Analytical Biochemistry 527 (2017) 33–44

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Determination of thiol-to-protein ratio and drug-to-antibody ratio by in-line size exclusion chromatography with post-column reaction

Kenichiro Furuki [a, b, *], Toshimasa Toyo'oka [b]

[a] Process Science Lab II, Biotechnology Labs, Astellas Pharma Inc., 5-2-3 Tokodai, Tsukuba-shi, Ibaraki 300-2698, Japan
[b] School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka-shi, Shizuoka 422-8526, Japan

ARTICLE INFO

Article history:
Received 2 March 2017
Received in revised form
11 April 2017
Accepted 17 April 2017
Available online 18 April 2017

Keywords:
Ultra-high-performance liquid chromatography
DTNB
Post-column reaction
Monoclonal antibody
Antibody-drug conjugate
Process analytical technology

ABSTRACT

An in-line size-exclusion (SE) ultra-high-performance liquid chromatography (UHPLC)- 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) method to quantify thiols in monoclonal antibodies (mAb) when manufacturing antibody-drug conjugates (ADCs) was developed. The mAbs are separated on an SE-UHPLC column and monitored with a UV detector at a wavelength of 280 nm. Eluents are channeled into a reaction coil and mixed with DTNB to form 5-thio-2-nitrobenzoic acid (TNB). Thiol concentration is calculated using absorption at 412 nm. Using optimized conditions, partially reduced mAbs can be separated from low-molecular weight contaminants and undergo the DTNB reaction. The standard curve of L-cysteine had good linearity between 100 and 1000 μM. The selectivity, linearity, repeatability, and robustness of this method were evaluated. The calculated free-SH:protein ratios of partially reduced mAbs were consistent between in-line SE-UHPLC-DTNB and conventional methods. The SE-UHPLC-DTNB method showed time- and temperature-dependent changes in the free-SH:protein ratio of mAbs during reduction. The changes in drug-antibody ratio (DAR) of ADCs during the conjugation reaction were also evaluated. This method is an inexpensive and versatile alternative to conventional methods of estimating the free-SH:protein ratio of mAbs and the DAR of ADCs. This method also minimizes assay time.

Introduction

Antibody-drug conjugates (ADCs) combine antibodies with bioactive compounds to create targeted therapies. ADCs were developed to address deficiencies in conventional antibody therapies, including poor cytotoxicity and low tumor penetration. ADCs are composed of three parts: a tumor-antigen-specific monoclonal antibody (mAb) that can be internalized, a bioactive small molecule that is usually cytotoxic, and a covalent linker that connects the mAb and small molecule [1,2]. ADCs have higher tumor cytotoxicity than mAbs alone and fewer off-target effects than drug alone [3].

Typically, the antibody is conjugated to the drug using lysine or cysteine residues [4–7]. To generate cysteine-linked ADCs, the inter-chain disulfide bonds of the mAbs are reduced and free thiols are conjugated with the linker and drug [3,7]. Frequently, mAbs are partially reduced, and free thiols covalently bind a maleimide-based linker [7,8]. These mAbs are partially reduced using calculated quantities of reducing agent, e.g., tris (2-carboxyethyl) phosphine (TCEP) to generate a specific number of free thiols [7,9]. Then, the drug, covalently bonded to the linker, is added. This reaction is not targeted, so it generates mixture of ADCs with varying numbers of drug per antibody. The reaction is quenched using N-acetyl-L-cysteine (NAC), which forms an adduct with the linker [10].

One of the challenges of ADC production is regulating the partial reduction process. The effectiveness of this process is evaluated by quantifying total thiols in an mAb and determining the free-SH:protein ratio. Currently, the number of total thiols in a sample is determined using a thiol-selective reagent. These reagents are usually fluorescent or colorimetric compounds that fluoresce or change absorbance in the presence of thiols [11–16]. In cases of non-fluorescent reagents, including 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) [12,14,17–19], 4,4'-dithiodipyridine (4-DTDP) [19–21], and n-octyldithionitrobenzoic acid [22], the reagent reacts with thiol, forming a adduct and ejecting a molecule that can be measured spectrophotometrically. Generally, DTNB is the preferred assay for quantifying thiols [12,14,17–19]. In the DTNB assay, the ejected molecule is 5-thio-2-nitrobenzoic acid (TNB), which has a

* Corresponding author. Process Science Lab II, Biotechnology Labs, Astellas Pharma Inc., 5-2-3 Tokodai, Tsukuba-shi, Ibaraki 300-2698, Japan.
E-mail address: kenfuruki@yahoo.co.jp (K. Furuki).

http://dx.doi.org/10.1016/j.ab.2017.04.008
0003-2697/

molar absorptivity coefficient of 14,150 M^{-1} cm^{-1} at 412 nm and neutral pH (Fig. S1) [23]. However, several compounds, including TCEP and NAC, can react with DTNB and absorb light at this wavelength, which interferes with quantification of total thiols in an mAb [24]. To eliminate interference, the mAb must undergo buffer exchange prior to performing a conventional DTNB assay. Unfortunately, the time required for buffer exchange is too long to evaluate reduction while ADCs are being produced.

One solution to this challenge is the quantification of analytes using HPLC. One study showed that thiols could be quantified using 4,4'-dithiodipyridine and reversed-phase (RP) HPLC [25]. While RP-HPLC can successfully separate mAbs from interfering molecules, such as reducing agents, the acidic conditions of RP-HPLC mobile phases reduce the absorptivity of TNB at 412 nm [25].

Another factor to consider when evaluating ADC production is the average drug-antibody ratio (DAR). DAR is a measure of drug distribution in ADC. The drug distribution can affect critical pharmacological properties such as efficacy, cell cytotoxicity, clearance and dispositon [6,26–28]. Immunoglobulin (Ig)G1 antibodies have two inter-chain disulfides in the hinge region and two that connect the light to the heavy chains. By contrast, IgG2 antibodies have six inter-chain disulfides and can form as many as three disulfide isoforms [29–32]. There are several methods available to determine DAR. Separation techniques such as hydrophobic-interaction or ion-exchange chromatography, capillary electrophoresis, or capillary isoelectric focusing have been used [6,27,33–37]. These techniques are usually coupled with absorbance-based detection rather than mass spectrometry because the separation buffers contain high concentrations of salt. RP-HPLC coupled with mass spectrometry is advantageous because it enables simultaneous desalting, separating the sample, getting the mass spectrum, and quantification [38,39]. Similar to the free-SH:protein ratio, the time required to determine DAR is too long to implement this analysis during ADC production.

In September 2004, the US Food and Drug Administration [40] issued a guidance document for developing process analytical technology (PAT). The purpose of PAT is to analyze and control manufacturing during production to ensure a high-quality final product. Numerous PAT techniques have been described for biologics, such as ADCs [41]. Implementation of PATs requires analytical tools that can provide real-time product-quality data. In turn, these data can be used to adjust production parameters and improve the output. PAT techniques often rely on mathematical relationships between in-line measurements and adjustable production parameters. To date, there are no rapid techniques to determine total thiol concentration in mAbs and ADC that can be developed into PAT.

To address this pressing need, we have developed a rapid analytical method to determine protein concentration and total thiol concentration using size-exclusion (SE)-UHPLC coupled with a simultaneous in-line DTNB assay. Protein concentration is determined by UV detector 1. DTNB is mixed with column eluent to determine total thiol concentration by UV detector 2 (Fig. 1).

Materials and methods

Reagents, materials, and apparatus

The following chemical substances were analytical grade, and suppliers are as follows: maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl-monomethyl-auristatin E (vcMMAE, Fig. S2), Sigma Aldrich (Steinheim, Germany); acetonitrile, Kanto chemical (Tokyo, Japan); TCEP and DTNB, Thermo Fisher Scientific (Waltham, MA, USA); NAC and L-cysteine, Wako Pure Chemical (Tokyo, Japan); trifluoro acetic acid (TFA), Nacalai Tesque (Kyoto, Japan).

Spectra and absorption measurements were collected in quartz cuvettes with a Shimadzu UV-5500 UV–Vis spectrophotometer (Kyoto, Japan). A Shimadzu UFLC-XR UHPLC system (Kyoto, JAPAN) equipped with two binary pumps, a column thermostat, and two UV detectors was used for chromatographic measurements. Data was acquired using LabSolutions ver. 5.86 (Shimadzu, Kyoto, Japan). In the UHPLC-coupled post-column system, protein concentration is measured based on the absorbance at 280 nm (UV detector 1) as the sample elutes off the column. A reaction module equipped with a reaction coil (volume 0.75 mL, Thermo Fisher Scientific) and column oven after the size exclusion mode UHPLC column was used. The eluent from the column is mixed with DTNB solution (mobile phase B) in a reaction coil for ≤ 1 min before collecting the absorbance at 412 nm (UV detector 2).

Humanized monoclonal antibodies, IgG1 (mAb A) and IgG2 (mAb B), were produced by the Process Sciences Department at Astellas Pharma, Inc. (Tsukuba, Japan).

Preparation of partially reduced mAb

mAbs were partially reduced with TCEP [42]. mAb A was diluted to a final concentration of 0.1 mM in 20 mM Tris, 5 mM EDTA, 150 mM NaCl, pH 7.5. mAb B was diluted to a final concentration of 0.1 mM in 25 mM sodium borate, 300 mM NaCl, 5 mM EDTA, pH

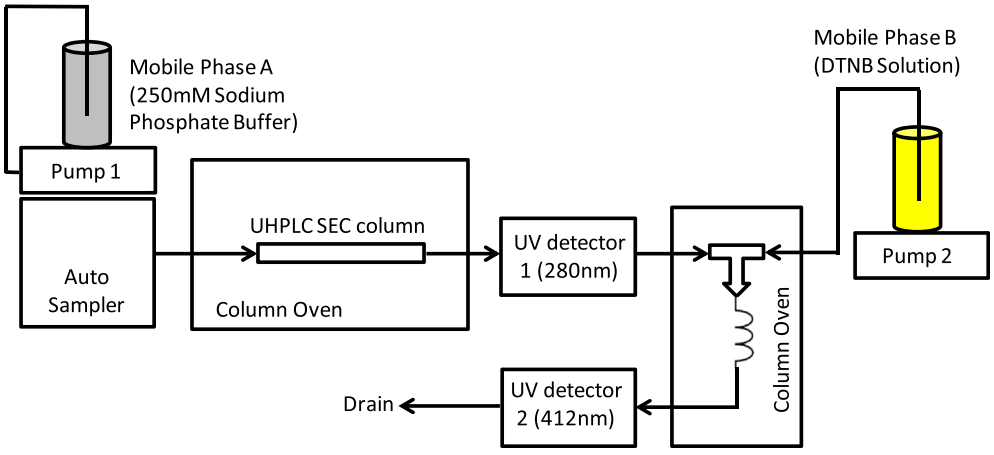

Fig. 1. Schematic of the instrumental set-up for the in-line SE-UHPLC-DTNB system. This system can simultaneously quantify protein and TNB concentrations.