Accelerating ADC Development with Mass Spectrometry

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Antibody-drug conjugates (ADCs), while affording exciting new opportunities for targeted therapy, are complex molecules that present significant analytical challenges. The drug-to-antibody ratio (DAR) and drug distribution profile (DDP) of an ADC are critical quality attributes that can be difficult to confirm using liquid chromatography alone. Fortunately, newer, advanced, high-throughput mass spectrometry methods are enabling rapid, sensitive, precise and accurate determination of the DAR, DDP and other important product quality attributes for various types of ADCs.

Introduction

The concept of delivering cytotoxic compounds specifically to cancer cells via their linkage to a "haptophore" was initially raised in 1913 by Paul Ehrlich [1]. The first antibody-drug conjugate (ADC) was approved by the US Food and Drug Administration (FDA) in 2013. By early 2020, eight FDA-approved ADCs were on the market [2]. These targeted therapies are attractive because, through binding of the antibody to specific antigens (usually on the surface of cancer cells, but in some cases on other cells or free-floating proteins), they enable the controlled delivery of small-molecule cytotoxic agents to a desired site of action, reducing the side effects associated with systemic delivery, increasing efficacy and potentially reducing the required dosage.

ADCs comprise a large biomolecule (antibody), some form of linker and a chemical therapeutic agent. The linker is designed to maintain conjugation while the ADC circulates through the body and only release the cytotoxic payload at the targeted site of action. Various conjugation methods, linkers and payloads have been leveraged, with second- and third-generation ADCs possessing improved in vivo performance. Conjugation is typically achieved using native or engineered surface lysine or cysteine residues.

The formation of thiol bonds with cysteine residues offers less heterogeneity than the formation of linkages with lysine residues. The site-specific approach allows precise control over the average drug to antibody ratio (DAR) and number of conjugation sites. Enzyme-mediated approaches are also used. As a result, the level of heterogeneity of ADCs depends strongly on the conjugation technique employed. In the worst case, upward of 109 unique conjugated species are obtainable for a maximum drug payload of 8 on the 60 potential conjugation sites of an antibody scaffold.

In all cases, the resulting ADC drug products are highly complex, heterogeneous molecules that have the potential to contain many product-related species that can impact the quality, efficacy and safety of these important therapies. Given this high level of complexity, full characterization of ADCs is essential not only for quality assessment, but for process optimization.

Determination of the DAR and DDP and identification of the conjugation sites are essential. In addition, correct estimation of the conjugation efficiency can be used to optimize the conjugation chemistry and thus increase the homogeneity and improve the product quality of ADCs.

Widely used methods for DAR and DDP determination include hydrophobic interaction chromatography (HIC) and reduction followed by reverse-phase high-performance LC (RP-HPLC) with UV detection. The former can reveal the degree of conjugation and often sufficiently resolve and characterize each DAR species, while the latter involves evaluation of the heavy and light chains based on their different retention times with and without conjugated payloads. Both methods are, however, time consuming.

Rapid yet sensitive and specific analytical techniques are therefore needed to accelerate the development of ADCs. Liquid chromatography in combination with high resolution mass spectrometry allows the calculation of the DAR of ADCs during product development using intact and/or subunit analysis. Bottom-up approaches that include enzymatic digestion of the protein into peptides before MS analysis are useful for determination of exact conjugation sites, but are less effective for overall DAR determination.

Liquid chromatography-mass spectrometry (LC-MS) methods leveraging subunit level and intact approaches combined with advanced instrument platforms and software systems can serve as robust methods for the rapid analysis of ADCs, providing the desired combination of speed, sample capacity and precision while affording valuable, reproducible, information-rich data. These methods are becoming of more interest as less hydrophobic cytotoxic payloads with good potency and greater monomeric stability when conjugated become more widely employed.

While LC-MS may not be universally appropriate for all ADCs, it does represent a fundamental, workhorse analytical platform for ADC characterization. The specific choice of analytical method must take into consideration the conjugation method and properties of the cytotoxic payload. Herein are presented several examples of the application of MS-based methods for accelerating ADC development:

- Size exclusion chromatography with serial ultraviolet detection and mass spectrometric detection (SEC-UV-MS) multi-attribute methods (MAMs) using intact and middle-up analytical approaches for high-throughput workflows
- LC-MS characterization of ADCs with cysteine and lysine side-chain conjugation
- LC-MS monitoring of maleimide ring-opening in cysteine-linked ADCs for conjugation process optimization
- Characterization of site-specific ADCs by subunit and peptide mapping analysis
- Native LC-MS analysis of intact cysteine-linked ADCs
- LC-MS analysis of nanobody-based ADCs

Experimental

For all of the studies, an Exion LC system was used in conjunction with either the SCIEX X500B QTOF system equipped with a Turbo V ion source or the SCIEX TripleTOF 6600 system equipped with an IonDrive Turbo V ion source.

- For LC-MS characterization of ADCs with cysteine and lysine side-chain conjugation and monitoring of maleimide ring-opening, a Phenomenex bioZen Intact XB-C8, 3.6 microm, 100 mm column was employed, also in conjunction with the SCIEX X500B QTOF system [3].
- For characterization of site-specific ADCs, a bioZEN 3.6 µm Intact C4 column was used for subunit analysis and a an Aeris 1.7 µm PEPTIDE XB-C18 column was used for peptide mapping. In both cases MS was performed using the SCIEX TripleTOF® 6600 system [4].
- For native LC-MS analysis of intact cysteine-linked ADCs, SEC was followed by MS on the SCIEX TripleTOF 6600 system [5].
- For LC-MS analysis of nanobody-based ADCs, a Phenomenex bioZen Intact XB-C8, 3.6 microm, 100 mm column was used for intact protein analysis and a bioZen Peptide XB-C18, 2.6 microm, 150 mm column was employed for peptide separation. Both were coupled to SCIEX X500B QTOF system [6].

The software used for data processing, interpretation and quantification included SCIEX OS software, BPV Flex software and BioPharmaView software.

More details on the exact experimental conditions can be found in the references provided for each study.

Results and Discussion

LC-MS Analysis of Cysteine- and Lysine Linked ADCs [3]

Characterization of thiol-conjugated ADC species was performed using a middle-up LCMS

workflow. Samples were deglycosylated and the subjected to Immunoglobulin G-degrading enzyme of Streptococcus pyogenes (IdeS) digestion followed by reduction with triscarboxyethylphosphine (TCEP) to cleave any remaining disulfide bridges, affording Fc/2, Fd, and light chain subunits with similar molecular weights. Rapid desalting chromatography step was performed prior to ionization, for total sample run times of just six minutes.

ADCs comprising trastuzumab conjugated to the representative payloads alpha-amanitin and the auristatins mc-MMAF and vc-MMAE were analyzed to demonstrate the validity of this approach. Light chain subunits were observed to have only one toxin linked, whereas the Fd subunits had one, two, or three total drug conjugations. Unlike DAR analysis with HIC-UV, this LC-MS approach yielded highly analyte-specific mass information that was agnostic to any altered UV characteristics due to conjugation.

Using this method for more hydrophobic toxins can be challenging, however, because these payloads can influence the ionization properties of the ADC. Analysis of trastuzumab conjugated to duocarmycin was possible by altering the ionization conditions. This approach was not successful when trastuzumab was conjugated to the pyrrolobenzodiazepine (PBD) SG3249 due to its propensity to form adducts. Therefore, if time is of the essence, HIC may be more suitable for this type of ADC; investing time in the development of LC-MS methods would be highly beneficial, however, given the more detailed information that can be obtained.

Conjugation through lysine residues generally yields more heterogenous DAR distributions with noticeably greater mass spectral complexity due to heterogeneous glycoform compositions. Adding a deglycosylation step to the sample preparation procedure can reduce the complexity of resulting spectra. This LC-MS approach was demonstrated for a lysine conjugate of cetuximab, which was determined to have between 4 and 13 payloads successfully attached with an average DAR of 9.

LC-MS monitoring of maleimide ring-opening in cysteine-linked ADCs [3]

LC-MS for DAR analysis has application beyond quality assessment of ADC products. It can also be useful for process and product development.

For instance, in cysteine-linked ADCs, the linkage is often formed via nucleophilic addition to form a maleimide-cysteine linkage. In some cases, this linkage reaction may be reversible or another nucleophile may be present that will cause a retro-Michael addition to take place. This undesirable reactivity can lead to poor in vivo stability and potentially reduced safety and efficacy.

The addition of certain functional groups next to the maleimide moiety can increase maleimide-cysteine linker stability by preventing these unwanted reactions. This approach requires monitoring with high analytical specificity, and LC-MS can be very useful for tracking the ring-opening process that occurs during maleimide-cysteine linkage formation.

To demonstrate this technique, ADCs comprising trastuzumab conjugated to three different maleimide-based MMAE linkers were analyzed via both HIC-UV and LC-MS. With the former method it was not possible to confirm specific ADC formation by mass. With the latter, the combination of mass analysis and chromatographic retention time allowed for differentiation of the three ADCs. LC-MS was also found to be suitable for monitoring the extent of ring opening over time under different reaction conditions (pH, temperature, etc.). Integration of LC-MS into design of experiment strategies can thus enable optimization of the conjugation process.

Characterization of site-specific ADCs by subunit and peptide mapping analysis [4]

Advances in MS instrumentation and software are increasing the applicability of MS for ADC analysis.

To demonstrate the effectiveness of the SCIEX TripleTOF 6600+ system with its IonDrive Turbo V ion source and BPV Flex software 2.1, which includes intact and peptide mapping workflows and automatic DAR determination, sequence confirmation and relative quantification of the conjugated peptides, three samples of the ADC comprising trastuzumab conjugated to Gly-Gly-Gly-Val-Cit p-aminobenzyl oxycarbonyl (PAB) monomethyl auristatin E (MMAE) (Gly-Gly-Gly-Val-Cit- PAB-MMAE) prepared under different conjugation conditions were evaluated. The payload was conjugated using transglutaminase (TG) to a highly specific conjugation site on the light chain (LC) of trastuzumab.

For subunit analysis, the ADCs were incubated with TCEP. The raw data spectra for the LC and heavy chain (HC) were reconstructed to a "zero charge state", allowing assignment of the major glycosylation forms of the HC and the drug-free and drug-conjugated forms of the LC. Samples for peptide mapping were subjected to reduction, alkylation and digestion. The results for the DAR calculation for both methods were consistent, indicating that subunit analysis provides a robust alternative to peptide mapping for a faster, simpler and highly

reproducible ADC characterization

Furthermore, in all of the ADC samples, the drug-free peptides were predominantly found in the deamidated form, the result of transglutaminase catalysis of the deamidation of glutamine residues in the presence of water at low pH. For the ADC produced after incubation of trastuzumab with TG in the absence of the drug, deamination was pronounced. Not surprisingly, pre-incubation with TG and the drug led to less deamidation, while no pre-incubation resulted in limited deamidation occurring. The conjugation efficiency inversely correlated to the level of deamidation.

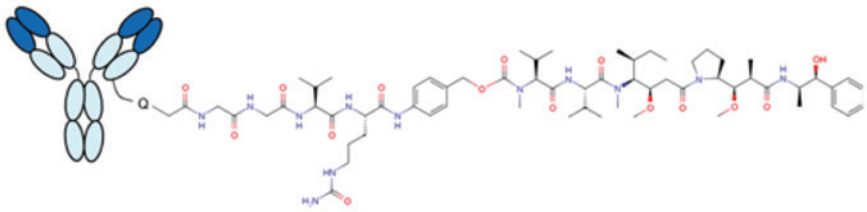
Native LC-MS analysis of intact cysteine-linked ADCs [5]

While subunit analysis can provide very useful information as outlined in the previous sections, native LC-MS analysis provides a non-denaturing approach to analyze intact ADCs without the need for digestion and reduction. Such analyses can be achieved by coupling SEC using volatile, aqueous buffers with MS analysis. Under these native-like conditions, ADCs maintain their 3-D structures and thus take up fewer charges during native analysis, resulting in less distribution of the signal and an increased spacing of different features of the sample on the m/z-axis. Introduction of artefacts due to sample processing is also less likely.

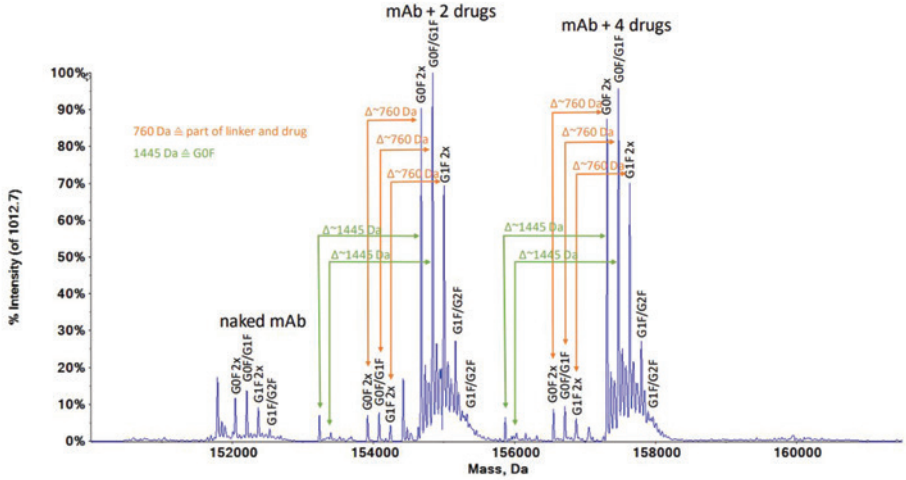
To demonstrate the power of native ADC analysis, a cysteine-linked ADC sample (mouse monoclonal antibody against hen egg lysozyme coupled with PAB MMAE. Using the TripleTOF 6600 system fitted with the IonDrive Turbo V source, which provides the soft conditions required to keep the molecule intact, the non-covalent sample was efficiently ionized, desolvated, and detected in its intact state. The high-quality raw spectrum was reconstructed to a "zero charge state" using BioPharmaView software, allowing assignment of the major proteoforms.

The protein was detected without any drug load (naked mAb), with two drugs and with four drugs attached. For each drug load, different glycoforms were observed as expected. The main proteoforms were automatically assigned and used to calculate a DAR value of 2.78. Additional peaks found in the reconstructed data were linked back to partial loss of the MC-vc- PAB-MMAE load and the loss of glycosylation.

Overall, processing of raw data for native ADCs with BioPharmaView software enables users to access critical quality attributes such as DAR with minimal hands-on time.



Schematic of conjugated trastuzumab with Gly-Gly-Gly- Val-Cit-PAB-MMAE.



Reconstucted TOF-MS data. Main glycoforms of naked mAb, mAb with drug load two and four can be found. In addition delta masses of 760 Da and 1445 Da being linked to the loss of the drug and a part of of the linker (orange arrows) and a loss of one G0F (green arrows) could be assigned.

The high-quality raw data also provides additional information on less abundant protein features, giving further insights into the complex molecular details of ADC samples. As a result, native SEC-MS supports comprehensive ADC analysis by rapidly providing orthogonal information to other analytical tools.

LC-MS analysis of nanobody-based ADCs [6]

In addition to antibodies, other antibody-related products can be used to develop targeted, conjugated therapies. Nanobodies, single-domain antibodies derived from heavy-chain-only antibodies commonly found in camelids that are a fraction of the size of typical IgG-based antibodies, have attracted attention because they have higher thermal and chemical stability and better tissue penetration, lack glycosylation and are easier to manufacture in prokaryotic cells.

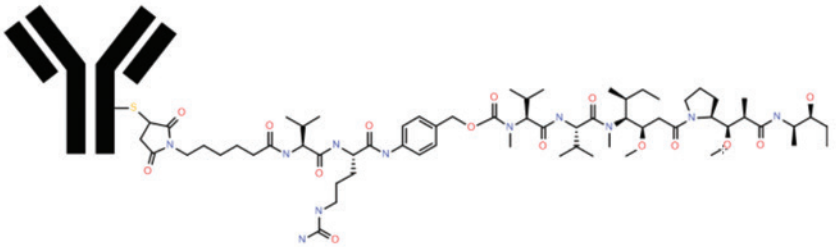
There is therefore a need to characterize these new entities with respect to their integrity, structure and modifications in order to assist in product development and to assure final product quality, and LC-MS provides a highly informative approach. Intact protein analysis allows for fast and streamlined molecular weight confirmation and an initial assessment of potential byproducts, and conjugates can be easily and reliably quantified. In addition, peptide mapping allows for exact localization and relative quantifications of even very low abundant modifications, as well as confirmation of correct disulfide bond formations.

These capabilities were demonstrated by evaluating four different nanobodies conjugated to either biotin via free amines at the protein N-terminus or lysine side chains or the dye Alexa Fluor 488 (AF-488) via the thiol groups of free cysteines.

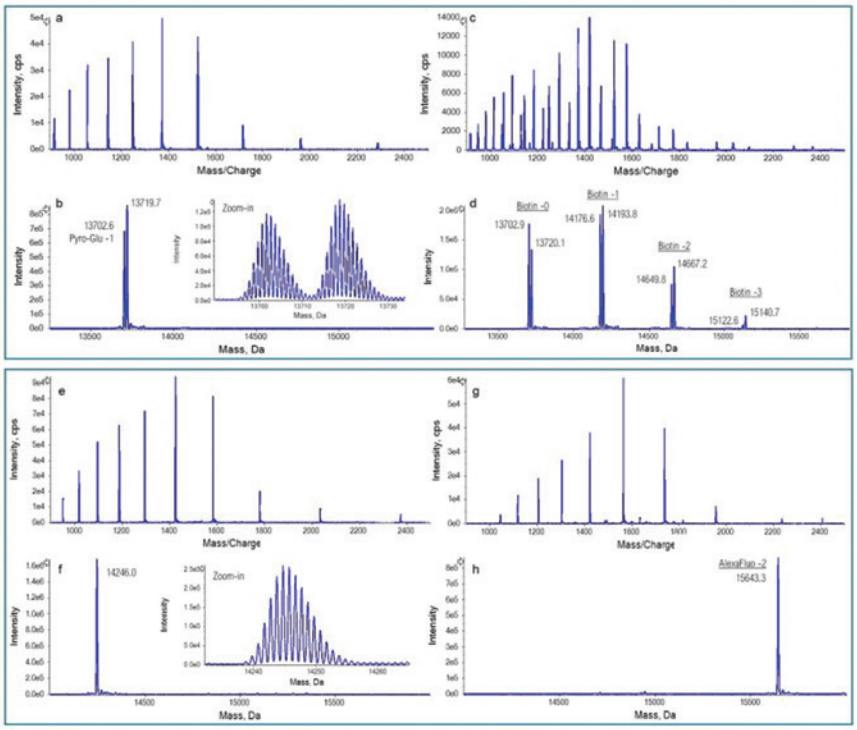
For intact analysis, the TOF-MS data of each nanobody and its labeled form were acquired with isotopic resolution using a generic LC-MS method. The data for each nanobody sample (naked and conjugated) were reconstructed, allowing for confirmation of the expected masses with very high accuracy. The method was also used to monitor different conjugation conditions and their impact on the mean biotin load for one of the nanobodies, enabling optimization from less than 1 to close to 2.

Peptide mapping was performed after digesting the nanobody conjugates under non-reducing conditions using a generic LC-MS/MS workflow. An excellent

sequence coverage up to 100%, supported by high quality MS/MS data, allowed for the confirmation of the sequence and for identification of all labeling sites. For the biotin conjugates, the percentage of biotin labeling at N-terminal peptides and lysine residues was determined and showed biotin binding at a multiplicity of sites with variable efficiency. This information is critical to verify that the labeling is directed to locations that minimally interfere with the nanobody antigen binding and help in refining the labeling approach to achieve optimal nanobody-conjugate functionality. AF-488 binding was found to be focused at two engineered cysteine residues.



Conjugation example via cysteines for MC vc-PAB-MMAE.



Representative experimental and reconstructed spectra of nanobodies and their conjugates. Top panel, left: TOF-MS (a) and reconstructed (b) spectra of the naked V_HH1 nanobody. Top panel, right: TOF-MS (c) and reconstructed (d) spectra of the biotin-conjugated V_HH1 nanbody with different loads. Bottom panel, left: TOF-MS (e) and reconstructed (f) spectra of the naked V_HV2 nanobody. Bottom panel, right: TOF-MS (g) and reconstructed (h) spectra of the AF488-conjugated V_HH2 nanobody with two labels per nanobody

Conclusions

The examples presented herein highlight the utility of LC-MS methods for the rapid characterization of ADCs and the value of MS as an orthogonal approach with utility during process / product development and for product quality determination. Both intact and subunit methods can be employed for the evaluation of many types of ADCs, including nanobody conjugates. The specific method of choice is dictated by the nature of the ADC and in particular the payload. In most cases, sample preparation and analysis times are minimal, yet more information than can generally be obtained by more traditional chromatography methods leveraging UV detection is obtained. LC-MS should therefore be considered as an important tool for accelerating ADC development.

Acknowledgments

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