Method development and validation of capillary sodium dodecyl sulfate gel electrophoresis for the characterization of a monoclonal antibody

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ABSTRACT

A capillary sodium dodecyl sulfate gel electrophoresis (cSDS) method has been developed and qualified for purity and impurity analysis of monoclonal antibodies. This method was optimized and qualified for the analysis of monoclonal antibody (mAb1) under reduced and non-reduced conditions.

Some of the sample preparation parameters including sample buffer pH, incubation temperature and duration, alkylation conditions with iodoacetamide (IAM), and reduction conditions with 2-mercaptoethanol (2-ME) were optimized. It was observed that under slightly acidic conditions (pH 5.5–6.5) the thermally induced fragmentation of non-reduced mAb1 was greatly decreased. As such, a citrate–phosphate buffer at pH 6.5 was used for sample preparation to replace the original Beckman sample buffer (pH 9.0). The optimal sample preparation conditions were found to be as follows: (1) incubation temperature and duration (reduced and non-reduced), 65 °C for 5 min; (2) alkylation condition, 10 μL of 0.25 M IAM; (3) reduction condition, 10 μL of 5-fold diluted 2-ME.

The method was qualified by evaluating specificity, accuracy, precision, limit of quantitation (LOQ), and linearity. The method exhibited no interference from sample buffer matrix. The method was found to be linear, accurate, and precise in the range of 0.25–3.0 mg/mL protein concentration. The LOQ of the method was determined to be 0.02 mg/mL for reduced and non-reduced mAb1. In addition, some aspects of sample stability were examined during qualification.

1. Introduction

Immunoglobulin G (IgG) is a major class of antibody molecules and one of the most abundant proteins in the blood serum [1]. IgG consists of two heavy chains (HC) and two light chains (LC) with a molecular weight of approximately 150,000 Da [1]. mAb1 is an engineered human IgG monoclonal antibody that acts as a toll-like receptor three antagonist. The primary clinical indication of interest for mAb1 is asthma, and secondary indications under consideration include sarcoidosis, inflammatory bowel disease, rheumatoid arthritis, and chronic obstructive pulmonary disease. Like any biopharmaceutical products, mAb1 must be well charac-

terized and subject to rigorous analytical testing for release prior to commercialization. High performance size exclusion chromatography (HP-SEC) is a common method for protein characterization based on its molecular weight [2]. However, HP-SEC is low in sensitivity and resolving power for low molecular weight species. In the 1960s, the discovery that the molecular weight of proteins can be determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revolutionized protein characterization and boosted the use of electrophoresis [3,4]. Over the decades, SDS-PAGE has become one of the most extensively used analytical techniques for characterization of complex protein mixtures [5,6]. SDS-PAGE has substantial resolving power with good sensitivity, especially with silver staining technology.

Although fairly sensitive, SDS-PAGE procedure suffers from some limitations including manual operation, long run time, and inaccurate quantification [7]. Capillary gel electrophoresis (CGE) is the automated and instrumental version of slab gel electrophoresis. The advantages of CGE over slab gel electrophoresis are automated sample injection, on-line detection, software-based data processing, high efficiency, and high throughput. Different modes of CE, such as capillary zone electrophoresis, micellar electrokinetic chromatography, and capillary gel electrophoresis (CGE), etc., are available for various applications. CGE has become an impor-

tant separation technique in analytical biochemistry and molecular biology [8]. In 1983 Hjerten successfully transitioned slab gel SDS-PAGE to CGE format (cSDS) using a 150 μm capillary filled with polyacrylamide [9]. Since the introduction of cSDS, numerous applications and developments have been reported [10–12]. The separation mechanism for cSDS is similar to SDS-PAGE except for the sieving gel matrix. For both cSDS and SDS-PAGE, proteins are denatured in SDS to produce SDS–protein complexes before analysis. The formation of SDS–protein complexes is achieved through hydrophobic bindings at a ratio of 1.4 g of SDS per gram of protein [13]. This binding ratio is relatively independent of the protein sequence when its molecular weight is greater than 15,000 Da. As a result, the native charge of protein is mostly masked by the negative charge of SDS, so the SDS–protein can be separated based on the mobility differences [14]. The mobility of SDS–protein complexes is inversely proportional to the logarithm of the effective molecular weight. When a voltage (reverse polarity) is applied to the capillary, all SDS–protein complexes will migrate towards the anode due to their negative charges contributed by SDS.

The replacement of slab gel with cSDS involves a change of sieving matrix. In cSDS, solutions of noncrosslinked linear polymer networks are commonly used due to their low viscosity or high flexibility [15]. These solutions of linear polymers can be replaced after each analysis simply by rinsing the polymer network from the capillary via pressure or vacuum. Replacing these low-viscosity "gels" not only avoids contamination and extends the lifetime of the capillary but also improves precision and robustness of the assay. Many different polymer networks have been used in cSDS applications [16]. Liu et al. at Beckman–Coulter developed a replaceable polymer matrix for an IgG purity assay based on the previous work done by Demorest and Karger [16]. Other polymer matrix based separation gels are also commercially available from some manufacturers. These separation gels are gaining popularity in biopharmaceutical laboratories due to their ease of use, but only limited applications of cSDS for the analysis of monoclonal antibodies have been reported [17–19]. There have been some ongoing efforts in the biopharmaceutical industry to adopt the cSDS technique in quality control (QC) laboratories as a purity assay for therapeutic protein and antibody product release [13]. However, many challenges, such as assay robustness and reproducibility, were encountered during the method validation and implementation using commercially available assay kits [20]. Although other cSDS methods using laser-induced fluorescent detection for protein characterization have been reported [21–24], because of the added complexity of chemical derivatization, those methods are not widely accepted in the QC environment in the biopharmaceutical industry. It is of great interest to further investigate this method and make it more robust, reproducible, and QC friendly.

In this study, the cSDS assay was optimized for the analysis of mAb1 drug substance under reduced and non-reduced conditions. Many of the sample preparation parameters including sample buffer pH, incubation temperature and time, alkylation conditions, and reduction conditions were investigated. The optimal sample preparation conditions were established and the method was qualified for potential use of QC release.

2. Materials and methods

2.1. Reagents and solutions

All chemicals and reagents used in this study were of analytical grade. 2-mercaptoethanol (2-ME), carbonic anhydrase, citric acid monohydrate, iodoacetamide (IAM), iodoacetic acid (IAA), N-ethylmaleimide (NEM), bovine serum albumin (BSA), β-galactosidase and 10% SDS stock solution were purchased from

Sigma–Aldrich (St. Louis, MO, USA). Phosphoric acid (H_3PO_4), methanol (MeOH), sodium phosphate dibasic dihydrate, and sodium chloride (NaCl) were provided by J.T. Baker (Phillipsburg, NJ, USA). Sodium hydroxide (NaOH) solution (0.1 M), hydrochloric acid solution (HCl) (0.1 M), sieving gel, 10 kDa internal standard (5 mg/ml protein in 0.5% SDS, 0.2% sodium azide), and sample buffer (0.1 M Tris–HCl, 1.0% w/v SDS, pH 9.0) were provided by Beckman–Coulter (Fullerton, CA, USA). The Beckman gel buffer consists of 0.2% SDS and a proprietary polymer buffer formulation (at pH 8.0). The SDS-polymer buffer creates a physical gel of an entangled polymer network for separation of the SDS–protein complexes. Water with a conductivity value lower than 5 μS/cm was obtained using a Milli-Q water purification system from Millipore (Billerica, MA, USA). mAb1 formulated bulk (50 mg/mL) and mAb1 research reference standard (10 mg/mL) were manufactured in house (Centocor R&D, Malvern, PA).

2.2. Preparation of solutions

A 0.25 M IAM solution was used as the alkylation reagent. The solution was prepared by dissolving 46 mg of IAM in 1 mL of water. A 0.25 M IAA and a 0.25 M NEM solution were also prepared for comparability studies. Stock solutions of carbonic anhydrase (5 mg/mL), BSA (5 mg/mL), and β-galactosidase (5 mg/mL) were prepared by dissolving 5 mg of respective material in 1 mL water. The 10 kDa internal standard (1 mg/mL) was prepared by diluting the stock standard (5 mg/mL) 5-fold in water. A 2-ME diluted solution was prepared by 5-fold dilution of 2-ME in water. A 0.1 M citric acid was prepared by dissolving 2.1 g of citric acid monohydrate in 100 mL of water. A 0.2 M sodium phosphate stock solution was prepared by dissolving 3.56 g of sodium phosphate dibasic dihydrate in 100 mL of water. Sample buffers at various pH and concentrations were prepared by adding appropriate 0.2 M sodium phosphate and 0.1 M citric acid and 10 mL of 10% SDS stock solution and diluting in water to each 100 mL of the total volume.

2.3. Preparation of samples

mAb1 formulated bulk was diluted to 10 mg/mL with deionized water before subsequent preparation. Samples were diluted to a final concentration of 2.5 mg/mL per the following procedure: 25 μL of the sample solution (10 mg/mL) was combined with 10 μL of IAM (non-reduced) or 2-ME (reduced), 10 μL of 10,000 Da internal reference standard, and 55 μL of the sample buffer to a total volume of 100 μL. A system suitability sample was prepared by mixing 5 μL each of BSA, carbonic anhydrase, and β-galactosidase; 2.5 μL of mAb1 research reference standard; 10 μL of IAM; 10 μL of 10,000 Da internal reference standard; and 67.5 μL of sample buffer. Each of the samples and system suitability solution was mixed, centrifuged at 5000 × g for 20 s and incubated at 65 °C in a water bath for 5 min. Each mixture was cooled to room temperature, centrifuged at 5000 × g for 5 min, and 90 μL was transferred from each mixture to a sample vial for injection.

2.4. Capillary sodium dodecyl sulfate gel electrophoresis (cSDS) analysis

The cSDS experiments were performed on a Beckman Proteome-Lab PA800 CE system equipped with a photodiode array detector and 32 Karat data acquisition software from Beckman–Coulter. Bare fused-silica capillaries (30.2 cm × 50 μm, i.d.) were also supplied by Beckman–Coulter. The detection window was placed at 10.2 cm from the outlet of the capillary. The capillary temperature was maintained at 25 °C for all the experiments. A voltage of −15 kV (reverse polarity) was applied during electrophoretic separations. Samples were injected electrokinetically at −5 kV for 15 s. Detec-

Table 1
Area percentage of IgG and impurity using sample buffers with different pH and varied ionic strength.

Sample buffer	IgG peak area	IgG area (%)	Impurity peak area	Impurity area (%)
HP-SEC[a]	16,474	98.47	140	0.83
100 mM Tris–HCl, 1.0% SDS, pH 9.0 (original Beckman buffer)	51,972	96.35	1227	2.27
100 mM Tris–HCl, 1.0% SDS, pH 7.5 (Beckman buffer with pH adjusted)	17,283	98.34	224	1.27
50 mM citrate–phosphate, 1.0% SDS, pH 6.75	26,126	98.22	319	1.20
75 mM citrate–phosphate, 1.0% SDS, pH 6.75	12,702	98.35	152	1.18

[a] Courtesy of Johnson and Johnson R&D.

tion was performed at 220 nm. Data were collected at a sampling rate of 5 Hz. Corrected areas (areas/migration time) were used for all the calculations. The current was also monitored to ensure that the Joule heating was below the upper limit in an Ohm's law plot.

2.5. Capillary conditioning

New capillaries were conditioned by rinsing with 0.1 M NaOH for 10 min, 0.1 M HCl for 5 min, water for 5 min, and running buffer for 10 min using 70 psi pressure. After the rinse, a voltage of −15 kV (reverse polarity) was applied to the capillary filled with running buffer for 5 min. To ensure reproducibility, between injections, the capillary was rinsed with 0.1 M NaOH for 2 min, 0.1 M HCl for 1 min, water for 1 min, and running buffer for 10 min using 20 psi pressure.

3. Results and discussion

3.1. Effect of sample buffer pH

Sample buffer pH has a great effect on the fluorescent labeling of a monoclonal antibody (for the cSDS analysis) because the derivatization rate is highly dependent on the acid–base properties of the target sites [21,22]. It was therefore necessary to study the effect of sample buffer pH on reduced and non-reduced mAb1 without fluorescent labeling. In addition, from the HP-SEC analysis, the area percentage (A%) for intact IgG analyzed under native conditions (no sample treatment) was 98.5%, and the largest impurity was 0.8%, while the A% of the intact IgG for non-reduced mAb1 obtained from the cSDS analysis using the Beckman sample buffer (pH 9.0) was only 96.4% and the impurity observed in the cSDS analysis was 2.3% (Table 1). The disagreement on the results from these two orthogonal methods indicates that it is of interest to further study whether the impurities were related to cSDS artifacts due to the sample preparation using high pH sample buffer.

In order to analyze intact IgG molecule (i.e., unfragmented and unaggregated), it is necessary to block the free thiol (SH) groups of cysteine to prevent the molecules from forming inter-molecular disulfide bonds and to decrease the production of free light chain and heavy chain fragments from the reduction of the existing disulfide bonds by nearby thiol groups of cysteine residues [25]. Disulfide bonds are usually formed from the oxidation of thiol groups:
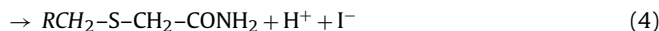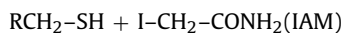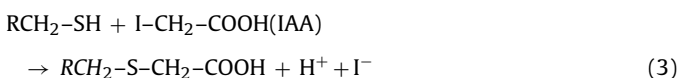
$$2R–SH \rightarrow R–S–S–R + 2H^+ + 2e^- \tag{1}$$
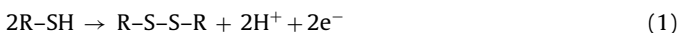
Reversible thiol/disulfide exchange reactions occur by the nucleophilic attack of a thiol on one of the two sulfurs of a disulfide [26]:

$$R–S–S–R + R–'SH \Leftrightarrow R–'S–S–R + R–SH \tag{2}$$
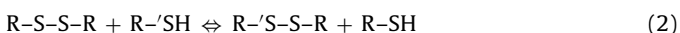
Thiol groups may be blocked by alkylation using alkylating reagents such as iodoacetic acid (IAA, pK_a 3.12), IAM, or N-ethylmaleimide (NEM). IAM is a widely used reagent for alkylation of cysteine [26].

The alkylation reactions with IAA and IAM can be written as:

$$RCH_2–SH + I–CH_2–COOH(IAA)$$
$$\rightarrow RCH_2–S–CH_2–COOH + H^+ + I^- \tag{3}$$

$$RCH_2–SH + I–CH_2–CONH_2(IAM)$$
$$\rightarrow RCH_2–S–CH_2–CONH_2 + H^+ + I^- \tag{4}$$

As can be seen from the reaction schemes above, alkylation and disulfide bond formation are favored under basic conditions. The protonated thiol (–SH) is unreactive, i.e., thiols cannot attack disulfide bonds, only thiolates can. Hence, thiol–disulfide exchange is inhibited at low pH (<7.0) where the protonated thiol form predominates relative to the deprotonated thiolate (The pK_a of a typical thiol group is roughly 8.3.

For cSDS analysis, the alkylation reactions (3) and (4) are preferable, and disulfide bond or thiol–disulfide exchange reactions (1) and (2) are undesirable. For example, a basic sample buffer condition (pH >7.0) will favor both alkylation and disulfide bond formation and thiol–disulfide exchange. On the other hand, an acidic sample buffer (pH <7.0) will prevent disulfide bond formation and thiol–disulfide exchange but may hinder alkylation depending on the reaction kinetics. It was reported that at pH 8.0, the reaction constant of a typical alkylation of thiol with IAM was $4.6 M^{-1} s^{-1}$ [27], while for thiol–disulfide exchange reaction the second order rate was $8600 M^{-1} s^{-1}$ [28]. Because of the slower alkylation reaction rate, using a high concentration of alkylating agent does not ensure adequate suppression of the thiol/disulfide exchange [27]. The large difference on reaction kinetics between alkylation and thiol/disulfide rearrangement indicates that an acidic sample buffer may be used without affecting the alkylation reaction.

To determine the effect of sample buffer pH, mAb1 formulated bulk samples were prepared and analyzed using the following buffers: (1) 100 mM Tris–HCl, pH 9.0 (Beckman buffer); (2) 100 mM Tris–HCl, pH 7.5 (Beckman buffer with pH adjusted); (3) 50 mM citrate–phosphate, pH 6.75; (4) 75 mM citrate–phosphate, pH 6.75. The samples were alkylated using 10 μL IAM and incubated at 65 °C for 2 min. The results are shown in Table 1. By decreasing the sample buffer pH from 9.0 to 7.5, the A% of IgG was increased from 96.4% to 98.3%. It was observed that the peak areas varied when using different buffer solutions. This was due to the difference in conductivity of each sample buffer.

For electrokinetic injection, the amounts (moles) of each ion injected are [29]:

$$Moles\ injected = \mu_{app} \left(E \frac{k_b}{k_a} \right) t\pi r^2 C$$

where μ_{app} ($m^2 V^{-1} s^{-1}$) is the apparent mobility of analyte, E is the applied electric field (V/m), r is the capillary radius (m), C is the sample concentration (mol/m^3), k_b is the conductivity of the running buffer, and k_a is the conductivity of the sample solution. The ions injected into a capillary are inversely proportional to the conductivity of the sample solution.

To confirm the pH effect on purity, more citrate–phosphate buffers were prepared, and the conductivity was maintained at the same level by adjustment with 1 M sodium chloride. When the pH was decreased from 8.0 to 6.5, the A% increased about 0.4%. Further lowering of the pH from 6.5 to 5.5, did not change the A% of IgG significantly. Based on the experimental data, the sample buffer with pH 6.5 was chosen for the future experiments. Under pH 6.5, over

Table 2
Area percentage of IgG and impurity using sample buffers with different pH with the same conductivity.

Sample buffer	IgG peak area	IgG area (%)	Impurity peak area	Impurity area (%)
25 mM citrate–phosphate, 1.0% SDS, pH 5.5	40,638	98.39	408	1.00
25 mM citrate–phosphate, 1.0% SDS, pH 6.0	49,388	98.28	521	1.04
25 mM citrate–phosphate, 1.0% SDS, pH 6.5	49,483	98.30	512	1.02
25 mM citrate–phosphate, 1.0% SDS, pH 7.0	52,678	97.91	688	1.28
25 mM citrate–phosphate, 1.0% SDS, pH 8.0	52,923	97.91	687	1.27
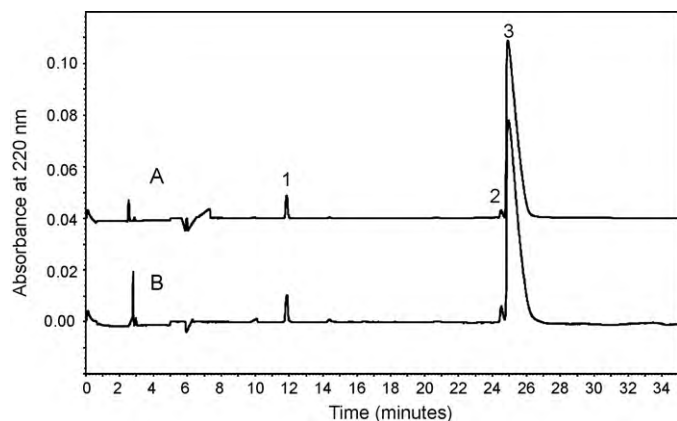
Fig. 1. Effect of sample buffer pH on cSDS separation of non-reduced mAb1. Peak identification: 1 – internal standard with molecular weight of 10,000 Da; 2 – largest impurity peak; 3 – mAb1 IgG. Peaks earlier than peak 1 were from sample buffer blank. Other impurity peaks were not labeled but integrated for total peak areas. A – 25 mM citrate–phosphate sample buffer at pH 6.5. Relative area (%) of IgG (peak 3) was 98.3% and relative area of impurity (peak 2) was 1.0%; B – original Beckman sample buffer (100 mM Tris–HCl, 1.0% SDS, pH 9.0). Relative area (%) of IgG (peak 3) was 96.4% and relative area of impurity (peak 2) was 2.3%. Separation conditions: 30 cm × 50 μm bare silica, electrokinetic injection (−5 kV for 15 s), separation voltage: −15 kV for 35 min.

98% of the thiol (pK_a ∼8.3) groups remain protonated, which will prevent disulfide bond formation effectively. The effect of sample buffer pH on cSDS separation of non-reduced mAb1 is shown in Fig. 1 and Table 2.

3.2. Alkylation and reduction conditions

Common alkylating agents are IAM, IAA, or NEM. The choice of reagent is governed by applications. Although NEM has been reported to have greater reactivity than that of IAM and IAA [27], equivalent results were obtained between NEM and IAM in this study. However, IAA produced more fragments than IAM. The alkylation conditions with IAM were then further examined by incubating mAb1 at 65 °C for 2 min using various volumes of 0.25 M IAM. It was found that the mAb1 purity increased as the volume of 0.25 M IAM increased from 0 μL to 10 μL, which confirmed that blocking thiol groups by alkylation was necessary (data not shown). Further increases in the volume of 0.25 M IAM did not change the

mAb1 purity. Based on these results, 10 μL of 0.25 M IAM was a choice of alkylation condition for this application.

Sample incubation temperature and time were evaluated under reduced and non-reduced conditions. Under reduced conditions, samples were incubated at 65 °C, 70 °C, and 75 °C for 2 min and 5 min. Under non-reduced conditions, samples were incubated at 60 °C, 65 °C, 70 °C, and 75 °C for 2 min and 5 min. Each sample was analyzed in duplicate. The results showed that incubation at 65 °C for 5 min resulted in high purity with little fragmentation for reduced samples. For non-reduced samples, incubation at 65 °C for 2 min and 5 min yielded comparable results based on the t-test analysis (data not shown). Therefore, 65 °C for 5 min was chosen as an incubation condition for both reduced and non-reduced samples.

Reduction conditions were studied using 2-ME as the reducing agent. mAb1 FB was reduced using 10 μL of 2-ME at various concentrations diluted in water. The samples were incubated at 65 °C for 5 min. Adequate reduction was obtained using 10 μL of 5-fold diluted 2-ME, which is equivalent to 2 μL of pure 2-ME.

3.3. Identification of the aglycosylated heavy chain (AGHC)

Aglycosylation of the heavy chain has been previously observed during the analysis of monoclonal antibodies under reduced conditions [30]. Although mAb1 is a glycoprotein and the AGHC could be considered to be part of the product, the amounts of AGHC need to be controlled to ensure product consistency. The aglycosylated heavy chain (AGHC) peak appeared at approximately 56,500 Da and migrated earlier than the native heavy chain. During the method development of cSDS assay for mAb1, a peak prior to the heavy chain was observed in the electropherograms of reduced mAb1 research reference standard samples. This peak was characterized to be an AGHC peak using the experiments described as follows.

Reduced mAb1 heavy chain contains a majority of glycoproteins. If some of the oligosaccharides are removed from the glycoproteins, the deglycosylated protein will co-migrate with AGHC during the cSDS separation. In this experiment, Glyko enzyme peptide N glycosidase F (PNGase F) was used as a deglycosylation agent to remove oligosaccharides from glycoproteins. PNGase F is a specific enzyme that cleaves N-linked glycans from glycoprotein. A PNGase F treated sample was prepared by adding 5 μL of 5 U/mL PNGase F to 100 μL of reduced

Table 3
Identification of aglycosylated heavy chain.

	HC[a] area	HC (%)	AGHC[b] area	AGHC (%)	Theoretical	Recovery
None PNGase treated	32,673	91.71	2956	8.30	NA[c]	NA
PNGase treated	12,578	41.74	17,545	58.27	NA	NA
Spike 10[d]	29,907	86.75	4569	13.25	14.12	93.9
Spike 15	26,309	84.43	4852	15.57	17.03	91.4
Spike 20	27,894	81.96	6141	18.04	19.95	90.5

[a] Heavy chain.
[b] Aglycosylated heavy chain.
[c] Not applicable.
[d] Spike 10, Spike 15, and Spike 20 are spike levels of 10%, 15%, and 20% of AGHC, respectively.

Table 4
Accuracy/linearity data for mAb1 purity under non-reduced conditions.

%Nominal conc.	Theoretical conc. (mg/mL)	Injection #	IgG peak area	IgG (%)	Relative error (%)	r^2 (peak area vs. conc.)
20	0.50	1	7339	97.57	−0.23	0.994
		2	7279	97.93	0.14	
		3	6782	98.28	0.50	
		Average	7133	97.93		
		RSD (%)	4.3	0.4		
40	1.00	1	14,280	97.98	0.19	
		2	14,983	97.99	0.20	
		3	14,362	97.71	−0.09	
		Average	14,542	97.89		
		RSD (%)	2.6	0.2		
80	2.00	1	30,389	97.79	0.00	
		2	30,350	97.92	0.13	
		3	29,455	98.02	0.23	
		Average	30,065	97.91		
		RSD (%)	1.8	0.1		
100	2.50	1	36,955	97.74	NA	
		2	35,540	97.93	NA	
		3	35,616	97.71	NA	
		Average	36,037	97.79 (Theoretical)	NA	
		RSD (%)	2.2	0.1		
120	3.00	1	41,122	97.98	0.19	
		2	36,907	97.82	0.03	
		3	41,652	98.12	0.33	
		Average	39,894	97.97		
		RSD (%)	6.5	0.2		

sample followed by incubation at 37 °C overnight. Upon completion of the incubation, three spiked samples were prepared by adding 10 μL, 15 μL, and 20 μL of the PNGase F treated sample to each non-treated sample. The PNGase treated sample was analyzed along with non-treated and spiked samples. The recovery data and migration time data confirmed that the peak that migrated prior to the heavy chain was from AGHC (see Fig. 2 and Table 3).
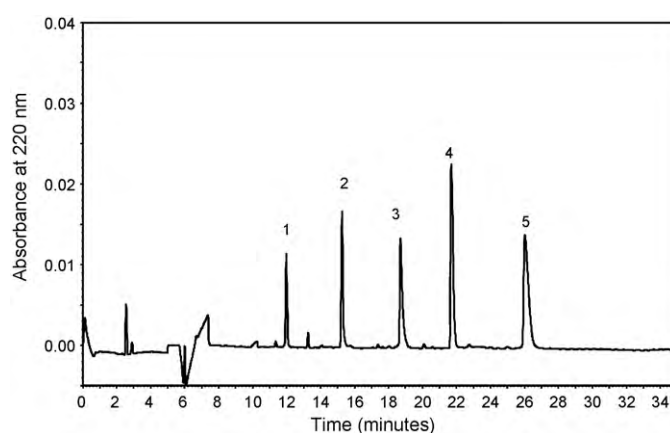
Table 5
Accuracy/linearity data for mAb1 purity under reduced conditions.

%Nominal Conc.	Theoretical Conc. (mg/mL)	Injection #	LC peak area	HC peak area	Purity (%)	HC:LC ratio	Relative error (%)
10	0.25	1	1529	3481	100.0	2.28	0.51
		2	1498	3408	100.0	2.28	0.51
		3	1233	2778	100.0	2.25	0.51
		Average	1420	3222			
		RSD (%)	11.5	12.0			
20	0.50	1	2348	5339	99.76	2.27	0.26
		2	2339	5385	99.78	2.30	0.28
		3	2327	5305	100.0	2.28	0.51
		Average	2338	5343			
		RSD (%)	0.5	0.8			
50	1.25	1	[a]				
		2	7184	16,974	99.54	2.36	0.04
		3	6967	16,459	100.0	2.36	0.51
		Average	7076	16,717	99.80	2.36	
		RSD (%)	NA	NA			
80	2.00	1	10,914	25,270	99.50	2.32	0.00
		2	10,856	25,094	99.51	2.31	0.01
		3	[a]				
		Average	10,885	25,182			
		RSD (%)	NA	NA			
100	2.50	1	13,323	30,374	99.54	2.28	NA
		2	13,315	30,361	99.43	2.28	NA
		3	13,195	30,098	99.52	2.28	NA
		Average	13,278	30,278	99.50		
		RSD (%)	0.5	0.5			
120	3.00	1	15,868	35,899	99.54	2.26	0.04
		2	15,555	35,154	99.44	2.26	−0.06
		3	15,300	34,707	99.62	2.27	0.12
		Average	15,574	35,253			
		RSD (%)	1.8	1.7			
r^2 (peak area vs. conc.)			0.999	0.998			

[a] Missing injection.
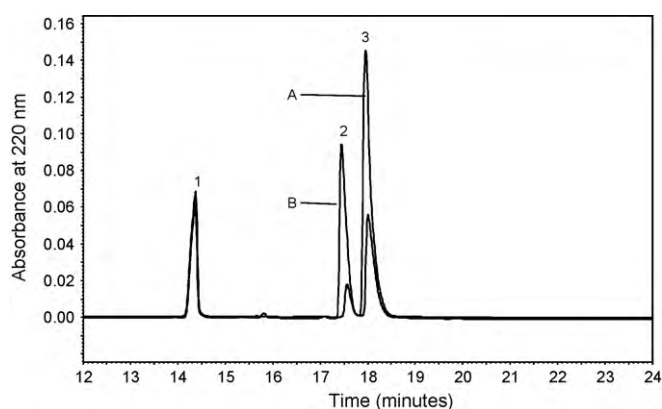
Fig. 2. Comparison of electropherograms of PNGase F treated reduced mAb1 vs. non-treated reduced mAb1. Peak identification: 1 – light chain; 2 – aglycosylated heavy chain; 3 – heavy chain. A – reduced mAb1 not being treated by PNGase F; B – reduced mAb1 treated by PNGase F. Separation conditions: 30 cm × 50 μm bare silica, electrokinetic injection (−5 kV for 15 s), applied separation voltage: −15 kV for 35 min, buffer: 25 mM citrate–phosphate, 1.0% SDS, pH 6.5.

3.4. Performance characteristics

3.4.1. Specificity

Specificity of the test method for mAb1 was demonstrated by investigation of possible matrix interference of the mAb1 formulation buffer. Reduced and non-reduced mAb1 formulated bulk, mAb1 formulation buffer, and water samples were prepared and analyzed. No detectable peaks were found in the formulation buffer or the water, indicating that no detectable interferents were present in the sample matrix. The matrix effect on peak area and relative migration time (RMT) was also studied. The RMT was calculated against the internal standard peak. A mAb1 FB sample was diluted in water and formulation buffer and analyzed under reduced and non-reduced conditions.

3.4.2. Accuracy

Accuracy of mAb1 purity was evaluated by measuring peak areas of IgG at concentrations ranging from 0.25 mg/mL to 3.0 mg/mL, which correspond to 10–120% of nominal concentration (2.5 mg/mL). Appropriate dilutions were made in water from a mAb1 (50 mg/mL) sample. Reduced and non-reduced samples

Fig. 3. Typical electropherogram of system suitability sample. Compound identification: 1 – internal reference standard (10 kDa); 2 – carbonic anyhydrase (31 kDa); 3 – bovine serum albumin (66 kDa); 4 – β-galactosidase (116 kDa); 5 – mAb reference standard (148 kDa). Separation conditions as in Fig. 2.

were prepared and analyzed in triplicate. Accuracy was determined as follows. For non-reduced samples, the IgG purity (%) from each sample concentration was compared to that from the nominal sample concentration of 2.5 mg/mL. For reduced samples, the purity (%) of the heavy chain and light chain at each sample concentration was compared to that from the nominal sample concentration of 2.5 mg/mL. Accuracy was expressed as error% and calculated according to the formula:

$$\text{Error}(\%) = 100(\text{Measured purity} - \text{theoretical purity})/$$
$$\text{theoretical purity at } 2.5 \text{ mg/mL}$$

Data for the accuracy of purity for mAb1 samples are shown in Tables 4 and 5; the error was <0.55% at all concentrations tested.

3.4.3. Precision

Precision was examined in terms of repeatability and intermediate precision. Repeatability was demonstrated by preparing six samples in duplicate from the same lot of mAb1 FB under both non-reduced and reduced conditions. Relative standard deviation (RSD) was calculated on the average values ($n = 6$). For non-reduced samples, repeatability was calculated for the molecular weight, peak

Table 6

Precision data for mAb1 purity under non-reduced conditions.

Day 1	Replicate	MW[a] (kDa)	IgG peak area	IgG (%)
	1	155	46,964	97.87
	2	153	48,090	97.96
	3	153	46,370	98.03
	4	151	48,583	97.77
	5	152	44,479	97.94
	6	149	50,875	97.96
	Average ($n = 6$)	152	47,560	97.92
	RSD ($n = 6$) (%)	1.4	4.6	0.1
Day 2	1	155	52,095	97.95
	2	153	52,437	97.88
	3	152	48,281	97.77
	4	155	36,113	97.56
	5	151	46,124	97.78
	6	149	53,699	97.8
	Average ($n = 6$)	152	48,125	97.79
	RSD ($n = 6$) (%)	1.6	13.6	0.1
Intermediate Precision (2 days)	Average ($n = 12$)	154	47,843	97.86
	RSD ($n = 12$) (%)	1.4	9.7	0.1

[a] Molecular weight.

Table 7
Precision data for mAb1 purity under reduced conditions.

Day 1	Replicate	LC[a]		HC[c]		Purity (%)	HC/LC
		MW[b] (kDa)	LC peak area	MW (kDa)	HC peak area		
	1	26	15,807	62	36,489	98.96	2.31
	2	26	15,919	62	36,670	98.94	2.30
	3	26	15,961	62	36,896	99.02	2.31
	4	26	16,066	62	37,045	99.11	2.31
	5	26	15,436	61	36,025	99.82	2.33
	6	26	15,890	61	36,547	99.11	2.30
	Average ($n = 6$)	26	15,847	62	36,612	99.16	2.31
	RSD ($n = 6$) (%)	0.0	1.4	0.4	1.0	0.3	0.5
Day 2	1	26	17,449	61	40,032	99.21	2.29
	2	26	16,357	61	37,605	99.13	2.30
	3	26	16,431	61	37,557	99.12	2.29
	4	26	16,390	61	37,559	99.27	2.29
	5	26	16,057	60	36,966	99.16	2.30
	6	26	16,194	60	37,331	99.13	2.31
	Average ($n = 6$)	26	16,480	61	37,842	99.17	2.30
	RSD ($n = 6$) (%)	0.0	3.0	0.7	2.9	0.1	0.3
Intermediate precision (2 days)	Average ($n = 12$)	26	16,163	61	37,227	99.17	2.30
	RSD ($n = 12$) (%)	0.0	3.0	1.0	2.7	0.2	0.5

[a] Light chain.
[b] Molecular weight.
[c] Heavy chain.

area, and IgG purity. The molecular weight was determined from the molecular weight standard curve of mobility vs. log (molecular weight) (Fig. 3). For reduced samples, the RSD (%) was calculated on molecular mass (HC and LC), peak area (HC and LC), purity, and the HC/LC ratio. Repeatability data under non-reduced conditions are shown in Table 6. The RSD for the molecular mass, peak area, and IgG purity was 1.4%, 4.6%, and 0.1%, respectively (day 1). Repeatability data for reduced conditions are presented in Table 7. The RSD of the molecular masses for the LC and HC were 0.0% and 0.4%, respectively. The RSD of peak areas for the LC and HC were 1.4% and 1.0%, respectively. The RSD of purity (%) was 0.3%, and the RSD of HC/LC ratio was 0.5% (day 1). The raw peak areas obtained for replicate 1 in day 2 appear to be outliers.

Intermediate precision was determined by performing the repeatability study on a different day using a different instrument and reagents and comparing the results with the first day. For non-reduced sample, the RSD ($n = 12$) of molecular masses, peak areas, and IgG purities were 1.4%, 9.7%, and 0.1%, respectively. For reduced sample, the RSD ($n = 12$) of molecular mass, peak areas, purities, and HC/LC ratios were 0.5% (LC) and 1.0 (HC), 3.0% (LC) and 2.7% (HC), 0.2%, and 0.5%, respectively. Intermediate precision data are shown in Tables 6 and 7.

3.4.4. Limit of quantitation

The limit of quantitation (LOQ) of the assay was determined by measuring the diluted mAb1 as a surrogate for impurity. A mAb (50 mg/mL) sample was diluted 5-fold to obtain the stock concentration of 10 mg/mL. Further dilutions were made to obtain the concentrations from 0.01 mg/mL to 0.5 mg/mL, corresponding to 0.1% to 5% w/w of the nominal sample concentration of 10 mg/mL. Reduced and non-reduced samples were prepared in triplicate and analyzed according to the optimized conditions. The relationship between peak area and theoretical protein concentration was found to be linear for both the non-reduced mAb1 ($r^2 = 0.9969$, $n = 18$), and the light ($r^2 = 0.9959$, $n = 18$) and heavy chains ($r^2 = 0.9998$, $n = 18$) of the reduced mAb1. The observed concentrations of non-reduced IgG, reduced HC and LC were calculated from their regression plots. The accuracy at each concentration was calculated according to the

formula:

$$\text{Accuracy (\%)} = \text{Measured concentration} /$$

$$\text{Theoretical concentration} \times 100$$

The HC and LC theoretical concentrations were based on the ratio of HC/LC = 2.3. The LOQ was determined to be 0.02 mg/mL for both non-reduced and reduced conditions. The LOQ value was based on the acceptable precision (RSD < 5%) and accuracy (90–110%).

3.4.5. Linearity for mAb1

The linearity of the assay for mAb1 was examined using the data from the accuracy study. The linear regression analysis was performed using the peak areas of non-reduced IgG, reduced HC, and reduced LC vs. corresponding protein concentrations. The assay was found to be linear for determining mAb1 purity in the range of 10–120% of the nominal sample concentration of 2.5 mg/mL.

3.4.6. Linearity for impurities

Linearity of impurity was evaluated using the data from the LOQ determination. mAb1 was employed as a surrogate impurity to assess the linearity. The linear regression analysis was performed using the peak areas of non-reduced IgG, reduced HC, and reduced LC vs. corresponding protein concentrations. The assay was found to be linear for determining impurities in the range of 0.1–5% of the nominal sample concentration of 2.5 mg/mL.

4. Concluding remarks

This study optimized the cSDS assay for the analysis of mAb1 drug substance under reduced and non-reduced conditions. Sample preparation parameters including sample buffer pH, incubation temperature and duration, alkylation conditions with iodoacetamide (IAM), and reduction conditions with 2-mercaptoethanol (2-ME) were investigated. It was observed that a slightly acidic sample buffer (pH 5.5–6.5) greatly decreased thermally induced fragmentation of non-reduced mAb1. As such, a citrate–phosphate buffer at pH 6.5 was used for sample preparation to replace the original Beckman sample buffer (pH 9.0). The optimal sample

preparation conditions were established and the method was qualified for potential use in a QC environment.

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