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Antibody-drug conjugate characterization by chromatographic and electrophoretic techniques

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ABSTRACT

Due to the inherent structure complexity and component heterogeneity of antibody drug conjugates (ADCs), separation technologies play a critical role in their characterization. In this review, we focus on chromatographic and electrophoretic approaches used to characterize ADCs with respect to drug-to-antibody ratio, drug distribution and conjugation sites, free small molecule drugs, charge variants, aggregates and fragments, etc. Chromatographic techniques including reversed-phase, ion exchange, size exclusion, hydrophobic interaction, two-dimensional liquid chromatography, and gas chromatography as well as capillary electrophoretic techniques including capillary electrophoresis sodium dodecyl sulfate, capillary zone electrophoresis and capillary isoelectric focusing are reviewed for their applications in the characterization of ADCs.

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1. Introduction

Since their introduction into the mainstream pharmaceutical industry in the mid 1990's, antibody drug conjugates (ADCs) have presented a significant technical challenge from the analytical and quality control perspective. ADCs are comprised of three major portions: a monoclonal antibody, an efficacious and extremely potent drug, and a linker that is used to tether the other two components together as depicted in Fig. 1. ADCs utilize the specificity of monoclonal antibodies to deliver the potent small molecule drug to a specific tissue or target, thereby enabling the delivery of an efficacious dose of the therapeutic agent to the target while minimizing off-target side effects [1–7]. As more ADCs become commercially approved and their indications expand beyond oncology, the need for a more complete understanding of the molecules and control of the critical quality attributes (CQAs) become essential.

The CQAs of ADC product include those of a standard monoclonal antibody; for example appearance, pH, osmolality, identity, protein concentration, size variants, charge variants, oxidized variants, potency, particulates, leachables, bioburden, endotoxins, and sterility etc, as well as several other CQAs that are more ADC-centric including drug-to-antibody ration (DAR), drug distribution, free drug species, conjugated drug impurities and residual conjugation solvents. ADC unique CQAs were thoroughly discussed in a previous review paper [8].

Separation-based techniques play a key role in the characterization and control of CQAs of pharmaceutical therapeutics, including ADCs. HPLC is the most versatile separation technique finding application in both small and large molecules, because of the availability of various modes of separation (reversed-phase, size exclusion, ion exchange, mixed-mode, HILIC, etc.) and detectors (UV–vis, fluorescence, light scattering, charged aerosol detection, mass spectrometry, etc.). For monoclonal antibody therapeutics, SEC is a fundamental characterization technique for aggregates and fragments [9–12], ion exchange chromatography (IEX) is commonly used for charge variants [13–16], and RPLC is used for peptide mapping and others. More ADC-centric assays include the DAR by HIC [8] and the free drug assay by RPLC [17,18].

Capillary electrophoresis (CE) finds its main application in large molecule therapeutics, where its electrophoretic separation mechanism offers a distinct and often superior separation of macromolecules compared to classic chromatographic

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Nomenclature

AAC	Antibody-antibiotic conjugate
ADA	Anti-drug antibody
ADC	Antibody-drug conjugate
API	Active pharmaceutical ingredient
CQA	Critical quality attributes
DAR	Drug-to-antibody ratio
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
RPLC	Reversed-phase liquid chromatography
IEX	Ion-exchange chromatography
CEX	Cation exchange chromatography
SEC	Size exclusion chromatography
HIC	Hydrophobic interaction chromatography
MS	Mass spectrometry
LC–MS	Liquid chromatography–mass spectrometry
ESI-TOFMS	Electrospray ionization time-of-flight mass spectrometry
2D-LC	Two-dimensional liquid chromatography
GC	Gas chromatography
CE	Capillary electrophoresis
CE-SDS	Capillary electrophoresis-sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
CZE	Capillary zone electrophoresis
CIEF	Capillary isoelectric focusing
iCIEF	Imaged capillary isoelectric focusing
UV–Vis	Ultraviolet–Visible
LIF	Laser induced fluorescence
MALS	Multi-angle light scattering
CDR	Complementarity determining region
ELISA	Enzyme-linked immunosorbent assay
RSD	Relative standard deviation
LC	Light chain
HC	Heavy chain

techniques. Several modes of CE, including capillary electrophoresis sodium dodecyl sulfate (CE-SDS), capillary zone electrophoresis (CZE), and capillary isoelectric focusing (CIEF)/imaged capillary isoelectric focusing (iCIEF) are commonly utilized in characterization of the CQAs of monoclonal antibody drugs such as charge variants, identification, and positional isomers/purity etc [19–21].

As a complex molecule, many unique technologies are also being developed and applied to ADCs, enabling a greater understanding of the different components of the ADC. These advancements include de-conjugating the small molecule portion of an ADC for further characterization and investigating the in vivo stability of ADCs by advanced mass spectrometry [22–24]. In addition, advanced separation technologies, such as two-dimensional chromatography [25–27], are also being applied to ADCs to break down their complexity and gain further knowledge through orthogonal separations.

In this review, we aim to report the major analytical chromatographic and electrophoretic technologies that are used to characterize ADCs. We will cover the recent advancement and applications of these techniques that enable a more comprehensive understanding of ADCs from both the large molecule and small molecule perspectives. For other ADC characterization techniques, readers are suggested to read recent reviews for mass spectrometry by Beck et al. [24] and bioanalytical assay by Stephan et al. [28]. For the characterization of general protein biopharmaceuticals, refer to a recent review by Fekete et al. [29].

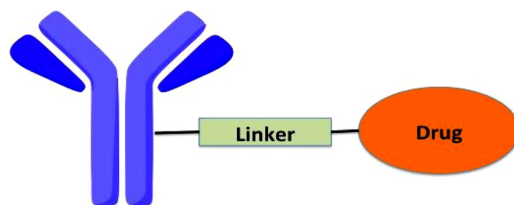

Fig. 1. Depiction of a typical ADC comprised of a monoclonal antibody, linker, and small molecule drug.

2. Chromatographic methods

2.1. Reversed-phase liquid chromatography (RPLC)

RPLC is one of the most versatile/popular chromatographic techniques for pharmaceutical analysis, providing excellent sensitivity, selectivity, accuracy, and robustness [30,31]. When interest lies in the ADC as a whole, RPLC can be used to determine isomeric distribution, compare conjugate heterogeneity, and conduct peptide mapping [32–34]; whereas when the focus is on the small molecule side, RPLC can be utilized for free drug analysis [35–46], residual solvent analysis, as well as to study the stability of the small molecule drugs [22]. When it comes to characterization assay of ADCs, RPLC is mostly used in comparability testing instead of as a primary QC tool.

2.1.1. Isomeric distribution determination

ADCs resulted from typical conjugation techniques, regardless of the conjugation site (lysine vs. cysteine) and linker type (cleavable vs. non-cleavable), are highly heterogeneous, consisting of ADC species with different numbers of drug molecules attached at various locations [47]. These species may have very different pharmacokinetic and toxicological properties that can directly affect their safety and efficacy. RPLC, especially under reducing conditions and combined with other analytical techniques (e.g., CE) is a powerful tool to determine isomeric distribution and assess conjugate heterogeneity [32–34].

RPLC has been used to determine the isomeric distribution of several ADCs containing auristatin drugs conjugated to interchain cysteines on native and engineered antibodies [32–34]. To minimize the heterogeneity of partially loaded cAC10-vc-MMAE conjugates, four different conjugation strategies were explored and the isomeric distributions of resulting conjugates were analyzed (Fig. 2) [32]. Since each isomer yielded a unique fragment pattern (distinct distribution of light chains and heavy chains with different number of drugs), the isomeric populations of all conjugates were readily determined by RPLC, except E4 that required non-reducing and denaturing CE to separate its antibody species [32]. Using the same RPLC method, the heterogeneity and isomeric distribution of engineered cAC10-vc-MMAE conjugates were also investigated and compared with native cAC10-vc-MMAE conjugates [33]. RPLC analysis of these engineered conjugates (solvent-accessible cysteines in cAC10 were replaced with serine) generated less peaks (less heterogeneity), whereas native cAC10-vc-MMAE conjugates produced more peaks (more heterogeneity) [33]. Using a shorter column with smaller particle size, the isomeric distribution of an anti-CD22-MC-MMAF conjugate was studied by RPLC [34].

2.1.2. Peptide mapping

Owing to the high reproducibility of the proteolytic fragment retention times, RPLC peptide mapping plays a major role in understanding protein structure [48], though other separation techniques (e.g., CE) are also available [49]. Peptide mapping through RPLC is typically achieved by site-specific protein cleavage with a proteolytic enzyme followed by gradient elution of

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