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STANDARDIZATION OF A SPECTROPHOTOMETRIC PROCEDURE TO QUANTIFY THE ACTIVATION OF PNEUMOCOCCUS SEROTYPE 5 POLYSACCHARIDE

ESTANDARIZACIÓN DE UN PROCEDIMIENTO ESPECTROFOTOMÉTRICO PARA CUANTIFICAR LA ACTIVACIÓN DE POLISACÁRIDO DE NEUMOCOCO SEROTIPO 5

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ABSTRACT

For the preparation of polysaccharide-protein conjugate vaccines, the hydrolysis and activation of capsular polysaccharides constitute a critical step for obtaining monovalent conjugates. This work describes the standardization of an analytical method for the quantification of aldehyde groups in the capsular polysaccharide of *Streptococcus pneumoniae* serotype 5, modified via periodate oxidation. The procedure is based on the reaction with Purpald's reagent. The proposed method was demonstrated to be linear ($r^2 > 0,98$, $r > 0,99$), with a fit coefficient of variation and a relative standard deviation of slope of less than 5 % and 2 %, respectively. It was precise, showing a relative standard deviation below 3 % under intermediate precision conditions, and robust against variations in NaOH concentration. The results in this study provide evidence for the safe use of the assay as process control in the modification step, of serotype 5 polysaccharide in the QuimiVio® vaccine.

Keywords: *Streptococcus pneumoniae*; Purpald assay; spectrophotometric; validation; aldehyde groups.

RESUMEN

En la preparación de vacunas conjugadas polisacárido-proteína, la hidrólisis y activación de los polisacáridos capsulares resulta un paso clave para la obtención de conjugados monovalentes. Este trabajo describe la estandarización de un método analítico para la cuantificación de grupos aldehídos en el polisacárido capsular del serotipo 5 de *Streptococcus pneumoniae* modificado, mediante oxidación periódica. El procedimiento se basa en la reacción con el reactivo de Purpald. El método demostró ser lineal ($r^2 > 0,98$; $r > 0,99$), con un coeficiente de variación y una desviación estándar relativa menor al 5 y 2 %, respectivamente; preciso, con una desviación estándar relativa inferior al 3 % en condiciones de precisión intermedia; y robusto, ante variaciones en la concentración de NaOH. Los resultados ofrecen las evidencias para un uso seguro del ensayo el cual puede aplicarse como control de proceso en la etapa de modificación del polisacárido del serotipo 5 en la vacuna QuimiVio®.

Palabras clave: *Streptococcus pneumoniae;* ensayo de Purpald; espectrofotométrico; validación; grupos aldehído.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a microorganism responsible for a large part of otitis media, meningitis, pneumonia, and generalized sepsis worldwide. It is the main cause of morbidity and mortality in young children and the elderly.[1,2,3] Pneumococcal conjugate vaccines have proven to be the most effective prevention method for the incidence of this bacterium. These vaccines are capable of inducing a much more specific and long-lasting dependent T response.[4]

There are several methods for obtaining conjugate vaccines, such as peptide coupling, reductive amination, or the use of functionalized spacer arms, among others. These methods aim at the formation of the covalent bond between a capsular polysaccharide of the bacterium (usually selected as antigen) and a carrier protein. In all cases the presence of complementary reactive groups is necessary. Polysaccharides, due to their structural composition, have less diversity of reactive groups than proteins.[5]

To obtain a monovalent conjugate of serotype 5 of pneumococcus, the oxidation of the polysaccharide, via sodium periodate, is one of the most widely used methodologies. In this controlled reaction, aldehyde groups are formed through the oxidation of two neighboring hydroxyl groups, with the consequent breaking of the carbon-carbon bond.[6]

A large number of procedures for the determination of aldehydes have been described. The most widely used methods for the determination of formaldehyde use reagents such as chromotropic acid (4,5-dihydroxynaphthalene 2,7-disulfonic acid) or acetylacetone.[7] Other methods reported for the determination of aldehyde groups are the Park Johnson assay[8] and the assay based on the selective reaction of aliphatic aldehydes with 3-methyl-2-benzothiazolone hydrazone (MBTH) reported by Jacobsen *et al.*[9] The use the Purpald reagent (4-amino-3-hydrazino- 5-mercapto-1,2,4-triazole) is one of the simplest methods with acceptable results in scenarios of high structural complexity [10]. In addition, the assay results are more stable over time, more sensitive, and have less interference than other methods. An analytical procedure based on the reaction with the Purpald reagent was developed and standardized to determine the levels of activation with aldehyde groups in the activated polysaccharide of *Streptococcus pneumoniae* serotype 5.

MATERIALS AND METHODS

Samples and reagents

Streptococcus pneumoniae oligosaccharides, serotype 5, were supplied by the Research Division of Finlay Vaccine Institute (BioCubaFarma, Havana, Cuba). Formaldehyde standard (CH_2O, 39 g/mol, 37 %) and Sodium Hydroxide (NaOH, 40 g/mol) were supplied by Merck Ltd (Darmstadt, Germany). Purpald reagent ($C_2H_6N_6S$, 146.172 g/mol, 99 %) was supplied by Sigma (Merck KGaA, Darmstadt, Germany). All the reagents used were of analytical quality.

Analytical procedure

In a test tube of 8 mL, 200 µL of sample, distilled water as blank, or formaldehyde standard were added followed by 200 µL of the freshly prepared 146 mM 4-amino-3-hydrazino- 5-mercapto-1,2,4-triazole in 1M NaOH (Purpald solution). The solutions were stirred vigorously and left to rest for 30 min. at room temperature. Finally, all tubes were completed with 600 µL of distilled water and the absorbance (Abs) was measured in a UV-Vis spectrophotometer (JENWAY 6705, 1 nm, Germany) at 550 nm. Standardization parameters such as specificity, linearity, accuracy, and precision[6,7] were evaluated.

Standardization's parameters

Specificity: Formation of the reaction complex of the aldehyde group with the Purpald reagent. Samples with aldehydes groups in their structure were evaluated for quadruplicate capsular polysaccharide serotype 5 (CPs-5), oxidized polysaccharide (PsOx-5) of serotype 5, and oxidize polysaccharide serotype 23F (PsOx-23F). In addition, three different samples with or without aldehydes residues (anhydrous glucose, acetone and *Neisseria meningitidis* C group capsular polysaccharide) were also tested. Only samples with aldehyde groups in their structure should have significant absorbance.[11,12]

Linearity: quadruplicate six-point calibration curves were obtained in a concentration range from 5 to 160 nmol/mL of formaldehyde solution 500 nmol/mL. The line of best fit was determined by the least squares method, as well as, its correlation coefficient and the variation of the response factor. In addition, the statistical significance of the slope and the intercept was evaluated.[12,13]

Precision: the precision of the analytical procedure was determined under conditions of repeatability and intermediate precision.

Repeatability: six determinations of a sample of *Streptococcus pneumoniae* serotype 5 oligosaccharides with aldehyde groups were performed in triplicate at two times on the same day by the same analyst. The mean and the relative standard deviation were calculated.[12,14]

Intermediate precision: the determination of a sample of *Streptococcus pneumoniae* serotype 5 oligosaccharides with aldehyde groups was carried out in triplicate by two analysts during three different days. The mean and the relative standard deviation were calculated.[12,15]

Accuracy: the standard addition method was used. The recovery percentage was compared against 100 % for a 95 % of confidence level. Statistical analysis from the use of the Student t statistic: Null hypothesis (Ho): mean % recovery = 100 % recovery, Alternative: medium % recovery ≠ 100 % recovery.[12]

Robustness: robustness assessment was considered during the development phase. For the evaluation of this parameter, the influence of the plus or minus 10 % variation of the sodium hydroxide concentration on the concentration of the carbonyl groups was studied by evaluating the difference through a multiple comparison of the obtained results.

For the evaluation of all the parameters studied in the standardization of the procedure, the Microsoft Excel version 2016 (Microsoft, USA) and STATGRAPHICS 5 PLUS version 5.1 (StatPoint, Inc, USA) packages were used for processing.

RESULTS AND DISCUSSION

Specificity

The results of the specificity for the analytical procedure were achieved by evaluating samples with and without aldehydes in their structure, and are reported in Table 1.

For those samples without aldehyde in their structure, it is not possible to measure an absorbance higher than the blank at the wavelength of the procedure. The results confirm that the by-products of polysaccharide activation, the polysaccharide matrix, or other possible interferences (reduced sugar, ketone group, hemiacetals groups to monosaccharides),[12] do not affect the quantification results of generated aldehydes. The specificity results are analogous with those reported in similar published studies for others saccharides. For the Purpald reaction to be used in polysaccharide quantification, a previous periodate oxidation is essential to form the aldehyde groups.[7,16] This reaction is also useful for differentiating aldehyde carbonyl groups from ketone groups.[17]

Table 1- Result of the study of Specificity

Samples	Abs_1	Abs_2	Abs_3	Abs_4
CPs-5	0,263	0,293	0,248	0,269
PsOx-5 (2 mmol/L)	0,073	0,051	0,063	0,077
PsOx-23F	0,722	0,708	0,753	0,742
Anhydrous glucose	-0,062	-0,041	-0,049	-0,046
Acetone	-0,105	-0,108	-0,103	-0,106
CPs-Men C	-0,064	-0,066	-0,077	-0,068

Legend: $Abs_1 \ldots Abs_4$ (Absorbances); CPs-5 (*Streptococcus pneumoniae*, serotype 5 capsular polysaccharide); PsOx-5 (2 mmol/L) (*Streptococcus pneumoniae*, serotype 5 pneumococcal oligosaccharides oxidized); PsOx-23F (*Streptococcus pneumoniae* serotype 23F pneumococcal oligosaccharides oxidized); CPs-MenC (*Neisseria meningitidis* serogroup C capsular meningococcal polysaccharide).

Linearity study

The acceptance criterion in this study was the linearity of the calibration curve obtained with formaldehyde samples in quadruplicate with Purpald reagent at six different concentrations (Table 2).

Table 2- Results of linearity parameter

Parameters to evaluate	Acceptance Criteria	Results
Line equation	y = bx + a	y= 0,0065x+0,0036
Linear correlation coefficient	r ≥ 0,99	0,9999
Determination Coefficient	$r^2 \geq 0,98$	0,9998
Response factor relative standard deviation RSDf (%)	< 5	2,39
Statistical significance of intercept,	a=0	[-0,0030; 0,0060]
Relative standard deviation of slope Sbrel (%)	Sbrel ≤ 2	0,39

The equation of the function that describes the best adjustment of the calibration curve corresponds to that of a linear function. The correlation and determination coefficients suggest a good correlation between the data and a good linear regression. The determination coefficient shows more than 98 percent of the aleatoric and instrument errors are introduced by the calibration curve. It is observed that the standard deviation of the experimental slope is lower than the accepted values and the zero value is included in the confidence interval of the intercept for 95 % confidence. Therefore, the intercept is considered not significantly different from zero. The absorbance response to changing the formaldehyde concentration

is acceptable. The obtained results demonstrate compliance with the Lambert-Beer law.[18]

Precision

For precision assessment under repeatability conditions, six replicates of a pneumococcal serotype 5 polysaccharide sample oxidized by periodic oxidation were evaluated. The results are shown in Table 3. The table describes the evaluation of the precision under repeatability conditions, calculating the experimental value of the relative standard deviation (RSD), which is less than the acceptance criteria for a pectrophotometric method (RSD 3 %).[12,14,19] The intra-assay precision results for the Purpald-based modify 5 serotype polysaccharides assay align well with those reported by other author groups in similar studies.[20,21] Therefore, it is possible to state that the procedure under the evaluated conditions is precise under repeatability conditions.

Intermediate precision

The results of the Intermediate precision, performed by 2 analysts on 3 different days are shown in Table 4 by comparing the values of RSD expressed in percentage between the days, analysts, and the global RSD.

The results shown in Table 4 illustrate that all the RSDs are less than 3 % (between the analysts, days, and finally the global).[12,14,20] The inter-assay precision results for the carbonyl determination of the modified polysaccharide serotype 5 samples using the Purpald reagent are comparable to the results obtained from the standardization of other analytical procedures and those published by other research groups for similar studies [20,21]. Consequently, in the conditions assessed, the method is precise at intermediate precision conditions.

Table 3- Precision study under repeatability conditions

C1 (nmol/mL)	C2 (nmol/mL)	C3 (nmol/mL)	C_{mean} (nmol/mL)	SD	RSD (%)	Acceptance criteria
182,273	176,212	179,242	176,210	4,121	2,34	**RSD (%)** ≤ 3
179,242	176,212	183,788				
179,242	176,212	180,758				
177,344	171,094	174,219				
167,969	171,094	174,219				
174,219	175,781	172,656				

Legend: $C_1 \ldots C_3$, C_{mean} (Concentrations); SD (Standard Deviation); RSD (Relative Standard Deviation)

Table 4- Results of the intermediate precision study

	Day 1	Day 2	Day 3	intra- analyst average (n=18)	SD	RSD (%)
Analyst 1	192,051	191,344	191,113	190,142	3,355	**1,8**
	183,333	190,806	192,118			
	182,308	192,957	193,122			
	191,538	191,882	185,083			
	186,923	191,882	190,108			
	192,564	190,806	192,620			
average	188,120	191,613	190,694			
SD	4,583	0,815	2,954			
RSD (%)	**2,4**	**0,4**	**1,5**			
	Day 1	**Day 2**	**Day 3**	**intra- analyst average (n=18)**	**SD**	**RSD (%)**
Analyst 2	187,725	194,570	191,147	190,386	3,026	**1,6**
	191,429	192,419	191,924			
	186,667	192,957	189,812			
	187,725	196,183	191,954			
	184,550	191,882	188,216			
	187,725	187,725	192,344			
average	187,637	192,623	190,899			
SD	2,230	2,869	1,593			
RSD (%)	**1,19**	**1,49**	**0,83**			
Inter-analyst	**Day 1**	**Day 2**	**Day 3**	**global average (n=36)**	**SD**	**RSD (%)**
average	187,878	192,880	190,797	190,264	3,151	**1,7**
SD	3,4455	2,079	2,265			
RSD (%)	**1,83**	**1,08**	**1,19**			

Legend: n (number of replicates); SD (Standard Deviation); RSD (Relative Standard Deviation)

Accuracy

An add-recovery test was performed to study the accuracy of the procedure, considering that the procedure is exact if there is no statistically significant difference between the average recovery and 100 % at a confidence level of 95 %. The results obtained are shown in Table 5.

Table 5- Results of the Accuracy study

C_{Sample} (nmol/mL)	$C_{Standard}$ (nmol)	C_{S+S} nmol/mL	Recover (%)
47,474	20	66,797	96,618
47,474	20	67,005	97,659
47,474	20	67,266	98,963
47,474	20	68,047	102,867
		Average	**99,027**
		SD	**2,368**
		RSD %	**2,2**

Legend: C_{Sample}, $C_{Standard}$ (concentration of sample and standard, respectively); C_{S+S} (summatory concentration of sample and standard); SD (Standard Deviation); RSD (Relative Standard Deviation).

Average recovery results were compared to 100 % recovery using a t-student test at a confidence level of 95 %, a very common accuracy evaluation methodology in numerous studies.[22,23] The achieve p-value equal to 0,285451, was greater than 0,05 values, indicating that there are no statistically significant differences. The accuracy results, obtained through the add-recovery experiment, show acceptable recovery percentage.[11,12,14] Hence, the proposed assay demonstrated to be accurate.

Robustness

For the study of robustness, the incidence of the variation of 10 % of the NaOH concentration was evaluated, from the values established in the procedure (Figure 1).

Figure 1 shows the results of the robustness assessment when the concentration of NaOH is varied by 10 %. A multiple comparison of the aldehyde group determinations was applied when varying the NaOH concentration, and there were no statistically significant differences between the results in the three determinations for 95 % confidence level. It can be stated that the robustness of the method is to a 10 % variation of the NaOH concentration.

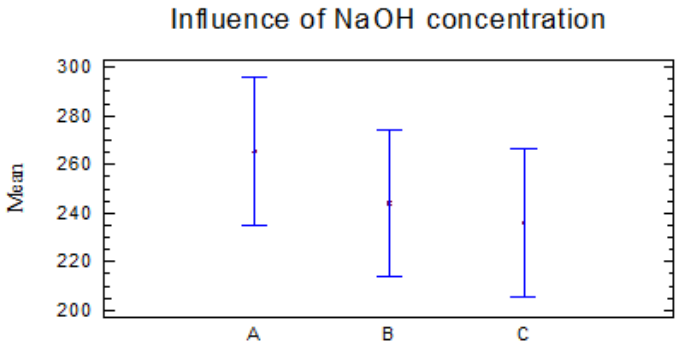

Fig. 1- Study of the influence of NaOH concentration

CONCLUSIONS

Purpald's method for determining carbonyl residues of aldehydes is simple and very useful in highly complex matrices. One such scenario is the activation of capsular polysaccharides via periodic oxidation. These biomolecules often contain hemiacetal residues in their structures, which generate interferences in traditional methods for aldehyde determination. The standardization study demonstrated that the proposed method, for quantification of carbonyl groups in oxidized samples of *Streptococcus pneumoniae* type 5, is linear, and precise under conditions of repeatability and intermediate precision. In addition, the method is specific for carbonyl groups of aldehydes and accurate. The robustness study by varying the NaOH concentration did not reveal significant changes for the assay results.

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INTEREST CONFLICT

The authors declare that there are no conflicts of interest relevant to the content of this article.

AUTHOR'S CONTRIBUTION

Bárbara Baró Bicet: participated in conceptualization, data curation, research, methodology, and visualization.
Felix Cardoso San Jorge: participated in conceptualization, data curation, formal analysis, methodology, visualization, writing (original draft), and review/editing.
Raine Garrido Arteaga: participated in formal analysis, review, and visualization.
Darielys Santana Mederos: participated in conceptualization, research, and visualization.
Jessy Pedroso Fernández: participated in conceptualization, formal analysis, methodology, writing (original draft), review, and visualization.
All authors reviewed and approved the final version of this manuscript.