Journal of Chromatography A, xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Chromatography-based methods for determining molar extinction coefficients of cytotoxic payload drugs and drug antibody ratios of antibody drug conjugates

Chunlei Wang*, Sike Chen, Janet Caceres-Cortes, Richard Y.-C. Huang, Adrienne A. Tymiak, Yingru Zhang

Research & Development, Bristol-Myers Squibb Company, Route 206 and Province Line Road, Princeton, NJ 08543, United States

ARTICLE INFO

Article history:
Received 15 January 2016
Received in revised form 24 May 2016
Accepted 25 May 2016
Available online xxx

Keywords:
Molar extinction coefficient (MEC)
Drug antibody ratio (DAR)
Chemiluminescence nitrogen detector (CLND)
Antibody drug conjugate (ADC)
UV spectrophotometry

ABSTRACT

UV spectrophotometry is widely used to determine the molar extinction coefficients (MECs) of cytotoxic drugs as well as the drug antibody ratios (DARs) of antibody drug conjugates (ADCs). However, the unknown purity of a drug due to interfering impurities can lead to erroneous MECs and DARs. Hence, reliable methods to accurately determine purity and the MECs of drugs with limited quantity is urgently needed in Drug Discovery. Such a method has been developed. It achieves absolute purity and accurate MEC determination by a single automated HPLC analysis that uses less than 5 μg of material. Specifically, analytical HPLC separation with online UV detection was used to resolve impurities and measure absorbance from only the compound of interest. Simultaneously, an online chemiluminescence nitrogen detector (CLND) was used to determine the concentration of the analyte. The MECs were then calculated from the absorbance and concentration results. The accuracy of the method was demonstrated using caffeine and a commercial cytotoxic drug, DM1. This approach is particularly suited to analyzing mixtures or samples with low purities. Excellent reproducibility was demonstrated by analyzing a proprietary drug with linker synthesized from different batches with very different levels of purity. In addition, the MECs of drug with linker, along with ADC peak areas measured from size exclusion chromatography (SEC), were used to calculate DARs for 21 in-house ADCs. The DAR results were consistent with those obtained by MS analysis.

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1. Introduction

Antibody-drug conjugates (ADCs) represent a rapidly growing class of anticancer therapy agents [1–4]. ADCs are designed to exploit the specificity of monoclonal antibodies for targeted delivery of chemically linked cytotoxins, often referred to as payloads, to cancer cells where the target antigen is overexpressed. This antigen-specific delivery improves anticancer efficacy, minimizes systemic toxicity, and therefore expands the narrow or nonexistent therapeutic index of potent cytotoxins [5]. Recently, the promise of the ADC approach has been further validated by clinical approval of two ADC therapies, *i.e.*, Adcetris [6] and Kadcyla [7]. Currently, there are more than 30 ADCs under clinical evaluation [8], and many more under preclinical development.

The development of ADCs involves simultaneous optimization of their constituent moieties, *i.e.*, antibodies that are specific to tumor antigens, highly potent cytotoxins, and stable linkers that are cleavable for cytotoxin drug release [4]. In addition, the drug to antibody ratio (DAR) is one of the most important quality attributes also to be examined [9–11], because it affects ADCs' physical stability [12], safety profile [13], *in vitro* and *in vivo* efficacy [14], and pharmacokinetic properties [5,15].

Several methods have been used to measure the DAR of ADCs, including UV spectroscopy, mass spectrometry (MS), and hydrophobic interaction chromatography (HIC) [11]. HIC methods were reported only for cysteine-linked conjugates, where ADCs with different drug loads were separated to obtain both average DAR as well as the distribution of protein:antibody ratios [11]. Lysine-linked conjugates, on the other hand, are much more heterogeneous and usually cannot be chromatographically resolved based on drug loads. In comparison, MS analysis can provide DAR and its distribution for both cysteine and lysine conjugation chemistries [16–22]. For successful data interpretation, however, pretreatment

* Corresponding author.
E-mail address: chunlei.wang@bms.com (C. Wang).

http://dx.doi.org/10.1016/j.chroma.2016.05.086
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such as deglycosylation is often needed to eliminate sample complexity and improve MS sensitivity [18–20]. Additional enzymatic digestion can further improve MS signal and reduce ionization bias [21,22]. Among available methods for DAR determination, UV spectroscopy is the simplest and requires no sample preparation. The average DAR is calculated based on the molar extinction coefficients (MEC) of drugs with linker, antibodies and the absorbance of their conjugates [11,23]. The UV method can be used only when the UV spectra of the antibody and the drug-linker have distinct absorption maxima [11,23]. The larger the difference in UV spectra, the more accurate the determined DAR. The quantitative aspect between the UV spectra similarity and DAR error margin is the current ongoing work and will be reported in a separate study. The MEC for an antibody can be calculated reliably based on its primary sequence [24,25], whereas the MEC for a drug with linker (drug-linker) need to be determined experimentally.

Accurate MEC measurement based on Beer's Law using a UV spectrophotometer with a cuvette requires a pure compound as a reference standard. UV-absorbing impurities interfere with determining absorbance for the compound of interest. In addition, the presence of any impurity or residual solvent would lead to an error in sample concentration obtained by weighing. While rigorous measurements are achievable in later stages of drug development, this high purity requirement creates significant challenges at the early lead selection stage [10], when drug-linker libraries are initially synthesized at or below a single digit milligram scale, and obtaining pure form of every library compound is not practical [26]. In order to obtain accurate MECs from samples at these early stages of discovery, one must first determine the absolute purity or concentration of the drug-linker, followed by an accurate MEC determination of the drug-linker without any interference from impurities. This, in turn, can enable accurate DAR calculations (with the premise that impurities in the drug-linker do not conjugate with antibodies) and the integrity of the structure activity relationship (SAR) during ADC drug discovery. Fishkin has recently described accurate MEC measurement for a 50 μg maytansinoid sample by first derivatizing it with BODIPY fluorophore and then removing impurities that may interfere with absorption measurement by semipreparative separations [10]. The quantification is based on the characteristic absorbance of BODIPY fluorophore and the 1:1 stoichiometry between the maytansinoid and BODIPY derivatization. The method successfully eliminated weighing errors and absorbance interferences from impurities. However, this approach was only applicable to thiol containing drugs and the derivatizing chromophore cannot be generalized for all classes of drugs [10].

In this study, we report a chromatography-based approach to determine absolute purity and MEC for crude cytotoxic drug-linkers by a single HPLC experiment. The UV absorbance was measured by an HPLC diode array detector (DAD), and the concentration was determined by an online "universal" concentration detector, a chemiluminescence nitrogen detector (CLND). The CLND achieves absolute quantification based on its equimolar response to nitrogen atoms in organic molecules. Indeed, online quantification by CLND has been used to determine relative response factors of different compounds by HPLC UV detection [27]. In comparison, we derive equations to calculate absolute MECs from HPLC peak area and explore its application to ADC characterization. The feasibility of the proposed method using modern HPLC systems was demonstrated using a caffeine standard. An example to accurately determine MECs of three model compounds simultaneously in a mixture was shown. For its application on ADC discovery, the MECs of a commercial cytotoxic drug, DM1, was determined and compared with the reported literature values. Reproducibility of the MEC determination was then evaluated using a BMS proprietary ADC drug-linker from different synthetic batches of different quality. In addition, size exclusion chromatography was used to measure ADC absorbance ratios at different wavelengths. The drug-linkers' MECs and ADC peak areas were used for DAR calculations of 21 ADCs. A final discussion about potential errors of the reported methods was also discussed.

2. Material and methods

2.1. Material

TraceCERT grade Caffeine, 2,5- and 3,5-dinitrobenzoic acids, Chromasolv gradient grade methanol, ReagentPlus grade trifluoroacetic acid (TFA), and puriss grade formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). High purity deionized water was supplied by the Barnstead Nanopure system from Thermo Scientific (Waltham, MA, USA). The cytotoxic drug mertansine, also known as DM1, was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), catalog number sc-482549, lot H0715. All drug-linkers and ADCs used in this study were generated in house. The antibodies were from the IgG1 subclass, and the payloads were from the tubulysine and pyrrolobenzodiazepine families.

2.2. UV spectroscopy

The MECs of model compounds were individually determined by traditional UV spectroscopic method on a Jasco (Easton, MD, USA) V-530 UV/Vis spectrophotometer. The MEC of caffeine was measured in water/methanol 50/50 with 0.1% TFA. The MECs of 2,5- and 3,5-dinitrobenzoic acid was measured in water/methanol 45/55 with 0.1% TFA, matching the isocratic HPLC mobile phase solvent composition.

2.3. Chromatography

Five different HPLC systems, including Agilent (St. Clara, CA, USA) 1100, 1200, 1260, 1290 II, and Shimadzu (Columbia, MD, USA) 10A, were used for MEC determination from certified caffeine standards. The caffeine standards were analyzed on different columns, including Waters (Milford, MA, USA) Sunfire C18, CSH C18 and Agilent Zorbax Bonus-RP, using different gradient profiles with water-methanol-0.1%TFA solvent systems to achieve caffeine elution at the solvent composition of 30, 40, 50 and 60% organics.

The mixture of caffeine, 2,5- and 3,5-dinitrobenzoic acids were analyzed on a Sunfire C18 column, 5 μm 2.1 × 150 mm, using an Agilent 1200 system, equipped with a G1379B degasser, a G1312B binary pump, a G1367C autosampler, a G1330B thermostat, a G1316A column compartment, a G1315C diode array detector with a G4301-60100 flow cell, 13 μL 10 mm path length, and an Antek 8060 CLND (PAC, Houston, TX, USA). The separation was achieved using isocratic 55% methanol in water with 0.1% TFA at 0.1 mL/min (flow rates up to 0.3 mL/min may also be used). The CLND was operated with a furnace temperature of 1050 °C, ozone and oxygen settings at 250 mL/min, and inlet and makeup helium flows at 50 mL/min.

The BMS cytotoxic drug-linkers were analyzed on the same Agilent 1200 system. The chromatography was run on a Zorbax Bonus-RP column, 3.5 μm 3 × 150 mm, using water-0.2%FA (A) and methnol-0.2%FA (B) solvents at 0.3 mL/min. The generic gradient profile was 10–100% solvent B in 30 min. The commercial cytotoxic drug, DM1, was also analyzed under the same condition, and the results were compared to available values reported in the literature.

The size exclusion chromatography (SEC) of ADCs were analyzed on a Waters BEH200 SEC column, 1.7 μm 4.6 × 150 mm, using an

Please cite this article in press as: C. Wang, et al., Chromatography-based methods for determining molar extinction coefficients of cytotoxic payload drugs and drug antibody ratios of antibody drug conjugates, J. Chromatogr. A (2016), http://dx.doi.org/10.1016/j.chroma.2016.05.086

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