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A practical approach to optimization and validation of a HPLC assay for analysis of polyribosyl-ribitol phosphate in complex combination vaccines

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Abstract

The use of multi-factor statistical experimental design methodology minimized the vaccine material and laboratory resources required for optimization and validation of an HPLC assay for quantitation of deploymerized and total PRP. Components of the assay selected for optimization were adjuvant dissolution, ultracentrifuge conditions including ultracentrifuge model, sample diluent, mobile phase and column oven temperature. Previous experience has shown these components of the assay to be most troublesome and therefore required optimization prior to validation. Specificity, linearity, precision, accuracy and ruggedness were confirmed through a validation of the optimized assay. The validation also established the assay to be stability indicating, by showing that changes to the integrity of the PRP-OMPC conjugate could be detected.

Keywords: PRP; Assay validation; Assay optimization; Combination vaccine; HPLC; Design of experiment

1. Introduction

Polyribosyl-ribitol phosphate (PRP) conjugate vaccines protect against *Haemophilus influenzae* type b infection, a causative agent of bacterial meningitis and other serious systemic bacterial diseases in young children worldwide. It has been documented in the literature that PRP needs to be conjugated to a protein carrier in order to be immunogenic in infants [1]. In a multi-valent combination vaccine containing aluminum adjuvant, different species of PRP can be found: unconjugated PRP (also referred to as free-PRP), depolymerized-PRP (smaller chain-lengths of PRP conjugate), and full chain-length of PRP-conjugate. Depolymerized (d-PRP) and free-PRP are created by hydrolysis of the phosphodiester bond between the PRP monomers releasing PRP fragments at a rate that is affected by temperature and by interactions with the adjuvant or divalent cations present in the vaccine [2–4]. As the amount of d-PRP and free-PRP increases in the vaccine, the product is thought to be less immunogenic and consequently, these species become an important stability attribute to monitor [5–7]. The measurement of d-PRP and free-PRP is challenging and becomes increasingly so with the addition of other antigens, such as recombinant hepatitis B surface antigen, acellular pertussis components, diphtheria and tetanus toxoids, and polio antigens. A modified high performance anion exchange chromatography method with pulse amperometric detection (HPAEC-PAD) was developed [8] and coupled with ultracentrifugation was used for quantitation of d-PRP and free-PRP. This method has been successfully established for monovalent Liquid PedvaxHIB® and bivalent COMVAX® vaccines. In combination vaccines, components are either adsorbed to the aluminum adjuvant or unadsorbed. Unadsorbed components are found, to some extent, in the vaccine supernatant even after ultracentrifugation. These components must be separated from PRP in a selective and reproducible manner. Thus, to reduce the time and resources necessary to perform the optimization, statistical multi-factor experimental designs were used to evaluate many aspects of the HPAEC-PAD application. The results of the assay validation confirmed specificity, accuracy, linearity and precision. Further, ruggedness to different operators and to the ultracentrifuge model was verified. The

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optimized assay was, therefore, established as a valid method for measuring total and unconjugated PRP content in a combination vaccine. The unconjugated assay can be used to monitor stability of the final product since it meets the FDA guidelines on stability indicating assays. "A validated quantitative analytical procedure must be able to detect changes with time of the pertinent properties of the drug substance and drug product". This was evidenced through the validation experiments whereby a wide range of depolymerized PRP concentrations were accurately and precisely quantitated by the method and verified with real time stability data (data not shown).

2. Methods and materials

2.1. Materials

The multi-valent vaccine consists of PRP-OMPC, Polio, Pertussis, Diphtheria, Tetanus and Hepatitis B (heretofore to be referred to as the "combination vaccine"). The vaccine matrix without PRP-OMPC was provided by the Merck/sanofi-pasteur collaboration. The surrogate marker for d-PRP and free-PRP was native PRP (raw material used for the production of PRP-OMPC). PRP-OMPC was also used as the reference standard starting at 15 μg PRP/mL and serially diluting to 0.94 μg PRP/mL. The internal standard used in assay is α-D-glucosamine 1-phosphate purchased from Sigma-Aldrich.

2.2. HPAEC-PAD

Measurement of PRP content was performed using a high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method. The system consisted of a Dionex GP40 or GP50 gradient pump connected to a Thermal Separations AS3500 autosampler. A borate trap between the pump and autosampler was used to remove any interfering borate from the eluents. The autosampler was connected to a Dionex ED40 electrochemical detector equipped with an integrated pulse amperometry option through a Dionex PA10 guard and analytical columns. The columns were held in a Dionex LC30 chromatography oven at ambient room temperature then 30 °C after optimization of the oven temperature. The mobile phase used for separation was initially 28 mM sodium hydroxide/100 mM sodium acetate then optimized to 32 mM sodium hydroxide/120 mM sodium acetate. Separation of the analyte was completed by running the mobile phase for 23 min followed by 2.5 min gradient change to the regeneration phase of 250 mM sodium hydroxide/1M sodium acetate which ran 10 min and followed by a 2.5 min gradient change back to the mobile phase then re-equilibration of the columns for 10 min prior to the next sample injection. The flow rate used for all analysis was 1.2 mL/min.

2.3. Ultracentrifugation

Separation of d-PRP and free-PRP from conjugated PRP was performed using either a Beckman Optima TLX ultracentrifuge with a 120.2 rotor with initial settings of 600,000 × *g* at 5 °C for 30 min. To convert revolutions per minute (RPM or "speed") to a gravitational force (×*g*) Eq. (1) was used:

$$\text{Gravitational force}\,(\times g) = 1.12 \times \text{Radius} \times \left(\frac{\text{RPM}}{1000}\right)^2, \quad \text{Radius} = 38.9\,\text{mm} \tag{1}$$

A Sorvall Kendro Discovery M150 micro ultracentrifuge was also used but conversion from *g* force to RPM was not required on this instrument.

2.4. Experimental strategy for separation and quantification of PRP components

Percent d-PRP and free-PRP is a ratio between these species to the total amount of PRP. A sample is first subjected to dissolution of the adjuvant followed by ultracentrifugation to separate d-PRP and free-PRP from PRP-conjugate. The ultracentrifuged supernatant containing the d-PRP and free-PRP and the non-centrifuged total PRP sample are then hydrolyzed in 0.3 M sodium hydroxide to yield the constituent disaccharide (ribosyl-ribitol-phosphate). The hydrolysate is passed through a 10,000 MWCO microfilter to remove protein. The filtrate is quantitated in parallel with the whole sample by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The reference standard was treated the same as the vaccine samples with sample concentrations obtained through interpolation from the standard curve.

2.5. Adjuvant dissolution optimization

The various species of PRP found in combination vaccines passively adsorb to the aluminum adjuvant. To quantitate PRP components accurately, the adjuvant must first be dissolved. This is accomplished through the use of a dissolution buffer consisting of sodium hydroxide and sodium citrate. To achieve an optimum mix of the dissolution reagents, a four-parameter central composite experimental design (CCD) [9,10] was performed using sodium hydroxide concentration, sodium citrate concentration, incubation time, and incubation temperature as the parameters. Table 1 lists the parameters and ranges used in the design.

The reagents were combined in water to make a concentrated dissolution buffer, which was mixed with the vaccine sample to achieve the final concentration of hydroxide and citrate specified by the experimental design. Absolute dissolution bias was

Table 1
Parameters for adjuvant dissolution optimization and associated ranges

Parameter	Range
NaOH (mM)	0, 50, 100, 150, 200
Citrate (mM)	0, 25, 50, 75, 100
Time (min)	0, 10, 20, 30, 40
Temperature (°C)	11, 24, 37, 50, 63

calculated, according to Eq. (2), as a means of determining if the selected conditions efficiently dissolved the adjuvant without causing a change in PRP content from the control.

$$\text{Absolute dissolution bias} = \left| \left(\frac{\text{Percent unconjugated from sample}}{\text{Percent unconjugated from control}} \right) - 1 \right| \times 100 \quad (2)$$

Design-Expert software by StatEase [11] was used to design the experiment and analyze the data. The interaction graphs generated by Design-Expert show the least significant difference intervals for the points annotated on the graphs.

2.6. Ultracentrifugation and ultracentrifuge model optimization

Settings previously established for separation of d-PRP and free-PRP from conjugated-PRP in monovalent and bivalent vaccines (600,000 × *g* at 15 °C for 20 min) were used during preliminary experimental studies. It was deemed necessary to consider a reduction in these conditions, especially force, which was set at the maximum operating boundary. If ultracentrifugation conditions are not set correctly, it is likely that the amount of d-PRP in a vaccine sample will not be reported accurately. Robustness around each parameter was needed for implementation of the assay in a quality control laboratory. To optimize the ultracentrifugation conditions efficiently, a three-parameter central composite design with time, temperature and force as parameters was employed. Also, robustness to the ultracentrifuge model was assessed through the use of two different models. Table 2 lists parameters and ranges used in the design.

To aid with the numerical optimization of the conditions, percent recovery bias was calculated using Eqs. (3) and (4) and served as a metric for assessing accuracy.

$$\text{Percent recovery} = \left(\frac{\text{Concentration observed in vacine matrix sample}}{\text{Concentration observed in saline control}} \right) \times 100 \quad (3)$$

$$\text{Percent recovery bias} = |100 - \text{percent recovery}| \quad (4)$$

Design-Expert software by StatEase was used to design the experiment and analyze the data.

Table 2
Parameters for ultracentrifugation optimization and associated ranges

Parameter	Range
Time (min)	5, 15, 30, 45, 55
Temperature (°C)	5, 10, 18, 25, 30,
Force (10,000 × g)	34.8, 40, 47.5, 55, 60
Ultracentrifuge	Beckman Optima TLX or Sorvall Discovery M150

2.7. Sample diluent

The diluent used for diluting both the sample and reference standard must be convenient to use and readily available. Two choices prevail, aluminum adjuvant diluent used in production of adjuvant adsorbed PRP-OMPC and saline. The aluminum adjuvant diluent under goes the same adjuvant dissolution process as the sample to create the assay diluent. An assessment on the suitability of each was performed based on an evaluation of linearity. Linearity was confirmed if the dilution effect associated with the samples was less than 20% in absolute value. The "dilution effect" per two-fold dilution was calculated according to Eq. (5) (also see Klein et al. [12]).

$$\%\text{Dilution effect} = 100(2^{|b-1|} - 1) \quad (5)$$

where *b* is the slope from the regression of the natural log of the observed concentration against the natural log of the expected concentration [13,14].

The relative retention time of PRP and the internal standard was also assessed to determine if a significant change in the ionic environment has occurred that may have an adverse impact on the integration and quantitation of the different peaks. The relative retention time (RRT) was calculated according Eq. (6).

$$\text{RRT} = \frac{\text{PRP peak retention time}}{\text{Internal standard retention time}} \quad (6)$$

The acceptable range of relative retention time for each run was determined according to Eqs. (7) and (8) where the control is completed in the current assay diluent.

$$\text{Average RRT of control} = \frac{\text{Average of PRP peak retention time}}{\text{Average of internal standard retention time}} \quad (7)$$

$$\text{Target RRT range} = \text{Average RRT of control} \pm 15\% \quad (8)$$

2.8. Mobile phase optimization

The mobile phase used for PRP-OMPC monovalent and bivalent vaccines showed less than optimal separation of the PRP peak from other components in the combination vaccine matrix. In this regard, a combination of sodium hydroxide and sodium acetate was evaluated in a two-parameter central composite experimental design. Table 3 lists the parameters and ranges used in the design.

Resolution asymmetry [15,16] for the internal standard and PRP peaks were the responses to be optimized. The central composite design was constructed using JMP Statistical Discovery Software® [17].

Table 3
Parameters for mobile phase optimization and associated ranges

Parameter	Range
NaOH (mM)	23, 25, 30, 35, 37
NaOAc (mM)	80, 86, 100, 116, 120

2.9. Column (oven) temperature optimization

The column oven holds the analytical and guard columns as well as the analytical electrodes. As the temperature increases, more active sites on the column resin are made available to the analyte, thus, resulting in a larger peak response. Conversely, as the temperature decreases the resin contracts and fewer active sites are available to the analyte, thus, resulting in less peak area. To prevent signal variation within an analytical run from temperature change, optimization was conducted by studying column oven temperatures from 25 to 50 °C in 5 °C increments. To estimate variability, the root mean square error (RMSE), obtained from the calibration curve, was assessed using Eq. (9) for each temperature condition.

$$\text{RMSE} = \sqrt{\frac{1}{n-2}\sum (y - \hat{y})^2} \tag{9}$$

where n is the number of observed concentrations, y the observed logarithm of the PRP to internal standard (IS) ratio and $\hat{y}$ is the predicted logarithm of the PRP/IS ratio obtained from performing a linear regression analysis of the logarithm of the PRP/IS ratio on the logarithm of the concentration for each temperature separately. A large RMSE means a large deviation from the linear fit. The combined effect of 25 and 30 °C was then compared to combined effect of 35–50 °C using Eq. (10).

$$\text{Combined RMSE} = \sqrt{\frac{(\text{RMSE}_1)^2 + (\text{RMSE}_2)^2 + \cdots + (\text{RMSE}_m)^2}{m}} \tag{10}$$

where m is the number of temperature conditions and RMSE is the RMSE for each temperature condition determined from Eq. (9). The variability was assessed within the normal assay range as well as an extended assay range in order to determine if very dilute samples would behave the same as more concentrated samples.

2.10. Validation

Validation studies were conducted using the optimized conditions and based on the principles of validation described in the ICH guidelines "Text on Validation of Analytical Procedures" [18], "Q2B, Validation of Analytical Procedures: Methodology" [19] and in Klein et al. [12]. Samples were prepared by spiking native PRP or PRP-conjugate into the vaccine matrix at similar levels in addition to the saline blank and vaccine matrix without the PRP antigen to ascertain background interference. A total of six runs were performed by two different analysts on three different HPLC systems. Two different ultracentrifuges were used for the depolymerized PRP validation. Key analytical parameters, including, specificity, accuracy, linearity, precision, detection limit and quantitation limit were evaluated. Also, ruggedness to operator and ultracentrifuge model were examined.

3. Results

3.1. Adjuvant dissolution optimization

Results from the experimental design showed that the interaction between sodium hydroxide and citrate was critical as well as the interaction between time and temperature. The parameter estimates are shown in Table 4. All the individual factors were significant with the exception of citrate, which was only marginally significant.

The critical interactions are shown in Figs. 1 and 2. As seen in Fig. 2, the significance of the sodium hydroxide by citrate interaction is illustrated by observing that low sodium hydroxide levels with high level of citrate produced the least dissolution bias; however, high levels of sodium hydroxide regardless of the level of citrate produce the most dissolution bias. In general to reduce the absolute dissolution bias, sodium hydroxide should be low in concentration and sodium citrate should be high in concentration.

The incubation time should be short (10 min) and temperature kept near room temperature (24 °C) as shown in Fig. 2. These settings were predicted to give a dissolution bias of approximately ±20% between percent unconjugated results obtained with the optimized conditions as compared to results obtained with the current dissolution conditions.

Final optimized adjuvant dissolution conditions were 51 mM NaOH/73 mM Na citrate (final concentration) at room temperature (20–25 °C) for 11 ± 3 min.

Table 4
Parameter estimates obtained from evaluation of the recovery bias data

Parameter	Coefficient estimate	p-Value
NaOH	1.12	<0.001
Citrate	−0.092	0.0659
Time	0.24	<0.001
Temperature	0.44	<0.001
NaOH × citrate	0.21	0.0014
Time × temperature	0.010	0.839

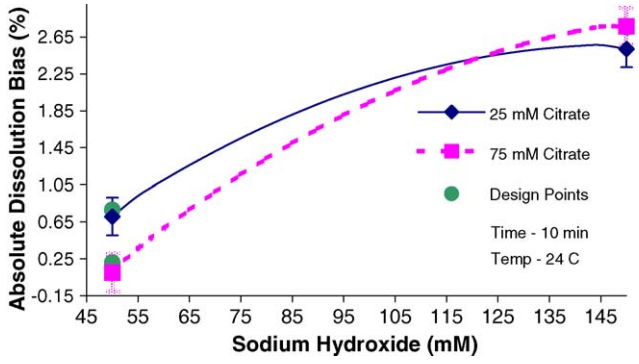

Fig. 1. Sodium hydroxide is the x-axis and absolute dissolution bias is the y-axis. The solid line (diamond ends) is the effect of the least amount of citrate (25 mM) combined with varying amounts of sodium hydroxide and the dashed line (square ends) is the effect of the most citrate (75 mM) combined with varying amounts of sodium hydroxide. The vertical bars represent the least significant difference intervals.

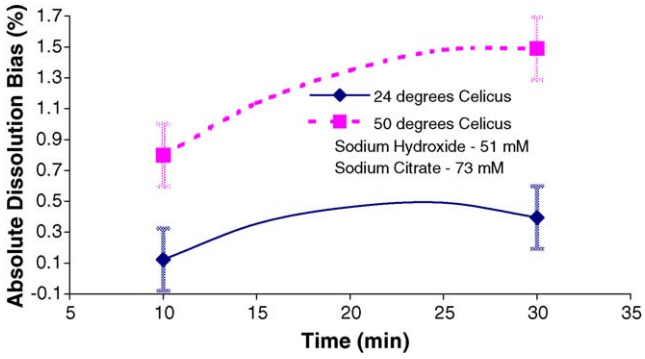

Fig. 2. Time is the *x*-axis and absolute dissolution bias, as calculated in Eq. (2), is the *y*-axis. The solid line (diamond ends) is the effect of the lowest incubation temperature (24 °C) combined with varying incubation times and the dashed line (square ends) is the effect of the highest incubation temperature (50 °C) combined with incubation times. The vertical bars represent the least significant difference intervals.

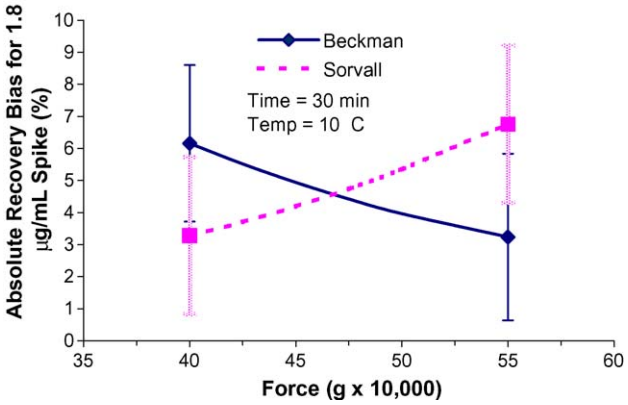

Fig. 3. Interaction of ultracentrifuge and force with 1.8 μg PRP/mL spike. The *x*-axis is force and the *y*-axis is the percent recovery bias calculated for the 1.8 μg PRP/mL spikes. The solid line (diamond ends) represents the Beckman model and the dashed line (square ends) represents the Sorvall model. The vertical bars represent the least significant difference intervals.

3.2. Ultracentrifugation and ultracentrifuge model optimization

Analysis using Design Expert indicated that the Sorvall showed less bias when compared to the Beckman. In order to use either the Sorvall or Beckman, conditions needed to be established that would give similar results between ultracentrifuges without increasing the bias. From Table 5, the interaction between time and force as well as the interaction between force and centrifuge were significant. Individual parameters were not significant (p-value > 0.05). Using Eq. (4) and the optimization feature in Design Expert, the model predicted a recovery bias of about 5% for either ultracentrifuge model when the conditions were set at $475{,}000 \pm 10{,}000 \times g$ at 10 ± 3 °C for 30 ± 1 min (about where the two curves intersect in Fig. 3). Roughly the same level of bias (about 8%) could also be achieved with the temperature set to 10 °C time at 15 min and force at $510{,}000 \times g$. Although it is desirable to have a shorter centrifugation time, using a higher force with the shorter time interval might have an adverse effect on aged samples (samples >24 months) and yield falsely elevated d-PRP/free-PRP values. The combination vaccine is sufficiently new that older material does not currently exist to test this hypothesis.

Verification runs were performed to confirm the predication obtained from the model The data showed less then a 2% recovery bias (data not shown) for either ultracentrifuge. Final optimized ultracentrifugation conditions were $475{,}000 \pm 10{,}000 \times g$ at 10 ± 3 °C for 30 ± 1 min, which are compatible with both the Sorvall and Beckman ultracentrifuge models.

Table 5
Parameter estimates obtained from evaluation of the recovery bias data

Parameter	Estimate	p-Value
Time	−0.52	0.4110
Temp	−0.65	0.2989
Force	−0.13	0.8348
Centrifuge	−0.63	0.2199
Time × force	−1.88	0.0373
Force × centrifuge	1.60	0.0169

3.3. Sample diluent

The dilution effect [12] is a useful practical metric for assessing the ability of the assay to accurately measure a diluted (or spiked) sample *relative* to an appropriate control and can be related to parallelism between the dilution profiles of the reference standard and test samples. Perfect dilutability ($b = 1$) implies a dilution effect of 0%. The slope was calculated based on the regression of the natural log of the concentration of each dilution curve to the natural log of the concentration of the control PRP-OMPC reference standard in assay diluent. The average slopes and dilution effects are shown in Table 6 below. The average dilution effects are less than 2%, which indicates excellent "dilutability". Changing the sample diluent to saline did not affect the linearity of the standard curve. Fig. 4 shows the comparison between the samples diluted in saline and those diluted in assay diluent.

Table 7 summarizes the relative retention time (RRT) for the six runs, which were performed on two different HPLC systems over several days by two analysts using independent sample preparations. The target RRT was set from the sample diluted in

Table 6
Comparison of each serial dilution curve to reference standard PRP-OMPC in assay diluent (control)

Sample	Average slope	Average % dilution effect
PRP-OMPC in assay diluent	1.020	NA
PRP-OMPC in saline	0.998	0.868
Combination vaccine in saline	1.002	1.283
Combination vaccine in current assay diluent	1.010	1.632
Native PRP In saline	1.007	1.170
Native PRP in current assay diluent	1.017	1.831

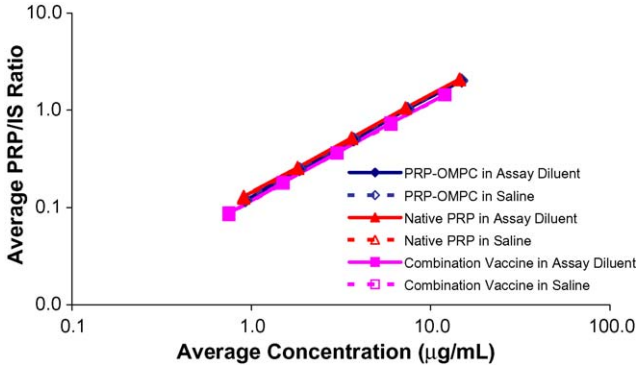

Fig. 4. Comparison of dilution curves in assay diluent and saline.

Table 7
Relative retention time summary

System number	Run	Target RRT range	Minimum RRT	Maximum RRT
1	1	1.25–1.69	1.46	1.47
	2	1.28–1.74	1.46	1.51
	3	1.27–1.71	1.46	1.50
2	1	1.29–1.75	1.51	1.53
	2	1.28–1.74	1.50	1.51
	3	1.27–1.71	1.47	1.51

The target RRT range as calculated in Eq. (8) is determined from the sample diluted in assay diluent while the minimum and maximum RRT were determined from the same sample diluted with saline.

adjuvant diluent and analyzed in the same run as the same sample diluted in saline. For the sample diluted in saline the RRT range fell within the target RRT, which indicates minimal impact on the ionic environment of the column necessary for separation of the analytes of interest. Sample diluent did not affect either dilutability or relative retention time; as such, saline was selected as the diluent for routine use in the assay.

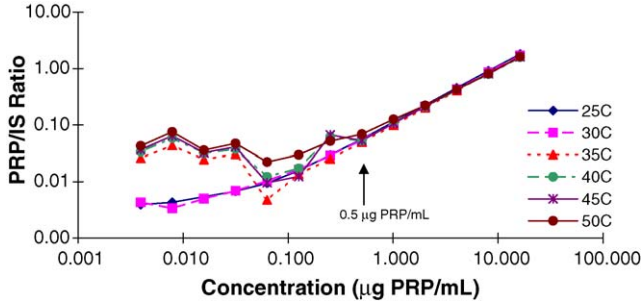

Fig. 6. PRP-OMPC (concentration range 12.0–0.05 μg PRP/mL) shown at each temperature investigated.

3.4. Mobile phase optimization

Table 8 summarizes statistically significant effects (p-value < 0.05) for each of the parameters evaluated. It is clear that the concentration of sodium acetate impacts both pre and post PRP resolution as well as asymmetry. Sodium hydroxide has a significant impact on the pre resolution but minimal effect on the peak asymmetry or post-PRP resolution. The final optimized mobile phase was 32 mM sodium hydroxide/120 mM sodium acetate. Both the internal standard and the PRP are well separated from other matrix components with symmetrical peaks as shown in Fig. 5.

3.5. Column (oven) temperature optimization

Fig. 6 shows the effect of varying column oven temperature on conjugated PRP in a monovalent vaccine. For all temperatures above 30 °C, the log PRP/IS ratio linearly decreases to a concentration of 0.5 μg PRP/mL and then it plateaus. The effect is more prominent in the monovalent vaccine, results were also observed for the combination vaccine (data not shown).

Since the results indicated that temperatures at 25 and 30 °C behaved in a similar fashion and temperatures at or above 35 °C followed a similar trend, a comparison between temperatures at

Table 8
Significant terms (p-value < 0.05) from mobile phase optimization for each response evaluated

Significant factor	Pre-PRP resolution estimate (p-value)	Post-PRP resolution estimate (p-value)	Asymmetry estimate (p-value)
NaOAc	−0.86 (0.002)	0.46 (0.004)	−0.39 (0.056)
NaOH	−1.12 (0.095)	0.62 (0.112)	0.27 (0.613)
$NaOAc^2$	0.004 (0.0009)	−0.001 (0.016)	−0.0003 (0.033)
$NaOH^2$	0.02 (0.043)	−0.006 (0.215)	−0.004 (0.530)

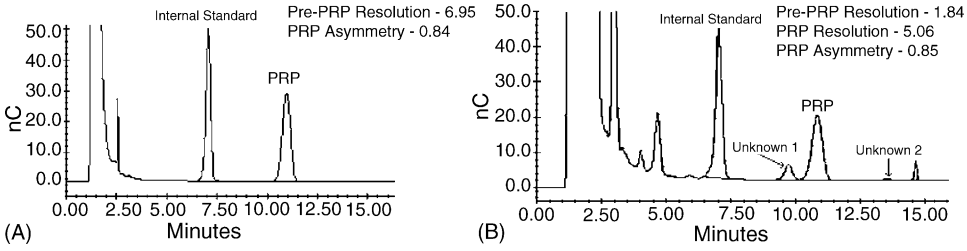

Fig. 5. Vaccine analyzed with optimized mobile phase. Panel A is PRP-OMPC at 7.5 μg PRP/mL and Panel B is the combination vaccine. Panel A: PRP-OMPC reference standard. Panel B: combination vaccine.

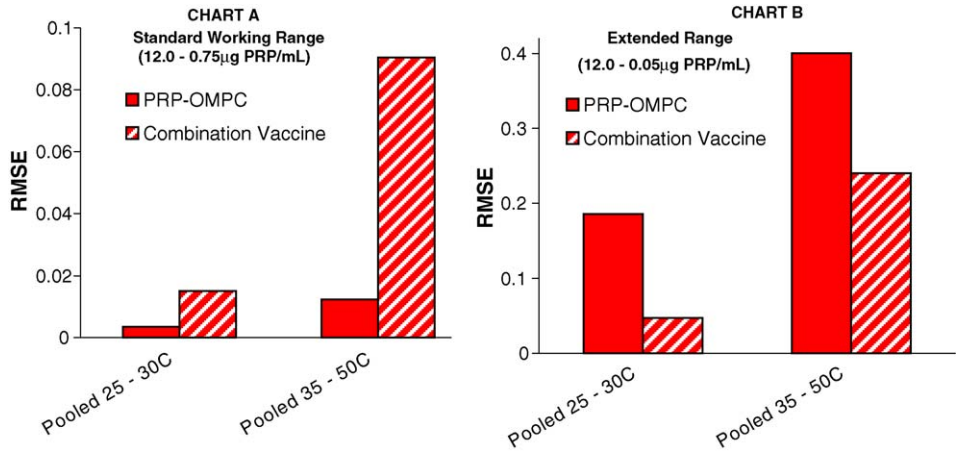

Fig. 7. Effect of oven temperature on the variability (as measured by the RMSE, see Eqs. (9) and (10)) around the standard curve. The Chart A shows the comparison made within the standard working range of the assay and Chart B shows the extended range of the assay. Note the difference in scale between the two charts. More variability was found around the extended standard curve.

30 °C and below and temperatures 35 °C and above was made with the findings shown in Fig. 7.

The data indicates that as the temperature in the column increases (≥35 °C) the system looses sensitivity in that lower concentrations of PRP look the same to the system. Within the standard working range, the combination vaccine showed more variability (RMSE) at the elevated temperature (≥35 °C) in contrast to the monovalent vaccine, which showed greater variability (RMSE) at the elevated temperature in the extended range. This apparent difference might be due to co-elution of vaccine components at the higher temperatures in the combination vaccine; thus, potentially masking the loss of sensitivity to PRP response in the lower concentration range. Therefore, the temperature selected for routine use in the assay was 30 °C.

3.6. Validation

The optimization work aided in characterizing the assay prior to validation and guided in establishing the specification range of critical assay validation parameters. The optimized method was found to be rugged to operator and ultracentrifuge model. The spiking of native PRP into the sample facilitated the determination of the ability of the assay to detect PRP degradation products in the vaccine. The optimization data in conjunction with the validation data shows the assay is capable of detecting change in the PRP moiety. The validation established that the assay is stability indicating, in that it can detect changes to the integrity of the PRP-OMPC conjugate. The critical validation parameters and results are summarized in Table 9. The assay was found to be specific since no signal was detected in either the vaccine matrix without PRP-OMPC or in the saline blank. The assay was found to be rugged to ultracentrifuge models and analysts in that no statistical difference (p-value > 0.05) was found between ultracentrifuges or analysts. The limits of quantitation and detection were calculated to be 0.15 μg PRP/mL. The dilution effect which measures linearity through an assessment of parallelism, is the proportional change in a sample's dilution-adjusted concentration when it is diluted two-fold. For the assay the dilution effect was 0.8% for the total PRP measurement, 1.6% for the Beckman and 0.9% for the Sorvall ultracentrifuge models. Accuracy, assessed by spike recovery, was demonstrated since all recoveries to be within acceptable ranges. Precision was confirmed since, the R.S.D. of all individual samples were below or equal to the protocol target criterion of 20% R.S.D. The overall run-to-run variability for both the vaccine matrix without PRP-OMPC and the saline control fell within the target criteria of 20% R.S.D. as well. Furthermore, the confidence interval on the ratio of the variability of the vaccine matrix without PRP-OMPC to the Saline Control includes 1. Thus, there is no evidence to suggest a statistically significantly larger inter-run variability for the vaccine matrix without PRP-OMPC relative to that of the saline control.

Table 9
Summary of validation parameters and results

Parameter	Result
Specificity	No signal in either saline or vaccine matrix without PRP
Ruggedness	No statistical difference was found between ultracentrifuges or analysts
LOD/LOQ	0.15 μg PRP/mL
Linearity	1.6% dilution effect
Accuracy	% Recovery 93–121%
Precision	≤20% R.S.D.

4. Conclusion

By employing several experimental designs, critical aspects of the HPAEC-PAD application for the quantitation of total and unconjugated PRP in a combination vaccine were optimized in a rapid manner. It is important for assay characterization to be able to distinguish between those parameters that must be tightly controlled from those that are less critical. As observed with the evaluation of different ultracentrifuge models, the design found a difference in recovery bias between the two models. This difference, however, was not of practical significance as shown with

the assay validation data. In addition to understanding significant interactions, robustness settings could be identified for critical preparation steps. Moreover, the validation supported the selection of the assay conditions by confirming that the assay was specific, accurate, linear, precise, and rugged.

Quantitating total and unconjugated PRP in vaccines is important for the assessment of product release and stability. These measurements are especially challenging in combination vaccines where other antigens or excipients can interfere with the analysis. This challenge is compounded when the assay is run in a quality control environment where many different operators will perform the assay. The use of statistical multi-factor experimental design to optimize assay conditions significantly reduced the overall assay development time while providing essential information on key assay characteristics.

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