

METHODS OF ASSESSING ANTIBODY-DRUG CONJUGATES**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/556,153 filed September 8, 2017, the contents of which are incorporated herein by reference.

BACKGROUND

[0002] Antibody-drug-conjugates (ADCs) are an emerging class of drug molecules. Their ability to locate to a specific target and deliver a potent drug makes them an attractive option for developing a target based therapeutic product. ADCs are produced by chemically linking potent drug molecules to a monoclonal antibody via a selected chemical linker. The average number of drug molecules that are conjugated to the monoclonal antibody is called drug-to-antibody ratio, ("DAR"). DAR is an important quality attribute of ADC products, because it can impact product efficacy, safety and/or stability. Accordingly, methods of assessing DAR of ADC products in a reliable and high throughput manner are desirable.

DETAILED DESCRIPTION

[0003] The present disclosure provides methods of assessing DAR of ADC products that provide advantages over known methods. Specifically, methods of the disclosure can be used in high-throughput applications and/or without having to dilute ADC samples during the assessment.

UV-Vis and Beer-Lambert's Law

[0004] DAR has traditionally been measured using UV-Vis spectroscopy (see, e.g., Chen, Methods Mol. Biol. 1045:267-73 (2013)). The basis for this analysis is the Beer-Lambert law, a direct proportional relationship between the absorbance and concentration of a substance:

$$A = \epsilon cl,$$

where A is the absorbance, ϵ is the extinction coefficient (a physical constant of the substance), l is the path length through the cell containing the analyte, and c is the concentration.

[0005] DAR measurement for an ADC product using UV-Vis spectroscopy relies on the difference in absorption maxima for the antibody (e.g., 280 nm) and the absorption maxima for the drug (e.g., at 252 nm). For example, the average DAR can be calculated using the difference in the measured absorption at 280 nm and 252 nm for the conjugated material. Although the UV-Vis method is widely used in industry, it lacks the throughput needed for formulation screening studies. It also cannot be used without sample dilution, leading to errors associated with sample dilution.

[0006] The present disclosure is thus based, at least in part, on alternative methods for measuring DAR using size exclusion chromatography (e.g., UPLC) and slope spectroscopy. These methods were characterized and compared to UV-Vis spectroscopy with respect to reproducibility, precision and sensitivity. The data generated support use of UPLC-based DAR methods to overcome the throughput limitations of traditional UV-Vis methods. Further, the slope spectroscopy based method can be used to analyze ADC samples without sample dilution.

UPLC-based methods

[0007] In one embodiment, size exclusion is used to determine DAR. In some embodiments, the methods disclosed herein comprise applying a sample comprising an antibody-drug conjugate to a size-exclusion chromatography matrix. In some embodiments, the methods disclosed herein comprise applying to and running a sample comprising an antibody-drug conjugate through a size-exclusion chromatography matrix. In some embodiments, a total amount of ADC sample is applied to a size exclusion matrix for analysis. For example, the following UPLC-based methodology was used to assess DAR.

Column:	Waters Acquity UPLC BEH200 SEC (Part# 186005225) 4.6mm x 15cm, 1.7 μ m particle size, max pressure: 1034 bar 2 columns attached inline
Mobile Phase:	Perchlorate SEC Buffer: 10 mM Phosphate, pH 6.0 1M NaClO ₄

Gradient Info:	Isocratic
Flow Rate:	0.4 mL/min
Method Run Time:	12 minutes
Column Temperature:	Not controlled
Wavelength of Detection	280 nm and 252 nm (or $A_{\max}$ for conjugated drug)
Target Injection Amount	6-150 μ g ADC (no dilution)

[0008] Data collected at 280 nm were integrated using Empower's Apex Track integration method with peak shoulder detection. The retention time integration range is molecule dependent, but is usually within 3-9 minutes. The peak with largest height and area was classified as "native", "main" or "monomer" peak. Any peaks eluting earlier than the "native" peak were classified as "HMW" peaks. Any peaks eluting later than the "native peak" were classified as "LMW" peaks.

[0009] The relative percentage of each species was calculated from the ratio of the area of individual peaks to the total area of all peaks. The relative percent area of the following was reported as an indicator of purity: % Total HMW, % Native (or Main or Monomer), and % Total LMW. The total area of all peaks was summed and used in subsequent DAR calculations. However, in some embodiments, only the area of the native peak is used.

[0010] Data collected at 252 nm were integrated using Empower's Apex Track integration method with peak shoulder detection. The retention time integration range is molecule dependent, but is usually within 3-9 minutes. The total area of all peaks was summed and used in subsequent DAR calculations. However, in some embodiments, only the area of the native peak is used.

[0011] DAR was determined from the total peak area at 280 nm ($A_{\max}$ for the ADC) and the total peak area at 252 nm ($A_{\max}$ for the drug). Although 252 nm is a common $A_{\max}$ for drug conjugates used for ADCs, an appropriate wavelength can be selected for a specific conjugate, e.g., using known methods. The amount of drug bound to the antibody can be determined by the difference in total peak areas at these two wavelengths, using the naked antibody as a reference standard, if applicable.

[0012] The following two equations (which were derived from the Beer-Lambert law) were verified and demonstrated consistency.

Equation 1:

$$\text{DAR} = \frac{\epsilon_{252\text{nm}}^{\text{mAb}} * \text{Total Area}_{280\text{nm}} - \epsilon_{280\text{nm}}^{\text{mAb}} * \text{Total Area}_{252\text{nm}}}{\epsilon_{280\text{nm}}^{\text{drug}} * \text{Total Area}_{252\text{nm}} - \epsilon_{252\text{nm}}^{\text{drug}} * \text{Total Area}_{280\text{nm}}}$$

[0013] Equation 1 does not require the use of a naked antibody reference standard. However, systematic determination of extinction coefficients (ϵ) for both the antibody and the drug at 252 nm is required. The extinction coefficient at a given wavelength can readily be calculated from the Beer-Lambert law by using a solution of either the antibody or the drug having a known concentration and measuring the absorbance at the given wavelength.

Equation 2:

$$\text{DAR} = \frac{\epsilon_{280\text{nm}}^{\text{mAb}}}{\text{Total Area}_{280\text{nm}}^{\text{mAb}}} * \frac{\text{Total Area}_{252\text{nm}}^{\text{ADC}} * \text{Total Area}_{280\text{nm}}^{\text{mAb}} - \text{Total Area}_{280\text{nm}}^{\text{ADC}} * \text{Total Area}_{252\text{nm}}^{\text{mAb}}}{\epsilon_{252\text{nm}}^{\text{drug}} * \text{Total Area}_{280\text{nm}}^{\text{ADC}} - \epsilon_{280\text{nm}}^{\text{drug}} * \text{Total Area}_{252\text{nm}}^{\text{ADC}}}$$

[0014] Equation 2 does not require extinction coefficient determination for the antibody at 252 nm, but it does require collection of UPLC data for a naked antibody reference standard.

[0015] Although UPLC has been exemplified, other size exclusion chromatography techniques can be used in methods described herein. Size exclusion chromatography generally refers to separation of molecules by size, where the chromatographic elution time is characteristic for a particular molecule. Additional methods include, e.g., SEC-HPLC, reversed phase (RP) HPLC, RP-UPLC.

[0016] In some embodiments, an ADC sample is not diluted prior to analysis by size exclusion chromatography (e.g., HPLC or UPLC). In some embodiments, an ADC sample does not require dilution prior to analysis by size exclusion chromatography as a total amount of the ADC sample is applied to the size exclusion chromatography matrix. In some embodiments, a sample containing about 1 $\mu\text{g}/\mu\text{L}$ to about 500 $\mu\text{g}/\mu\text{L}$ ADC is analyzed.

Slope spectroscopy-based methods

[0017] In some embodiments, DAR is determined by calculating the concentrations of antibody and drug in an ADC sample. For example, slope spectroscopy is a known method for determining the absorbance of a solution at various path lengths. The values of the absorbance at various path lengths can then be used, based on the Beer-Lambert law, to calculate the concentration of a compound in the solution. Methods and systems employing slope spectroscopy are known (see, e.g., US Publ. No. 20120130649) and commercially available (see, e.g., SoloVPE (C Technologies, Inc., Bridgewater, NJ)). Such methods and systems were adapted to measure concentrations of antibody and drug in ADC preparations, from which a DAR was determined.

[0018] For example, an ADC sample can be placed in a vessel; a probe can be moved relative to the vessel such that the probe makes contact with the bottom of the vessel; the probe can be moved relative to the vessel such that the probe moves from the bottom of the vessel through the sample by a predetermined increment such that a preselected path length through the solution is obtained; an absorbance reading can be taken at an absorption maxima for the antibody; the probe can be moved repeatedly relative to the sample and a measurement can be taken; a regression line can be generated from the absorbance and path length such that a slope of the regression line is obtained; and the concentration of the antibody can be determined by dividing the slope of the regression line by the extinction coefficient of the antibody. The steps can then be repeated using the absorption maxima for the drug to determine the concentration of the drug. DAR can be calculated from the determined drug concentration and antibody concentration.

[0019] In some embodiments, an ADC sample is not diluted prior to analysis by slope spectroscopy. In some embodiments, a sample containing about 0.1 $\mu\text{g}/\mu\text{L}$ to about 500 $\mu\text{g}/\mu\text{L}$ ADC is analyzed.

Antibody-Drug Conjugates

[0020] The term “antibody-drug conjugate” as used herein, refers to a protein that is created by linking an antibody to a biologically active cytotoxic payload or drug. Antibody-drug conjugates (ADC) are generally produced through chemical modification/coupling reactions known to those skilled in the art. Any antibody-drug conjugate can be analyzed using the methods described herein.

[0021] In some embodiments, an antibody-drug conjugate includes an anti-tumor antibody (see, e.g., Adler et al., Hematol. Oncol. Clin. North Am. 26:447-81 (2012); Li et al.,

Drug Discov. Ther. 7:178-84 (2013); Scott et al., Cancer Immun. 12:14 (2012); and Sliwkowski et al., Science 341:1192-1198 (2013)). Table 1 presents a non-comprehensive list of certain human polypeptide antigens targeted by known, available antibody agents, and notes certain cancer indications for which the antibody agents have been proposed to be useful. Any of the antibodies in Table 1 can be included in an antibody-drug conjugate assessed using methods of the disclosure.

Table 1:

Human Antigen	Antibody (commercial or scientific name)	Cancer indication
CD2	Siplizumab	Non-Hodgkin's Lymphoma
CD3	UCHT1	Peripheral or Cutaneous T-cell Lymphoma
CD4	HuMax-CD4	
CD19	SAR3419, MEDI-551	Diffuse Large B-cell Lymphoma
CD19 and CD3 or CD22	Bispecific antibodies such as Blinatumomab, DT2219ARL	Non-Hodgkin's Lymphoma
CD20	Rituximab, Veltuzumab, Tositumomab, Ofatumumab, Ibritumomab, Obinutuzumab,	B cell malignancies (Non-Hodgkin's lymphoma, Chronic lymphocytic leukemia)
CD22 (SIGLEC2)	Inotuzumab, tetraxetan, CAT-8015, DCDT2980S, Bectumomab	Chemotherapy-resistant hairy cell leukemia, Hodgkin's lymphoma
CD30	Brentuximab vedotin	
CD33	Gemtuzumab ozogamicin (Mylotarg)	Acute myeloid leukemia
CD37	TRU-016	Chronic lymphocytic leukemia
CD38	Daratumumab	Multiple myeloma, hematological tumors
CD40	Lucatumumab	Non-Hodgkin's lymphoma
CD52	Alemtuzumab (Campath)	Chronic lymphocytic leukemia
CD56 (NCAM1)	Lorvotuzumab	Small Cell Lung Cancer
CD66e (CEA)	Labetuzumab	Breast, colon and lung tumors
CD70	SGN-75	Non-Hodgkin's lymphoma
CD74	Milatumzumab	Non-Hodgkin's lymphoma

CD138 (SYND1)	BT062	Multiple Myeloma
CD152 (CTLA-4)	Ipilimumab	Metastatic melanoma
CD221 (IGF1R)	AVE1642, IMC-A12, MK-0646, R150, CP 751871	Glioma, lung, breast, head and neck, prostate and thyroid cancer
CD254 (RANKL)	Denosumab	Breast and prostate carcinoma
CD261 (TRAILR1)	Mapatumumab	Colon, lung and pancreas tumors and haematological malignancies
CD262 (TRAILR2)	HGS-ETR2, CS-1008	
CD326 (Epcam)	Edrecolomab, 17-1A, IGN101, Catumaxomab, Adecatumumab	Colon and rectal cancer, malignant ascites, epithelial tumors (breast, colon, lung)
CD309 (VEGFR2)	IM-2C6, CDP791	Epithelium-derived solid tumors
CD319 (SLAMF7)	HuLuc63	Multiple myeloma
CD340 (HER2)	Trastuzumab, Pertuzumab, Ado-trastuzumab emtansine	Breast cancer
CAIX (CA9)	cG250	Renal cell carcinoma
EGFR (c-erbB)	Cetuximab, Panitumumab, nimotuzumab and 806	Solid tumors including glioma, lung, breast, colon, and head and neck tumors
EPHA3 (HEK)	KB004, IIIA4	Lung, kidney and colon tumors, melanoma, glioma and haematological malignancies
Episialin	Epitumomab	Epithelial ovarian tumors
FAP	Sibrotuzumab and F19	Colon, breast, lung, pancreas, and head and neck tumors
HLA-DR beta	Apolizumab	Chronic lymphocytic leukemia, non-Hodkin's lymphoma
FOLR-1	Farletuzumab	Ovarian tumors
5T4	Anatumomab	Non-small cell lung cancer
GD3/GD2	3F8, ch14.18, KW-2871	Neuroectodermal and epithelial tumors
gpA33	huA33	Colorectal carcinoma
GPNMB	Glembatumumab	Breast cancer
HER3 (ERBB3)	MM-121	Breast, colon, lung, ovarian, and prostate tumors
Integrin $\alpha V \beta 3$	Etaracizumab	Tumor vasculature

Integrin $\alpha 5\beta 1$	Volociximab	Tumor vasculature
Lewis-Y antigen	hu3S193, IgN311	Breast, colon, lung and prostate tumors
MET (HGFR)	AMG 102, METMAB, SCH900105	Breast, ovary and lung tumors
Mucin-1/CanAg	Pemtumomab, oregovomab, Cantuzumab	Breast, colon, lung and ovarian tumors
PSMA	ADC, J591	Prostate Cancer
Phosphatidylserine	Bavituximab	Solid tumors
TAG-72	Minretumomab	Breast, colon and lung tumors
Tenascin	81C6	Glioma, breast and prostate tumours
VEGF	Bevacizumab	Tumour vasculature

[0022] In some embodiments, an antibody-drug conjugate includes a drug that is one or more of pro-apoptotic, cytostatic and/or cytotoxic agents, for example specifically including agents utilized and/or recommended for use in treating one or more diseases, disorders or conditions associated with undesirable cell proliferation. In many embodiments, a drug is a chemotherapeutic agent useful in the treatment of cancer. In some embodiments, a chemotherapeutic agent may be or comprise one or more alkylating agents, one or more anthracyclines, one or more cytoskeletal disruptors (e.g., microtubule targeting agents such as taxanes, maytansine and analogs thereof), one or more epothilones, one or more histone deacetylase inhibitors (HDACs), one or more topoisomerase inhibitors (e.g., inhibitors of topoisomerase I and/or topoisomerase II), one or more kinase inhibitors, one or more nucleotide analogs or nucleotide precursor analogs, one or more peptide antibiotics, one or more platinum-based agents, one or more retinoids, one or more vinca alkaloids, and/or one or more analogs of one or more of the following (i.e., that share a relevant anti-proliferative activity). In some particular embodiments, a chemotherapeutic agent may be or comprise one or more of Actinomycin, All-trans retinoic acid, an Auiristatin, Azacitidine, Azathioprine, Bleomycin, Bortezomib, Carboplatin, Capecitabine, Cisplatin, Chlorambucil, Cyclophosphamide, Curcumin, Cytarabine, Daunorubicin, Docetaxel, Doxifluridine, Doxorubicin, Epirubicin, Epothilone, Etoposide, Fluorouracil, Gemcitabine, Hydroxyurea, Idarubicin, Imatinib, Irinotecan, Maytansine and/or analogs thereof (e.g., DM1), Mechlorethamine, Mercaptopurine, Methotrexate, Mitoxantrone, a Maytansinoid,

Oxaliplatin, Paclitaxel, Pemetrexed, Teniposide, Tioguanine, Topotecan, Valrubicin, Vinblastine, Vincristine, Vindesine, Vinorelbine, and combinations thereof.

[0023] In some embodiments, an antibody-drug conjugate assessed using a method of the disclosure is hLL1-doxorubicin, hRS7-SN-38, hMN-14-SN-38, hLL2-SN-38, hA20-SN-38, hPAM4-SN-38, hLL1-SN-38, hRS7-Pro-2-P-Dox, hMN-14-Pro-2-P-Dox, hLL2-Pro-2-P-Dox, hA20-Pro-2-P-Dox, hPAM4-Pro-2-P-Dox, hLL1-Pro-2-P-Dox, P4/D10-doxorubicin, gemtuzumab ozogamicin, brentuximab vedotin, trastuzumab emtansine, inotuzumab ozogamicin, glembatumomab vedotin, SAR3419, SAR566658, BIIB015, BT062, CMC-544, SAR3419, CDX-011, SGN-75, SGN-CD19A, AMG-172, AMG-595, BAY-94-9343, ASG-5ME, ASG-22ME, ASG-16M8F, MDX-1203, MLN-0264, anti-PSMA ADC, RG-7450, RG-7458, RG-7593, RG-7596, RG-7598, RG-7599, RG-7600, RG-7636, ABT-414, IMGN-853, IMGN-529, IMGN-901, vorsetuzumab mafodotin, or lorvotuzumab mertansine (see, e.g., Sassoon et al., *Methods Mol. Biol.* 1045:1-27 (2013); Bouchard et al., *Bioorganic Med. Chem. Lett.* 24: 5357-5363 (2014)).

Applications

[0024] Methods of the disclosure have a variety of applications and include, e.g., quality control at different stages of manufacture of a drug substance or drug product, analysis of an ADC preparation prior to and/or after completion of a drug substance or drug product manufacture (e.g., prior to or after distribution to a fill/finish environment or facility), prior to or after release of a drug substance or drug product into commerce (e.g., before distribution to a pharmacy, a caregiver, a patient, or other end-user). In some instances, an ADC preparation is a drug substance (an active pharmaceutical ingredient or “API”) or a drug product (an API formulated for use in a subject such as a human patient). In some instances, an ADC preparation is from a stage of manufacture or use that is prior to release to caregivers or other end-users; prior to packaging into individual dosage forms, such as syringes, pens, vials, or multi-dose vials; prior to determination that the batch can be commercially released, prior to production of a Certificate of Testing, Material Safety Data Sheet (MSDS) or Certificate of Analysis (CofA) of the preparation.

[0025] Assessments from methods described herein are useful for guiding, controlling or implementing a number of activities or steps in the process of making, distributing, and monitoring and providing for the safe and efficacious use of an ADC preparation. Thus, in an embodiment, e.g., responsive to the evaluation, e.g., depending on whether a criterion is met

(e.g., a particular DAR, average DAR, and/or DAR range), a decision or step is taken. Methods described herein may include making a decision: (a) as to whether an ADC preparation may be formulated into drug substance or drug product; (b) as to whether an ADC preparation may be reprocessed (e.g., the preparation may undergo a repetition of a previous process step); and/or (c) that the ADC preparation is not suitable for formulation into drug substance or drug product. In some instances, methods comprise: formulating as referred to in step (a), reprocessing as referred to in step (b), or rendering the preparation unusable for commercial release, e.g., by labeling it or destroying it, as referred to in step (c).

CLAIMS

What is claimed:

1. A method of determining a ratio of drug to antibody (DAR) in a sample comprising an antibody-drug conjugate, comprising:

applying the sample to a size exclusion chromatography matrix;

detecting absorbance response of the sample at a first light wavelength (λ_1), wherein the first light wavelength is a predetermined absorbance maxima of the antibody;

detecting absorbance response of the sample at a second light wavelength (λ_2), wherein the second light wavelength is a predetermined absorbance maxima of the drug;

determining a total absorbance of the sample at the first light wavelength and a total absorbance of the sample at the second light wavelength, each total absorbance calculated by integrating peaks of absorbance responses over a time interval; and

calculating the DAR using the following Equation 1:

$$\text{DAR} = \frac{\epsilon_{\lambda_2}^{\text{mAb}} * \text{Total Area}_{\lambda_1} - \epsilon_{\lambda_1}^{\text{mAb}} * \text{Total Area}_{\lambda_2}}{\epsilon_{\lambda_1}^{\text{drug}} * \text{Total Area}_{\lambda_2} - \epsilon_{\lambda_2}^{\text{drug}} * \text{Total Area}_{\lambda_1}}$$

wherein $\epsilon_{\lambda_1}^{\text{mAb}}$ is an extinction coefficient of the antibody at the first light wavelength; $\epsilon_{\lambda_2}^{\text{mAb}}$ is an extinction coefficient of the antibody at the second light wavelength; $\epsilon_{\lambda_1}^{\text{drug}}$ is an extinction coefficient of the drug at the first light wavelength; $\epsilon_{\lambda_2}^{\text{drug}}$ is an extinction coefficient of the drug at the second light wavelength; Total Area $_{\lambda_1}$ is the total absorbance of the sample at the first light wavelength; and Total Area $_{\lambda_2}$ is the total absorbance of the sample at the second light wavelength.

2. The method of claim 1, further comprising calculating the extinction coefficient of the antibody at the first light wavelength or at the second light wavelength based on absorbance of a solution of the antibody with a known concentration.

3. The method of claim 1, further comprising calculating the extinction coefficient of the drug at the first light wavelength or at the second light wavelength based on absorbance of a solution of the drug with a known concentration.
4. The method of any one of claims 1-3, wherein the size exclusion chromatography comprises ultra-performance liquid chromatography (UPLC), a reversed phase (RP) UPLC, or a high performance liquid chromatography (HPLC).
5. The method of any one of claims 1-4, wherein the sample includes 1 $\mu\text{g}/\mu\text{L}$ to 500 $\mu\text{g}/\mu\text{L}$ of antibody-drug-conjugate (ADC).
6. The method of any one of claims 1-5, wherein the antibody is an anti-tumor antibody.
7. The method of any one of claims 1-6, wherein the drug is a pro-apoptotic, cytostatic or cytotoxic agent.
8. The method of any one of claims 1-7, wherein integrating peaks of absorbance responses includes detecting a peak shoulder of each peak of absorbance response.
9. The method of any one of claims 1-8, wherein integrating peaks of absorbance responses includes summing the area of high molecular weight (HMW) peaks, low molecular weight (LMW) peaks and a main peak.
10. The method of any one of claims 1-9, wherein the time interval is between 3 to 9 minutes.