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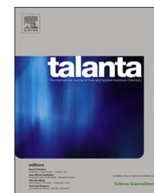

High-precision quantitation of a tuberculosis vaccine antigen with capillary-gel electrophoresis using an injection standard

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ABSTRACT

Analysis of proteinogenic vaccine antigens in a quality control environment requires an accurate, precise, and reliable method for protein separation and quantitation. While having multiple advantages over the classical SDS-PAGE, capillary gel electrophoresis (CGE) has not yet become a standard tool in vaccine antigen analysis. Here we report on development of a CGE-based method for quantitative analysis of a tuberculosis vaccine fusion antigen protein, H4, currently in clinical trials. We demonstrate that our method can monitor antigen purity and relative quantity with greater precision and accuracy versus SDS-PAGE. In addition, due to use of direct light-absorbance detection, the CGE method is suitable for absolute quantitation, an application for which SDS-PAGE is limited due to the need for staining and limited dynamic range of detection. To further improve the performance of our quantitation method, we introduced Bovine Serum Albumin (BSA) as an injection standard to correct for signal variance associated with the injected sample volume. We found that, for our specific application, BSA was more appropriate as an injection standard versus one provided in a commercial kit, in terms of precision and accuracy for quantitation of H4. In addition to providing better method performance versus SDS-PAGE, CGE is also faster and less resource-intensive. We conclude that CGE should be considered as a replacement for traditional SDS-PAGE methods for vaccine antigen quantitation in a quality-control environment.

1. Introduction

Mycobacterium tuberculosis (Mtb) is an infectious bacterium that causes tuberculosis (TB) leading to the highest death rate of all infectious diseases [1]. Mtb usually attacks the lungs, but may also be prevalent in other human organs [2]. The infection is easily spread from an actively infected person by transmission of respiratory fluids as a result of coughing or sneezing. While most infected individuals do not experience symptoms, 1 in 10 of these individuals eventually progress to active infection with a 50% chance of dying when the infection is left untreated [1]. The current available vaccine against TB, *Bacillus Calmette-Guérin* (BCG) vaccine, consistently provides protection against the most severe childhood cases of the infection, but does not provide life-long protection [3]. For this reason there are numerous vaccine candidates being tested in clinical trials to support the development of a booster vaccine in order to continue providing immunity and protection against TB disease [4–6]. One such candidate is the recombinant fusion protein HyVac (H4) (Statens Serum Institut, Copenhagen, Denmark) with a molecular size of 41.3 kDa consisting of protein antigens Ag85B and TB10.4. These two antigens are highly expressed at the early stages of infection by Mtb and are immunogenic in both Mtb positive and BCG vaccinated subjects [7,8]. H4 has been shown to boost a BCG-induced immune response and, thereby, prolong protective immunity against TB [8].

During clinical development, vaccine products are tested for identity, purity, and antigen quantity. Control of these critical quality attributes is important to ensure consistency and quality of the vaccine products from the early stages of development to commercialization. Fast and reliable quantitation of protein antigens in vaccine product stages is critical for product development and quality control. For decades, SDS-PAGE has been the method of choice for protein analysis [9,10]. While SDS-PAGE is attractive as a general separation technique due to its robustness and low cost, it has some limitations due to inadequate quantitative capabilities, longer experimental time, as well

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as offline detection, which contribute to both lowered accuracy and precision [11]. These limitations prompted researchers, specifically industrial, to consider replacing SDS-PAGE with a less experimentally demanding automated counterpart, SDS capillary gel electrophoresis (CGE) [11–13]. As a result of this effort, a number of CGE methods have been developed and validated for vaccine product development and quality control [14–18]. The availability of commercial kits (from Sciex, Agilent technologies and Bio-Rad Laboratories) that include water-soluble linear polymers, such as linear polyacrylamide, used as sieving matrices for CGE, has led to increased reproducibility and robustness of this methodology making it desirable for analysis of proteins in Biopharmaceutical industries. Proteins are reduced with β-mercaptoethanol (BME) and denatured with SDS which imparts a negative charge, with the charge being nearly proportional to their molecular weight (MW). The negatively charged proteins move from the negative to the positive electrode, but with differential retardation by a sieving matrix. Larger proteins move slower than smaller ones allowing for efficient separation of proteins with molecular weights in the range of 10–225 kDa.

Compared to SDS-PAGE, CGE requires less time and provides higher accuracy and precision in quantitation of protein amount. This is mainly due to automation of injection and online detection offered by CGE compared to manual sample injection, staining, de-staining and off-line data analysis (densitometry) with SDS-PAGE [18]. In CGE, though only one sample is analyzed at a time in a single-capillary instrument, multiple samples can be analyzed sequentially in automated systems. For example, automated sequential analysis of up to 24 samples can be executed with the PA800 plus system (Sciex). While advantageous versus manual loading in SDS-PAGE, automated injection in CGE is not as reproducible as injection in Size Exclusion – High Performance Liquid Chromatography (SE-HPLC), which is an industry standard [19]. Lower reproducibility of CGE is especially noticeable for small sample injection volumes [20]. Despite greater injection reproducibility, SE-HPLC suffers from lower resolution than CGE, so both methods are often used as orthogonal modes of separation for protein purity analysis. In industrial settings this limitation of injection volume reproducibility is especially important, as high levels of precision and accuracy are required for routine analysis of products such as vaccine antigens. For increased injection precision in CGE methods, it has been reported by Cianciulli *et al.* that the use of injection standards is beneficial in certain cases [20].

In this report, we discuss our efforts to further improve the accuracy and the precision of CGE in analysis of a vaccine antigen by inclusion of an injection standard. We hypothesized that a universal 10 kDa protein standard used in a commercial CGE kit for MW standardization [21] may not be ideal for use as an injection standard due to it being processed differently from the target analyte during sample preparation. To overcome this problem, we suggest the use of an alternative injection standard that more closely mimics the physicochemical properties of the analyte. We selected BSA as an injection standard and this allowed improved recovery and increased repeatability of the method when the same sample is injected at different times within the run sequence.

In summary, we describe details of CGE method development for both quantity and purity analysis of TB H4 vaccine antigen using BSA as an injection standard. Our results suggest that this new approach of analysis with CGE can serve as a basis for developing highly accurate and precise quantitation and purity tests for protein antigens in vaccine products.

2. Materials and methods

2.1. Chemicals and materials

SDS-MW gel buffer (PN 390953), SDS-MW sample buffer (PN 390953), 10 kDa internal standard (PN A26487), SDS-MW size standard (PN A22196), and bare-fused silica capillary of 50 µm ID (375 µm OD) (PN 338451) were purchased from Sciex (Concord, Canada). Acidic wash solution (0.1 M HCl) and basic wash solution (0.1 M NaOH) were from Fluka (Toronto, Canada). 10 mM Tris-HCl pH 8.3 was prepared from the 1 M Tris-HCl pH 8.3 (PN T1083) from Teknova (Toronto, Canada). β-mercaptoethanol (BME) (PN M6250) was purchased from Sigma (Toronto, Canada). Pierce™ Bovine Serum Albumin standard ampules at 2 mg/mL (PN 23209) were purchased from ThermoFisher Scientific (Toronto, Canada). Ultra-pure filtered water was used for all of the experiments (Millipore, Canada). H4 antigen samples were prepared at Sanofi Pasteur Ltd. (Toronto, Canada).

2.2. Sample preparation

H4 samples were prepared by mixing 45% (v/v) of SDS-MW sample buffer, 2 mg/mL BSA to final concentration of 100 µg/mL, 5 mg/mL 10 kDa internal standard (IS) to a final concentration of 100 µg/mL, H4 protein diluted in 10 mM Tris-HCl pH 8.3 to the desired protein concentration, and 1% (v/v) BME. The mixed solutions were heated at 75 °C for 3 min, cooled to room temperature and centrifuged prior to analysis. The tested H4 samples had a final concentration between 15 µg/mL and 160 µg/mL. SDS-MW size standard was prepared as suggested by the SDS-MW analysis kit (Sciex) protocol except that 1% (v/v) BME instead of the suggested 5% (v/v) BME was used for reducing proteins; samples were heated for 3 min at 75 °C after the addition of BME. For purity analysis, H4 samples were prepared in the same manner, but without the addition of BSA or IS as injection standards. The tested H4 samples had a final concentration between 70 and 1000 µg/mL. For the BSA spiking experiment, 1000 µg/mL BSA solution was prepared by diluting a 2000 µg/mL BSA stock solution with 10 mM Tris-HCl buffer (pH 8.3). Using the 1000 µg/mL BSA Solution, different concentrations of BSA (0, 7, 13, 33, 67, 100, 133 and 167 µg/mL) were spiked into H4 samples to a final protein concentration of 650 µg/mL.

2.3. Instrumentation and the CGE method

All experiments were performed on a Sciex PA 800 plus (formerly Beckman Coulter) CE system with a photo diode array detector (Sciex, Concord, Canada). Data processing was performed by the 32Karat software provided with the CE system. Separation of the proteins was performed in a 50 µm ID bare-fused silica capillary with a total length of 30.2 cm (20 cm effective length); the negative electrode was at the inlet of the capillary. One capillary was used for Intermediate precision determination. The CGE method for preconditioning of the capillary was performed as per instructions of the SDS-MW kit (Sciex) with 0.1 M sodium hydroxide, 0.1 M hydrochloric acid, water, and SDS-MW gel buffer, at 70 psi (483 kPa) for 3, 1, 1, and 10 min, respectively. Hydrodynamic injection of the samples was performed by using 0.7 psi (4.8 kPa) for 240 s, while electrokinetic injection was done at an electric field of 170 V/cm for 20 s. Unless otherwise stated, electrophoretic separation was carried out at 500 V/cm for 30 min at 25 °C, with the sample-storage garage temperature set to 10 °C (the minimum temperature that can be maintained reliably). Absorbance signals were recorded at 220 nm.

2.4. Data analysis and reporting

To increase the precision of the method and control for run-to-run variability of the amount of sample injected, H4 samples were analyzed in the presence of 100 µg/mL BSA used as a normalization and injection standard. For the relative quantity assay, the reportable value for H4 was recorded as the velocity-corrected peak area of H4 divided by the velocity-corrected peak area of BSA. From this point onward within the document, velocity-corrected peak area will be referred to as

peak area. The molecular size of the analyzed proteins was assigned by using the SDS-MW size marker and the Quality function in 32Karat software (compared against a calibration curve of log MW vs. migration time) where the migration time of different sized proteins is proportional to the log of the molecular weight. Robustness was assessed from the multifactorial method development experiments by varying sample preparation parameters: heating temperature, heating time, and sample buffer volume, as well as CE method parameters: capillary temperature, injection time, injection pressure, separation voltage, and separation pressure. The method repeatability and linearity were determined based on 5 concentration levels (15, 35, 75, 105, and 135 μg/mL H4). An H4 concentration of 20 μg/mL was selected as limit of quantitation (LOQ), since it is the lowest amount of analyte which can be quantitatively determined with suitable precision and accuracy (precision 1.9% and accuracy 101.9%).

Relative recovery calculation:

Relative Recovery(%) = (Mean H4 reportable value from 3 determinations/ Theoretical H4 reportable value) × 100,

example of Theoretical H4 reportable value calculation at 40% target load:

Theoretical H4 reportable value = Mean H4 reportable value from 3 determinations at 100% target load × 0.4

Relative purity:

Relative Purity = (Target antigen peak area/ total integrated peak area in the electropherogram) × 100

3. Results and discussion

3.1. TB antigen quantitation

In our laboratory, SDS-PAGE was used for quantitation of H4 vaccine antigen relative to an in-lane normalization standard, BSA (results not shown). We aimed to assess the applicability of the CGE method, typically used for the analysis of monoclonal antibodies, to the analysis of purified TB vaccine antigen H4. While the CGE method is faster and more robust than the alternative SDS-PAGE method, an inherent issue with any CE method is the irreproducibility of small-volume injection. This challenge is typically overcome by introducing an injection standard into the sample. Here we tested both the 66.4 kDa BSA and the 10 kDa Internal Standard (IS) which is provided with the SDS-MW kit (Sciex, Concord, ON). Reproducibility of electrokinetic injection (as recommended by the kit manufacturer) can be affected by variations in sample conductivity due to differences in sample composition; therefore, for relative quantitation method we used hydrodynamic injection by pressure pulses of 0.7 psi (4.8 kPa) × 240 s. To prevent degradation prior to analysis, samples were stored in a cooling garage inside the CE instrument at 10 °C. Analyses were conducted under separation conditions recommended by the SDS-MW kit manufacturer.

We first studied how redox conditions influenced the quality of analysis. Electropherograms obtained with the reduced H4 sample showed a single peak with a migration time of approximately 16 min (Fig. 1), likely corresponding to monomeric H4. Under non-reducing conditions, H4 oligomerizes resulting in appearance of peaks with migration times greater than 20 min along with a lower amount of the H4 monomer peak at 16 min. This result suggests that the H4 sample should be fully reduced for high-precision CGE analysis; therefore, all further experiments were performed under reducing conditions.

We then compared IS and BSA as injection standards in quantitation of H4 by CGE. A target load of 100 μg/mL of H4 is defined as 100% and a peak area for this concentration was determined. Then CGE analysis was performed in triplicate with 100 μg/mL of each 10-kDa IS and BSA for 5 concentrations of H4: 40, 70, 100, 130, and 160 μg/mL (Fig. 2**A**). Recovery values were calculated as the ratio between peak area for a given H4 concentration (either corrected with the injection standard or not) and peak area for 100 μg/mL H4 (either corrected with the injection standard or not). Relative recoveries of H4 for all conditions and H4 concentrations studied ranged between 92% and 104% (Fig. 2**B**), which was within the desired range of 90–110% [19]. While the recoveries were acceptable for all conditions, there was a systematic bias – the dependence of the relative recovery on H4 concentration (slope = 0 is expected for absence of bias). The magnitude of this bias is reflected by the slope of a straight line used to fit the dependence. The bias was significantly greater for non-corrected and IS-corrected data (slopes of 0.10 and 0.09, respectively) than for BSA-corrected data (slope of 0.03), indicating that BSA was a better injection standard than the 10-kDa IS for accurate recovery. In addition to improving accuracy, BSA-corrected data improved repeatability compared to non-corrected data; RSD for the corrected data was 1.7% while for non-corrected data was 4.3% (n = 3). It is worth mentioning that IS-correction, while being inefficient for improving recovery accuracy, improved repeatability to approximately the same level as BSA-correction.

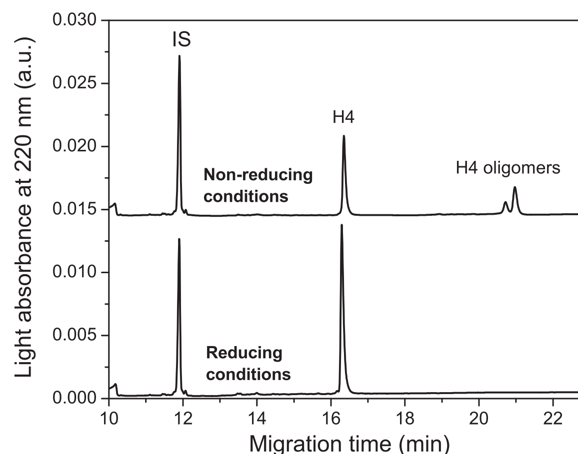


Fig. 1. CGE analysis of 100 μg/mL H4 (41.3 kDa) solution in the presence of 100 μg/mL 10-kDa IS under non-reducing (top trace) and reducing (bottom trace) conditions. The top trace is offset vertically for clarity of presentation. Reducing conditions were created by adding to the sample 1% (v/v) BME and incubating for 3 min at 75 °C; for the non-reducing conditions BME was replaced with the SDS sample buffer. The samples were stored in an instrument cooling garage at 10 °C. Injection into a capillary was done by pressure pulse of 0.7 psi × 240 s. Separation was carried out at 500 V/cm with a negative electrode at the inlet and a capillary temperature set to 25 °C.

While the exact reason for an overall better performance of BSA than IS as an injection standard is not known, both BSA and H4 are somewhat larger, folded proteins, tend to form oligomers and contain multiple disulfide bonds requiring the use of a reducing agent in the CGE analysis. Thus BSA may have more similar properties to the H4 analyte tested versus IS, which is desirable for an internal protein standard [20]. BSA is also reliable and convenient as an injection standard since it is stable at room temperature and is commercially available as a precisely formulated solution (2.00 ± 0.03 mg/mL, calibrated by direct comparison to purified BSA from the National Institute of Standards and Technology). BSA is available in single-use vials and as such, it is recognized as the industry standard for protein quantitation [22]. Similar to H4, BSA contains disulfide bonds, that are disrupted in the presence of the reducing agent BME. IS, whose sequence is not provided by the vendor, does not appear to have disulfide bonds as demonstrated by lack of mobility shift under reduced and non-reduced experiments (Fig. 1). Only one peak was observed for BSA, while for IS, more than one peak was detected by CGE. Upon degradation, H4 may produce impurities that can overlap with IS peak. Unlike IS, BSA is larger than H4 so it does not interfere with the H4

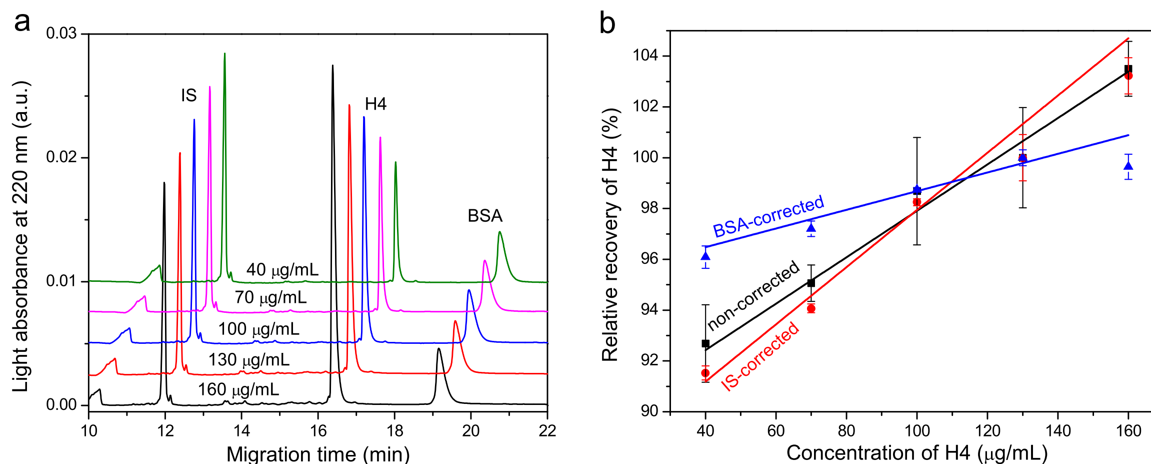


Fig. 2. Effect of an injection standard (either 10-kDa IS or BSA) on the accuracy of relative recovery of H4. Panel **a** shows representative CGE electropherograms of samples containing 100 μg/mL of each 10-kDa IS and BSA and varying concentrations of H4 (shown in the panel). The traces are offset vertically and horizontally for clarity of presentation. Peak areas from such electropherograms were used to create panel b. Panel **b** shows the influence of a method of data processing (non-corrected, IS-corrected, and BSA-corrected peak areas of H4) on the dependence of relative H4 recovery on H4 concentration. Point and error bars correspond to averages and respective standard deviations for a set of 3 experiments.

endogenous impurities.

Our results show that the use of BSA as a standard compensates for variation in the injected volume when different antigen amounts are tested, but also improves the repeatability of the method, i.e. the run-to-run variance of the same sample tested on the same day. Run-to-run variance could arise from differences in capillary preconditioning, sample adhering to the capillary, or reduced sample stability in the sample compartment while awaiting injection in the run sequence. To further demonstrate that BSA improves the precision of the method due to compensating for variances in the order of the sample injected in a sequence of runs, two samples of 100 μg/mL H4 were prepared: one containing 100 μg/mL BSA and another without BSA. Each sample was injected in three consecutive runs at the beginning, middle and end of a sequence, while different concentrations of H4 in the presence of 100 μg/mL BSA where tested in between (results not shown). Correction of H4 peak area by normalizing it to the BSA peak area improves the precision of the method throughout the sequence of experiments as indicated by the low RSD values of 0.4%, 0.6% and 0.3% (for the beginning, middle, and end of a sequence of experiments, respectively). By contrast, samples analyzed without normalization of H4 with BSA resulted in higher RSD values of 1.1%, 0.8% and 1.0%, respectively. Accuracy was marginally improved with BSA normalization, resulting in a relative recovery range of 98–101% compared to non-normalized recoveries of 97–103%, with a higher precision for BSA normalized results as indicated by the lower RSD values as stated above. These results show that inclusion of BSA as an injection standard improves both accuracy and precision of the method due to compensation for variances in sample injection that arise from: (i) different concentrations of H4 and (ii) different order of the sample tested in a sequence of experiments.

3.2. Calibration curve for H4 absolute quantitation

Due to its limitations, SDS-PAGE is currently used only as a relative quantitation method for H4 stability analysis. As an alternative to SDS-PAGE, we have developed a method for accurate quantitation of H4 in μg amounts by using CGE with an H4 calibration curve. As we have demonstrated that there was greater precision using BSA as an injection standard, 100 μg/mL BSA was used for H4 peak area normalization for building a calibration curve (Fig. 3) as well as for H4 peak area normalization in the analysis of test samples. A 5-point calibration curve ranging from 15 to 135 μg/mL H4 prepared in duplicate was used to determine the concentrations of four H4 samples obtained by dilution of the stock solution (concentration range from 20 to 110 μg/mL). The concentrations were determined with high accuracy as indicated by the relative recovery values ranging between 99% and 101% with RSD values below 3% for $n = 2$ (Table 1).

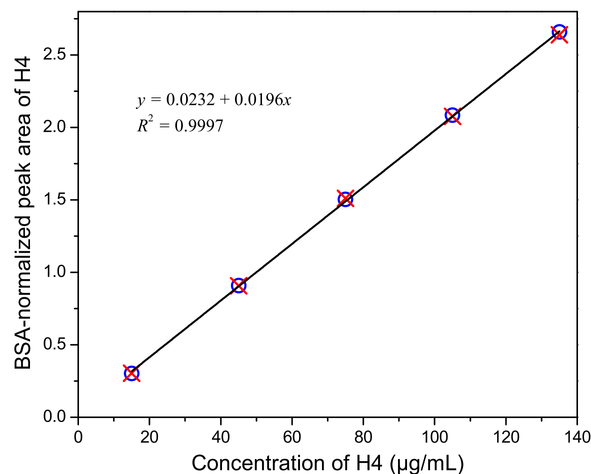


Fig. 3. A 5-point calibration curve for determination of H4 concentration by using a BSA (100 μg/mL)-corrected peak area of H4. Measurements were conducted in duplicates shown by 5 open circles and 5 crosses. The line is the best fit of the 10 experimental points with a linear function; the inset shows the fitting parameters.

Table 1
Expected and measured H4 concentrations for a set of dilutions of the stock H4 solution. Expected concentrations are obtained by dividing the stock concentration by a dilution factor. Measured concentrations were determined by using a calibration curve shown in Fig. 3. Relative recovery values were found by comparing measured concentrations to expected concentrations.

Expected H4 concentration (μg/mL)	Measured H4 concentration (μg/mL)	Relative recovery (%)	RSD (%, n = 2)
20.00	19.73	98.63	1.46
50.00	50.44	100.89	2.58
95.00	96.23	101.30	2.29
110.00	110.99	100.90	1.94

3.3. Optimization of the CGE quantitation method

Using full factorial Design of Experiments (DoE) we have optimized the CGE separation of H4 antigen for precise and accurate quantitation

of the protein for stability monitoring. Factors such as electric field used for separation, capillary temperature, and the amount of SDS-MW sample buffer were investigated. The parameters tested were electric fields of 430, 630 and 230 V/cm; capillary cartridge temperatures of 20, 25, and 30 °C; and SDS-MW sample buffer amounts of 30%, 57%, and 83% (v/v). The experiments were performed with 100 and 20 µg/mL H4. Targeted responses such as accuracy (recovery of the 20 µg/mL H4 relative to 100 µg/mL), precision of the H4 to BSA peak area ratio (as a coefficient of variation, CV, n = 6), precision (CV) of migration time, and peak area for both H4 and BSA at 20 and 100 µg/mL H4 were measured and assessed to determine the effect of the different conditions on the H4 analysis. All experiments included co-injection of 100 µg/mL BSA with H4.

Our experiments showed good method robustness with respect to changes of the varied experimental conditions; the results did not change drastically with changing experimental conditions within the specified ranges of their variation. We, nevertheless conducted formal optimization to find the optimum set of parameters even if the improvement was minor. The optimal method as interpolated from the results is one that utilizes 45% (v/v) SDS-MW sample buffer, separation with 465 V/cm and a capillary temperature of 23 °C. Comparing the optimized method with the original method, where separation was carried out at 500 V/cm; and a capillary temperature of 25 °C, the optimized method showed improved resolution and baseline separation between the H4 main peak and the smaller-size impurities (Fig. 4).

The optimized CGE H4 quantitation method with a lower capillary cartridge temperature and a lower separation voltage improved the resolution of the main H4 peak relative to the lower size impurity peak, but increased the run time by two minutes. Due to the colder capillary temperature, the injection volume of the sample might be slightly lower as inferred from a slight decrease in the signal intensity for the protein peaks. The final optimized method with 23 °C capillary cartridge temperature, and 465 V/cm electric field, with samples solubilized in 45% (v/v) SDS sample buffer, was shown to improve the resolution of the protein peaks, thus, improving peak detection, integration, and quantitation. Precision (CV) values for the repeatability (n = 6) and intermediate precision (n = 9) of the method were determined to be less than 2%.

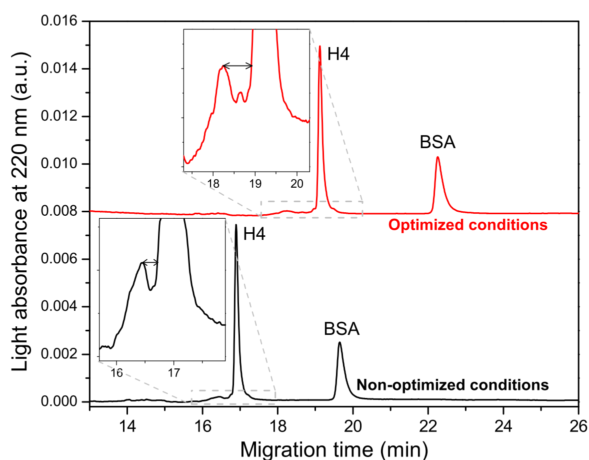


Fig. 4. Optimization of the CGE-based determination of relative quantity of H4. A solution containing 100 µg/mL of each H4 and BSA was prepared by solubilizing the proteins in 45% (v/v) SDS sample buffer and 1% (v/v) BME. It was heated at 75 °C for 3 min. Samples were injected using by pressure pulses of 0.7 psi × 240 s. Non-optimized conditions were: capillary temperature of 25 °C and electric field of 500 V/cm. Optimized conditions were capillary temperature of 23 °C and electric field of 465 V/cm. The resolution between the minor peak and H4 peak with the optimized conditions was 2.5, while the resolution between these peaks with the non-optimized conditions was 1.1. The improvement of resolution is minor but, statistically significant.

3.4. Antigen purity assessment

In addition to establishing a method for determining antigen quantity, the feasibility of using CGE for analysis of H4 vaccine antigen purity, a critical quality attribute, was also examined. A faster, more accurate and robust CGE purity method compared to traditional SDS-PAGE, would be an asset in a quality control environment. Using a similar approach to the one described above for H4 antigen quantitation, we have developed a CGE method for determining the purity of the H4 as the main component of the vaccine product.

For the purity assay, electrokinetic injection was used, since this method has higher injection efficiency and resulted in higher signal intensity versus hydrodynamic injection. For the purity assay, it was important to increase the LOQ of the H4 peaks detected to ensure detection of all the impurities present in the sample. Electrokinetic injection showed high reproducibility as discussed below.

3.5. Optimization of the CGE purity method

Using full factorial DoE, we have optimized the CGE separation of the H4 antigen for precise purity assessment of the protein. In order to accurately quantitate not only the target protein but also potential product and/or process-related impurities such as host cell proteins or degradation products, higher method sensitivity and dynamic range are required. For this reason, the factors of injection time (10 and 40 s), electric field used for electrokinetic injection (65 and 330 V/cm), capillary temperature (20 and 30 °C), and electric field used for separation (330 V/cm and 1 kV/cm) as well as sample-heating time (5 and 15 min) and temperature (65 °C and 100 °C) were included in the full factorial design. DoE experiments were performed at two levels. At level one (L1), H4 test samples had no BSA spiked (H4 total concentration in CE vial was 650 µg/mL). At level two (L2), H4 test samples were spiked with BSA to a total protein concentration of 650 µg/mL. The BSA spike concentration was calculated in order to achieve BSA relative peak area of 10% which was equal to 123 µg/mL BSA concentration. The BSA spike was used as a mock impurity, co-injected with H4 during DoE analysis to assess the ability of the method to detect an impurity at a level of 10% of total protein. The influence of different factors was investigated on various method responses including H4 relative purity (reportable value) at L1 and L2, reportable value precision at two levels, peak resolution and BSA relative peak area at L2. Peak resolution was measured between the H4 and the BSA peaks. Based on the DoE results, conditions predicted (using the DoE model) to have minimum %CV with a reportable value for purity ≤ 92% were chosen. A purity level of 92% was selected as the upper limit since it was observed from experimental results that the conditions with greater than 92% relative purity had lower sensitivity with respect to detecting impurities. The optimal method as determined from the DoE employed the following conditions: 45% (v/v) SDS sample buffer, heating at 69 °C for 6 min, electrokinetic injection with 130 V/cm for 40 s, capillary cartridge temperature of 24 °C, and separation electric field of 530 V/cm.

Using the optimized purity method, BSA amounts which resulted in a relative peak area of 0.5% to 13% of the total area of all peaks in the electropherogram were spiked into H4 samples (Fig. 5) and the accuracy, linearity, and overall method precision were assessed. As BSA and H4 had different peak area responses, it was important to take this into account when calculating the theoretical relative peak area (purity) of H4 upon spiking BSA. For this purpose, the ratio of peak area per µg/mL for each protein was calculated. The peak area per µg/mL of H4 was calculated using results obtained from nine independently prepared replicates (no BSA spike). In this series of experiments, determinations were performed by two different analysts: analyst 1 performed three determinations on day 1, and analyst 2 performed three determinations on day 2 and three determinations on day 3. For BSA, the peak area per µg/mL was calculated using 100 µg/mL BSA

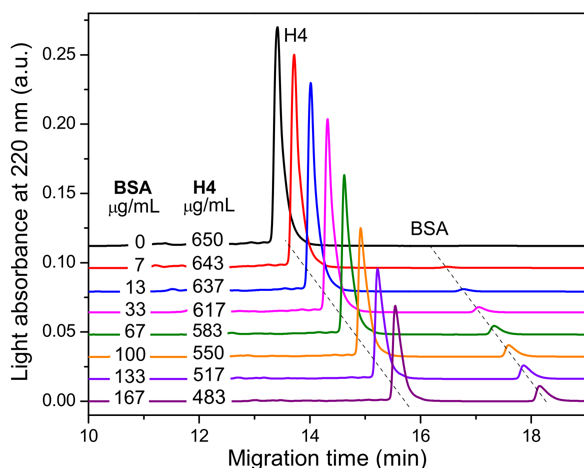


Fig. 5. Electropherograms for assessment of accuracy, linearity, and overall method precision. BSA was spiked into H4 solutions to mimic impurity with relative peak area ranging from 0.5% to 13% of the total area of all peaks in the electropherogram. The sum concentration of H4 and BSA was maintained at 650 µg/mL; individual concentrations for every sample are shown in the plot.

solution (no H4). The results for BSA were obtained from six independently prepared replicates by analyst 2 on day 4. The peak area per µg/mL for H4 main peak, H4 impurities and BSA peak were 59.533, 7.165 and 21.595, respectively. These values were utilized to calculate H4 relative purity and BSA relative peak area at different H4 and BSA concentrations. The BSA and H4 theoretical relative peak area at each level as well as theoretical H4 relative purity were calculated as follows:

$$Theoretical\ BSA\ peak\ area\ (at\ each\ level)\ =\ BSA\ concentration\left(\frac{\mu g}{mL}\right)\times BSA\ peak\ area\ per\ \frac{\mu g}{mL} \quad (1)$$

$$Theoretical\ H4\ main\ peak\ area\ (at\ each\ level) = H4\ concentration\left(\frac{\mu g}{mL}\right)\times H4\ main\ peak\ area\ per\frac{\mu g}{mL} \quad (2)$$

$$Theoretical\ H4\ impurities\ peak\ area\ (at\ each\ level) = H4\ concentration\left(\frac{\mu g}{mL}\right)\times H4\ impurities\ peak\ area\ per\ \frac{\mu g}{mL} \quad (3)$$

$$Total\ theoretical\ peak\ area\ =\ Theoretical\ BSA\ peak\ area + Theoretical\ H4\ main\ peak\ area + Theoretical\ H4\ impurities\ peak\ area \quad (4)$$

$$Theoretical\ H4\ relative\ purity = \frac{Theoretical\ H4\ main\ peak\ area}{Total\ theoretical\ peak\ area}\times 100 \quad (5)$$

Linearity of both, the H4 relative purity and BSA relative peak area responses were investigated. For each replicate, the experimentally determined relative purity of H4 or BSA relative peak area values were plotted against the theoretical values and analyzed by linear regression. Both plots show high linearity with $R^2 \geq 0.99$ (Fig. 6).

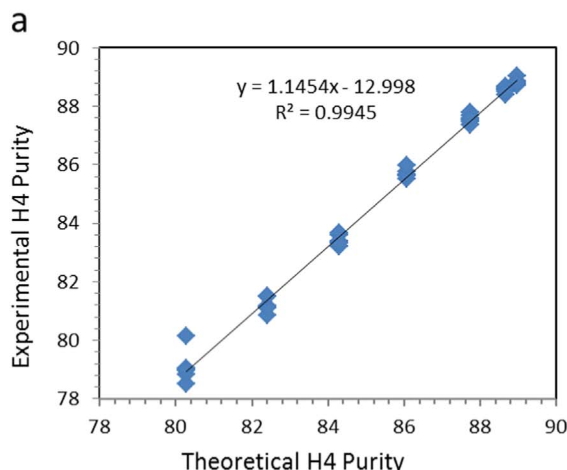
H4 relative purity by CGE was determined to be 89% with repeatability (CV) of 0.14% (n = 6), and intermediate precision (CV) of 0.13% (n = 9). By comparison, SDS-PAGE-determined relative purity of H4 from the same lot was 93% (Fig. 7). This purity decrease may be due to the different detection modes of Coomassie staining for SDS-PAGE and measuring the absorbance at 220 nm for CGE. This could lead to underestimation of contributions from minor impurities and, consequently, overestimation of H4 purity by SDS-PAGE. A reproducible profile of distinct low intensity impurity bands below the main H4 band was observed on SDS-PAGE (Fig. 7).

In addition, the CGE method was accurate within the tested range of H4 concentrations with a H4 purity value relative recovery (purity value relative recovery = Experimental H4 relative purity /Theoretical H4 relative purity × 100) of 100% for 650 µg/mL H4 and CV value of 0.1% (n = 6, intermediate precision), and 99% for 483 µg/mL H4 concentration (spiked with 167 µg/mL BSA) with a CV value of 0.7% (n = 6, intermediate precision).

BSA (66.4 kDa) does not interfere with the H4 antigen main peak (41.3 kDa) or with detected impurity peaks, and we have shown that amounts as low as 0.5% BSA co-injected into the capillary with H4 are consistently detected and quantitated using the developed CGE method. Thus, BSA is suitable as a mock impurity.

4. Conclusions

CGE is a well-established analytical method used in many QC laboratories for over a decade [18]. In this study, we applied CGE to quantitation and purity determination for the investigational TB vaccine antigen protein, H4. We have introduced the use of BSA as an injection standard, to ensure high precision and accuracy of vaccine antigen analysis. The developed CGE H4 quantitation method was



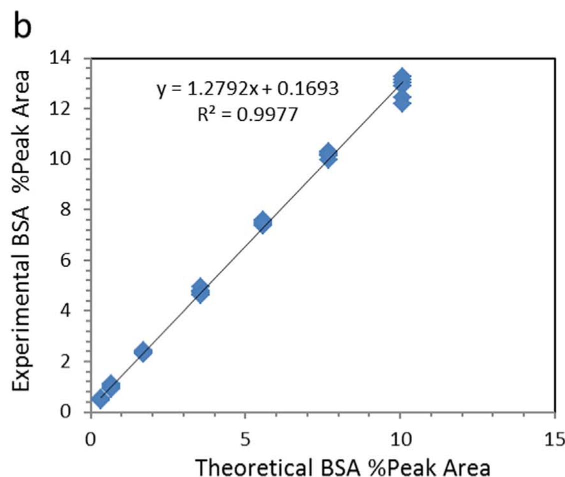


Fig. 6. Demonstration of method linearity: experimental *versus* theoretical H4 relative purity (a) and experimental *versus* theoretical BSA relative peak area (b). Both plots show high linearity with $R^2 \geq 0.99$.

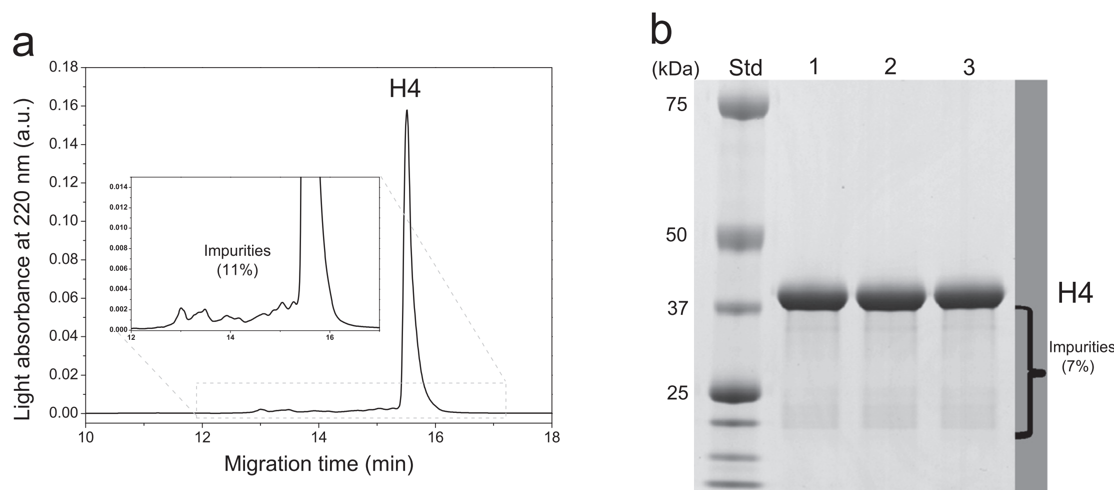


Fig. 7. Comparison of CGE (a) and SDS-PAGE (b) purity assays. CGE analysis was carried out under the following conditions: injected with 130 V/cm for 40 s, capillary cartridge temperature of 24 °C, and separation electric field of 530 V/cm, sample cooling garage temperature set to 10 °C and H4 concentration of 650 µg/mL. The inset shows the zoomed-in impurity region. SDS-PAGE conditions were: H4 load of 5 µg into lanes 1–3, Coomassie staining for visualization.

shown to be robust and accurate with an intermediate precision (CV) of 2% compared to 6% when using SDS-PAGE for relative protein quantitation. Due to the high precision of the method, we were also able to use the CGE method for protein quantitation in µg amounts when applying a calibration curve with relative recovery values of 98–101% for antigen concentrations between 20 and 110 µg/mL. Furthermore, due to its lower LOQ relative to the traditional SDS-PAGE method, the CGE method is more accurate for protein purity assessment. The ability to measure stability of vaccine antigens is highly important to establish a product expiry date and for this reason a rapid, reliable quantitation method such as CGE with high accuracy, precision, and specificity is ideal for testing of vaccine antigens during vaccine development and clinical trial testing. With the developed CGE method we are able to replace the existing SDS-PAGE assay for assessing product stability and purity. In addition, the approach of using BSA as an injection standard will be transferable to analyses of other vaccine antigens and potentially complex protein mixtures with molecular weights being either smaller or larger than the molecular weight of BSA (66.4 kDa).

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