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Quantitative Proton Nuclear Magnetic Resonance evaluation and total assignment of the capsular polysaccharide *Neisseria meningitidis* serogroup X

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ABSTRACT

Neisseria meningitidis constitutes the main cause of meningococcal disease in infants. Serogroups A, B, C, W135, Y, and X have the higher incidence in young children and teenagers. The use of polyvalent conjugate carbohydrate-based vaccines has decreased the meningococcal infection around the world. Recently, the serogroup X has been found to be responsible of different outbreaks of meningococcal diseases, mainly in "Meningitis Belt" of Africa and the structure of the repetitive unit of the capsular polysaccharide has been confirmed through a monodimensional ^{13}C NMR study. No further characterization studies have been carried out, especially with the use of other nuclei. In this paper a novel method for quantification of the *N. meningitidis* serogroup X by proton qNMR is reported. Deep characterization of the serogroup X polysaccharide was also carried out by combination of correlation experiments involving ^{13}C, ^{1}H, and ^{31}P nuclei.

1. Introduction

Neisseria meningitidis is one of the main pathogens responsible of meningococcal disease in infants and adolescents. Thirteen serogroups have been reported, however six of them (A, B, C, W135, Y and X) are the main cause of outbreaks, endemic and epidemic meningococcal diseases [1]. The serogroup X was first described in 1966 by Bories et al. [2] This strain causes substantial diseases in the "Meningitis Belt" of Africa but only rarely causes meningococcal disease in other parts of the world. Different outbreaks by this group have been reported in Niger, Ghana, Kenya, Burkina Faso, Togo, and Uganda since 2006 [3–5]. Several vaccines are employed to prevent the meningococcal infections from serogroups A, B, C, W135, and Y but none of them induce protection against X serogroup [6]. This serogroup has become a critical target for the next generation of meningococcal vaccines. Importantly, in 1974 Bundle et al. confirmed the structure for the repetitive unit of native polysaccharide through a monodimensional ^{13}C NMR study [7]. The serogroup X polysaccharide (PsX) is a homopolymer of linked 2-acetamido-2-deoxyglucosyl phosphate α1→4[→4)-α-D-GlcpNAc-(1→OPO_3→]; (see Fig. 1) No further characterization studies have been carried out especially with the use of other nuclei. However, many NMR correlations experiments developed since 1980 and some capabilities of modern NMR spectrometers can now expand the earlier studies [8,9]. Hence, a comparative structural characterization of PsX is recommended. In the last decade, the application of quantitative Proton Nuclear Magnetic Resonance had an increasing impact on the pharmaceutical industry. The qNMR method involves NMR experiments with several modified parameters in order to obtain spectra with quantifiable signals (99.9% recovery of thermal equilibrium magnetization). These parameters depend essentially on the relaxation times of the nuclei to be evaluated for the analyte and the reference [10,11]. This paper discusses a novel Proton Nuclear Magnetic Resonance method for the quantification of *N. meningitidis* serogroup X capsular polysaccharide. In addition, the total NMR assignment of the PsX as result of combining correlation experiments of ^{1}H, ^{13}C, and ^{31}P nuclei is reported as a complementary study.

Abbreviations: ^{1}H NMR, Proton Nuclear Magnetic Resonance; ^{13}C NMR, Carbon 13 Nuclear Magnetic Resonance; qNMR, Quantitative Nuclear Magnetic Resonance; QNP, Quadruple Nucleus Probe; HSQC, Heteronuclear Single Quantum Coherence; PENDANT, Polarization Enhancement During Attached Nucleus Testing; DQF-COSY, Double Quantum Filtered Correlation Spectroscopy; GARP, Globally optimized Alternating-phase Rectangular Pulses; LB, Line Broadening.

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[1] In memory of Dra. Violeta Fernández Santana, deceased on November 20th, 2011.

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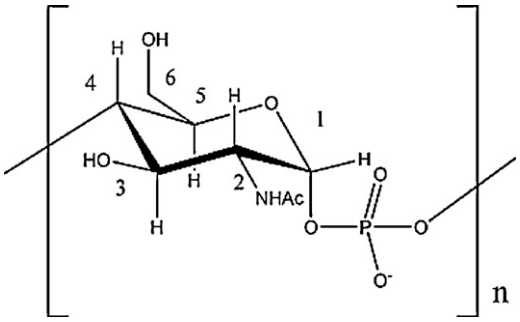

Fig. 1. Chemical structure of PsX repetitive unit.

2. Materials and methods

2.1. Sample preparation

The capsular polysaccharide of *N. meningitidis* serogroup X was supplied by the Technological Development Department from the Finlay Institute, Havana, Cuba as a result of collaborative scientific project between Norwegian Institute of Public Health (NIPH), Norway and Finlay Institute. One aliquot was taken from a stock solution to determine the PsX content by the colorimetric assay. From the colorimetric result several aliquots were also taken to prepare three sets of samples for qNMR with seven different quantities of PsX per set [(A) 5.84 mg, (B) 7.61 mg, (C) 11.12 mg, (D) 15.55 mg, (E) 16.46 mg, (F) 19.69 mg, and (G) 21.96 mg]. The aliquots were diluted in 0.6 ml of deuterium oxide for a lyophilization step. This stage allows the exchange of hydroxyl protons by deuterium. The resultant powder was again dissolved in 0.6 ml of deuterium oxide with 3-(trimethylsilyl) 2,2,3,3-tetra-deuteropropionic acid sodium salt (TPS-d_4) (1 mg/ml) as reference. For phosphorus spectra, one capillary with triphenylphosphine (PPh_3) as reference was inserted into the 5 mm NMR tube. All the solvents and reagents were of spectroscopic quality.

2.2. Colorimetric assay

Primary quantitative evaluation was carried out for a stock solution of PsX according to the total phosphorus determination proposed by Chen et al. [12].

2.3. Instrument settings

NMR analyses were carried out on a Bruker Avance DPX 250-MHz instrument operating with a 5 mm QNP *z*-axis gradient probe at 27 °C. The ^{13}C NMR edited spectrum was obtained through a PENDANT experiment. The edited HSQC (^{1}H–^{13}C) delay for single quantum evolution was optimized for $^{1}J_{H-C}$ = 150 Hz. The HSQC (^{1}H–^{31}P) delay for developing the heteronuclear correlation was optimized for $^{3}J_{H-P}$ = 7 Hz. DQF-COSY experiments were run in the magnitude mode [13]. The TSP-d_4 non-deuterated methyl signal was referenced at δ 0.00 ppm to calibrate the spectra. The phosphorus spectra were calibrated referencing the PPh_3 signal at δ −5.00 ppm [13].

The optimized proton qNMR experiment was set without sample spinning, a recycled time of 8 s, pulse duration P1 adjusted as reported by Ernst and Anderson [14] and corresponding to 88°, and ^{13}C decoupling using the GARP sequence. To obtain the spectra, each Free Induction Decay (FID) was processed with a Fourier Transform apodizated by an exponential function (LB 0.3 Hz). To guarantee the signal integration process baseline adjustment was required. The spectral data were acquired and transformed under TopSpin 1.3 software pack.

2.4. Statistical analysis

The statistical evaluation of the proposed method was tested by the study of the following parameters: specificity, linearity, precision (intra and inter day), accuracy, and ruggedness. All statistical calculations were carried out according to International Conference on Harmonization (ICH) guidelines [15,16].

3. Results and discussion

Unlike the only approach for the PsX assignment carried out by ^{13}C NMR by Bundle et al. [7], we have made a total assignment of the PsX repeating unit using also ^{1}H and ^{31}P NMR. In addition, we have developed an assay for the quantitative evaluation of the polysaccharide optimizing several parameters of the ^{1}H NMR spectrum.

3.1. Assignment of the polysaccharide by ^{1}H, ^{13}C, and ^{31}P NMR

The ^{13}C PENDANT (Fig. 2a) spectrum shows some peaks as doublets due to the heteronuclear coupling between carbon and phosphorus nuclei [7,13]. The singlet of the carbonyl group is a negative peak at low field (δ 175.1 ppm). The other negative peak is the methylene carbon (C-6) at δ 60.9 ppm. The doublet of the anomeric carbon (C-1) appears in the expected region at δ 94.6 ppm and $^{2}J_{C-P}$ = 5.9 Hz. The methyl of the N-acetyl methyl group shows a singlet at high field (δ 22.7 ppm). Carbon C-2 is a doublet at δ 54.2 ppm ($^{3}J_{C-P}$ = 8.5 Hz). The other carbons (C-3, C-4, and C-5) show close doublets at δ 70.6, 74.5, and 72.6 ppm with coupling constants of $^{3}J_{C-P}$ = 1.5 Hz, $^{2}J_{C-P}$ = 6.0 Hz and $^{3}J_{C-P}$ = 5.1 Hz, respectively. These last assignments were compared with the literature [7] and confirmed through the combination of the edited HSQC (^{1}H–^{13}C), HSQC (^{1}H–^{31}P) and the DQF-COSY experiments; see Table 1.

The ^{1}H NMR spectrum shows the anomeric proton (H-1) as doublet of doublet at δ 5.57 ppm due to the heteronuclear ($^{3}J_{H-P}$ = 6.4 Hz) and the homonuclear coupling constants ($J_{H-1-H-2}$ = 2.9 Hz) [7]. These values confirm the stereochemistry of the glycosidic bond [17]; see Fig. 2b. The singlet at δ 2.09 ppm belongs to the methyl of N-acetyl methyl group. The assignments of the region between δ 4.20 and 3.70 ppm were carried out step by step combining the bidimensional experiments. The only negative cross peak in the edited HSQC (^{1}H–^{13}C) is the heteronuclear correlation of the methylene (H-6a → C-6 and H-6b → C-6) [13]. The shift of H-2 (δ 4.07 ppm) was also assigned from the edited HSQC (^{1}H–^{13}C) [13]; (Fig. 3a). The signal of H-4 was unambiguously assigned at δ 4.11 ppm using the HSQC (^{1}H–^{31}P) spectrum. The shift of H-5 at δ 4.05 ppm was provided by the COSY experiment through the scalar coupling cross peak between H-6 → H-5. The analogous cross peak for H-3 → H-4 correlation shows the final assignment of H-3 at δ 3.99 ppm (data not shown) [17]. H-3, H-4, and H-5 assignments are shown in Fig. 3b. The phosphorus assignment at δ −2.0 ppm was also made; (see Fig. 3c).

3.2. Specificity, structural analysis and spin lattice relaxation time (T_1) calculation

We have selected the region between δ 1.99 and 2.19 ppm (R-1) for quantitative evaluation of PsX due to this intense signal without interference. In addition, the region between δ −0.10 and 0.10 ppm (R-2) was selected for evaluating the reference; (Fig. 4). The specificity was demonstrated checking the no interference of any other component in the evaluated regions ensuring the specific contribution of the analyzed compounds PsX and TSP-d_4; see Fig. 3.

The conditions for acquiring the FID of qNMR were optimized using an inversion-recovery experiment to measure spin lattice relaxation times of evaluated protons (T_1) and to calculate the Ernst angle in order to provide a quantitative magnetization recovery.

Table 1
Results of the structural characterization of PsX.

Nucleus observed	δ ^{13}C NMR [Bundle et al.] (ppm)	$^{x}J_{C-P}$ (Hz)[a]	δ ^{1}H NMR (ppm)	$^{3}J_{H-P}$ (Hz)
1	94.6 [95.2]	5.9 [5.0]	5.57	6.4
2	54.2 [55.0]	8.5 [8.0]	4.07	–
3	70.6 [71.5]	1.5 [1.7]	3.99	–
4	74.5 [70.3]	6.0 [5.9]	4.11	5.4
5	72.6 [73.0]	5.1 [5.0]	4.05	–
6	60.9 [65.3]	–	3.89	–
Methyl (NHAc)	22.7 [23.0]	–	2.09	–
C=O (NHAc)	175.1 [175.6]	–	–	–
Phosphate	31 P NMR δ −2.00 ppm			

[a] $x = 2$ for C-1 and C-4 nuclei; $x = 3$ for C-2, C-3 and C-5 nuclei.

The higher value calculated for relaxation time was 1.5 s for the methyl nucleus of TSP-d_4.

3.3. Calculations from the spectra of proton qNMR

To determine the content of PsX in the samples, an absolute method was used (Eq. (1)) [18].

$$mPsX_{measured} = \frac{I1}{I2} \times \frac{M_{PsX}}{M_{TSPd_4}} \times \frac{m_{TSPd_4}}{m_{sample}} \times 3 \times P_{TSPd_4} \quad (1)$$

The constant "3" in Eq. (1) is the ratio value of the three methyl groups in TSP-d_4 referred to the only N-acetyl methyl group of the PsX, "M" is the molar mass of PsX repetitive unit and of the reference (TSP-d_4), "m" is the mass of the reference and of the total sample, "P''_{TSPd_4}" is the purity of the reference reported by the supplier and "$I1, I2$" are the integral values from R-1 and R-2 regions in the qNMR spectrum, respectively (Fig. 4).

Table 2
Result for *mPsX*.

$mPsX_{theoretical}$ (mg)	$mPsX_{measured}$ (mg)		
	Set 1	Set 2	Set 3
5.84	5.66	5.61	5.73
7.61	7.76	7.79	7.60
11.12	11.05	11.23	11.25
15.55	15.51	15.61	15.57
16.46	16.68	16.43	16.81
19.69	19.11	19.38	19.73
21.96	22.16	21.90	21.39

The results achieved in the colorimetric assay were employed as $mPsX_{theoretical}$ for statistical evaluation. The $mPsX_{measured}$ was calculated according Eq. (1) for each of the prepared three sets of samples; see Table 2.

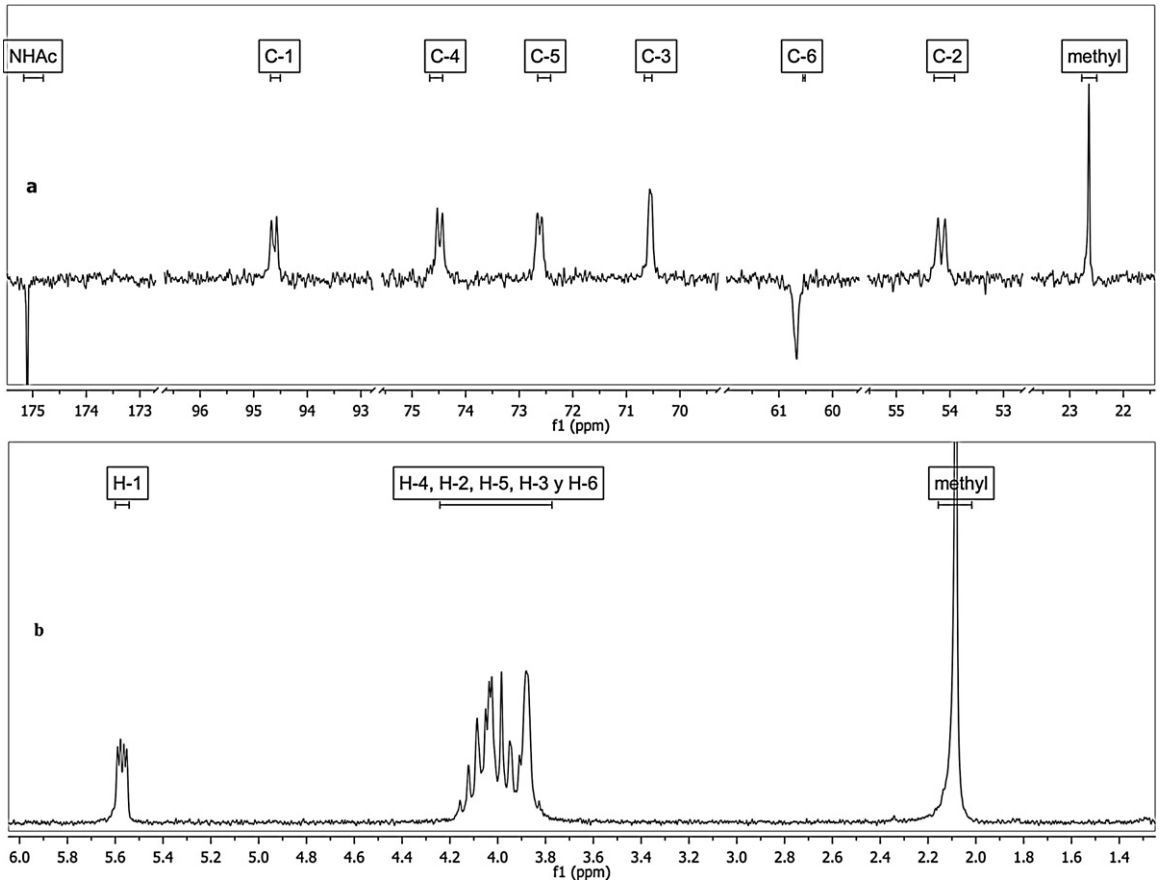

Fig. 2. Monodimensional spectra. (a) PENDANT spectrum and (b) proton spectrum.

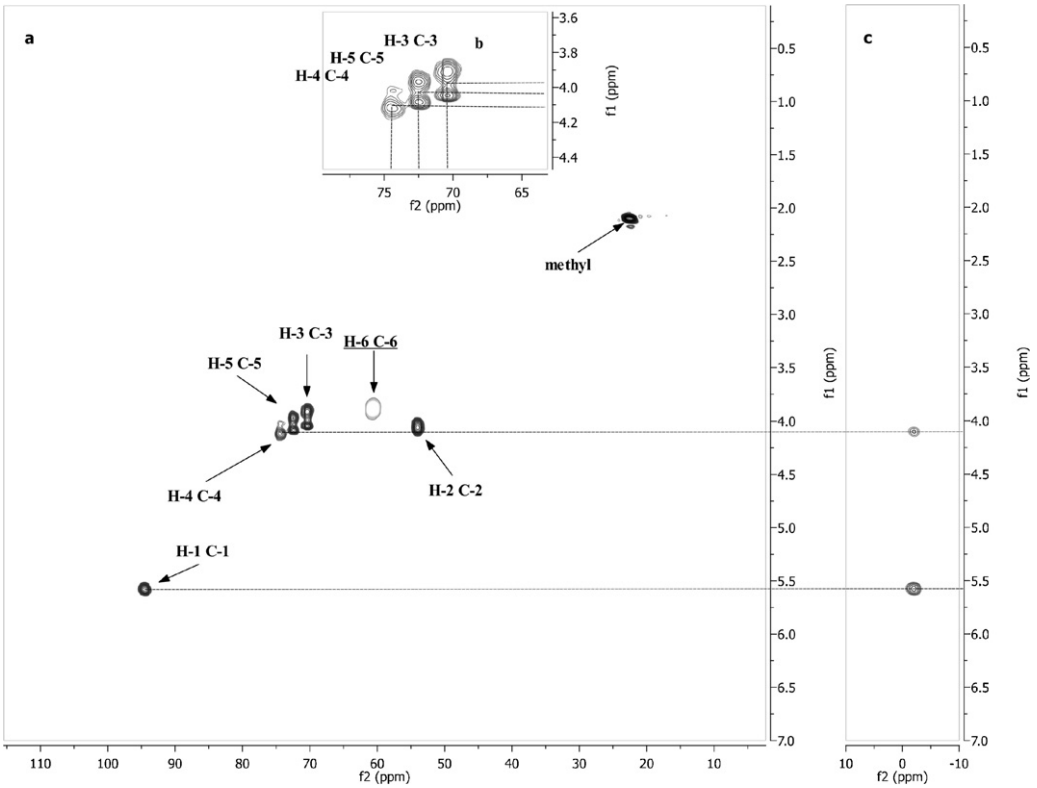

Fig. 3. Heteronuclear correlation spectra of PsX. (a) Edited HSQC ($^{1}H–^{13}C$). Positive peaks (multi-contour peaks) negative peak (single-contour peak) and (underlined assignment) and (b) expanded region of the closed peaks in the edited HSQC ($^{1}H–^{13}C$). (c) HSQC ($^{1}H–^{31}P$) spectrum.

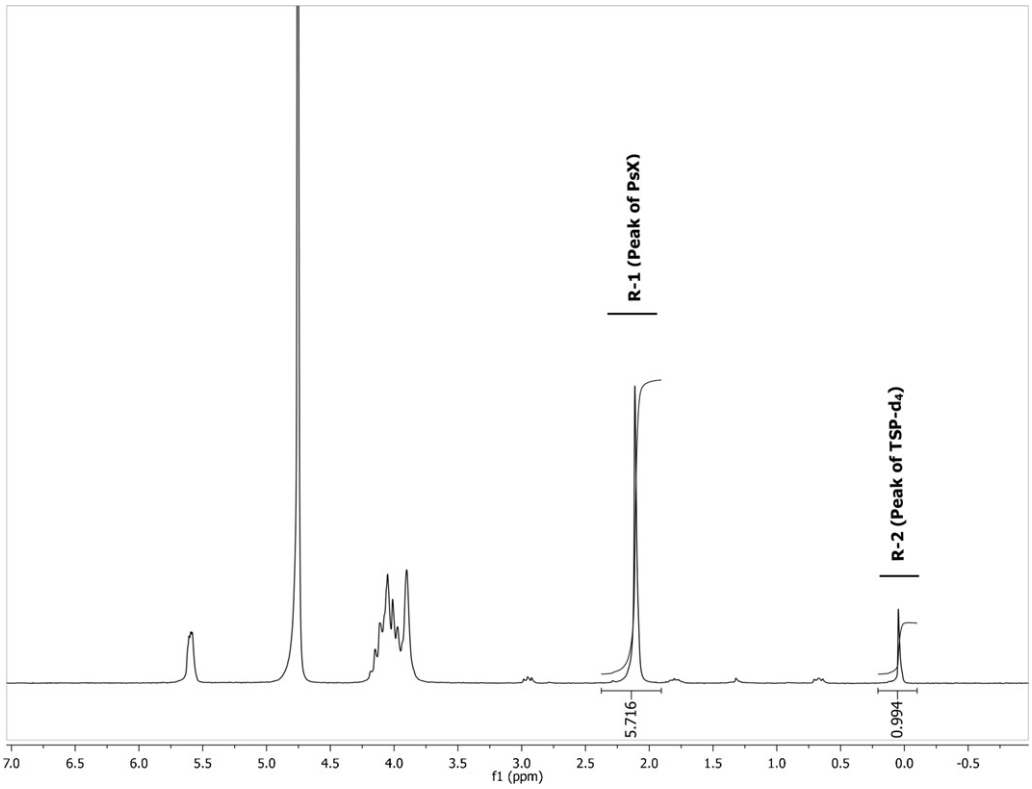

Fig. 4. Processed ^{1}H qNMR spectrum of PsX.

3.4. Accuracy, range and linearity

The accuracy was evaluated by determining the recovery percent of the known quantities of PsX in the samples and the confidence intervals for 95% of reliability (Eq. (2)).

$$\%PsX_{recovery} = mPsX_{measured} \times \frac{100}{mPsX_{theoretical}} \quad (2)$$

$mPsX_{measured}$ is the calculated PsX value from the qNMR spectral analysis using Eq. (1) and $mPsX_{theoretical}$ is the expected value for each sample.

The assessment was carried out with one set of seven samples (21 determinations, three replicates per sample). The mean for $\%PsX_{recovery}$ calculated was 100.04%, between 99.22% and 100.86% for 95% confidence [15].

In the linearity study, three sets of seven samples at different concentrations of PsX were analyzed to determine the slope for the curve obtained by a linear regression adjustment and the proportional bias. Xu et al., have also employed the proportional bias as linearity criterion (*prop. bias* ≤ 10%) [19,20] (Eq. (3)).

$$prop.\,bias = (2^{|m-1|} - 1) \times 100 \quad (3)$$

"*m*" is the estimated slope of the linear regression of $mPsX_{theoretical}$ versus $mPsX_{measured}$.

The method was linear in the range between 9.73 and 32.82 mg/ml of PsX in D_2O with an adjusted R^2 coefficient of 0.9998. The proportional bias for the linearity evaluation was 0.22%; see Fig. 5 and Table 3.

3.5. Intermediate precision and repeatability

The precision evaluation was based on Relative Standard Deviation (*%RSD*) calculation for the results of 3 replicates measured under repeatability conditions ($\%RSD_{repeatability}$) (Eq. (4)). Two additional replicates were acquired for a second analyst. The generated data was combined with the repeatability assay in order to calculate the RSD% under inter-operator variations (intermediate precision) ($\%RSD_{precision}$, Eq. (5)) [19,20].

$$\%RSD_{repeatability} = (e^{S_{intra}} - 1) \times 100 \quad (4)$$

$$\%RSD_{presicion} = (e^{\sqrt{S_{intra}^2 + S_{inter}^2}} - 1) \times 100 \quad (5)$$

S_{intra} and S_{inter} are the estimated intra-assay and inter-assay standard deviations, respectively.

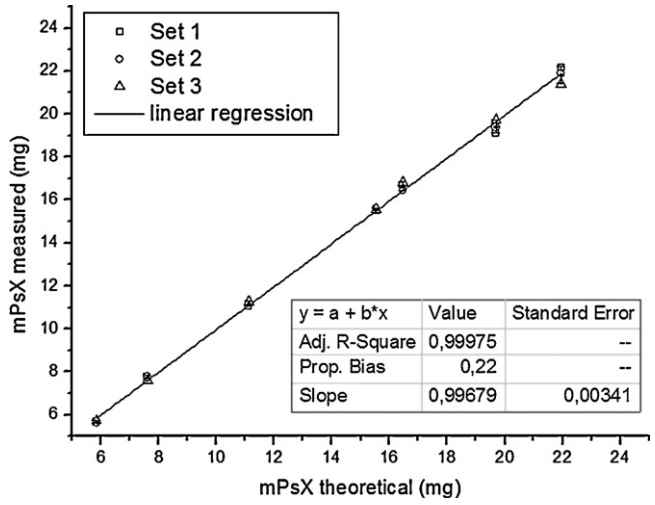

Fig. 5. Linear regression plot of linearity assessment.

For the repeatability assessment data from 21 determinations (similar to accuracy assay) were processed. The generated results were used to calculate the relative standard deviation. In addition, 14 determinations were carried out by a second analyst (B) as variation to assess intermediate precision. The resultant values of $\%RSD_{repeteability}$ and $\%RSD_{presicion}$ were 3.42% and 4.48%, respectively (Table 3).

3.6. Quantification limit (QL)

Many instrumental methods employ Eq. (6) for determination of QL. ICH advises as alternative assessment for those assays where the signal to noise ratio (s/n) can be evaluated that it must be better than 10:1 [15]. For evaluating the assay under s/n criterion, several dilutions of the analyte must be acquired to reach a ratio close to the ICH recommendation. Diehl et al. proposed a s/n ratio of at least 250:1 for a precision better than 1% for qHNMR methods [21].

$$QL = 10 \times \frac{\sigma}{m} \quad (6)$$

where σ is the standard deviation of the response and m is the slope of the calibration curve.

In order to ensure that the evaluated range was above the QL, we focused on evaluating the s/n ratio employing the R-1 region of the less concentrated sample (A) and a signal free noise range from 8 to 6 ppm in the spectrum. Due to the fact that our lowest PsX concentration (9.73 mg/ml) was 36 times higher than the ICH recommendation, it was not necessary to calculate the actual QL for the method (Table 3).

3.7. Ruggedness

The ruggedness was checked by evaluating three different batches of PsX by three analysts on two days. The results were expressed as averaged inter-analyst percentage difference and averaged inter-day percentage difference. Both results were calculated according to Eq. (7).

$$\%Difference = (e^{|\bar{x}-\bar{y}|} - 1) \times 100 \quad (7)$$

where x is the within-sample mean of the logarithm-transformed result for operator A (or day 1) and y is the analog result for operator B (or day 2).

The maximum percentage difference (*%Difference*) inter-days was 1.41% and the percentage difference from analyst (A) to (B) was 0.09%. The acceptance criterion was similar to that employed by Xu et al. [19]; (Table 3).

3.8. Analytical advantages achieved

First, a full and detailed assignment of the PsX repetitive unit was achieved with the aid of three different nuclei. Second, the validation study was carried out by means of quantitative Proton Nuclear Magnetic Resonance. The method will also allow monitoring the purity of the PsX in several stages of the production scheme of the vaccine candidate.

The colorimetric assay has been widely used for quantification of polysaccharides with phosphate residue in the repeating unit. However, the method has a low specificity, since it detects the total phosphorus content. Hence, to obtain successful results, extreme care is recommended with glassware and its cleaning [12]. The proposed proton qNMR method is useful since it uses undoubtedly the signal of the polysaccharide unit.

Table 3
Summary of results for statistical assessment.

Parameter assessed	Acceptance criteria	Result
Linearity	*Prop. bias* $\leq$ 10%	0.22% bias
Accuracy	The average percentage recovery versus expected values: $\%PsX_{recovery}$ 100 ± 20%	100.04% recovery
Precision	Repeatability: $\%RSD_{repeteability} \leq 5\%$	3.42% *RSD*
Precision	Intermediate precision: $\%RSD_{precision} \leq 10\%$	4.48% *RSD*
Range		(9.73–32.82) mg/ml
QL	Will be determined according to adequate accuracy, precision and linearity	9.73 mg/ml (s/n 360:1)
Ruggedness	Inter-day difference: %*Difference* $\leq$ 10% on average	1.41% *Difference*
Ruggedness	Inter-analyst difference: %*Difference* $\leq$ 10% on average	0.09% *Difference*

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