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A high-throughput hydrophilic interaction liquid chromatography coupled with a charged aerosol detector method to assess trisulfides in IgG1 monoclonal antibodies using *tris*(2-carboxyethyl)phosphine reaction products: *Tris*(2-carboxyethyl)phosphine-oxide and *tris*(2-carboxyethyl)phosphine-sulfide

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ABSTRACT

A robust, high-throughput method using hydrophilic interaction liquid chromatography (HILIC) coupled with a charged aerosol detector (CAD) is reported as a novel approach for trisulfide quantitation in monoclonal antibodies (mAbs). The products of mAb reduction using *tris*(2-carboxyethyl)phosphine (TCEP) include a species (TCEP(S)) that is stoichiometrically produced from trisulfides. The TCEP reaction products are chromatographically separated, detected, and quantified by the HILIC—CAD method. The method was qualified to quantify trisulfides across a range of 1–40% (mol trisulfide/mol mAb). In all tested matrix components, assay linearity and intermediate precision were established with correlation coefficients (R^2) > 0.99, and relative standard deviations (RSD) < 10%. A method comparability study was performed using peptide mapping LC–MS as an orthogonal measurement. For the range of 1–40% trisulfides, the analysis demonstrates that, on average, HILIC—CAD reads between 0.95 and 1.10 times the value of LC–MS with 95% confidence. Applications of the HILIC—CAD method include trisulfide determination in purified mAbs to be used in the production of cysteine-linked antibody-drug conjugates, and in cell culture development studies to understand sources of, and strategies for control of, trisulfides.

1. Introduction

A trisulfide is a post-translation modification formed by the insertion of an extra sulfur atom into a disulfide bridge. For proteins, the modification was first discovered in cysteine bridges (Cys^{53}–Cys^{165} and Cys^{182}–Cys^{189}) of biosynthetic human growth hormone produced in *Escherichia coli* [1,2] and in the non-recombinant protein Cu-Zn superoxide dismutase [3]. More recently, trisulfides have been identified and characterized in recombinant monoclonal antibodies (mAbs) of all immunoglobulin G (IgG) sub-classes [4–7]. Using peptide mapping liquid chromatography mass spectrometry (LC–MS) for mAb characterization, mAb trisulfides have been found in the inter-chain disulfide bridges, predominantly in the heavy chain-light chain (HC–LC) bridges [5,8–10]. Low levels of mAb trisulfides have been detected in the HC—HC hinge disulfide bridges [6] at 5–10% of the amount found in HC-LC bridges. There are no reports of mAb trisulfides in the intra-chain disulfide bridges, perhaps due to their buried or sterically hindered nature. Most recently, for cysteine-linked antibody-drug conjugates (ADCs) produced using a *tris*(2-carboxyethyl)phosphine (TCEP) reduction step, the presence of trisulfides can lead to a variable drug-to-antibody ratio (DAR) [4].

Previously described methods for mAb trisulfide quantitation have involved liquid chromatography-electrospray ionization mass spectrometry. The quantitation of trisulfides in the intact and enzymatically-digested mAb was performed using a variety of mass analyzers, such as time-of-flight, quadrupole, and orbitrap [4–6,11]. Specifically for peptide mapping LC–MS analysis, trisulfides are assessed through extracted ion chromatogram comparison of trisulfide peptides and the corresponding disulfide peptides. While trisulfide site specificity can be determined by LC–MS, all cysteine bridges must be analyzed to obtain total moles of trisulfides per moles of mAb. In addition, mass spectrometry requires extensive instrument maintenance, training, software and validation, relative

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to other forms of analysis. For these reasons, an alternative method for quantitation is needed for robust, high-throughput analysis of total trisulfide levels.

The high-throughput method presented here is based on reaction products formed during mAb reduction with *tris*(2-carboxyethyl)phosphine (TCEP) [4], a common reducing agent used in the manufacture of cysteine-linked ADCs [12–14]. Based on reaction stoichiometry, for each disulfide bond reduced by TCEP, two free thiols are made available for drug conjugation, and one equivalent of *tris*(2-carboxyethyl)phosphine-oxide (TCEP[O]) is produced, as shown in Equation (1);

$$\mathrm{TCEP} + \mathrm{mAb(S\text{-}S)} + \mathrm{H_2O} \rightarrow \mathrm{mAb(SH)_2} + \mathrm{TCEP[O]} \quad (1)$$

However, experimental results for drug-to-antibody ratio (DAR) derived from mAbs that contained trisulfide bonds diverged from the expected 2:1 stoichiometry. This is due to the presence of trisulfides. For every trisulfide that reacts with TCEP, a disulfide bond is formed along with an equivalent of *tris*(2-carboxyethyl)phosphine-sulfide (TCEP[S]), as shown in Equation (2);

$$\mathrm{TCEP} + \mathrm{mAb(S\text{-}S\text{-}S)} \rightarrow \mathrm{mAb(S\text{-}S)} + \mathrm{TCEP[S]} \quad (2)$$

The resulting mAb(S-S) formed in Equation (2) will require an additional equivalent of TCEP to yield two free thiols, therefore two moles of TCEP are needed to fully reduce a mAb trisulfide, as shown in Equation (3);

$$2\mathrm{TCEP} + \mathrm{mAb(S\text{-}S\text{-}S)} + \mathrm{H_2O} \rightarrow \mathrm{mAb(SH)_2} + \mathrm{TCEP[S]} + \mathrm{TCEP[O]} \quad (3)$$

As shown in Equation (3), TCEP[S] formation is stoichiometrically equivalent to mAb trisulfide content as was previously confirmed by 1D and 2D NMR experiments [4].

Based on this relationship, a robust quantitative method for TCEP[O] and TCEP[S], produced from reduction of mAb samples, was developed using hydrophilic interaction liquid chromatography (HILIC) coupled with a charged aerosol detector (CAD). For each mAb sample, a predefined quantity of TCEP is added, all of which reacts to produce either TCEP[O] or TCEP[S]. Based on the molar ratio of TCEP[O] and TCEP[S] that is formed, the level of trisulfides can be determined. The method provides an alternative to the previously described LC–MS method for trisulfide quantitation [5].

Unlike NMR or LC–MS based trisulfide analyses, HILIC—CAD is a fast and simple method that can be used for high-throughput quantitation of total trisulfides in purified proteins. This method can be used as a tool to aid in cell culture process development, or to quantify the trisulfide levels in antibody intermediates intended for use in ADC manufacturing.

2. Materials and methods

2.1. Reagents and chemicals

HPLC-grade acetonitrile (MeCN) was purchased from EMD Millipore (Billerica, MA). Ammonium formate, *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), and hydrogen peroxide solution (30% in water v/v) were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen sulfide in water (125 mM) was purchased from Ricca Chemicals (Arlington, TX). *Tris*(hydroxymethyl)aminomethane (TRIS) and sodium chloride (NaCl) were purchased from Mallinkrodt Baker (Phillipsburg, NJ). Monoclonal antibodies [**mAb-1**, **mAb-2**, and **mAb-3** (standard IgG1 sub-class)] were produced in Chinese hamster ovary (CHO) cells and were purified and formulated at Genentech (South San Francisco, CA). Ultrapure water was obtained using a Millipore Milli Q® system (EMD Millipore; Billerica, MA).

2.2. Standards and stock solutions

The water content by Karl Fischer and percent purity by GC were obtained from the TCEP·HCl lot certificate of analysis, and used to calculate the mass required to equal 14.33 mg of pure TCEP·HCl. A 5 mM TCEP·HCl stock solution was prepared by dissolving the TCEP·HCl in 10 mL of ultrapure water, and was used for reduction of all mAbs. 1.01 mM TCEP·HCl stock solution was prepared by diluting the 5 mM TCEP·HCl stock solution 1.01:5 with ultrapure water. 1 mM oxidized TCEP (TCEP[O]) was generated by adding 10 μL of hydrogen peroxide (30%/water v/v) to 1 mL of 1.01 mM TCEP·HCl stock solution. A 5 M NaCl stock solution was prepared by dissolving 14.6 g of NaCl in 50 mL of ultrapure water.

Tris(2-carboxyethyl)phosphine sulfide (TCEP[S]) standard for chromatographic and CAD optimization was generated by reaction of TCEP with allyl trisulfide using a previously published protocol [4].

2.3. HILIC—CAD trisulfide analysis

2.3.1. Standard and sample preparation

Serial dilution of the 1 mM TCEP[O] stock solution in water produced TCEP[O] standards of the following concentrations: 600 μM, 450 μM, 300 μM, 150 μM, 75 μM, 60 μM, 45 μM, 30 μM, 15 μM, 6 μM, and 3 μM. The standards were diluted 1:1 with MeCN, and analyzed by HILIC—CAD.

Experimental mAb samples were adjusted to a target concentration of 20 mg/mL prior to TCEP reduction. Samples greater than 20 mg/mL were diluted with the appropriate formulation buffer, while samples less than 20 mg/mL were concentrated using 10 kDa molecular weight cut-off Amicon filters. Four milligrams of mAb (200 μL of 20 mg/mL solution) were reduced with 2.00 molar equivalents of TCEP (5 mM stock solution) and incubated in the dark at ambient temperature for 90 min. After reduction, NaCl (5 M stock solution) was added to a final concentration of 100 mM, and incubated at 37 °C for 15 min. TCEP[O] and TCEP[S] were separated from the reduced mAb using 10 kDa molecular weight cut-off filters. The filtrate was diluted 1:1 with MeCN, and analyzed by HILIC—CAD.

2.3.2. TCEP[O] and TCEP[S] separation

Chromatographic analyses were performed using an Agilent 1290 series UHPLC system (Agilent Technologies, Palo Alto, CA) consisting of a quaternary pump, a thermostatted auto sampler, and a thermostatted column oven. The analytes were separated on an Acclaim Trinity P1 column (150 mm x 3.0 mm i.d., 3 μm particle size, Thermo Scientific) [12] in series with a guard cartridge of the same stationary phase (10 mm × 3.0 mm i.d, 3 μm particle size, Thermo Scientific). Sample injection volume was 80 μL and standard injection volume was 50 μL, and the temperature of the column oven was maintained at 30 °C. The mobile phases were (A) MeCN and (B) 350 mM ammonium formate, pH 4.0 in Milli-Q water with the following linear gradient flow program: 0 min, 65% A; 10 min, 10% A; 10.1 min, 0% A; 13 min, 0% A; 13.1, 65% A; 16 min, 65% A. The total chromatographic run time was 16 min with a constant flow rate of 0.4 mL/min. The first 6.5 min of the run, containing the majority of the matrix components, was diverted away from the CAD detector and sent directly to waste.

2.3.3. TCEP[O] and TCEP[S] detection

Analyte detection was conducted using a Corona Veo RS charged aerosol detector (Thermo Scientific). The operating conditions were as follows: Evaporator temperature, 60 °C; Gas regulation mode,