretic methods have been employed to evaluate the charge variant profile of ADCs[15-20]. Whole-column imaging-detection capillary isoelectric focusing (icIEF) have been utilized for monitoring the charge heterogeneity of mAbs and ADCs due to its advantages such as higher resolution, speed, and quantitative analysis[20-21].

Tomaymycin is an anti-cancer antibiotic produced by Streptomyces achromogenes[22]. In this study, tomaymycin molecules were linked to two monoclonal antibodies through non-cleavable or cleavable linkers. The objective of this work was to study the impact of the nature of the antibody and the linker on the charge distribution profile of the tomaymycin conjugates using icIEF method developed in another study[23].

METHODOLOGY

Chemicals

The ICE280 chemical test kit, ICE280 electrolyte solution Kit, 1% and 0.5% methylcellulose, and p*I* markers (6.61, 7.05, 8.18 and 9.5) were obtained from Convergent Bioscience. The Pharmalyte solutions (3-10 and 8-10.5) were obtained from GE Healthcare. Urea, sucrose, histidine, and phosphoric acid were purchased from Sigma.

mAbs and ADCs

Two monoclonal antibodies, namely mAb-1 (anti-EphA2) and mAb-2 (anti-CD19), were investigated in this study. The naked antibody solutions of both mAbs were prepared in a phosphate buffer at a pH of 6.5 with an approximate concentration of 10mg/mL.

The two mAbs were conjugated to tomaymycin molecules via two types of linkers: cleavable and non-cleavable linker. These ADCs were formulated in a buffer containing 10mM histidine, 10% sucrose, and N-methyl-2-pyrrolidone (NMPs%) at a pH of 6.5 with an concentration of approximately 2mg/mL.

Sample preparation

The protein sample was prepared by diluting to the desired final concentration in a solution containing 0.35% methylcellulose, 4% pharmalytes (3–10) and pharmalytes (8–10.5) in a 1:1 ratio, 2M urea and p*I* markers (6.61, 8.81, 7.05, and 9.5 Following the preparation, the sample was centrifuged at 6000rpm for 3minutes to remove any precipitates. Subsequently, the clarified sample was transferred to a glass autosampler vial and centrifuged again to eliminate any remaining bubbles. Finally, the prepared sample was placed in the autosampler carousel for subsequent analysis.

icIEF instrument

The icIEF analysis was performed using an iCE280 instrument equipped with a PrinCE autosampler, manufactured by Convergent Bioscience. The capillary column utilized in the analysis had dimensions of 50mm length, 100µm inner diameter (ID), and 200µm outer diameter (OD). This transparent capillary column was embedded into a glass cartridge, and its inner surface was coated with a fluorocarbon material to minimize electroosmotic flow. For the analysis of both the ADCs and mAbs, a cathodic solution consisting of 100mM NaOH and 0.1% methylcellulose, as well as an anodic solution containing 80mM H_3PO_4 and 0.1% methylcellulose, were employed. The protein focusing time

was carried out for either 7 or 10minutes at a voltage of 3000V. Detection of the focused proteins was achieved using a CCD camera operating at a wavelength of 280nm.

RESULTS and DISCUSSION

mAbs are protein molecules that often exhibit a significant level of charge heterogeneity. This heterogeneity can arise from modifications that occur during various stages from production to storage[13]. Characterizing the charge variant profile of therapeutic mAbs is crucial for ensuring purity and batch-to-batch consistency[11-14].

In this study, two specific monoclonal antibodies were investigated: mAb-1, an anti-CD19 antibody targeting the CD19 cell surface antigen, and mAb-2, an anti-EphA2 antibody designed to recognize and bind to EphA2.

The charge heterogeneity profiles of these mAbs were analyzed using icIEF, and the results are presented in (Figure 1). It was observed that mAb-1 exhibited greater homogeneity and had a more acidic nature compared to mAb-2.

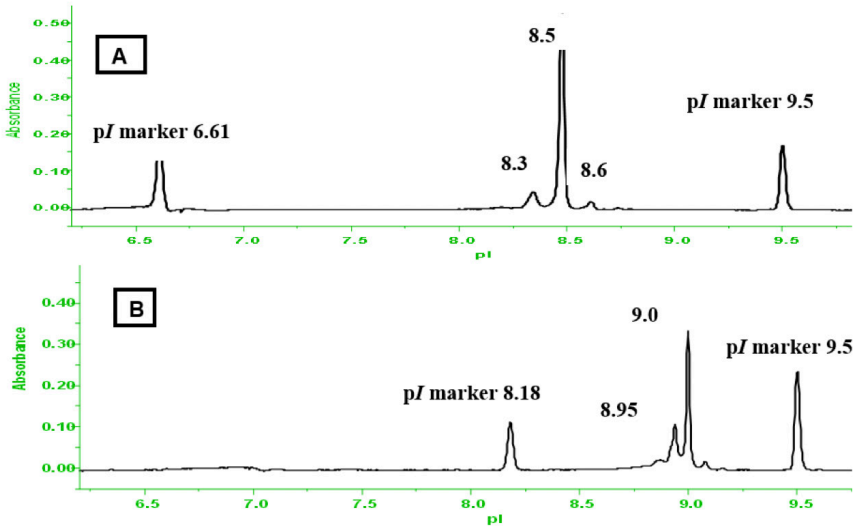

Figure 1. Analysis of unconjugated antibodies by icIEF: (A) mAb-1 and (B) mAb-2. Experimental conditions; final concentration 0.2 mg/mL in 0.35% methyl cellulose, 2% 3–10 pharmalytes and 2% 8–10.5 pharmalytes (1:1 ratio) and 2M urea. pI markers: 6.61, 8.18, 9.50. Focusing time: 7min at 3000 V. Detection I: 280 nm.

Table 1 provides further details regarding the charge variants of the two mAbs. For mAb-1, a single main peak corresponding to a specific charge variant with a p*I* value of 8.5 was observed, accounting for 85% of the total. On the other hand, mAb-2 displayed two distinct charge variants, with p*I* values of 9.00 (60%) and 8.95 (30%). The presence of the more acidic charge variant in mAb-2 (p*I* 8.95) suggests the possibility of deamidation occurring at one or two asparagine (Asn) residues, leading to a shift in the p*I* value compared to the main charge variant (p*I* 9.00) [24]. This observation aligns with previous studies that have reported similar p*I* values for charge variants of anti-EphA2 mAbs, albeit with higher percentages (9.0 at 64% and 8.9 at 36%)[19].

Table 1. Charge variant profile of unconjugated antibodies (mAb-1 and mAb-2), including charge variant number, p*I* range, Δ p*I*, And % Area of major species.

Unconjugated antibody	Charge variant Number	p*I* range	Δ p*I*	p*I* and % Area of major species
mAb-1	3	8.3-8.6	0.3	8.5: 85%
mAb-2	4	8.9-9.1	0.2	8.95: 30%, 9.00: 60%

Overall, both mAbs exhibited an acceptable level of charge homogeneity, and the observed degree of charge heterogeneity is consistent with what is typically observed in therapeutic monoclonal antibodies in previous studies[6, 16, 19, 25-27].

Antibody-tomaymycin conjugate charge profile

Two monoclonal antibodies (mAb-1 and mAb-2) that were characterized using icIEF were conjugated to tomaymycin molecules using either non-cleavable or cleavable linkers. The effects of the linker type on the charge variant profile of the resulting ADCs were evaluated using the icIEF method.

Cleavable conjugates

The tomaymycin molecules were conjugated to the amino groups of the mAb Lys residues using an optimized cleavable linker that contained a hindered disulfide bond (Figure 2). Conjugating the Lys residues of the mAb can reduce the solubility of the mAb conjugates due to the decreased net charge of the resulting conjugate[14]. To improve the solubility of the resulting ADCs, the cleavable linker incorporates an amino group. This amino group is expected to introduce additional positive charges to the antibody-tomaymycin conjugates and potentially alter their charge profile. The charge variant profiles of the cleavable antibody-tomaymycin conjugates are depicted in (Figure 3).

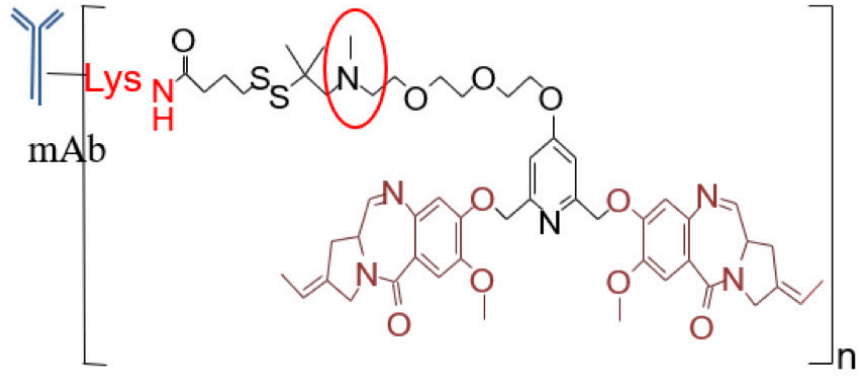

Figure 2. Chemical structure of tomaymycin molecules conjugated to mAbs Lys residues through cleavable linker. The cleavable linker contains a hindered disulfide bond and an amine group.

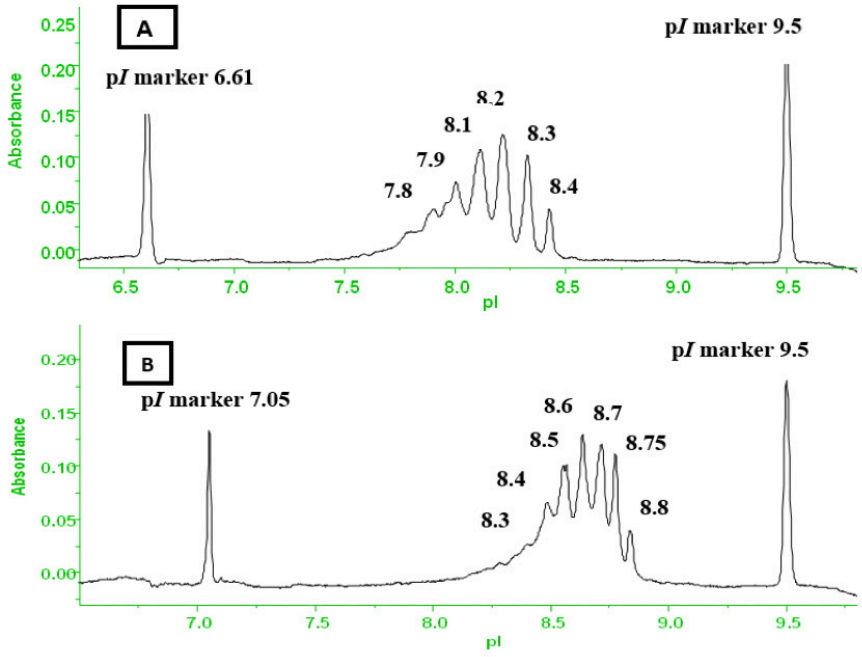

Figure 3. Analysis using icIEF of (a) cleavable mAb-1 tomaymycin conjugate, (b) cleavable mAb-2 tomaymycin conjugate. Experimental conditions; final concentration 0.5 mg/mL in 0.35% methyl cellulose, 3–10 pharmalytes (2%) and 8–10.5 pharmalytes (2%) in 1:1 ratio and 2M urea. pI markers: 6.61, 7. 05, 9.50. Focusing time: 10 min at 3000 V. Detection I: 280 nm.

As anticipated, the cleavable antibody-tomaymycin conjugates exhibited greater heterogeneity compared to the corresponding unconjugated antibodies[11]. While the unconjugated antibodies had a narrow p*I* range (Δp*I*: 0.2 for mAb-1 and Δp*I*: 0.3 for mAb-2) (Table 1), the cleavable antibody-tomaymycin conjugates displayed a wider p*I* range (Δp*I*: approximately 0.5) and the same number of charge variants (7) (Table 2). This charge heterogeneity in the ADCs is due to the covalent binding of the tomaymycin-linker to the free amino groups of the mAb Lys residues. Typically, mAbs contain up to 80 Lys residues[6], resulting in a heterogeneous mixture of unconjugated mAbs and mAbs conjugated with varying numbers of drugs in random combinations at different sites on the antibodies[6,7].

Table 2. Charge variant profiles of cleavable tomaymycin conjugated mAb-1 and mAb-2, including charge variant number, p*I* range, Δ p*I*, and % area of major species

cleavable tomaymycin conjugates	Charge variant Number	p*I* range	Δ p*I*	p*I* and % Area of major species
mAb-1	7	7.8-8.4	0.6	8.1: 21%, 8.2: 22%, 8.3: 18%, 8.4: 6%
mAb-2	7	8.4-8.9	0.5	8.5:21%, 8.6:21%, 8.75: 13%, 8.8: 4.8%

The cleavable antibody-tomaymycin conjugates exhibited increased acidity (p*I* range for tomaymycin-conjugated mAb-1: 7.8 to 8.4 and mAb-2: 8.4 to 8.9) compared to their unconjugated counterparts (p*I* range for mAb-1: 8.3 to 8.6 and mAb-2: 8.9 to 9.1). The decrease in the p*I* range of the cleavable ADCs is attributed to the reduction in positive charges when the tomaymycin-linker is attached to the mAb Lys residues. The number of Lys amino groups conjugated to the tomaymycin-linker affects the p*I* values of the resulting charge variants, with an increase in modified amino groups leading to more acidic variants. Previous studies have demonstrated that chemical conjugation of mAbs with various drugs using Lys residues can alter the electrostatic properties of the mAb surface and decrease the p*I* values[11-13,28].

The findings regarding the charge variant profiles of the investigated cleavable ADCs align with previous study on cleavable maytansinoid antibody conjugates, which exhibited higher heterogeneity and acidity compared to the unconjugated monoclonal antibodies[19]. Similar observations were made by Baylon et al., where the chemical conjugation of IgG1 Fc with Alexa Fluor 350 via Lys residues resulted in a decrease in the p*I* of the conjugated species compared to the unconjugated species[29].

The p*I* values of the charge variants in the cleavable mAb-1-tomaymycin conjugates were lower than those in the cleavable mAb-2-tomaymycin conjugates. This can be attributed to the fact that mAb-1 is inherently more acidic than mAb-2. Consequently, the p*I* values of the charge variants in the mAb-1-tomaymycin conjugates shifted towards lower values compared to the charge variants in the mAb-2-tomaymycin conjugates.

Evaluating the level of unconjugated antibodies in the ADC conjugates is crucial, as residual unconjugated antibody can directly impact the efficacy of the ADC[11,30]. Table 2 demonstrates that the percentage of charge isoforms corresponding to unconjugated antibodies was approximately 0% in the ADC, indicating the successful conjugation process of the mAb with tomaymycin.

Non-cleavable conjugates

The tomaymycin molecules were conjugated to the amino groups of Lys (Lys) residues in the two monoclonal antibodies under investigation using non-cleavable linkers, as depicted in (Figure 4). The charge heterogeneity profiles of the non-cleavable tomaymycin-conjugated antibodies are presented in (Figure 5). Detailed information on the charge profiles of the non-cleavable monoclonal antibody tomaymycin conjugates, including the number of isoforms, p*I* range, Δp*I*, and p*I* and % area of major species, can be found in (Table 3).

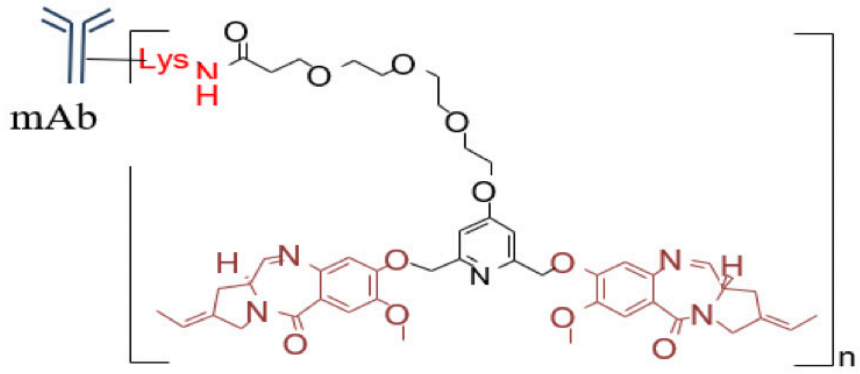

Figure 4. Chemical structure of tomaymycin molecules conjugated to mAbs Lys residues through non-cleavable linker.

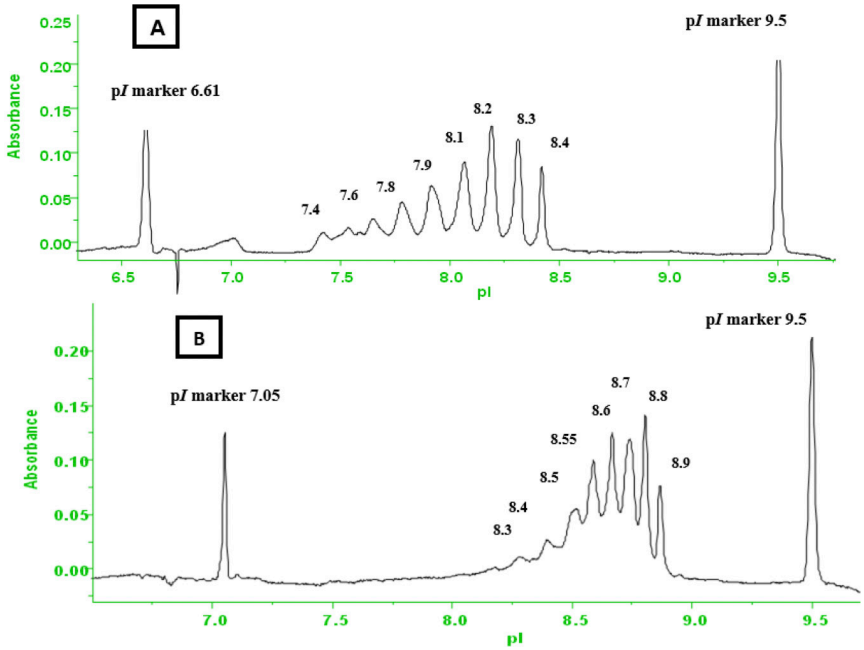

Figure 5. Analysis using icIEF of (a) non-cleavable tomaymycin mAb-1 conjugate, (b) noncleavable tomaymycin mAb-2 conjugate. Experimental conditions; final concentration 0.5 mg/mL in 0.35% methyl cellulose, 2% 3–10 pharmalyte and 2% 8–10.5 pharmalyte in 1:1 ratio and 2M urea. p*I* markers: 6.61, 7.05, 9.50. Focusing time 10 min at 3000 V. Detection I: 280 nm.

Table 3. Charge variant profiles of non-cleavable tomaymycin conjugated mAb-1 and mAb-2, including charge variant number, p*I* range, Δ p*I*, And % Area of major species

Non-cleavable tomaymycin conjugate	Charge variant number	pI range	Δ p*I*	p*I* and % area of major species
mAb-1	9	7.4-8.4	1	8.1: 27%, 8.2: 27%, 8.3: 17%, 8.4: 10%
mAb-2	8	8.2-8.9	0.7	8.6:20%, 8.7: 22%, 8.8:17%, 8.9: 7.5%

Similarly for the cleavable tomaymycin conjugated antibodies, it was observed that the non-cleavable conjugates exhibited greater heterogeneity and acidity compared to their corresponding unconjugated antibodies. The pI ranges for the mAb-1 and mAb-2 tomaymycin conjugates with non-cleavable linkers were 7.4 to 8.4 (Δp*I*: 1) and 8.2 to 8.9 (Δp*I*: 0.7), respectively. The increase in charge heterogeneity of the non-cleavable conjugates (wide Δp*I*) and the decrease of the isoforms p*I* values was were attributed to the number of drugs attached to the free amino groups of Lys residues in the antibodies. The percentage of

charge isoforms corresponding to the naked antibodies was approximately 0% for non-cleavable conjugates.

Comparison of charge profiles of cleavable and non-cleavable conjugates

When comparing the charge profiles of cleavable and non-cleavable conjugates, it was observed that the non-cleavable mAb-1 tomaymycin conjugates exhibited higher heterogeneity (Δp*I*: 1) compared to the cleavable conjugates (Δp*I*: 0.6), while the non-cleavable mAb-2 conjugates showed a slight increase in charge heterogeneity (Δp*I*: 0.6) compared to the cleavable conjugates (Δp*I*: 0.5).

The number of charge variants was higher for the non-cleavable conjugates (9 for mAb-1 and 8 for mAb-2) compared to the cleavable conjugates (7 for both mAb-1 and mAb-2).

The non-cleavable conjugates exhibited additional acidic variants (p*I* range: 7.4 to 8.4 for mAb-1 conjugates and 8.2 to 8.9 for mAb-2 conjugates) compared to the cleavable conjugates (p*I* range: 7.8 to 8.4 for mAb-1 conjugates and 8.4 to 8.9 for mAb-2 conjugates). These additional acidic isoforms suggested an increase in the number of drug-linker attached to the amino groups of Lys residues. Similar findings were reported by Lin et al., where the icIEF charge profile of mAb-DM4 conjugates exhibited a shift in p*I* values towards acidic values due to an increase in the number of conjugated DM4 molecules[31].

As mentioned earlier, the cleavable linker contained a basic group (amino group). Previous studies by Stan et al. indicated that conjugation of drugs to mAb carbohydrates did not affect the protein charge, but when the drug-linker possessed a charge, there was a change in the p*I* values of the resulting conjugates[32]. Therefore, it was expected that the additional amino groups in the cleavable linker would increase the p*I* of the charge variants in the cleavable conjugates. Interestingly, the percentages of the most basic species (p*I* 8.4 for mAb-1 and p*I* 8.9 for mAb-2 conjugates) were higher for the non-cleavable conjugates compared to the cleavable conjugates. These results indicate that the additional amino group in the cleavable linker did not significantly impact the p*I* of the charge variants in the conjugates.

Two monoclonal antibodies, designated as mAb-1 and mAb-2, were conjugated to tomaymycin molecules through either a non-cleavable or cleavable linker. The results obtained from icIEF demonstrated that mAb-1 exhibited greater homogeneity compared to mAb-2. Conjugation process led to a decrease in the p*I* values and an increase in charge heterogeneity of the tomaymycin-antibody

conjugate when compared to the unconjugated antibody. The impact of the unconjugated antibody's characteristics on the p*I* isoform profile of the antibody-drug conjugate (ADC) appears to be more significant than the nature of the linker (cleavable *vs* non-cleavable).

STATEMENT OF ETHICS

Ethical approval was not required to perform this study.

CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Ayat Abbood carried out the analysis and wrote the article.

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Not Applicable

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