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Article

Characterization of polyribosylribitol phosphate by high performance molecular exclusion chromatography with ultraviolet detection

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Abstract: Haemophilus influenzae type b is an important human pathogen causing several invasive diseases in children under five years of age, against which glycoconjugated vaccines based on polyribosylribitol phosphate have been licensed. Quimi-Hib® is the first and only vaccine against this pathogen using the polysaccharide obtained by chemical synthesis. The Active Pharmaceutical Ingredient is produced by the Center for Genetic Engineering and Biotechnology and is obtained from its conjugation to tetanus toxoid. In the present report, a characterization of polyribosylribitol phosphate was performed using the high performance molecular exclusion chromatography technique with ultraviolet detection at 215 nm. Three batches were evaluated in the study and the elution profile was determined on a Superdex™ 75 10/300 GL Increase column with a purity percent of 77.42 ± 8.97 and an average molar mass of 7381 Da ± 210.93. The main impurity present in polyribosylribitol phosphate is dimethyl sulfoxide, the solvent used in the activation reaction with the N-hydroxysuccinimidyl ester of β-maleimidopropionic acid. The polyribosylribitol phosphate was purified by filtration with a 2000 Da Amicon Ultra-15 to 99.1% purity and conjugated to tetanus toxoid. The yield of the conjugation reaction with the purified polysaccharide was 30.0% ± 1.77% which shows no significant difference with the control which was 33.7% ± 3.57% demonstrating that dimethyl sulfoxide does not affect the performance of the conjugation reaction.

Keywords: gel chromatography; polysaccharides; tetanus toxoid; dimethyl sulfoxide; conjugate vaccines; haemophilus influenzae type b

1. Introduction

Haemophilus influenzae serotype b (Hib) bacteria is a serious global human health problem and primarily affects children under 5 years of age [1]. The diseases it causes include meningitis, pneumonia, epiglottitis, and other respiratory tract diseases [2]. The global incidence of Hib-caused disease during 2000–2012 was 0.27 per 100,000 population according to HealthyPeople 2020 [1].

The active ingredient of commercial Hib vaccines is the polysaccharide polyribosylribitol phosphate (PRP). Initially, purified capsular polysaccharide was used to induce protective immunity; however, the response in children was very poor.

The solution to this problem was achieved by covalently linking the polysaccharide to carrier proteins such as: tetanus toxoid (PRP-TT) [3], diphtheria toxoid [4], CRM197 (non-toxic variant of diphtheria toxin) and the protein complex of the outer membrane of the Neisseria meningitidis serogroup B bacterium (PRP-OMP) [5]. With all these antigen preparations, a protective level of antibodies and a

high immune response was obtained from two months of age [6]. These vaccines are called glycoconjugate vaccines and have been reported to be highly effective and safe vaccines for all patients regardless of their age [7].

In the case of the Quimi-Hib® vaccine, the polysaccharide is obtained by chemical synthesis and is conjugated to tetanus toxoid (TT). The incidence of Hib infections in Cuba decreased significantly from 1.5 per 100,000 inhabitants in 1998 to 0.9 in 2001 after its introduction in the national vaccination schedule. In 2003, Quimi-Hib®, the first vaccine with a synthetic antigen in the world, was registered and marketed [8].

The Hib Active Pharmaceutical Ingredient (API) is produced on an industrial scale at the Center for Genetic Engineering and Biotechnology (CIGB). The performance of the conjugation reaction has been consistent in meeting the specifications of the PRP and conjugate as established by WHO for the production of Hib vaccines [9]. The quality of PRP is evaluated lot by lot using the following techniques and compared with the corresponding acceptance limits: ribose content (>32% on dry weight basis), phosphorus content (6.8% to 9.0% on dry weight basis), bacterial endotoxin content (<10 EU/µg PRP) and degree of polymerization and activation (>5 polymeric units). The identity and presence of impurities is evaluated by Nuclear Magnetic Resonance (NMRN1H13C). The acceptance limit established for contaminants in the last stage of synthesis is as follows: maleimido propionic acid (<0.8 ppm/20 µg of product), amino spacer antigen (<10% of signal at 3.3 ppm), *N*-hydroxysuccinimide (<0.8 ppm/20 µg of product), maleamic acid (<0.3 of signal 6.83 ppm) and cyclic phosphate (<10% of signal at 5.01 ppm) [9].

The purification of the PRP activated with the N-hydroxysuccinimidyl ester of β-maleimido propionic acid is carried out by filtration with a membrane of 1000 Da and another of 10000 Da which suggests that its molecular weight is in this range. In the evaluation by1H NMR, an intense signal is observed at 2.7 ppm, which corresponds to the presence of dimethyl sulfoxide (DMSO), the solvent used in the PRP activation reaction [9]. This solvent presents oxidizing properties in a pH range from 3.0 to 8.0 [10] which includes the conditions in which the conjugation reaction is carried out at pH 7.4 [6].

The present study aims to characterize PRP by high-performance molecular exclusion chromatography with ultraviolet detection to obtain the elution profile, molecular weight and purity using this technique. In addition, to purify the PRP and to evaluate the effect of impurities present in the PRP on the performance of the conjugation reaction.

2. Materials and methods

2.1. Samples, reagents and standards

The PRP and tetanus toxoid batches were supplied by the Finlay Vaccine Institute.

The control IFA was provided by CIGB's Quimi-Hib® vaccine IFA production facility.

The standard proteins used to perform the calibration curve were: ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), ribonuclease A (13,700 Da), aprotinin

(6512 Da) supplied by Sigma Aldrich (USA).

The reagents used for the conjugation reaction on the laboratory scale were of "for synthesis" quality and those used in the analytical assays were of "for analysis" quality. The dithiothreitol (DTT) used was supplied by Merck KGaA (Darmstadt, Germany), the N-hydroxysuccinimido ester of 3,3-dithiopropionic acid and *N*-ethylmaleinimido by SigmaAldrich (St. Louis, MO, USA) and dimethyl sulfoxide (DMSO) by Scharlau (Scharlab S.L., Spain). The EDTA used was supplied by Merck (Germany).

The supplier of sodium dihydrogen phosphate (NaH_2PO_4) was Merck (Germany) and of sodium chloride (NaCl), AppliChem (Germany).

Characterization of PRP by molecular exclusion chromatography was performed on Äkta pure 25 M equipment (USA) and a Superdex™ 75 10/300 GL Increase column (GE HealthCare, USA) with a fractionation range of 3000–70,000 Da was used. Samples of purified PRP, PRPTT conjugates and control IFA were evaluated on a high-performance liquid chromatograph coupled to a Prominence UV detector (SHIMADZU, Japan) and a Yarra™ SEC 2000 column (Phenomenex, USA) with a separation range of 1000–300,000 Da was used.

2.2. Analysis of PRP by molecular exclusion chromatography

The wavelength of maximum absorption of PRP was determined in an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Sweden) using a sample at 0.25 mg/mL taken from the PRP_2 batch.

The elution profile of PRP was determined on the Superdex™ 75 10/300 GL Increase column (GE HealthCare, USA). The mobile phase used was a buffer solution of NaH_2PO_4 (100 mM) and NaCl (150 mM), pH = 7.0. Three batches of PRP were analyzed and a volume of 10 µL was applied at a concentration of 5.0 mg/mL. The flow rate used was 0.8 mL/min at a maximum pressure of 1.8 MPa for 31 min at room temperature. Samples were measured at the wavelength of maximum absorption. The purity of PRP was determined as the percent area under the curve over the retention time.

The calibration curve to determine the molecular weight was performed by weighing 1 mg of each standard protein dissolved in 1 mL of type II reagent water, obtaining a final concentration of 1.0 mg/mL of each one. Of each standard protein, 50 µL were taken for a final volume of 200 µL of sample. The relative molecular weight of the PRP was determined through the best fit of the correlation curve between the retention time and the molecular weight of the standard proteins.

The evaluation of the impurities in the PRP (DMSO or others) was performed on the three batches of PRP on the Superdex™ 75 10/300 GL Increase column maintaining for the analysis the conditions used to determine the elution profile. The sample applied was 100 µL of pure DMSO (98%) at a concentration of 4.5% (0.049 g/mL) and an equal volume of dissolved PRP at a concentration of 1.0 mg/mL.

2.3. PRP purification

The PRP batches selected for this study were PRP_2 and PRP_3 at a concentration of 30.0 mg/mL in a volume of 1 mL. For purification the samples were filtered on a

2000 Da molecular sieve Amicon Ultra-15 (Germany) at 2330 x g, with a filtration time of 2.5 h. At the end of each wash, the volume was adjusted to 1 mL and PRP concentration was determined by orcinol assay [11] and purity by molecular exclusion chromatography on a Yarra™ SEC 2000 column (Phenomenex, USA). The sample volume applied was 50 µL at a concentration of 1 mg/mL. The flow rate used was 0.8 mL/min at a maximum pressure of 1.8 MPa for 17 min at room temperature. Samples were measured at the wavelength of maximum absorption of PRP. The mobile phase used was a buffer solution at pH = 7.0 (100 mM NaH_2PO_4, 150 mM NaCl). The purity of PRP was determined as the percent area under the curve over the retention time.

2.4. Conjugation of PRP to tetanus toxoid.

Conjugation was performed in two stages:

Step 1: A solution of tetanus toxoid was filtered with phosphate buffered saline, pH 8.2; with EDTA 86 g/L, 250 mL) with a 30,000 Da molecular sieve membrane. To the filtered solution (100 mg; 0.67 µmol) was added *N*-hydroxysuccinimido ester of 3,3-dithiopropionic acid (8.1 mg; 20 µmol) in dimethyl sulfoxide (500 µL) and $N_{2(g)}$ atmosphere. The mixture was stirred for 2 h at 20 °C. DTT (77.12 mg; 500 µmol) was then added under $N_{2(g)}$ atmosphere and the stirring was maintained for 1 h at 20 °C. The resulting solution was filtered with saline phosphate buffer, pH 7.4 with EDTA at 1.86 g/L, 250 mL) with a 30,000 Da molecular sieve membrane and evaluated for the presence of thiol groups in the permeate by the Ellman technique [12]. This was the criterion for stopping the filtration. Protein concentration was determined by the Lowry method [13,14].

Stage 2: To the tetanus toxoid solution previously modified by addition of thiol groups (5 mg, 1 mL) was added PRP previously dissolved in saline phosphate buffer, pH 7.4; 30 mg/mL at a 1:2.5 PRPTT ratio in $N_{2(g)}$ atmosphere. The solution was kept in agitation for 12 h at 20 °C. *N*-ethylmaleimido reagent (0.082 mg; 0.654 µmol) was added to inactivate the thiol groups that remained free in the conjugation reaction. The conjugate was filtered with a phosphate-salt buffer, pH 6.8 with a 30,000 Da molecular sieve membrane and the amount of PRP present in the permeate was evaluated by the phenolsulfuric acid technique [15] as a criterion for stopping the filtration. The concentration of protein and total and free carbohydrates in the conjugate was determined by Lowry [13,14] and orcinol [11] analytical techniques, respectively.

Conjugation to tetanus toxoid was done in duplicate with PRP_3 and $PRP_{3.1}$ (purified PRP_3) and the corresponding conjugates and starting compounds were analyzed by molecular exclusion chromatography on a Yarra™ SEC 2000 column (Phenomenex, USA), under the same experimental conditions mentioned above for PRP purity determination, after purification.

The reaction yield was determined as the ratio between the mass of bound PRP and the mass of starting PRP. The results obtained were compared by means of a simple analysis of variance (ANOVA) for a significance level of 0.05 and these in turn with the percentage of incorporation of the starting PRP_3 reported by the Quality Control Laboratory (CIGB, Cuba) which was 34.2%.

2.5. Statistical analysis

Statistical analysis and data processing were performed with Statgraphics Plus, Origin® 8 and Microsoft Excel spreadsheet software.

3. Results and discussion

3.1. PRP analysis by molecular exclusion chromatography

Spectrophotometric scanning of PRP_2 from 200 to 400 nm showed an absorption maximum around 215 nm wavelength as observed in **Figure 1**.

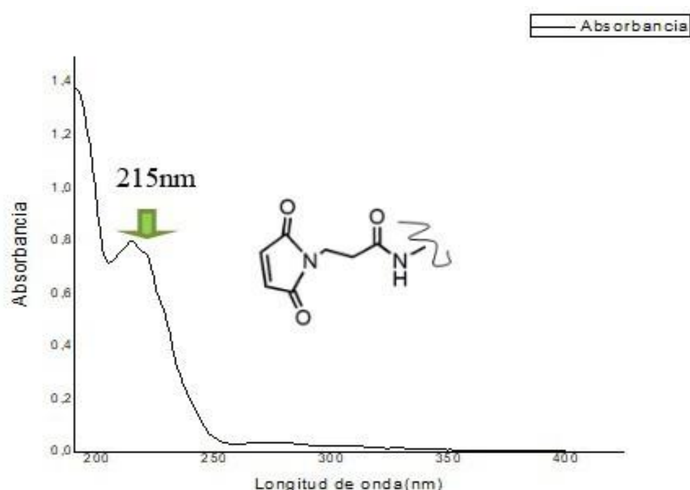

Figure 1. UV-Visible spectrum of PRP dissolved in water.

The wavelength value obtained is characteristic of the α, β-unsaturated ketone groups in the maleimido ring present in the PRP. The conjugation between the carbonyl group and the vinyl system, decreases the energy difference between the π and π* orbitals producing the bathochromic shift of the band towards the 210 to 235 nm region (ε_{max} = 8000 to 20,000). This band has an appreciable charge transfer character, i.e., in the excited state the electron density on the carbonyl increases at the expense of the π cloud of the vinyl [16].

Table 1 shows the most probable distribution of molecular weights according to retention time and % purity of the three PRP batches expressed as the area under the curve.

Table 1. Most probable distribution of molecular weights according to retention time and % purity of PRP_1, PRP_2 and PRP_3 samples evaluated on the Superdex™ 75 10/300 GL Increase column at 215 nm.

Samples	Population 1			Population 2			Population 3		
	TR_1 (min)	Purity (%)	MM_1 (Da)	TR_2 (min)	Purity (%)	MM_2 (Da)	TR_3 (min)	Purity (%)	MM_3 (Da)
PRP_1	21.75	86.96	7.409	24.52	1.75	3.588	26.23	11.30	2.293
PRP_2	21.67	69.16	7.576	24.46	0.72	3.645	26.07	30.13	2.402
PRP_3	21.88	76.14	7.157	ND	ND	ND	26.41	23.86	2.187
Media	21.77	77.42	7.381	24.49	1.24	3.616	26.23	21.76	2.294

STD: Standard deviation. MM: molecular mass. TR: retention time. ND: Not detected.

The relative molecular weight was determined by substituting the retention times (tr) in the equation of the calibration curve log MM = 6.344 − 0.113 tr, constructed from the curve of the protein standards. The coefficient of determination R^2 = 0.996, close to unity, evidenced the good fit of the model. The estimation of the PRP molecular weight was performed using the calibration curve constructed with the following protein standards: ovalbumin (tr = 14.81 min, 43,000 Da), carbonic anhydrase (tr = 16.69 min, 29,000 Da), ribonuclease (tr = 19.15 min, 13,700 Da) and aprotinin (tr = 21.99 min, 6512 Da).

From the mass values obtained for the most abundant population (population 1), the average apparent molecular mass of PRP was estimated, obtaining the value 7381 Da ± 210.93, which is within the declared size range for PRP obtained by chemical synthesis, between 1000–10,000 Da.

The remaining populations elute at the exclusion limit of the column, making it difficult to estimate the distribution of their molecular weights and, therefore, their correct identification.

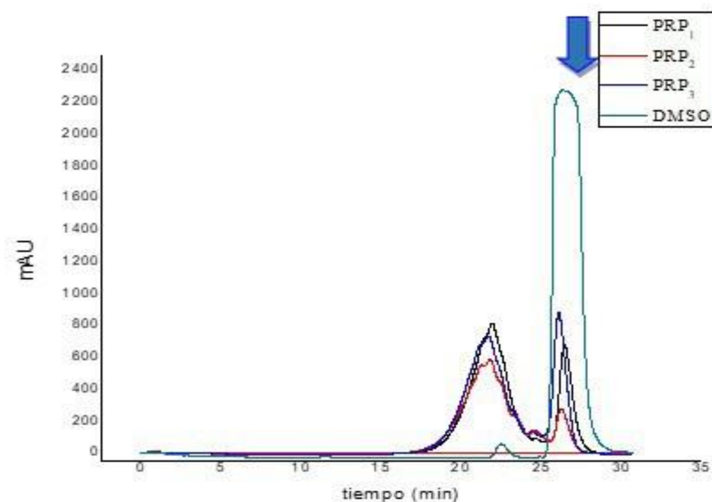

Figure 2. Identification of the characteristic signal of DMSO in samples from batches PRP_1, PRP_2 and PRP_3 by molecular exclusion chromatography with the Superdex™ 75 10/300 GL Increase column at 215 nm.

As background, it is known that in the PRP produced by the Finlay Vaccine Institute there is dimethyl sulfoxide given by the signal that appears at 2.7 ppm in the RNM^{1}H spectrum and that its presence in the reaction medium could provoke the oxidation of the thiol groups previously formed in the protein [10]. DMSO is miscible in water and exhibits oxidizing properties over a wide pH range from 3.0 to 8.0[17]. This reaction would compete with the thiol-maleimide interaction between the modified tetanus toxoid and the polysaccharide, decreasing the yields of the product of interest. Populations 2 and 3 observed in the chromatogram could correspond to the presence of DMSO in the sample, with a molecular size of 78.13 Da, a very small species that penetrates the pores of the stationary phase and elutes in a retention time not in accordance with its size. Of the remaining impurities that could be present in the evaluated batches, very few intense signals are observed in the1H and 13C NMR spectra, so they were not analyzed in this study.

The presence of DSMO in the three PRP batches was evaluated on the Superdex™ 75 10/300 GL Increase (**Figure 2**).

The chromatogram (**Figure 1**) shows that in the pure DMSO sample (98%) a signal appears at 26.32 min, which overlaps the second most predominant population in the PRP samples, with an average retention time of 26.23 min. This overlap of both signals with similar tr suggests that this signal corresponds to the DMSO impurity present in the PRP.

3.2. PRP purification

Purification of PRP was performed with the aim of removing the DMSO content present in the PRP. The PRP batches used in the study were PRP_2 and PRP_3. Samples taken from each wash were subjected to isocratic separation on a Yarra™ SEC 2000 column as it presents a wider separation range and allows the analysis of samples with molecular sizes larger than that of PRP such as the TT conjugate (150,000 Da). **Figure 3** shows the elution profiles of the samples resulting from the analysis of PRP_2 and PRP_3 in each filtration performed.

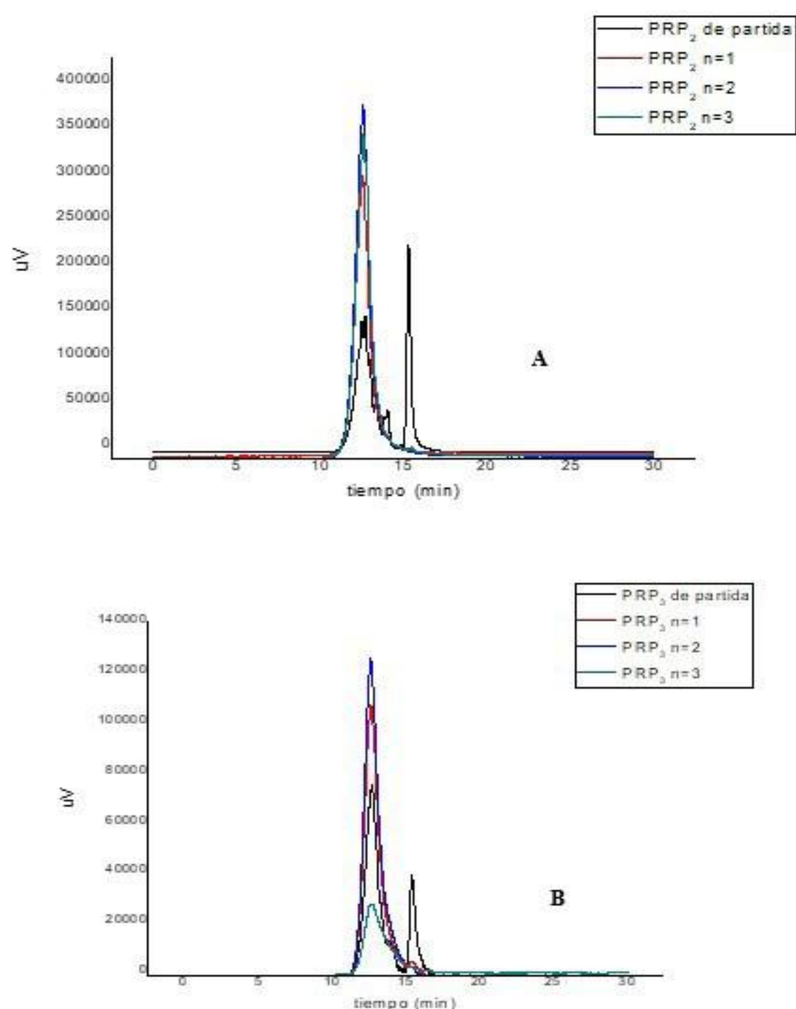

Figure 3. Elution profile of PRP (A: PRP_2 and B: PRP_3) purified on the Yarra™ SEC 2000 column at 215 nm, n: filtrations performed with water on a 2000 Da Amicon Ultra-15.

Figure 3 shows that with the Yarra™ SEC 2000 column the run time is shorter and, therefore, the tr decreases for each population. However, the purity value determined for PRP on the Superdex™75 10/300 GL Increase column at 215 nm is maintained (**Tables 1** and **2**).

The chromatograms show how the DMSO signal undergoes a considerable decrease from the first wash with water, a condition maintained by the sample in the rest of the washes performed. **Table 2** shows the results of the % purity of the purified PRP and the concentration of carbohydrates determined by the orcinol assay [11].

Table 2. Concentration of initial and purified PRP_2 and PRP_3 samples with 3 washes determined by orcinol assay [11] and % purity of PRP signal as analyzed on Yarra™ SEC 2000 column.

Samples	*n*	Volume (mL)	TR (min)	PRP concentration (mg/mL)	Purity (%)
PRP_2	0	1	12.51	30	63.34
$PRP_{2.1}$	1		12.51	19.2	100.00
$PRP_{2.2}$	2		12.53	15.4	100.00
$PRP_{2.3}$	3		12.50	10.1	99.68
PRP_3	0		12.69	30	76.20
$PRP_{3.1}$	1		12.68	20.59	99.10
$PRP_{3.2}$	2		12.56	14.69	99.87
$PRP_{3\,3}$	3		12.50	9.55	100.00

TR: retention time.

As both aspects, purity and concentration, are fundamental to obtain a product with high yields, it was decided that one wash is sufficient to obtain a PRP with a purity above 99% and a concentration close to 30.0 mg/mL, as established by the conjugation reaction on an industrial scale. Taking these elements into account, the PRP was purified by filtration with a wash and the tetanus toxoid conjugation reaction was performed.

3.3. Conjugation of PRP to tetanus toxoid

The conjugation reaction was performed with PRP_3 and $PRP_{3.1}$ and **Figure 4** shows the elution profile obtained on the Yarra™ SEC 2000 column at 215 nm for samples taken from PRP_3-TT, $PRP_{3.1}$-TT and IFA 19002 (control) conjugates and PRP_3 and modified tetanus anatoxin reactants.

The chromatogram shows very similar elution profiles between the PRP_3-TT and $PRP_{3.1}$-TT conjugates and between them and the IFA 19002 control, indicating that the industrial process was reproduced at laboratory scale. In this case, a single population predominates corresponding to the conjugate that was filtered through a 30,000 Da cut-off membrane where free PRP and other impurities are eliminated.

The average retention time was 6.10 min ± 0.003, which decreases compared to that obtained for the starting PRP, a product of covalent binding to TT with a reported molecular weight value of Da.

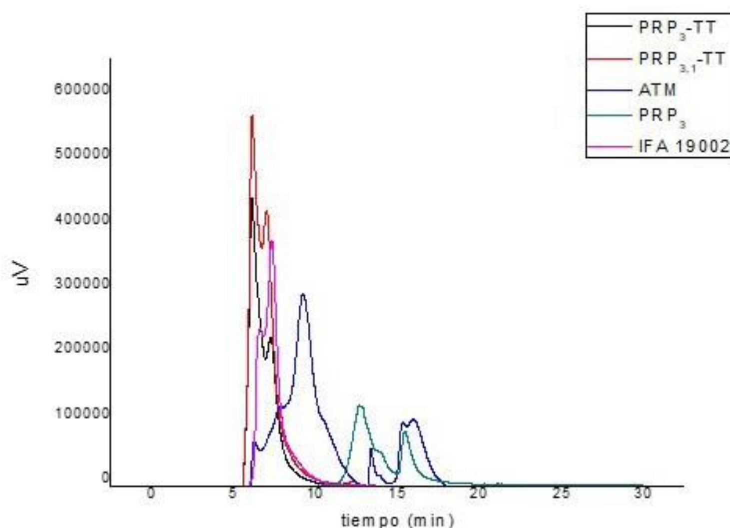

Figure 4. Elution profile of PRP_3-TT and $PRP_{3.1}$-TT conjugates and IFA 19002 (control and PRP_3 precursors and modified tetanus anatoxin) on the Yarra™ SEC 2000 column at 215 nm.

The average yield of the conjugation reaction with PRP_3 was 33.7% ± 3.57% and with purified PRP_3 ($PRP_{3.1}$) was 30.0% ± 1.77%. Comparison of these results by ANOVA showed that there are no significant differences between them or with the value reported by the Quality Control Laboratory (34.2%) for the conjugate obtained at production scale, with a confidence level of 95% ($p \geq 0.05$).

The results show that the presence of DMSO in the PRP does not affect the performance of the tetanus toxoid conjugation reaction in the process of obtaining the IFA of the Quimi-Hib® vaccine. That is, the concentration of DMSO in the sample is not sufficient to promote the formation of disulfide bonds in the protein.

4. Conclusions

The characterization of the PRP obtained by chemical synthesis at industrial scale by means of high performance molecular exclusion chromatography with ultraviolet detection, allowed determining that the PRP presents an absorption maximum at 215 nm, with a purity percentage of 77.42 ± 8.97 and an average molar mass of 7.381 Da ± 210.93 which is consistent with the expected size between 1000–10000 Da. The analysis of the elution profile showed that the main impurity present in the composition of the PRP is DMSO. Purification of PRP to 99.1% and subsequent conjugation to TT showed that the DMSO present does not affect the performance of the conjugation reaction.

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Conflict of interest: The authors declare no conflict of interest.

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