Characterization of Charge Variant Profile of Non-Cleavable Conjugated Antibodies

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***Abstract*—The objective of this work was to study the charge variant profile of some monoclonal antibodies (mAbs) conjugated to maytansine derivative (mAb-1) or tomaymycin molecules (mAb-2 and mAb-3) via non-cleavable linkers. The imaging capillary isoelectric focusing method (icIEF) was emolpyed for this purpose. Initially, the charge variant profiles were determined for the three naked mAbs. The analysis revealed that these mAbs exhibited major species as well as minor species. Specifically, mAb-1 and mAb-2 displayed two charge species (pI values: 9.00 and 8.95). In the case of mAb-3, a main charge variant (pI values: 8.50) along with two minor charge species (pI values: 8.30 and 8.60) were observed. The investigated non-cleavable conjugates were less homogen and less basic than corresponding naked antibodies. pI values ranged from 7.4 to 8.9 for the non-cleavable mAb-1 maytansinoid conjugates (ΔpI: 1.4), from 8.2 to 8.9 (ΔpI: 0.7) for the non-cleavable mAb-2 tomaymycin conjugates, and 7.4 to 8.4 (ΔpI: 1) for the mAb-3 conjugates. The icIEF profiles of both naked mAbs and ADCs showed good intra-day and inter-day repeatability.**

***Index Terms*— Antibodies, Conjugates, icIEF, Charge, Variant.**

I. INTRODUCTION

Antibody-drug conjugates (ADCs) are an important targeted therapies for cancer treatment (Dumontet et al., 2023). Ozogamicin was the first approved ADC by the Food and Drug Administration (FDA) in 2000 for acute myeloid leukemia (AML) treatment (Gottardi et al., 2020). Since then, the ADC landscape has expanded rapidly, with 14 ADCs currently approved and over 100 ADCs in clinical trials for cancer treatment (Yang et al., 2023).

ADCs harness the specificity of antibodies to target specific antigens expressed on the surface of cancer cells, while also delivering potent cytotoxic molecules to selectively eradicate cancer cells (Khongorzul et al., 2020). These cytotoxic molecules are covalently linked to mAbs through cleavable and non-cleavable chemical linkers (Baah et al., 2021, Yang et al., 2023, Su et al., 2021, Fu et al., 2022). Cleavable linkers release cytotoxic drugs in response to specific stimuli, such as enzymatic activity or changes in pH, within the tumor microenvironment (Baah et al., 2021,Yang et al., 2023, Su et al., 2021, Fu et al., 2022). On the other hand, release of cytotoxic drugs requires complete enzymatic degradation of non-cleavable conjugates (Baah et al., 2021,Yang et al., 2023, Su et al., 2021, Fu et al., 2022). Compared with cleavable linkers, non-cleavable linkers have several advantages, including a longer plasma half-life and decreased toxicity to normal cells (Baah et al., 2021,Yang et al., 2023, Su et al., 2021, Fu et al., 2022).

The drug molecules are often conjugared to mAbs through cysteine (Cys) and lysine (Lys) residues (Khongorzul et al., 2020). The resulting mixture contains multiple species that differ in the number of conjugated drugs (drug-to-antibody ratio, DAR) and the conjugation sites (Baah et al., 2021,Yang et al., 2023, Su et al., 2021, Fu et al., 2022).

The complexity of conjugated antibodies requires the use of analytical methods that enable to determine their characteristics and thus allow monitoring the occurrence of any change in their properties (Wagh et al., 2018). Charge variant characterization plays a key role in ensuring ADC quality (Zhang et al., 2019).

Charge variant profiles serve as fingerprints to assess batch consistency and stability of ADCs. Ion exchange chromatography (Zhang et al., 2019, Matsuda et al., 2020, Baek et al. 2020, Fekete et al. 2015] and capillary isoelectric focusing (cIEF) (Gahoual et al. 2016, Chen et al., 2016, Wu et al., 2022, Wu et al., 2021) are among the established methods used to monitor ADC charge variant profiles. Both techniques separate proteins based on the differences of isoelectric point (pI).

Imaged cIEF (icIEF) stands out for its high reliability, sensitivity, resolution, and reproducibility in protein pI determination and quantification (Wagh et al., 2018, Wu et al., 2022). Additionally, icIEF enables real-time monitoring of charge variant separation (Wagh et al., 2018, Wu et al., 2022).

Maytansine derivatives and tomaymycin, potent cytotoxic molecules, are frequently employed in ADC development. Maytansine derivatives inhibit tubulin polymerization in cancer cells, disrupting cell division (Cao et al., 2020). Tomaymycin, an anti-cancer antibiotic produced by Streptomyces achromogenes, exhibits potent antitumor activity (Tozuka et al., 1983).

The aim of this work was to study the charge variant profiles of maytansine derivative and tomaymycin conjugated to mAbs through a non-cleavable linker. For this purpose, the icIEF method developed in a previous study was used (Abbood, 2023).

II. MATERIALS AND METHODS

Chemical test and electrolyte solution Kits for ICE280 were sourced from Convergent Bioscience. 1% and 0.5% methylcellulose and pI markers (6.61, 7.05, 8.18 and 9.5) were purchased from Convergent Bioscience. Pharmalyte solutions of pH range (3-10 and 8-10.5) were obtained from GE

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Healthcare. Urea, sucrose, histidine, and phosphoric acid were procured from Sigma.

A. mAbs and ADCs

In this study, three mAbs were analyzed: mAb-1 and mAb-2 (anti-EphA2) and mAb-3 (anti-CD19),. The solutions of these mAbs were prepared in a phosphate buffer with pH 6.5 at a n approximate concentration of 10 mg/mL.

The mAb-1 was conjugated to maytansinoid molecules, while mAb-2 and mAb-3 were conjugated to tomaymycin molecules. The cytotoxic molecules were attached to the three mAbs via a non-cleavable linker. To formulate these ADCs at a concentration of 2mg/mL, a buffer consisting of 10mM histidine, 10% sucrose, and N-methyl-2-pyrrolidone (NMP) at pH of 6.5 was used.

B. Sample Preparation

A solution comcposed 0.35% methylcellulose, 4% pharmalytes (3–10) and pharmalytes (8–10.5) in a 1:1 ratio, 2M urea and pI markers (6.61, 8.81, 7.05, and 9.5 was used to prepare the mAbs and ADCs samples at the desired final concentration. Samples were centrifuged at 6000 rpm for 3 minutes. The clarified samples were then transferred to glass autosampler vials and centrifuged again to eliminate any air bubbles. Finally, the prepared samples were placed in the autosampler carousel for subsequent analysis.

C. icIEF Instrument

icIEF analyses were conducted using an iCE280 instrument, which was equipped with a PrinCE autosampler from Convergent Bioscience. The capillary column used in the analysis was 50mm long, with an inner diameter (ID) of 100µm and an outer diameter (OD) of 200µm. During the analysis of both ADCs and mAbs, a cathodic solution containing 100mM NaOH and 0.1% methylcellulose, as well as an anodic solution consisting of 80mM H3PO4 and 0.1% methylcellulose, were employed. The focusing time was either 7 or 10minutes at a voltage of 3000V. Detection of the focused proteins was accomplished by a CCD camera operating at a wavelength of 280nm.

III. RESULTS AND DISCUSSION

Charge variants contribute to the heterogeneity often observed in the production of therapeutic mAbs and ADCs (Baah et al., 2021,Yang et al., 2023, Su et al., 2021, Fu et al., 2022). The charge heterogeneity can result from some chemical changes that take place during cell culture, purification, and formulation. Characterizing the charge variant profile of therapeutic mAbs and ADCs is important to ensure their quality and thus their efficacy. This study initially focused on characterizing the charge variant profile of naked mAbs. Then, the charge variant profiles of ADCs were determined.

A. Unconjugated mAbs

The charge variant profile of three mAbs were characterized: mAb-1 and mAb-2, antibodies targeting the CD19 cell surface antigen, and mAb-3, antibody specifically designed to recognize and bind to EphA2. icIEF method was used to study the charge heterogeneity profiles of these mAbs and the finding are presented in Figure 1. Notably, mAb-1 and mAb-2 exhibited greater charge heterogeneity and displayed a more basic nature compared to mAb-3.

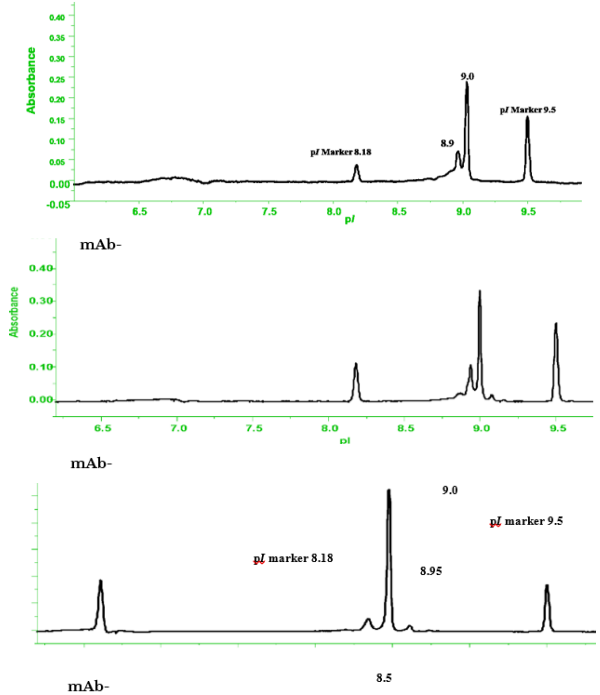

Figure 1. Charge variant profile of naked mAbs, icIEF conditions: concentration of naked mAbs: 0.2mg/ml diluted in 0.35% methyl cellulose, 4% 3–10 pharmalytes/ 8– 10.5 pharmalytes (1:1 ratio), 2M urea. pI markers: 6.61, 8.18, 9.50. Focusing time: 10min at 3000V.

A charge variant profile of mAb typically consists of major species accompanied by minor species that are either more acidic or more basic than the major species (Goyon et al., 2017, Wagner-Rousset et al., 2017). Figure 2 further illustrates the area percent of charge variants for the three mAbs.

mAb-1 exhibited two charge species (pI values and area percent: 9.00 (64%) and 8.95 (36%). The ΔpI was equal to 0.1. mAb-2 had three charge variants (ΔpI: 0.3). The pI values of the two major peaks were 9.00 (60%) and 8.95 (30%). These results align with previous findings on the charge variant profile characterization of therapeutic mAbs (Cai et al. 2011, Spanov et al. 2022, Beck et al. 2022, and Abbood 2023). The presence of the more acidic charge specie in both mAb-1 and mAb-2 (pI 8.95) compared to the main charge specie (pI 9.00) may be attributed to the deamidation of one or two asparagine (Asn) residues, resulting in a shift in the pI value (Spanov et al., 2017, Beck et al., 2022).

Regarding mAb-3, three charge species were observed (ΔpI: 0.3), of which the main peak had a pI value of 8.5 (85%) and two secondary peaks had pI values of 8.3 (13%) and 8.6 (7%). The specie with a high pI value (more acidic: pI 8.3) may be due to deamidation of the main specie (pI 8.5), while those with a lower pI value (more basic: pI 8.6) may be due to the presence of a C-terminal Lys, as suggested by several studies indicating that the presence of a C-terminal lysine is a key factor in the origin of basic species (Beck et al., 2022, Cai et al., 2011).

Intra- and inter-day repeatability studies of icIEF mAbs profile showed good repeatability of pI values (RSD% values less than 0.26%) and area percent (RSD% values less than 8%) of the charge species of the studied mAbs (Table 1).

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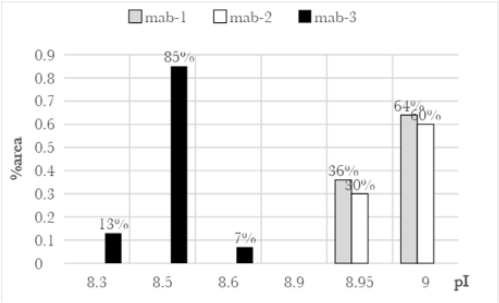

Figure 2. Area% of charge variant of studied unconjugated antibodies.

TABLE 1
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)			
	pI		Area%		pI		Area%	
	Mean	RSD%	Mean	RSD%	Mean	RSD%	Mean	RSD%
mAB-1	8.95	0.2	34%	3	8.95	0.25	35%	4
	9.00	0.25	64%	3	9.00	0.20	65%	4
mAB-2	8.95	0.15	30%	5	8.95	0.16	31%	6
	9.00	0.17	60%	5	9.00	0.18	60%	6
mAb-3	8.5	0.2	85%	6	8.5	0.1	86%	7

B. *Non-cleavable ADCs*

The characterized mAbs using icIEF were conjugated through the amino groups of Lys residues to maytansine derivatives (mAb-1) and tomaymycin (mAb-2 and 3) via a cleavable linker (Figure 3).

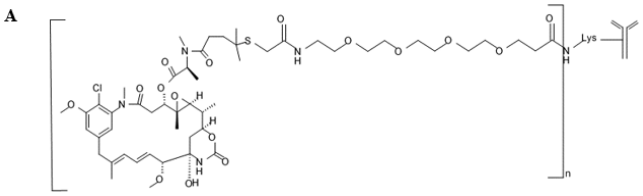

Figure 3. Structure of (A) non-cleavable maytansinoid ADCs (DM4-NHAc-(PEG)4), (B) non-cleavable tomaymycin ADCs (tomaymycin-pyridine-(PEG)4).

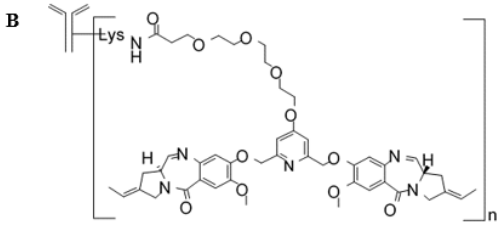

Figure 4 depicts the charge variant profiles of the non-cleavable antibodies conjugated to maytansine and tomaymycin. The results showed that analysed non-cleavable conjugated antibodies were less homogeneous and less basic than their corresponding naked mAbs.

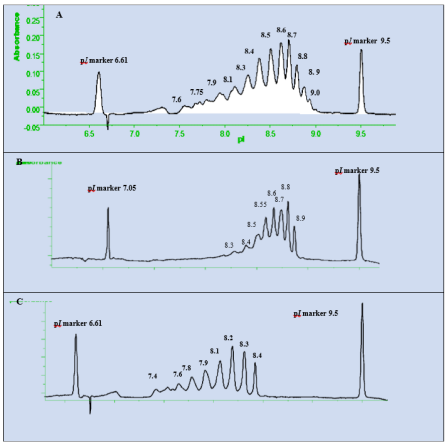

Fig. 3. Analysis of (a) non-cleavable maytansinoid mAb-1 conjugate, (b, c) non-cleavable tomaymycin mAb-2 conjugate. Experimental icIEF conditions; final concentration 0.5 mg/mL in 0.35% methyl cellulose, 2% 3–10 pharmalyte and 2% 8–10.5 pharmalyte in 1:1 ratio and 2M urea. pI markers: 6.61, 7.05, 9.50. Focusing time 10 min at 3000 V. Detection λ: 280 nm. Figure 5 provides a detailed datas regarding the area percent of charge variants for the non-cleavable ADCs. The ΔpI was equalto 1.4 for the non-cleavable maytansinoid mAb-1 pI ranged from 7.4 to 8.9. For the non-cleavable tomaymycin mAb-2 and mAb-3 conjugates, the pI ranges were 8.2 to 8.9 (ΔpI: 0.7) and 7.4 to 8.4 (ΔpI: 1), respectively.

The observed decrease in charge homogeneity of the non-cleavable conjugates, reflected by the broader ΔpI values, and the decrease in the specie pI values may be a result of varying number of drugs attached to the free amino groups of lysine (Lys) residues in the surface of antibodies. The non-cleavable mAb-1 conjugates exhibited a higher number of charge species (14) than the non-cleavable conjugates (8 for mAb-2 and 9 for mAb-3). It is worth noting that an antibody typically contains up to 80 lysine residues (Dennler et al. 2015). The observed charge variants in ADCs arose from the varying number of amine groups of lysine residues conjugated to the linker-drug. As the number of conjugated druge increses, the pI values of charge species decrease (more acidic). Similar findings have been reported for mAb conjugates (Lin et al. 2013). It is interesting to note that the percentage of charge species corresponding to the naked antibodies was low. These results indicated the success of conjugation process of mAbs.

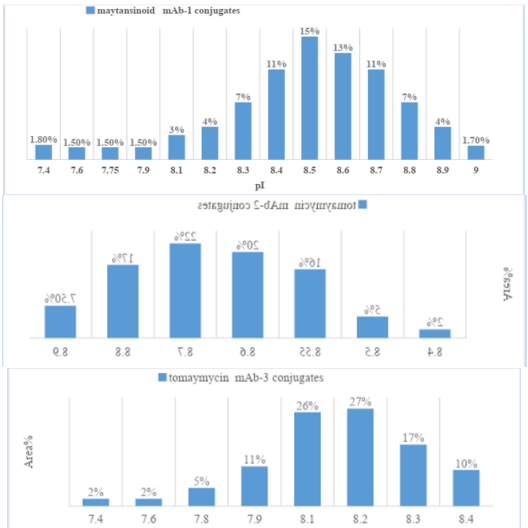

Fig. 4. % Area of major charge variant of non-cleavable conjugated antibody.

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RSD% values were less than 0.26% for pI values and 7% for area perecent of ADCs, indicating a good intra-day and inter-day repeatability (Table 2).

TABLE 2
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)			
	pI		Area%		pI		Area%	
	Mean	RSD%	Mean	RSD%	Mean	RSD%	Mean	RSD%
Maytansinoid mAb-1 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 mAb-2 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 mAb-3 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

CONCLUSION

The charge variant profiles of three mAbs and their non-cleavable conjugates were determined by icIEF. A comparison of the charge variant profiles of the naked mAbs revealed that mAb-3 possessed a more acidic and homogeneous profile compared to mAb-1 and mAb-2. Via non-cleavable linkers, maytansine derivatives were conjugated to mAb-1, while tomaymycin molecules were conjugated to mAb-2 and mAb-3. The resulting non-cleavable conjugated antibodies exhibited increased heterogeneity and acidity compared to their corresponding naked mAbs. The pI and area percent values for both naked mAbs and ADC charge variants exhibited good intra- and inter-day repeatability.

REFERENCES

1. Abbood A. Optimization of the Imaged cIEF Method for Monitoring the Charge Heterogeneity of Antibody-Maytansine Conjugate. J Anal Methods Chem, 2023;8150143. https://doi.org/10.1155/2023/8150143.
2. Baah S, Laws M, Rahman KM. Antibody-Drug Conjugates-A Tutorial Review. Molecules. 2021May;26(10):2943. doi: 10.3390/molecules26102943. PMID: 34063364; PMCID: PMC8156828.
3. Baek J, Schwahn AB, Lin S, Pohl CA, Pra MD, Tremintin SM, et al. New Insights into the Chromatography Mechanisms of Ion-Exchange Charge Variant Analysis: Dispelling Myths and Providing Guidance for Robust Method Optimization. Anal Chem. 2020Sep;92(19):13411–13419. https://doi.org/10.1021/acs.analchem.0c02775.
4. Beck A, Nowak C, Meshulam D, Reynolds K, Chen D, Pacardo DB, et al. Risk-Based Control Strategies of Recombinant Monoclonal Antibody Charge Variants. Antibodies (Basel). 2022 Nov 20;11(4):73. doi: 10.3390/antib11040073. PMID: 36412839; PMCID: PMC9703962.
5. Cai B, Pan H, Flynn GC. C-terminal lysine processing of human immunoglobulin G2 heavy chain in vivo. Biotechnol Bioeng. 2011 Feb;108(2):404-12. doi: 10.1002/bit.22933. PMID: 20830675.
6. Cao S, Dong YH, Wang DF, Liu ZP. Tubulin Maytansine Site Binding Ligands and their Applications as MTAs and ADCs for Cancer Therapy. Curr Med Chem. 2020;27(27):4567-4576. doi: 10.2174/0929867327666200316144610. PMID: 32175831.
7. Chen T, Chen Y, Stella C, Medley CD, Gruenhagen JA, Zhang K. Imaged capillary isoelectric focusing (icIEF) tandem high resolution mass spectrometry for charged heterogeneity of protein drugs in biopharmaceutical discovery. J Pharm Biomed Anal. 2016Oct;1032:39-50. https://doi.org/10.1016/j.jchromb.2016.07.023.
8. Dennler P, Fischer E, Schibli R. Antibody Conjugates: From Heterogeneous Populations to Defined Reagents. Antibodies. 2015; 4(3):197-224. https://doi.org/10.3390/antib4030197.
9. Dumontet C, Reichert JM, Senter PD, Lambert JM, Beck A. Antibody–drug conjugates come of age in oncology. Nat Rev Drug Discov. 2023 June;22:641–661. https://doi.org/10.1038/s41573-023-00709-2.
10. Fekete P, Beck A, Veuthey JL, Guillarme D. Ion-exchange chromatography for the characterization of biopharmaceuticals. J Pharm Biomed Anal. 2015Sep;113:43-55. https://doi.org/10.1016/j.jpba.2015.02.037.
11. Fu Z, Li S, Han S, Shi C, Zhang Y. Antibody drug conjugate: the "biological missile" for targeted cancer therapy. Signal Transduct Target Ther. 2022Mar:22;7(1):93. doi: 10.1038/s41392-022-00947-7. PMID: 35318309; PMCID: PMC8941077.
12. Gahoual R, Beck A, Leize-Wagner E, François YN. Cutting-edge capillary electrophoresis characterization of monoclonal antibodies and related products. J. Chromatogr. B Biomed. Appl. 2016Oct;1032:61-78. https://doi.org/10.1016/j.jchromb.2016.05.028.
13. Gottardi M, Sperotto A, Ghelli Luserna Di Rorà A, Padella A, Cangini D, Giannini MB, et al. Gemtuzumab ozogamicin in acute myeloid leukemia: past, present and future. Minerva Med. 2020 Oct;111(5):395-410. doi: 10.23736/S0026-4806.20.07019-6. Epub 2020 Sep 21. PMID: 32955828.
14. Goyon A, Excoffier M, Janin-Bussat MC, Bobaly B, Fekete S, Guillarme D, et al.. Determination of isoelectric points and relative charge variants of 23 therapeutic monoclonal antibodies. J Chromatogr B. 2017;1065:119–128. doi: 10.1016/j.jchromb.2017.09.033.
15. Khongorzul P, Ling CJ, Khan FU, Ihsan AU, Zhang J. Antibody-Drug Conjugates: A Comprehensive Review. Mol Cancer Res. 2020 Jan;18(1):3-19. doi: 10.1158/1541-7786.MCR-19-0582. Epub 2019 Oct 28. PMID: 31659006.
16. Lin J, Lazar AC. Determination of charge heterogeneity and level of unconjugated antibody by imaged cIEF. Methods Mol. Biol. (Clifton, N.J.), 2013; 1045: 295–302. https://doi.org/10.1007/978-1-62703-541-5_19.
17. Matsuda Y, Kliman M, Mendelsohn BA. Application of Native Ion Exchange Mass Spectrometry to Intact and Subunit Analysis of Site-Specific Antibody-Drug Conjugates Produced by AJICAP First Generation Technology. J Am Soc Mass Spectrom. 2020 Jul 1. doi: 10.1021/jasms.0c00129. Epub ahead of print. PMID: 32608232.
18. Spanov B, Govorukhina N, van de Merbel NC, Bischoff R. Analytical tools for the characterization of deamidation in monoclonal antibodies. Journal of Chromatography Open. 2022Nov;2:100025. https://doi.org/10.1016/j.jcoa.2021.100025.
19. Su Z, Xiao D, Xie F, Liu L, Wang Y, Fan S, Zhou X, et al. Antibody–drug conjugates: Recent advances in linker chemistry. Acta Pharm Sin B. 2021Dec;11(12):3889-3907https://doi.org/10.1016/j.apsb.2021.03.042.
20. Tozuka Z, Yazawa H, Murata M, Takaya T. Studies on tomaymycin. III. Synthesis and antitumor activity of tomaymycin analogs. J Antibiot (Tokyo). 1983 Dec;36(12):1699-708. doi: 10.7164/antibiotics.36.1699. PMID: 6662811.
21. Wagh A, Song H, Zeng M, Tao L, Das TK. Challenges and new frontiers in analytical characterization of antibody-drug conjugates. MAbs. 2018Feb/Mar;10(2):222-243. doi: 10.1080/19420862.2017.1412025. Epub 2018 Jan 5. PMID: 29293399; PMCID: PMC5825200.
22. Wagner-Rousset E, Fekete S, Morel-Chevillet L, Colas O, Corvaïa N, Cianférani S, et al. Development of a fast workflow to screen the charge variants of therapeutic antibodies. J. Chromatogr. A. 2017May:1498:147–154. doi: 10.1016/j.chroma.2017.02.065.
23. Wu J, McElroy W, Haitjema C, Lück C, Heger C. Platform Methods to Characterize the Charge Heterogeneity of Three Common Protein Therapeutics by Imaged Capillary Isoelectric Focusing. Methods Mol Biol. 2021;2261:93-103. doi: 10.1007/978-1-0716-1186-9_8. PMID: 33420987.
24. Wu J, McElroy W, Pawliszyn J, Heger CD. Imaged capillary isoelectric focusing: Applications in the pharmaceutical industry and recent innovations of the technology. TrAC Trends Analyt Chem. 2022May;150:116567. https://doi.org/10.1016/j.trac.2022.116567.
25. Yang Y, Wang S, Ma P, Jiang Y, Cheng K, Yu Y, et al. Drug conjugate-based anticancer therapy - Current status and perspectives. Cancer Letters. 2023Jan:215969. https://doi.org/10.1016/j.canlet.2022.215969.
26. Zhang Z, Zhou S, Han L, Zhang Q, Pritts WA. Impact of linker-drug on ion exchange chromatography separation of antibody-drug conjugates. MAbs. 2019 Aug/Sep;11(6):1113-1121. doi: 10.1080/19420862.2019.1628589. Epub 2019 Jun 25. PMID: 31238787; PMCID: PMC6748606.

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