Chapter 6
Bioanalytical Assay for Characterization of Antibody-Drug Conjugates (ADCs)

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6.1 Introduction

Antibody-drug conjugates (ADCs) are typically heterogeneous molecules composed of a cytotoxic drug linked to an antibody (a whole monoclonal antibody or its fragment) via a stable chemical linker (Polakis 2005; Teicher and Chari 2011). ADCs are becoming increasingly important for the treatment of cancer. The drug is considered to be stably associated with the antibody in the systemic circulation. When the antibody binds to the antigen-expressing tumor cells, the ADC is able to be internalized and trafficked to the lysosomes. The linker can be hydrolyzed by the low intralysosomal pH (pH 4.5–5.0) or digested by proteases, resulting in the release of the free drugs. ADCs have complex molecular structures that can incorporate the features of both large and small molecules. The small-molecule drugs are conjugated to an antibody via a variety of amino acid residue conjugation sites (Carter and Senter 2008; Junutula et al. 2008) which will result in heterogeneity of ADCs. These include conjugation of the linker drug at lysine residues and cysteine residues through reduction of the interchain disulfide bonds or at engineered cysteine residues (Fig. 6.1**).** The conjugation reaction can result in heterogeneous mixtures of ADC molecules with various drug-to-antibody ratios (DARs). If the conjugation is at the lysine residue, the distribution of DARs can range from 0 to 9 drugs, based on previous reports (Stephan et al. 2011). A range of drug numbers such as 0, 2, 4, 6, 8 will be conjugated at different disulfide bond where the cysteine residues are located. ADCs with mainly DAR 2 have been reported for conjugation at engineered cysteines (Stephan et al. 2011; Kaur et al. 2013).

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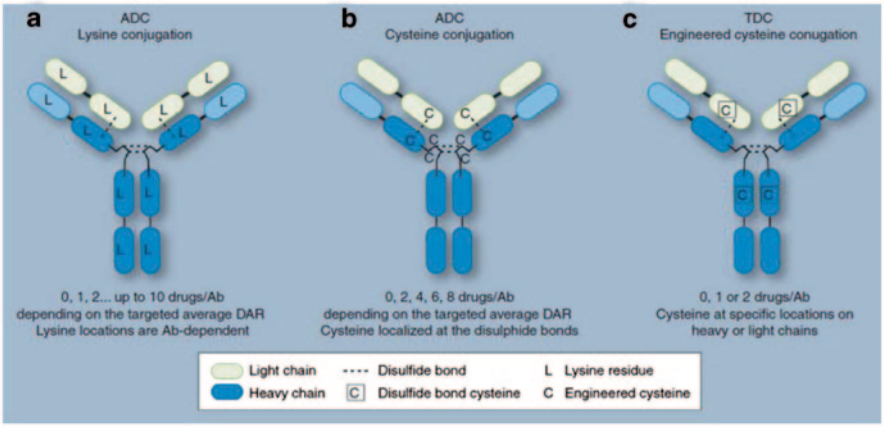

Fig. 6.1 Antibody–drug conjugate (ADCs) conjugation sites and drug load characteristics. **a** Conjugation through lysines, **b** Conjugation through reduced interchain disulfide bonds, **c** Conjugation through engineered cysteines. *Ab* Antibody, *ADC* Antibody–drug conjugate, *DAR* Drug-to-antibody ratio, *TDC* ThiomAb-drug conjugate. (Adapted by permission from Future Science Ltd: Stephan et al. 2011)

The complexity of ADCs may increase in vivo due to the biotransformation resulting from catabolism and metabolism. In addition to drug deconjugation by chemical or enzymatic cleavage, other biotransformation, such as adduct formation, generation of peptide fragments or linker drug fragments can also lead to increased complexity (Kaur et al. 2013; Shen et al. 2012). Due to the presence of mixtures with different DARs and the potential for biotransformation, it is important to develop an appropriate bioanalytical assay to accurately measure ADCs and all the analytes in the plasma or serum (Gorovits et al. 2013).

The species measured for ADCs generally include a combination of the total antibody, the conjugated antibody, the antibody-conjugated drug, and free (unconjugated) drug. The amount of total antibody can be used as an assessment of the protein component of the ADC. The content of conjugated antibody and antibody-conjugated drug can provide an assessment of the conjugate efficiency. Free drug could be used for evaluation of the safety (Gorovits et al. 2013). DAR is one of the most important markers for quality evaluation of an ADC in terms of potency and toxicity. Thus, reliable in vitro and in vivo methods to measure DAR distribution are highly required. In addition, a variety of methods have been utilized to analyze the distribution of drug-linked forms (e.g., fraction of antibodies containing different number of drugs) which is an important characteristic of ADC, because different forms may lead to different pharmacokinetic and toxicological properties (Hamblett et al. 2004; Wakankar et al. 2011). The information and typical bioanalytical methods for quantification and qualification of ADC are listed in Table 6.1. A single assay may serve multiple purposes and provide different information. However, a set of assay methods is required to comprehensively describe the concentration and composition of heterogeneous ADCs. The commonly used approaches and methods for bioanalytical characterization of ADCs are summarized in this chapter.

Table 6.1 Analytes and related parameters commonly assessed for antibody–drug conjugate (ADCs) bioanalysis

Analyte types and parameters	Details	Typical bioanalytical method
Total antibody	Conjugated and unconjugated antibodies (DAR ≥ 0)	LBA
Conjugated antibody	Antibody with drugs (minimum of DAR ≥ 1)	LBA
Antibody-conjugated drug	Total drug conjugated to antibody	MS/LBA
Free drug	Drug fallen off the antibody	MS/LBA
DAR	Average number of conjugated drug	HPLC/UV/Vis/HIC/MS
Drug distribution	Location of drug on the antibody	MS (LC-ESI-MS/MALDI-TOF-MS)/ HIC/ HPLC/IEC/SEC

LBA ligand-binding assay, *DAR* drug to antibody ratio, *MS* mass spectrometry, *HIC* hydrophobic interaction chromatography, *HPLC* high-performance liquid chromatography

6.2 Typical Bioanalytical Methods for ADCs

There are a variety of assay methods that have been used to analyze ADCs. These include ligand-binding assay (LBA), ultraviolet–visible spectrophotometry (UV/Vis), mass spectrometry (MS), and chromatography-based assays.

6.2.1 LBAs

LBA refers to an assay method based on the binding of a ligand to its specific receptor (Luckey et al. 1993). Large molecules have well-defined tertiary structures that are suitable for LBAs. LBA has been commonly used to determine the concentrations of ADC, total antibody (unconjugated and conjugated), and the free or released small-molecule drug. LBAs are generally performed using enzyme-linked immunosorbent assays (ELISAs), cell-based binding assays, or other types of binding assay.

6.2.1.1 ELISA-Based Binding Assays

ELISA is a test using the basic immunology concept of an antigen binding to its specific antibody, which allows detection of various substances including both small molecules (such as chemical drugs) and large molecules (such as peptides, proteins, or antibodies). It is commonly used to determine the ADC concentration based on the specific antigen which can capture the antibody. ELISA can be used to measure the total antibody including fully conjugated antibody, partially deconjugated antibody, and fully deconjugated antibody, using specific reagents which can bind to either the antibody or the small chemical drugs conjugated to the antibody. In

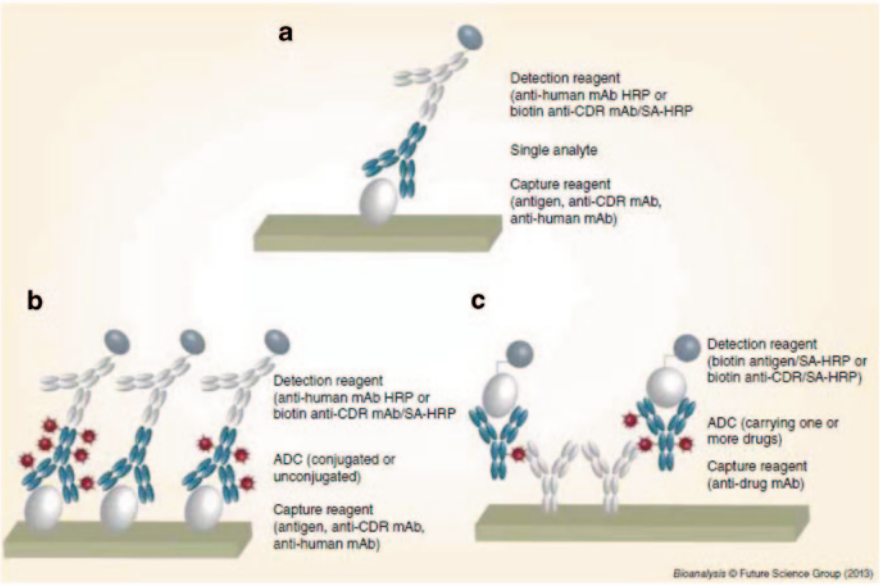

Fig. 6.2 ELISA-based binding assays. **a** Typical ELISA, **b** ELISA for ADC total-antibody measurement, **c** ELISA for ADC conjugated-antibody measurement. *ADC* antibody–drug conjugate, *Anti-CDR* anti-complementarity determining region, *HRP* horseradish peroxidase, *mAb* monoclonal antibody, *SA–HRP* streptavidin–horseradish peroxidase. (Adapted by permission from Future Science Ltd: Kaur et al. 2013)

addition, ELISA can been used to measure the concentration of the free drugs released from the ADC.

ELISA is the most common assay for measurement of total antibody. Figure 6.2a shows a typical ELISA binding assay that is used for large-molecule analysis. The capture reagent (antigen, anti-complementarity determining region monoclonal antibody (mAB), or antidrug mAB) is attached in a solid matrix. The target analytes can be recognized and bond to the capture reagent, while other molecules will be washed out. Then the amount of analytes can be quantified by a detection reagent. Figure 6.2b shows an example of a measurement of total antibody using reagents that bind to the antibody (Kaur et al. 2013). This format uses the specific antigen to capture either monoclonal or humanized antibody followed by detection of captured antibody with enzyme-conjugated anti-murine or human IgG (Stephan et al. 2011). This method has been applied to quantification of several ADCs, including huC242-DM1 (Tolcher et al. 2003), trastruzumab-DM1 (Lewis Phillips et al. 2008), anti-CD33-Calicheamicin (gemtuzumab ozogamicin) (Dowell et al. 2001), anti-MUC16-vc-MMAE (Junutula et al. 2008), and CR011-vc-MMAE (Pollack et al. 2007). When using this type of direct antigen coat approach, the purified protein as capture reagent must be available.

The antidrug antibody can be used as the capture reagent (Fig. 6.2c; Lewis Phillips et al. 2008; Advani et al. 2010**;** Xie et al. 2004). In addition, the antidrug antibody can also be utilized as a detection reagent (Junutula et al. 2008; Tolcher et al.

2003; Stephan et al. 2008; DiJoseph et al. 2004; Sanderson et al. 2005). The major difference is the assay sensitivity to drug load. The antidrug antibody is supposed to capture every ADC binding at least one drug when it is used as capture reagent and thus the assay should be insensitive to the drug load, while the signal intensity will be proportional to the amount of drug conjugated to the antibody if antidrug antibody is used as the detection reagent. (Stephan et al. 2011). As exemplified by the study performed by Kovtun et al. (2006), the concentration of cantazumab mertansine maytansinoid conjugate (huC242-DM1) is measured by using a murine anti-maytansinoid monoclonal antibody as the capture reagent. Then the conjugate from either the standard or the test samples are detected using the horseradish-peroxidase-labeled donkey antihuman IgG. The challenges to the assay using antidrug antibody as the capture antibody is that they may not be able to measure all the ADCs with different drug loads. As discussed by Xie et al. (2004), such assay may underestimate the loss of drug of molecules from the conjugate when the clearance rate for the huC242-DM1 is identified in a PK study. The antidrug antibodies have also been used as detection reagents for ADC quantification. For example, Stephan et al. (2008) have used a biotinylated anti-DM1 antibody or a biotinylated anti-MMAF antibody to detect anti-CD22-MCC-DM1, or MC-MMAF ADCs, respectively. The conjugated assays described above can provide signals dependent on the drug load with a signal proportional to the number of DM1or MMAF. Sanderson et al. (2005) have used an anti-idiotype cAC10 mAb as capture and an biotinylated anti-MMAE mAb antibody to detect the anti-CD30 ADC (cAC10-Val-Cit-MMAE) with 2, 4, and 8 val-cit-MMAE drug linkers per antibody (DAR 2, 4, 8). It has been found that the cAC10-Val-Cit-MMAE, with a DAR of 2, can generate lower conjugated antibody assay signal intensity than with a DAR of 4. This suggests that the ADC conjugate circulating concentration might be higher than what has been measured considering the potential drug loss in the circulation versus the original ADC used as the standard material. It is noteworthy that ADC ELISA is not capable of providing measurement of the DAR or the overall drug loading (Stephan et al. 2011).

ELISA can also be used to measure the concentration of the free drugs released from ADCs, although mass spectrometry is considered to be the most common method (Tolcher et al. 2003; Sanderson et al. 2005). After determining the amount of total mAb in plasma by the cAC10 mAb ELISA, Sanderson et al. have subsequently used an MMAE competition ELISA to detect the amount of MMAE released from ADC (cAC10-Val-Cit-MMAE) following in vitro incubation with cathepsin (Sanderson et al. 2005). For free MMAE competition ELISA, an anti-MMAE mAb (clone SG3.218) has been found to be the most sensitive reagent for the detection of free MMAE. To determine the sensitivity of the assay using mAb SG3.218 for capturing the free and horseradish peroxidase (HRP)-conjugated MMAE, a series of dilutions of free MMAE standards is mixed with HRP–MMAE conjugate using known concentrations to compete for binding to anti-MMAE mAb SG3.218-coated microtiter plates. The binding of the HRP–MMAE reporter is effectively competed by free drug in a dose-dependent manner.

ELISAs are high throughput and relatively inexpensive. However, this kind of measurement has some limitations (Polakis 2005). The reagents are susceptible to various interferences (Teicher and Chari 2011). It is not able to distinguish ADCs

with different DARs. Such information is very important because efficacy and safety of ADCs can potentially be compromised due to the release of conjugated drugs from the ADC in systemic circulation over time, which may result in a change in the DAR.

6.2.1.2 Cell-Based Binding Assays

Although ADC ELISA is very commonly used, it is not applicable when the antigen is not available. In this case, another common approach to characterize ADC-binding activity is the use of cells expressing either the endogenous or transfected antigen which could be recognized by the ADCs. In vitro cell binding experiments have been performed to determine the targeting effectiveness of ADC to antigen-positive cells. The cells need to express sufficient amount of target protein while the nonspecific binding to the cells has to be as low as possible to provide sufficient assay sensitivity. In general, the cells are placed in a 96-well plate and subsequently incubated for 30 min to several hours with serial dilutions of ADC. After incubation, the plate is washed and detected with a specific antispecies secondary antibody radiolabeled or conjugated to a fluorescent dye. In order to reduce possible antigen internalization upon ADC binding, the cell-based binding assays are performed at the temperature between 0 and 4 °C. When no purified antigen is available, the cell-based binding assay will have its own advantage although this approach seems to be a little cumbersome. At present, there are various types of cell-binding assays that can evaluate ADCs such as direct cell-binding assay (Sapra et al. 2005) and competitive cell-bind assay (McDonagh et al. 2006). Flow cytometry-based analysis can also be used in cell- binding assays (Junutula et al. 2008; Chen et al. 2007). Cell surface binding is usually analyzed by incubation of whole cells with either radiolabeled or fluorescent-labeled ligands followed by detection of cell-binding label. To decrease nonspecific binding to the cell, extensive washing is imperative with this strategy. Since the cell surface binding assay cannot exhibit sufficient reproducibility and accuracy due to variable cell loss from the plate during the multiple washing steps, an alternative procedure for measuring antibody binding to cell surface antigens using an immobilized plasma membrane fraction has been developed (Vater et al. 1995). In this method, isolated plasma membrane fraction exhibiting cell surface antigen is bound to a 96-well plate and incubated with antibodies that can recognize a certain cell surface protein. These approaches can provide the alternatives to mitigate the limitation of conventional cell-based assay.

6.2.1.3 Other Types of Binding Assays

Surface plasmon resonance (SPR) binding analysis methodology is another type of binding assay for detecting the interaction of two different molecules (Ramakrishnan et al. 2006; Schuck 1997), in which the antigen is immobilized onto the chip and has been used for various ADCs, including inotuzumab ozogamon (CMC-544) (Boghaert et al. 2008) and gemtuzumab ozogamicin (CMA-676) (DiJoseph et al.

2004). In principle, the detection system relies on the measurement of the changes in refractive index caused by the interaction of macromolecules on the biosensor chips. As SPR directly detects mass (concentration) with no need for special radio or fluorescent labeling of interacting components before measurement, it presents a great advantage in eliminating possible changes of their molecular properties. This method is very sensitive; however, these instruments need to be dedicated to handle toxic materials which can limit their use for ADC analysis. In order to prevent contamination of the instrument itself, a convenient alternative is the implementation of biolayer interferometry-based platforms. This optical technique analyzes the interference pattern of white light reflected from two surfaces: one is a layer of immobilized protein on the biosensor tip and the other is an internal reference layer (Stephan et al. 2011). The antigen immobilized on the biosensor tip surface can specifically capture ADC in the solution. This type of binding will result in a wavelength shift. Regardless of the format of the assay, the ADC binding should be performed along with the unmodified antibody control.

6.2.2 UV/Vis Spectroscopy

One of the most important attributes of an ADC is the average number of drugs that are conjugated to a single antibody (DAR). Various methods have been used to measure DAR, depending on the properties of the drug and how it is linked to the protein (i.e., lysine-linked or cysteine-linked). Ultraviolet–visible spectrophotometry (UV/Vis) is the simplest analytical method to determine DAR and ADC concentrations. Examples include the characterization of calicheamicin analogues (Hinman et al. 1993), cA10-Val-Cit-MMAE (Hamblett et al. 2004), and maytansinoid DM1 (Chari et al. 1992). The measure absorbance of the ADC and extinction coefficients of the antibody are used to determine the average DAR; however, the calculation based on UV absorption can be complicated by similarities in extinction coefficients of the antibody and drugs (Chen 2013).

6.2.3 MS Based Assays

Mass spectrometry (MS) is an analytical technique which can be used to determine the masses of molecules such as peptides and other chemical compounds by their mass-to-charge (m/z) ratios. MS-based assay has been utilized to characterize DAR fractions, determine the relative ratios of ADCs with different DARs, analyze free drug and metabolites, and monitor various ADC molecular entities (Kozak and Raab 2013). The process involves ionizing molecules to generate charged species or molecular fragment followed by measuring their m/z ratios. DARs are typically assessed by electrospray ionization mass spectrometry (ESI-MS). Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique to profile and monitor biomolecules. Matrix-assisted laser desorption-ionization time-of-flight

mass spectrometry (MALDI TOF-MS) is another reported new approach for determining drug loading (Quiles et al. 2009; Safavy et al. 2003). The observed mass shifts of the peak centroids are used to calculate the average drug loading and the peak profiles are used to mathematically model the drug distribution. IR MALDI has been used to evaluate the analysis of calicheamicin conjugates (Siegel et al. 1997). MALDI in combination with UV (UV MALDI-TOF MS) has been used to analyze the average DAR of lysine-linked conjugates prepared using activated paclitaxel (Safavy et al. 2003).

Although the MS-based assay can be used alone to characterize the ADCs without any additional step, such as separation and purification, it is usually coupled with chromatography technology, e.g., it is used in combination with reverse-phase high-performance liquid chromatography (RP-HPLC), liquid chromatography–mass spectrometry (LC–MS), and liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) for determination of the DAR which it is suitable for lysine-linked ADC. First, the ADC samples are desalted using RP-HPLC with a reversed-phase LC column and then the MS spectrum is processed, deconvoluted, and converted to a series of zero charge state masses that corresponds to the increasing number of drugs in the ADC (Beck 2014). The quantification of small molecules such as free drug or its metabolites is commonly analyzed by LC-MS or LC-MS/MS following extraction from tissues or plasma/serum, while both LBA (Buckwalter et al. 2004) and MS (Kaur et al. 2013; Alley et al. 2008) analytical platform can be applied for the unconjugated drug assay. The cAC10-vc-MMAE incubated in human, mouse, or dog plasma can be analyzed by LC-–MS/MS for determination of the release of free MMAE (Francisco et al. 2003). High-resolution accurate mass system such as affinity capture capillary LC-–MS can characterize drug release by quantifying DAR distributions of the ADC in plasma/serum in a semi-quantitative manner (Clark et al. 2013; Xu et al. 2011).

Recently, LC-MS with electrospray ionization coupled to time-of flight (TOF) or triple–quadrupole mass detectors (Wakankar et al. 2011) has been used to analyze ADC entities such as huN901-DM1(Wang et al. 2005), T-DM1(trastruzumab-MCC-DM1) (Junutula et al. 2010), and C242-DM4 (Lazar et al. 2005). Wang L et al. studied the drug distribution profile of huN901-DM1 using size-exclusion chromatography (SEC) coupled with ESI-TOF-MS (Wang et al. 2005). Figure 6.3 shows an example of the deconvoluted spectrum of deglycosylated huN901-DM1 using this method. The seven major peaks can be assigned to naked dghuN901 (0D) and dghuN901 with 1–6 convalently linked DM1 drugs (1D−6D) with the expected mass difference (852 Da) between drug-containing forms. For the reduced conjugate, the light and heavy chains can be separated by RP-HPLC. The number of linker attached to the light and heavy chains can be obtained directly from the deconvoluted MS spectrum. The three prominent peaks in the light-chain spectrum are for species with zero, one, and two linkers, while the four prominent peaks in the heavy chain spectrum correspond to chains with zero, one, two, and three attached linkers (Fig. 6.4). Therefore, both chains of the antibody are modified with attached linker and conjugated with DM1 drugs.

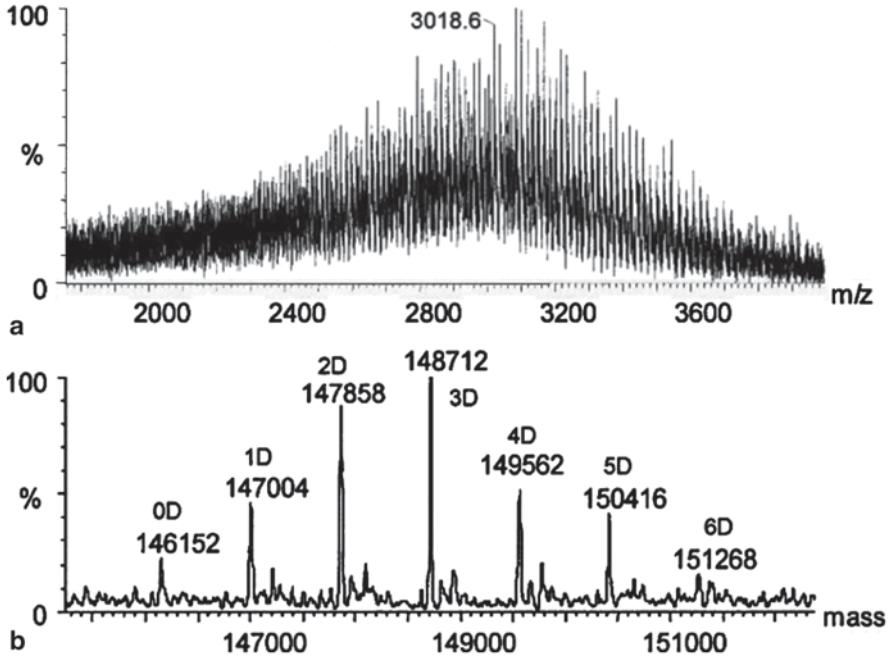

Fig. 6.3 ESI-TOF MS analysis of deglycosylated huN901-DM1. **a** Raw ESI-MS spectrum spanned an *m/z* range of 2000–4000, **b** Deconvoluted MS spectrum of seven prominent peaks which were assigned to naked dghuN901(0D) and dghuN901 with 1–6 covalently linked DM1 drugs (1D–6D). "D" in B designates DM1 drug. (Adapted by permission from John Wiley & Sons: Wang et al. 2005)

One of the challenges of LC-MS is that extra consideration is needed when preparing ADC samples. For example, protease inhibitor should be added to the homogenization buffer for preventing any degradation of antibody and link-drug when tissues are homogenized. Any pH alteration of the matrix, such as that used for protein precipitation by organic solvent or solid-phase extraction (SPE) along with LC conditions, may lead to a change in DAR or loss of the drug (Clark et al. 2013). Another issue is that conventional LC-MS for small-molecule drug measurement is to quantify a priori postulated forms of the drug released by the ADC from catabolism. However, it is theoretically possible that the released drugs may bind to the plasma peptides/protein or contain part of ADCs linker. Therefore, the putative released drug analyte may not be the major form of the released drug. The acidic conditions during electrospray ionization of the ADC which may cause the dissociation of drug-derivatized chains have limited the application of this technique to ADCs produced by conjugation with only cysteine residues (Clark et al. 2013; Wang et al. 2005; Lazar et al. 2005).

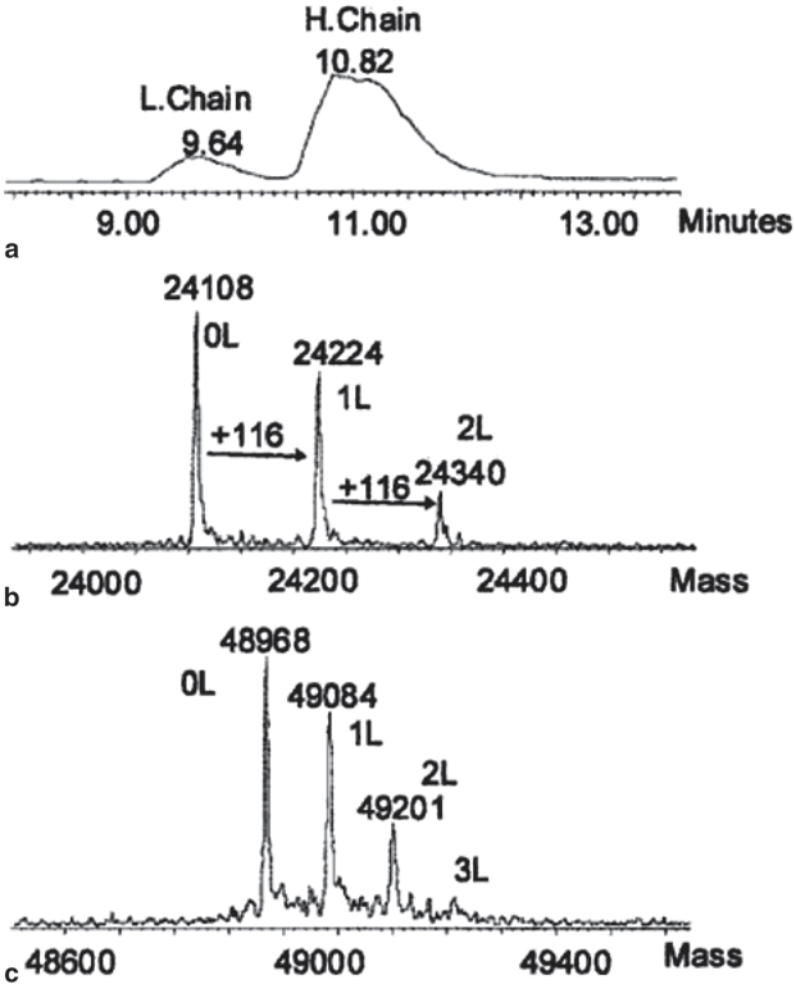

Fig. 6.4 RP-HPLC coupled with ESI-TOF MS analysis of deglycosylated and reduced huN901-DM1. **a** RP-HPLC separation of Light (*L*) chain and heavy (*H*) chain, **b** deconvoluted MS spectra of conjugated light chains with zero, one and two linkers (0L–2L), **c** deconvoluted MS spectra of conjugate heavy chains with zero, one, two, and three attached linkers (0L–3L). (Adapted by permission from John Wiley & Sons: Wang et al. 2005)

6.2.4 Chromatography-Based Assay

Although various types of binding assays have been implemented to characterize the ADC binding and biological activity, they do not allow a detailed evaluation of the ADCs as multicomponent mixtures. Therefore, additional analytical methods should be applied to profile and monitor various ADC molecular entities. Chromatography is a useful approach to separate ADCs followed by providing important

information such as the number and location of conjugation sites, amount of drug, and average DAR. Chromatographic methods generally capitalize on the increase of hydrophobicity imparted to the antibody by conjugation with the drug-linker and it is the most common methods to characterize drug-load distribution. The most frequently published methods are RP-HPLC, hydrophobic interaction chromatography (HIC), ion-exchange chromatography (IEC), and size-exclusion chromatography (SEC).

6.2.4.1 RP-HPLC

Reverse-phase chromatography can be used to separate, identify, quantify, and purify the individual components of a mixture. RP-HPLC is the most widely used analytical technique to separate the free drug released from ADC. The components are then monitored with a UV/Vis detector with the wavelength at the absorption maxima of the drug. The amount of free drug can be calculated based on the HPLC peak area with a standard curve constructed by plotting the peaks areas versus the corresponding nominal concentrations. RP-HPLC in combination with mass spectrometry (MS) has also been reported to monitor the free drug species (Francisco et al. 2003).

In addition, reverse-phase chromatography has been used to assess the drug-linker distribution on antibody heavy and light chains for cys-linked ADCs (McDonagh et al. 2006; Sun et al. 2005), which allows calculation of the average drug-load distribution of ADCs. The method involves a reduction reaction to completely dissociate the heavy and light chains of the ADC. Then the light and heavy chains and their corresponding drug-loaded forms can be separated by an RP column. The weighted average DAR is calculated by the percentage peak area from integration of the light and heavy chain peaks and the drug number (Beck 2014; Ouyang 2013). For example, the location of drug monomethyl auristatin E (MMAE) attachment to the anti-CD30 monoclonal antibody heavy and light chains can be determined by the RP-HPLC methods (Hamblett et al. 2004**;** McDonagh et al. 2006). It is important to mention that direct injection of protein-containing ADC samples onto RP-HPLC column may result in column deterioration due to the irreversible binding of proteins to the stationary phase. Therefore, using guard columns and various sample cleanup procedures to separate the free drug from the protein–drug conjugate in the sample prior to the RP-HPLC analysis is required. (Wakankar et al. 2011).

6.2.4.2 HIC

HIC is another powerful technique that has been used to separate ADC fractions containing various drug loads and to determine DAR and drug load distribution for Cys-linked ADCs. Separation is performed using a gradient elution with decreasing ionic strength and detection is achieved by monitoring the UV absorbance of the eluting species (Alley and Anderson 2013). The drug-loaded species can be resolved

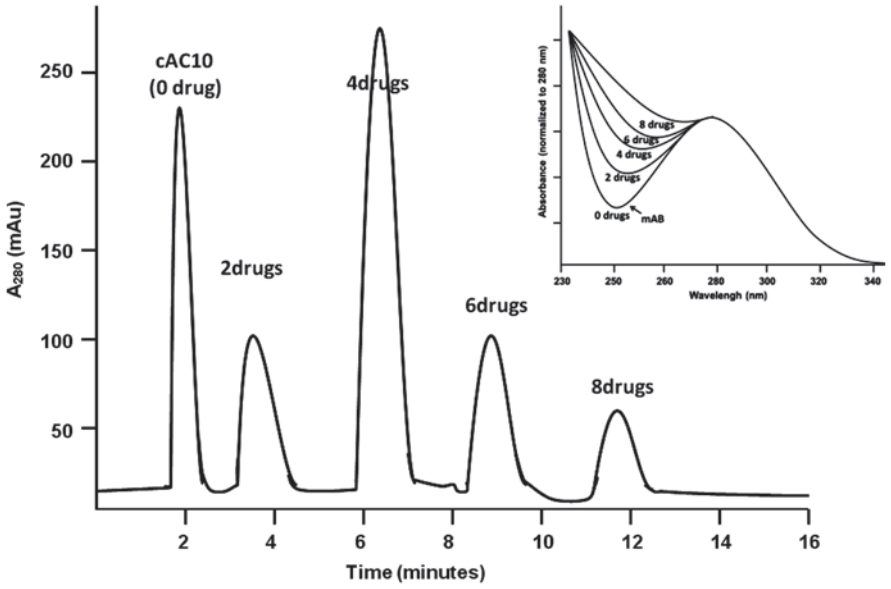

Fig. 6.5 HIC analysis of a cAC10-Val-Cit-MMAE yields five predominant peaks that correspond to mAb (cAC10) conjugated to zero, two, four, six, and eight drugs per antibody. Insert: an overlay of the UV spectra of the starting mAb and the HIC peaks showing the increase in absorbance at 248 nm relative to 280 nm as the level of conjugated drug-linker per mAb increases. (See also Hamblett et al. 2004; Sanderson et al. 2005)

based on their hydrophobicity. The hydrophobicity increases with the increasing number of conjugated drugs. For example, an ADC can bind up to eight drug molecules. The unconjugated form is the least hydrophobic, while the eight-drug form is the most hydrophobic. Thus, the unconjugated form will be eluted firstly and the eight-drug conjugation eluted last. The eluted components can be detected using a UV detector and the peak areas represent the relative percentage distribution of drug-loaded ADCs. Then the weighted average DAR can be calculated using the information of percentage peak area and the drug number (Beck 2014; Ouyang 2013). As shown in Fig. 6.5, Hamblett et al. used an HIC-HPLC method to purify cAC10-val-cit-MMAE into two, four, and eight drugs per antibody along with the corresponding UV spectra of the individual peaks (Hamblett et al. 2004). As attachment of drug MMAE results in greater absorbance at ~248 nm (λmax for MMAE) relative to 280 nm (λmax for cAC10), the five major peaks can be specifically identified. The separation by HIC allows isolation and purification of chromatographically pure species which can be further analyzed by ELISA and cell-based assay. In addition to profile the drug-load distribution, the average drug load for the ADC can be determined by the mean weighted peak area. HIC possesses a great advantage in preserving the integrity of the ADC. However, high salt is usually presented in the mobile phase initially which is necessary for protein binding and the compounds can be eluted with decreasing salt gradients. Thus, this technique cannot be directly coupled to mass spectrometer (Wakankar et al. 2011).

6.2.4.3 Other Types of Chromatography

Charge-based chromatography such as IEC has been used to determine the distribution of ADCs, while size-based chromatography such as SEC can also been used to analyze the drug distribution as well as characterize ADCs for protein fractionation, aggregation, and degradation during storage in liquid formulation (Kozak and Raab 2013; Cordoba et al. 2005; King et al. 2002).

6.3 General Assay Validation Considerations

The ADC is a complex molecule composed of a large molecule (antibody) and multiple molecule drugs. Therefore, an appropriate assay should be validated with the capability to detect both molecules precisely and accurately. Large molecules have well defined tertiary structure and are often more hydrophilic. In contrast, small molecules lack a tertiary structure and are often more hydrophobic (Dere et al. 2013). Due to the significant differences in physicochemical properties between small and large molecule, the combination of different approaches, such as LBAs for large molecule and LC-MS for small molecules, is usually employed for quantification of ADCs. Currently, there are no regulatory guidelines and best standard industry practices for bioanalytical methods of ADCs. However, the current widely accepted guidelines for large and small molecules can be a good reference. For example, the FDA guidance on bioanalytical method validation (US FDA 2013) have described the basic elements of assay validation (see Table 6.2)**.** Based on these guidelines, additional requirements unique to ADCs can be added.

Current guidelines used for validation of chromatographic assays can be applied when LC-MS methodology is used to measure unconjugated cytotoxic drug concentrations. As mentioned previously, ELISA is designed to measure the total antibody analyte, including conjugated, partially unconjugated, and fully unconjugated antibodies. Therefore, additional experiments should be performed during validation to demonstrate the ability of the assay to quantify both conjugated and completely unconjugated antibody with acceptable accuracy and precision. Another aspect unique to ADCs includes an assessment of the stability of free drug in the presence of ADC, because free drug concentration may increase due to the release of additional drug from the ADC under the storage conditions. Assessment of chemical stability is required for small molecules, the stability assessment for large molecule is more complicated and needs to be evaluated not only the physicochemical property but also biological integrity.

When the assay is applied to analyze the real sample (such as samples from a pharmacokinetic study), it should be noted that the calibration curve for LBA assays such as ELISA is usually prepared using the product reference standard that has a fixed DAR distribution. Such standards are only similar to the samples of initial time points. Moreover, most LBA calibration curves are inherently nonlinear over the nominal concentration range and a nonlinear regression is needed to achieve a

Table 6.2 Assay validation parameters for ligand binding and chromatographic assay

Validation process	Ligand-binding assays (LBA)	Chromatographic assays
Selectivity	Matrix effect	
	Cross-reactivity and interference (When possible, LBA should be compared with a validated reference method (i.e., LC-MS)	
Accuracy	A minimum of five determinations per concentration; a minimum of three concentrations in the range of study	
	Within 20% of actual value (except LLOQ) LLOQ should not deviate > 25%	Within 15% of actual value (except LLOQ) LLOQ should not deviate > 20%
Precision	A minimum of five replicates per concentration; a minimum of three concentrations	
	<20% CV (LLOQ <25% CV)	<15% CV (LLOQ<20% CV)
Recovery	Evaluation of samples at three concentrations	
Acceptance criteria for calibration curve	At least 75% of nonzero standards (at least six nonzero calibrator concentrations) should be within the below limits for the analytical run to qualify, including the LLOQ	
	Inherently nonlinear, have response–error relationship, more concentration points than for chromatographic assay LLOQ < 25%, ULOQ < 20%	LLOQ < 20% All other standards < 15%
Number of QC samples in a batch	The minimum number of QCs should be at least 5% of the number of unknown samples or six total QCs QC samples at the following three concentrations (within the calibration range) in duplicate should be added Low QC: 3 × LLOQ Medium QC: midrange of calibration curve High QC: near high end range	
Acceptance criteria for QC samples	At least 67% (four out of six) of the QC concentration results should be within 20% of nominal (theoretical) values. At least 50% of QCs at each level should be within 20% of their nominal concentrations Total error (accuracy and precision) < 30%	At least 67% (four out of six) of the QCs concentration results should be within 15% of nominal (theoretical) values. At least 50% of QCs at each level should be within 15% of their nominal concentrations
Reproducibility	Assessed by replicate measurements including QC and possibly incurred samples. Reinjection reproducibility should be evaluated	
Stability	a. Freeze and thaw stability; b. Bench-top Stability; c. Long-term Stability; d. Stock solution stability; e. Processed sample stability	

Table 6.2 (continued)

Validation process	Ligand-binding assays (LBA)	Chromatographic assays
Additional issues	A. Endogenous compounds: The biological matrix used to prepare calibration standards should be the same as the study samples and free of the endogenous analyte. The matrix should be demonstrated to have (1) no measurable endogenous analyte, (2) no matrix effect or interference when compared to the biological matrix. The endogenous concentrations of the analyte in the biological matrix should be evaluated prior to QC preparation B. Biomarkers: For validation of assays to measure in vivo biomarker concentrations in biological matrices such as blood or urine. To address the same questions as method validation for PK assays. The accuracy, precision, selectivity, range, reproducibility, and stability of a biomarker assay are important characteristics that define the method	
	Diagnostic kits: The LBA kits suitability for using in PK or PD studies should be demonstrated	

QC quality control, *LLOQ* lower limit of quantification, *ULOQ* upper limit of quantification, *PK* pharmacokinetics, *PD* pharmacodynamics

good fitting. Thus, it is important to verify that the calibration curve is appropriate for quantifying the dynamically changing mixture of ADC for PK evaluation (Dere et al. 2013).

The revised draft FDA guidance for bioanalytical method validation contains an additional section on endogenous compounds. Small-molecule assays often include a pre-assay extraction to alleviate problems from individual matrix variability. In contrast, assays to quantify large molecules are often developed to measure analyte in complex matrices without pre-extraction where endogenous protein may be present. Therefore, special considerations must be taken regarding matrix effect (DeSilva et al. 2003).

6.4 Challenges and Future Perspective

ADC bioanalysis is complicated compared with conventional large- or small- molecule bioanalysis. The multicomponent nature of ADCs which arises from the heterogeneity of the conjugation and the biotransformation in vivo represents a significant challenge for the development of reliable and accurate bioanalytical analysis. The DAR composition undergoes continuous change in the circulation due to drug deconjugation and degradation of the antibody, so the ADC reference standard used for assay quantification may not be identical to the ADCs mixture from in vivo samples (Gorovits et al. 2013). Therefore, additional attention for assay reagents is required to ensure that it can detect all DAR species formed in vivo without sacrificing selectivity. As described above, all the bioanalytical assays currently used to characterize ADCs have their own limitations, so developing a comprehensive

bioanalytical strategy is highly recommended for the ADCs. Meanwhile, as the special regulatory guidelines for validation of bioanalytical methods for characterizing ADCs are still being drafted, validation studies should be performed to improve the interpretation of the data so generated.

6.5 Conclusion

We have summarized the common assays that are commonly used to characterize the ADCs and highlighted some examples. Multiple methods are required to fully characterize physiochemical and pharmacokinetic properties of ADCs. The complexity of ADCs presents challenges in the development of bioanalytical methods, so new bioanalytical techniques will need to be developed to ensure assays' accuracy.

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