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Development of Quantitative Residual Acetate and Phosphate Assay in Multi Vaccine Products Using Capillary Zone Electrophoresis

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ABSTRACT

Some vaccine process manufacturing techniques require the use of acetate (Ac) and phosphate (Pi) containing buffers to complete biochemical reactions prior to downstream product purification. These two anions must be removed from the final bulk product, which necessitates the need for an assay to monitor Ac and Pi reduction throughout. Due to the separation speed, low volume consumption, and resolving power, a capillary electrophoresis (CE) method has been chosen to separate and quantitate the levels of these anions in final product and in-process intermediate samples. Furthermore, this optimized CE method has been successfully qualified and is routinely used to support vaccine process development.

INTRODUCTION

The advent of complex vaccine manufacturing technologies in vaccine development has presented new and unique analytical challenges that require rigorous analytical characterizations in addition to the standard release and stability assays [1].The World Health Organization (WHO) and Pharmacopoeia institutes provide guidance and recommendation of type of the release, stability, and characterization testing for almost all vaccine products [2,3].Of these, characterization and clearance confirmation of in-process residuals is important to the manufacturing and release of a safe and effective vaccine drug product. There are only limited publications regarding process residuals analysis in vaccine product manufacturing [4,5]. Downstream purification of complex vaccines is a multi-phase process that involves purification of the vaccine manufacturing product and formulation in final alum-based adjuvant. During the manufacturing and purification processes several chemicals are introduced, of which some need to be removed before the next step. Acetate and phosphate anions are two of the chemical additives that are required to be monitored during the purification process and in the final product of some complex vaccines before formulation. Furthermore, these two anion residuals could potentially be used as surrogate clearance for other small anion molecules. Starting material monitoring and in-process residual characterization using HPLC based methods alone or in combination with mass spectrometry (LC-MS) is well documented in pharmaceutical manufacturing [6,7].To the best of our knowledge, there is only one report available regarding characterization of residuals using capillary electrophoresis (CE)-based methods in vaccine development [8]. A CE-based method provides a fast separation time, high resolving power, low sample consumption, and very low waste generation. The most common CE technique used for anion separation is capillary zone electrophoresis methods (CZE) with indirect analyte UV detection [9-11]. The method discussed here uses commercially available anion separation kit CElixir™ OA from Microsolv Technology Corp. CElixir™ OA uses a patented dynamic capillary coating system to achieve a highly reproducible separation of anions at a pH of 5.4 [12-14].

In this paper, we report the use of this CElixir™ technology to separate and optimize the quantitative aspect of residual acetate and phosphate in multi vaccine products requiring the use of acetate and phosphate to facilitate biochemical reactions during the manufacturing. We also examine how this method is used both for in-process monitoring of acetate and phosphate and its use as a product release test. Furthermore, we discuss the evaluation of various analytical performance characteristics, such as linearity, accuracy, and the limit of quantitation (LOQ) and demonstrate the methods capability for validation.

MATERIALS AND METHODS

Reagents

The CElixir™ OA, pH 5.4 CZE kit was obtained from Microsolv Technology Corporation (Eatontown, NJ, USA). Phosphate and acetate standards were acquired from Fluka Analytical, a Sigma-Aldrich brand (St. Louis, MO, USA). Solutions of hydrogen bromide and sodium hydroxide for use in capillary regeneration were made using materials from Sigma-Aldrich (St. Louis, MO, USA).

Sample Types

Various vaccine intermediates were obtained for method development. The samples were purified using various chromatography steps by the Vaccine Process Research and Development group at Merck Research Laboratories (Merck & Co. Inc, West Point, PA, USA). Final aqueous product (FAP) samples were tested as well as in-process intermediates of various vaccine manufacturing products

CZE Procedure

All runs were conducted on either Beckman Coulter PA800 or P/ACE MDQ capillary electrophoresis instruments (Beckman Coulter, CA, USA). The CZE separation was conducted using a 60.2 cm length x 75 µm IDx375 µm OD bare-fused silica capillary (Beckman Coulter, CA, USA). The capillary length to the detector (Ld) is 50.2 cm. The capillary first is coated with a poly-cation (initiator), followed with a background electrolyte containing UV absorbing material at 254 nm (accelerator), and the sample is injected hydrodynamically at 1 psi for 15 seconds with a short water plug after it. The separation occurs for 7.0 minutes with 30.0 kV reverse polarity applied to the anode and cathode, the typical observed current is -4uA. The diode array detector (DAD) is employed for peak detection using 8 mm aperture. The capillary wall was regenerated after each separation by washing with a 0.1M solution of NaOH, H_2O, 0.1 M solution of HBr, H_2O for 3 min per cycle throughout instrument run sequences.

Standard and Sample Preparation

Commercially available acetate and phosphate standards are diluted in the range of 0.063 mM to 1 mM in CE grade water. Vaccine samples were diluted to the appropriate dilution based on the respective intermediate step or FAP being tested. Samples were then filtered to remove the vaccine product from the sample preparation matrix. The filtrate was transferred to PCR vials and injected in duplicate on the Beckman Coulter PA800 system.

RESULTS AND DISCUSSION

CZE Separation

At pH >3, silanol groups in bare fused silica capillary are ionized leaving the inner surface of the capillary negatively charged. The CElixir™OA, pH 5.4 kit contains an initiator solution that propagates the negatively charged silica capillary with polycations creating an electric double layer. The accelerator solution contains a negatively charged pyridine-dicarboxylic acid at pH 5.4 and is injected after the initiator to create a reproducible and robust electro-osmotic flow (EOF) towards the anode under reversed polarity or anodic separation conditions. Upon injection, negatively charged analytes, such as acetate and phosphate, will separate via both electrostatic and EOF forces towards the anode creating fast separation. Standards and samples are analyzed with indirect UV detection at 254 nm. The negatively charge pyridine-dicarboxylic acid accelerator solution acts as a chromophore and completely absorbs UV energy at 254 nm producing a full detector response. When negative ions such as phosphate or acetate passes the detection window and displace the negatively charge chromophore, the detector will sense a decrease in absorption and record negative peaks, however the computer instrument reverses the signal automatically to display positive peak output. Figure 1 illustrates an electropherogram for Ac and Pi ions separation at 1mM each with migration times of 4.8 min and 5.2 min, respectively. At pH 5.4 both phosphate and acetate bear one negative charge, however acetate has less mass and therefore faster mobility in the capillary since CZE separations are based on charge-to-mass ratios **(Figure 1)**.

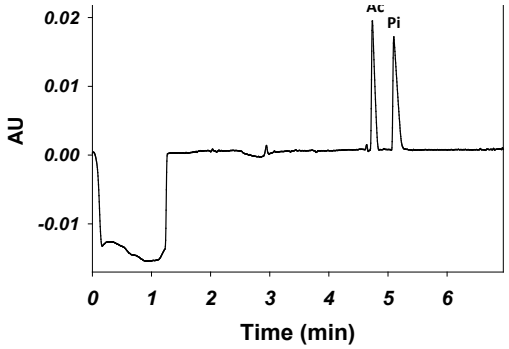

Figure 1. An electropherogram of standard acetate (Ac) and phosphate (Pi) anions each injected at 1 mM. Their migration times are about 4.8 and 5.2 min, respectively.

Optimization of Key Assay Parameters

Autosampler and Capillary Temperature

We had observed in our laboratories that auto-sampler storage of samples at room temperature, 25°C, will lead to dehydration of sample at a rate of approximately 4 µL per hour. This tendency causes an unpredictable increase in analyte concentration leading to overestimated and unreliable acetate and phosphate determinations. No sample dehydration is observed when the auto-sampler temperature is maintained at 12°C. It is expected that acetate and phosphate migration times depend on the temperature since the inverse viscosity dependence of electro-osmotic velocity of injected analytes15 Sufficient separation and migration of the analytes was observed at 20°C, therefore, the capillary temperature was not varied throughout development. However, the capillary temperature was varied as a part of our robustness study package during assay qualification.

Sample Filtration and Dilution

In order to analyze residual acetate and phosphate in vaccine products (MW >100 kDa), 100 uL of sample is transferred to either 30 kDa or 10 kDa MWCO filter and centrifuged to remove vaccine product. The filtrate is collected and 75 uL is transferred to a PCR vial for CE analysis. In general, the filter contains some chemicals used as preservatives that may interfere with acetate or phosphate peaks, hence it is important to screen various filters from different manufactures. (Figure 2A) illustrates various filters from different vendors were tested by adding 100 uL water and centrifuged. Four different filters, PALL 3K, YM30, PALL 10K, and Millipore 30K are shown in (Figure 2A), traces 2, 3, 4, and 5, respectively. This demonstrates that each filter has slightly different detectable residuals or no detectable residuals, such as in Millipore 30K (Figure 2A, Trace 5). The control sample (Figure 2A, Trace 1) that is not passed through the filter shows a clean trace; furthermore the Millipore 30K filter is comparable to the control hence this filter is chosen for subsequent study. The acetate and phosphate electropherogram using the Millipore 30K filter is shown in trace 6 (Figure 2A) illustrates that the PALL 3K (Trace 2) and PALL10K (trace 4) have residual peak that co-migrates with acetate at 4.8 min and YM30 (Trace 3) has a potential interference peak with phosphate at 5.3. Hence, it is critical to evaluate and find a filter with non-detectable residual peaks. Also, it is highly recommended that the filter is washed twice with water before use because different lots of filters from the same vendor may inadvertently contain unwanted chemicals (data not shown) **Figure 2A**.

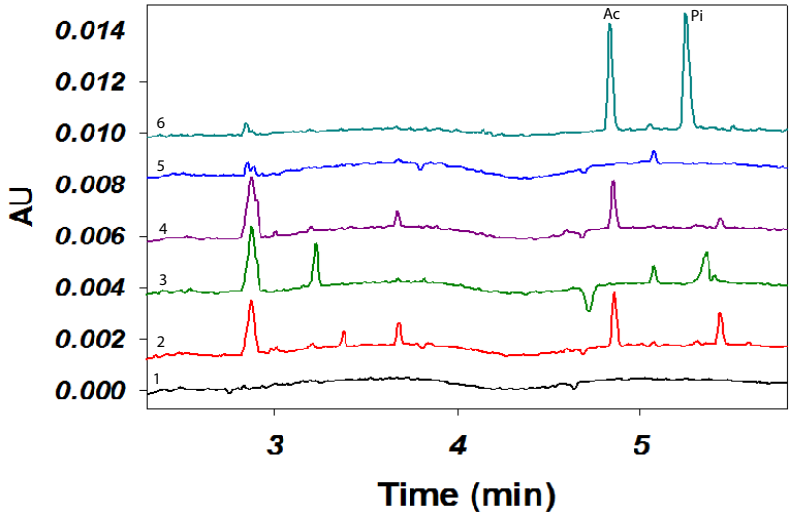

Figure 2A. Electropherograms of water injected from various filters screening; blank water without filter as control (trace 1), PALL 3K (Trace 2), YM30 (Trace 3), PALL 10K (Trace 4), and Millipore 30K (Trace 5). Trace 6 is acetate and phosphate standards that were filtered using Millipore 30K.

Regulatory provisions require the removal of residual metals and chemical additives, such as acetate and phosphate, resulting from biological manufacturing processes [16]. Multiple diafiltration steps are used during the purification of this complex vaccine product. The initial purification process uses acetate buffer with concentration range from 10 mM to 100 mM depending on vaccine type, while subsequent downstream purification steps buffer with phosphate in the concentration range of 10 mM to 400 mM is added. This results in the simultaneous introduction of phosphate and removal of acetate from the process. Since the relative concentration of acetate and phosphate may be different at each filtration step, separate dilution schemes were developed for quantitation of these analytes throughout downstream purification. Figure 2B demonstrates the separation observed when a D2 sample of vaccine intermediate is injected without dilution for the quantitation of acetate Figure 2B.

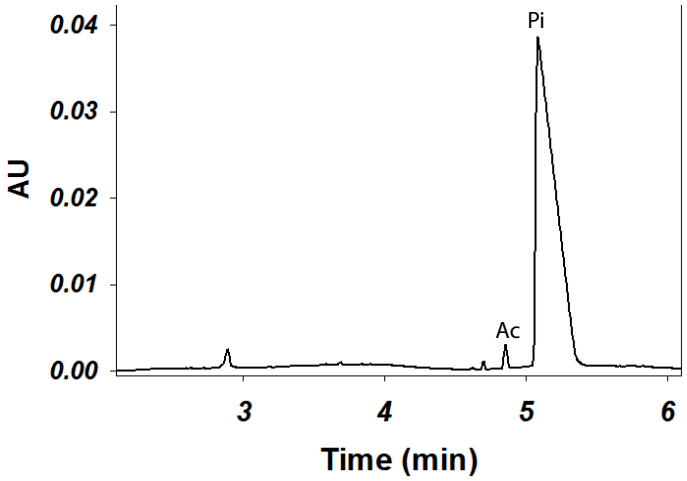

Figure 2B. Electropherogram of D2 step sample injected neat showing acetate peak at about 4.8 min and large phosphate peak at about 5.2 minn.

The D2 sample is the first purification step in which acetate is removed and phosphate is added for the subsequent downstream purification process. Hence the phosphate peak in the D2 sample is large and in order to measure phosphate in this sample type, it must be diluted 1:40 as shown in Figure 2C.

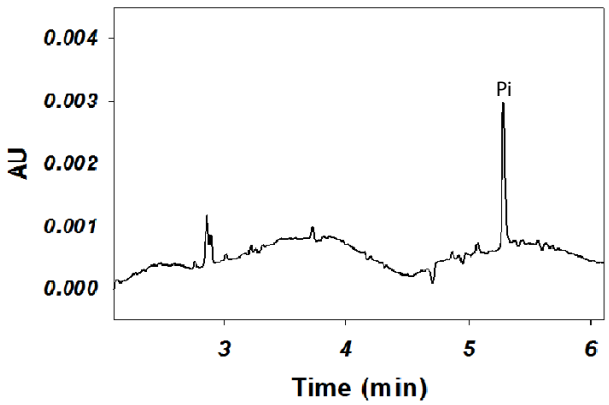

Figure 2C. Electropherogram of the same D2 step sample with 40x dilution to analyze the phosphate.

Finally, the vaccine FAP product is injected neat to confirm that acetate and phosphate concentrations are below the limit of quantitation (<LOQ) of the assay **(Figure 2D)**.The large peak at approximately 3.5 min is chloride ion since the final buffer of FAP sample contains NaCl **Figure 2D**.

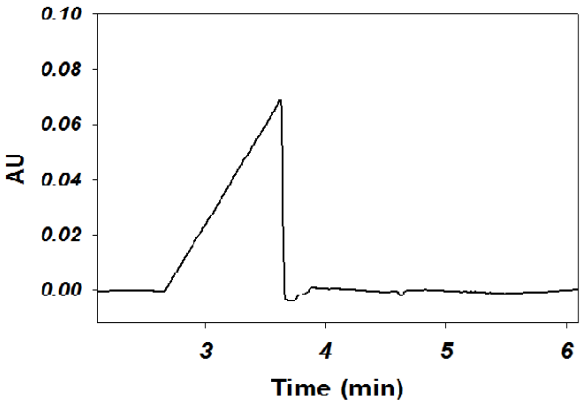

Figure 2D. Electropherogram profile of final aqueous product (FAP) sample illustrating the absence of acetate and phosphate peaks. The large chloride peak observed from 2.5 min to 3.5 min is attributed to the presence of NaCl in FAP sample.

Phosphate Peak Tailing

At the end of each run the capillary is conditioned by washing with 0.1M NaOH and H_2O for 0.5 min each and also with 0.1M HBr and H_2O cycle for 1 min. It is hypothesized that the acetate and phosphate ions could progressively bind to the poly-cation of

the initiator layer and cause peak broadening or tailing. Therefore, an HBr acid wash has been implemented to remove the poly-cation polymer in addition to a NaOH basic wash. However, peak tailing is still observed after 60 injections and gets worse at 90 injections as shown by the red trace in (Figure 3A) for phosphate.

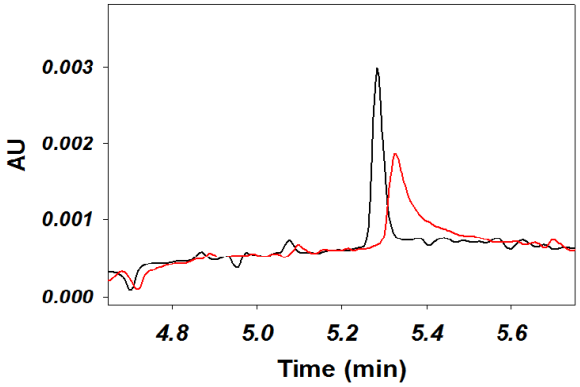

Figure 3A. The phosphate peak is shown at injection 1 (black trace) and peak tailing at injection 90 (red trace).

The peak tailing is not observed for acetate anion (data not shown). It was suspected that this phenomenon was likely due to insufficient reconditioning of the capillary after separation and the washing times were increased for all four solutions to 3 min each. Figure 3B shows an overlay of the phosphate and acetate standards at injection 1 (black trace) and 200 (red trace), the peak shape is consistent and no tailing is observed after lengthening the washing to 3 min.

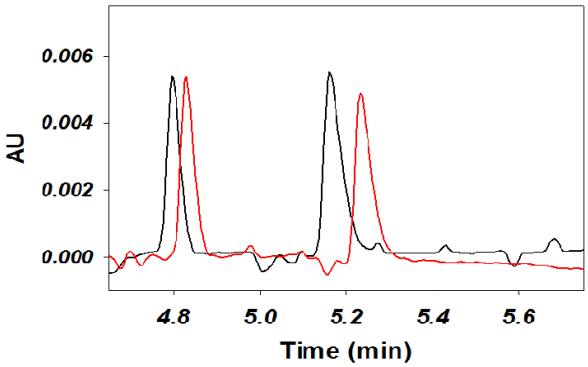

Figure 3B. Acetate and phosphate peaks in standard sample are shown at injection 1 (black trace) and injection 200 (red trace) without any peak tailing after increasing the NaOH and HBr washes to 3 min at the end of separation. The migration time shift is likely due buffer depletion.

Evaluation of Performance Characteristics

The International Conference on Harmonization suggests that qualification and validation are highly recommended for analytical procedures that are quantitative tests for impurities and/or limit tests for the control of impurities [17]. All standard qualifications are evaluated including accuracy, intra and inter-assay precision, repeatability, ruggedness, specificity, limit of detection (LOD), limit of quantitation (LOQ), and linearity.

Peak Migration Time Consistency

Peak migration time intra assay precision was initially assessed by evaluating 200 injections of the standards. The intra-assay migration time% RSD is 1.2% and 1.4% for acetate and phosphate, respectively, with buffer incrementation every 10 to reduce buffer depletion. The inter-assay migration time precision of experiments conducted on multiple days of 120 injections total yields% RSD of 6% for both acetate and phosphate. It is well documented that migration time reproducibility for capillary based methods in general is not as good as retention time reproducibility for HPLC based methods. There are many known factors that may contribute to migration time variability on capillary based methods, especially factors that affect the EOF [18].Of those factors impacting EOF, minimization of analyte and capillary wall interaction and maintaining a high charge density along the internal capillary wall throughout the separation are critical to maintaining consistent inter- and intra-day peak migration times [18,19]. In this study, when migration time shifting occurred, acetate and phosphate migration times shifted simultaneously within a given injection leading us to believe that the minor variability observed is due to variation bulk movement of charge rather than capillary wall interaction. Therefore, we attribute this phenomenon to a slightly variable EOF from injection to injection which may be caused by inconsistencies in the charge density of our electronic double-layer on the internal capillary wall over multiple injections. These migration time inconsistencies do not interfere with our ability to quantitate acetate and phosphate and do not deter from the methods ability to be used for product release.

Confirmation of Peak Identity

The peak identity for both anions was confirmed by observing the migration of samples spiked with 0.25 mM of commercially available acetate and phosphate standards on D2 sample containing both analytes. Because phosphate is present at a higher concentration it was diluted 40-fold before adding additional phosphate. Each spiked sample was overlaid with a sample run neat to confirm the peak identity (Figure 4A).

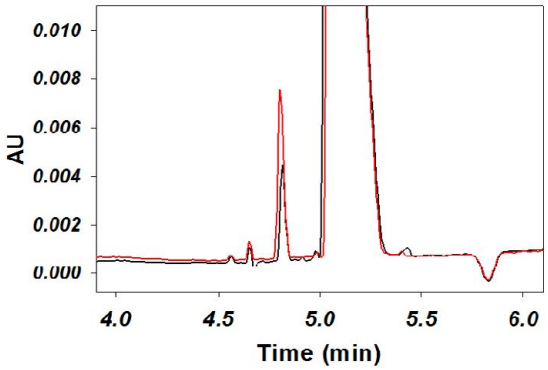

Figure 4A. Peak identity confirmation and spike recovery experiments (A) 0.25 mM acetate standard was spiked into D2 step sample showing peak at 4.8 min increases.

Figure 4B demonstrate the increase in signal intensity of both acetate and phosphate when each of these respective analytes was spiked into the vaccine sample.

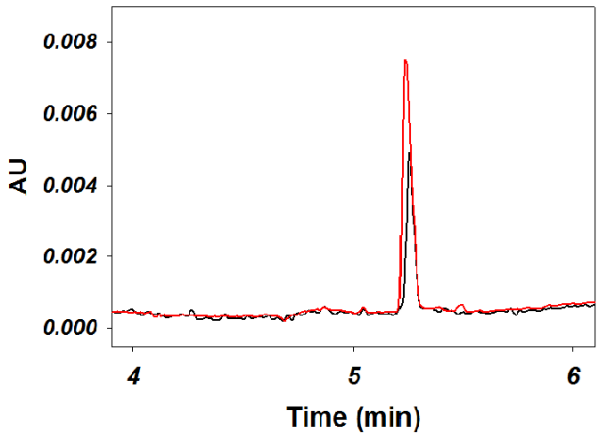

Figure 4B. 0.25 mM phosphate standard was spiked into 40x diluted D2 step sample showing peak at 5.2 min increases.

In addition, both analytes were spiked into the FAP sample as shown in (Figure 4C). **Figure 4C**

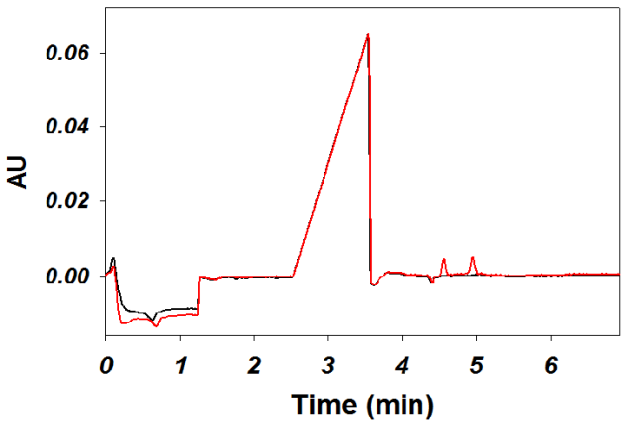

Figure 4C. : 0.25 mM acetate and phosphate standards were spiked into FAP sample.

Quantitation Parameters

Standard curve linearity was assessed over the selected concentration range (0.063 mM to 1 mM) for three individual experiments taking place on different days for both phosphate and acetate (Figure 5A).

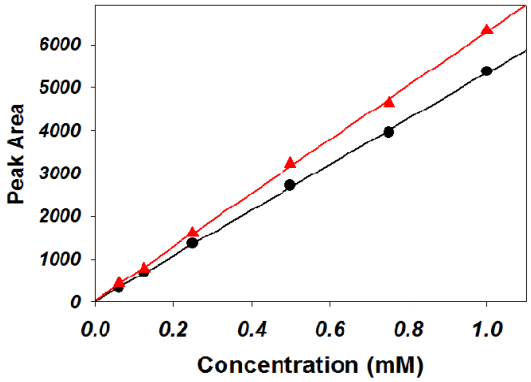

Figure 5A: Standard curves of acetate (● black) and phosphate (▲ red).

A standard curve was generated on each of three days. The coefficient of determination (R2) was measured for each standard curve generated. All curves displayed a coefficient of determination >0.998. Additionally, the inter-day %RSD of three independent concentration measurements within a given standard curve value was <10% for both anions.

The estimated method precision of the proposed new CZE method for acetate and phosphate was obtained by performing intra- and inter-day assay precision measurements of (n=3) process intermediate samples at the D2 step. The relative standard deviations (RSD) of acetate was <5% while phosphate was <10% the increase of %RSD of phosphate compared to acetate is likely caused by extra dilution needed to measure phosphate in this particular sample. The intra-assay peak area precision for 200 injections has RSD <5% for both acetate and phosphate as demonstrated at two concentration levels 0.25 mM and 1 mM shown in (Figure 5B).

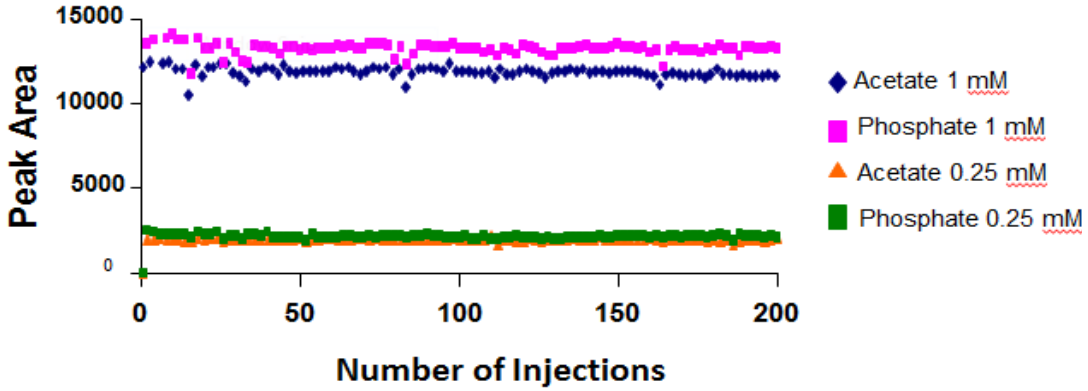

Figure 5B. Alternating injection between 0.25 mM (100 injections) and 1 mM (100 injections) acetate and phosphate standards to evaluate peak area precision of total 200 injections. The peak area % RSD is <5% at 0.25 mM and <3% at 1 mM acetate and phosphate concentration.

The accuracy of this method was determined by spiking acetate and phosphate reference standards into vaccine FAP samples (see Figure 4C). Two known acetate and phosphate standard concentrations at the low end (0.063 mM) and the middle (0.25 mM) were used. The % spike recovery for acetate ranges from 85% to 107%, while for phosphate the % spike recovery ranges from 101% to 104%.

The limit of detection (LOD) of both acetate and phosphate, with this new method was approximately 0.031 mM at a signal to noise ratio of 3, and the limit of quantification (LOQ) was about 0.063 mM at a signal to noise ratio of 10.

Determination of acetate and phosphate in the process samples

Using the method described above, the residual acetate and phosphate were analyzed in multiple process intermediate samples. Both acetate and phosphate are added as buffers at appropriate vaccine process manufacturing. Their clearances are performed through several diafiltration steps. Figure 6 shows a typical acetate and phosphate analysis in the sample matrix obtained from various manufacturing process steps. The initial step of adding acetate without phosphate into the sample matrix is called D1.

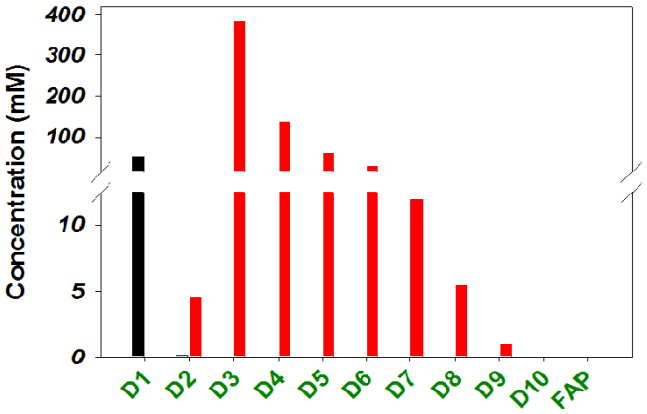

Figure 6: Bar graph representation of acetate (black bar) and phosphate (red bar) clearance during vaccine process. The initial sample (D1 step) was not tested and has no phosphate and contains nominal acetate concentration between 10 mM to 100 mM depending on the vaccine types. In general, acetate is cleared after D3 step and phosphate is after D10. No acetate and phosphate are detected in all FAP samples.

Acetate is then removed through several subsequent diafiltration steps while phosphate is being added at the D2 step and peaked at the D3 step. Phosphate removal starts at D4 through D10. Table 1 shows the typical acetate and phosphate results for a set of process intermediate samples (Table 1).

Table 1: Typical acetate and phosphate clearance.

Step	[Acetate] in mM	[Phosphate] in mM
D1	50	0
D2	0.19	4.5
D3	<0.063	383
D4	<0.063	137
D5	<0.063	58
D6	<0.063	27
D7	<0.063	12
D8	<0.063	5.4
D9	<0.063	0.97
D10	<0.063	<0.063
FAP	<0.063	<0.063

Although acetate is not known to be harmful, its residual removal is used as a surrogate assay for other small molecule substance clearances that are added at pre-D1 step. The phosphate clearance is critical for final bulk product, since alum adjuvant is added and it is known that phosphate reacts with alum. In addition, residual phosphate is also used as surrogate assay for other small molecules and metals that are added during process (D3 step).

CONCLUSION

The residual acetate and phosphate anion assay has been developed for evaluating vaccine process intermediates and final products using a CE method. Although there is ion chromatography (IC) assay for acetate and phosphate [20,21]. The method is relatively long and cumbersome to for routine testing. In addition this IC assay uses high pH eluent which needs to be tightly controlled due to the amperometric detection, slight variation in pH have been known to introduce extra source of assay variability. The CZE technique has been shown to be a reliable tool for quantitating and demonstrating clearance of residual acetate and phosphate for in-process and FAP samples in multi vaccine products. Demonstrating the removal of residual buffer components in final product release testing is critical to the success of all vaccine candidates for which additive buffers are used in the manufacturing, but are not desired in the final product. Furthermore, because of the anionic similarities of acetate and phosphate to other harmful small anion process residuals, this assay may also be used as surrogate to confirm the absence of these residuals in final aqueous products. We have also shown that this assay is fit for intended use in GMP regulated environments making a powerful alternative method to an expensive mass spectrometry or high solvent consumption HPLC method in residual analysis. The method is sensitive enough to be used in residual testing, has acceptable levels of accuracy and precision, and is robust. These method properties provide reliable results when testing in-process and FAP samples. .

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