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International collaborative study for the calibration of a replacement International Standard for the WHO 1st International Standard for *Haemophilus influenzae* type b polysaccharide

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Summary

In this report we present the results of a collaborative study for the preparation and calibration of a replacement International Standard (IS) for *Haemophilus influenzae* type b polysaccharide (polyribosyl ribitol phosphate; 5-D-ribitol-(1→ 1)-β-D-ribose-3-phosphate; PRP). Two candidate preparations were evaluated. Thirteen laboratories from 9 different countries participated in the collaborative study to assess the suitability and determine the PRP content of two candidate standards. On the basis of the results from this study, it is recommended that Candidate 2 (NIBSC code 12/306) is established as the 2nd WHO IS for PRP Standard with a content of 4.904 ± 0.185 mg/ampoule, as determined by the ribose assays carried out by 11 of the participating laboratories (expanded uncertainty calculated using a coverage factor of 2.23 which corresponds to an approximate 95% level of confidence).

Introduction

Haemophilus influenzae type b (Hib) can cause many invasive diseases such as severe pneumonia and meningitis, in addition to potentially severe inflammatory infections in various tissues. Although this problem occurs worldwide the burden of Hib disease is most significant in resource-poor countries. Vaccines are the only public health tools capable of preventing the majority of serious Hib disease. Hib conjugate vaccines are made from Hib capsular polysaccharide (PS) type b (polyribosyl ribitol phosphate; 5-D-ribitol-(1→ 1)-β-D-ribose-3-phosphate; PRP) conjugated to a carrier protein such as tetanus toxoid (TT), non- toxic cross-reacting material (CRM) or outer membrane proteins (OMP), to make them immunogenic in the infants. Hib conjugate vaccines exist as monovalent vaccines, combined with meningococcal C conjugate vaccine (Hib/MenC) or with diphtheria, tetanus and whole cell/acellular pertussis (DTwP or DTaP) -based vaccines, with or without injectable polio (IPV) and hepatitis B (HepB) vaccine components. In view of their demonstrated safety and efficacy, WHO recommends that Hib conjugate vaccines be included in all routine infant immunization programs. So far, 184 countries (95% of WHO member States) had included the vaccine in their routine immunization programs with the rest planning to introduce it in the near future. The introduction of Hib conjugate vaccines has resulted in a dramatic reduction in invasive Hib disease in young children worldwide (WHO, 2013).

The supply of effective vaccine is dependent on confirmation of vaccine potency and safety through quality control testing by manufacturers and, independently, by the National Control Laboratories (NCLs). Quality control testing of current Hib conjugate vaccines relies almost completely on physico-chemical methods to monitor consistency of production and identify any trends in batches over time; biological testing is carried out only to ensure safety. In addition, immunogenicity testing is carried out by manufacturers only during vaccine production and following changes to manufacturing processes to confirm induction of T cell-dependent response by the conjugates [WHO Recommendations (2000), Ph. Eur. 1219 (2013) and PA/PH/OMCL (04) 91 (2013)].

A wide variety of assays can be used for the quantification of the capsular polysaccharide, PRP in purified polysaccharide, bulk conjugates and final lot vaccines. These include the phosphorus assay, ribose determination by orcinol assay, high pH anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD) for PRP and immunochemical assays. All these assays would benefit from the presence of a PRP reference standard (Holliday & Jones, 1999). The first WHO International Standard (IS) for PRP (coded 02/208, 4.933 mg/ampoule) was established by the WHO Expert Committee on Biological Standardization (ECBS) in 2005 (WHO/BS/05.2018 & Mawas *et al*. 2007) based on an international collaborative study using the ribose assay and

has been used extensively for calibrating in-house reference preparations and as a working reference preparation by NCLs and vaccine manufacturers, according to the intention of WHO. The use of the IS in the 1st collaborative study showed a reduction in between-laboratory variation for the ribose, phosphorus and HPAEC-PAD assays, suggesting its suitability for the evaluation/quantification of PRP content in the vaccine and components. The continuing development of new Hib vaccines, the implementation of distribution of Hib vaccines throughout the world and the increasing number of NCLs testing and releasing Hib vaccines around the world increased the demand for this standard preparation. The current stock level is < 40 ampoules. As a result, a project was initiated at NIBSC to calibrate and establish a replacement standard. The proposal is to assign unitage to the candidate replacement standard using the ribose assay as was used for the 1st PRP IS and this proposal was endorsed by WHO at the ECBS in October 2012.

Two candidate materials for the replacement standard were provided by vaccine manufacturers to NIBSC for filling and freeze-drying. Both candidates were confirmed to be suitable for evaluation as candidate replacement standards after preliminary trial fill studies at NIBSC. A collaborative study (NIBSC code CS 484) was initiated with the primary aim of calibrating the candidate standards in SI units, using the ribose assay.

In total, thirteen laboratories participated in the study, including 6 manufacturers, 6 National Control Laboratories and one Pharmacopeia laboratory from 9 countries (Belgium, Canada, China, Cuba, France, Germany, India, Italy and the United Kingdom), with 11 of these performing the ribose assay. The participating laboratories are listed in Appendix A and are referred to throughout this report by a code number, not corresponding to the order of listing.

Materials and methods

The candidate standards

Two PRP preparations were provided by two different manufacturers and were identified as Candidate 1 (provided by the National Institutes for Food and Drug Control, China; NIFDC) and Candidate 2 (provided by Serum Institute of India; SII). NIBSC received 20g of dried unconjugated PRP from NIFDC on 19th April 2012 on dry ice and 3 litres of frozen bulk PRP from SII on 27th February 2013, at PRP concentration of 9.22 mg/ml, as determined by the manufacturers. The candidates were stored at -20ºC until processed. The materials provided were sterile and characterization details provided by the manufacturers are presented in Table 1. Details of manufacturing records including results of in-process controls are available from NIBSC upon request.

Table 1. Details of the PRP bulk materials

Bulk PRP	Candidate 1	Candidate 2
Amount provided	20 g	2.9 litres at 9.22 mg/ml (26.7g)
Ribose content (w/w)	37 %	37.4%
Phosphorus content (w/w)	8.0 %	8.0 %
Protein (w/w)	0.2 %	0.07 %
Nucleic acid content (w/w)	0.2 %	0.02 %
Molecular size distribution of the polysaccharide	K_D=0.08	59.6% before K_D of 0.3
Bacterial endotoxin	<10 EU/µg PRP	<0.1 EU/µg PRP
Free formaldehyde (w/v)	Not known	<0.00005 %
Buffer	None	None

The WHO recommendations for the preparation, characterization and establishment of international and other biological reference standards (WHO/BS/04.1995) were followed for the preparation of the candidate materials. Candidate 1 was dissolved in distilled water (DW) to a PRP concentration of 2.5 mg/ml and NaCl was added to a final concentration of 0.555 mg/ml. Candidate 2 was diluted to the same PRP concentration as candidate 1 in DW and NaCl was added to a final concentration of 0.555 mg/ml.

Filling (2 ml per 5 ml DIN ampoule) was performed at room temperature with constant stirring within the Centre for Biological Reference Materials (CBRM) at NIBSC on the 18th October 2012 for Candidate 1 and on the 4th April 2013 for Candidate 2. For freeze-drying, filled ampoules were loaded into a pre-cooled freeze-dryer (Serial CS100, Argentueil, France).The freeze-drying program was set up as follows: primary freezing at -50ºC for 4.5 h and hold for 4 h, followed by sublimation at -30ºC for 40 h, at a vacuum of 100 µbar. This was followed by a ramp over 15 h to a secondary drying temperature of 30ºC then a vacuum of 30 µbar was applied for 20 h at 30ºC. The ampoules were sealed and stored at -20ºC at NIBSC. The finished products were coded 12/218 and 12/306 for Candidates 1 and 2, respectively.

Characterization of the lyophilised candidate PRP standards

Freeze-dried candidate standards were examined for precision of the fill, appearance, residual moisture content and oxygen head space. The precision of the fill was determined by weighing ampoules after fill: representative ampoules were weighed at 1 min intervals throughout the production run. A total of 196 ampoules were weighed for 12/218 and a total of 124 ampoules were weighed for 12/306. Measurement of the mean oxygen head space served as a measure of ampoule integrity. Residual moisture content was measured using the coulometric Karl Fischer (KF) method for both candidates, with total moisture expressed as percentage of the mean dry weight of the ampoule contents. Results of characterization of the lyophilised candidates are presented in Table 2.

Table 2. Details of the filled and lyophilised materials

	Candidate 1	Candidate 2
Appearance	White cake	White cake
Nominal fill volume	2 ml	2 ml
NIBSC product code	12/218	12/306
No. of ampoules filled	3772	4287
Collaborative study code	Sample B	Sample D
Mean fill mass	2.01, n=196	2.01; n=124
CV of fill mass (%)	0.07	0.12
Mean dry weight	5.5 mg; n=6	6.0 mg; n=6
CV of dry weight (%)	1.39	3.55
Mean residual moisture (%)	1.28	1.45
CV of residual moisture (%)	19.5 n=12	45.4; n=12
Mean Oxygen headspace	0.34 % n=12	0.28; n=12
CV of oxygen space (%)	32.44	44.95
Microbial analysis	No contamination	No contamination

Collaborative study materials

Participants in the collaborative study were sent two duplicate sets of 5 preparations coded A-E, where ampoules B and D were the candidate standards 1 and 2 (NIBSC codes 12/218 and 12/306, respectively). Ampoule A was the 1st WHO IS for Hib PRP (NIBSC code 02/208), ampoule C was Meningococcal serogroup Y polysaccharide (NIBSC code 01/428; 1 mg/ampoule) and preparation E was a Hib-CRM_{197} bulk conjugate.

Upon receipt, participants were asked to reconstitute samples A-D with 1 ml sterile distilled water and store at -20ºC in small aliquots until further use. Prior to use in assays, samples would be thawed and kept at 4ºC for up to 1 week, while completing the assay(s). Further dilutions to give test solutions in the correct concentration range for the assay were to be made in the appropriate assay solution or buffer, according to the laboratory's protocol.

Study design

Participants were requested to test the two sets of samples separately one after the other, preferably in different weeks, using the in-house method commonly performed in their laboratories. Tests were to be carried out within 7 days of thawing the reconstituted sample. A value of 1-20 mg for the content of the preparations was provided to participants as a guideline to help in preparation of dilutions. Details of all the assay methods performed by the participants are shown in Tables 3-5.

Assays for evaluation of the candidate standards

All laboratories provided raw data and methodological details on the report sheet, with most supplying standard operating procedures. The **ribose, or orcinol, assay** for measuring the ribose content, and calculated PRP content of the candidate standards was performed by 11 out of the 13 participating laboratories (Table 3), including 5 laboratories that took part in the collaborative study for the 1st IS in 2004 (Labs 3, 4, 7, 8 and 12). The ribose assay is a spectrophotometric, or colorimetric test that relies on the reaction of the ribose, the constituent pentose of PRP, with the orcinol-ferric chloride-HCl reagent. The ribose assay was performed according to methods described in Ashwell (1957) and Kabat & Mayer (1961), and is a pharmacopeia compendium method (see, for example Ph. Eur. 20531). All methods were similar or identical to those performed for the 1st IS collaborative study with relatively small variations between them.

All laboratories used D-ribose as a quantitative standard and provided lot numbers and sufficient information to access certificates of analysis (CoA). Ribose standards were ≥ 99% pure, and CoAs provided moisture contents (% w/v) which ranged from 0-0.4%; some independent moisture content analyses were performed, giving values of 0.43% from a freshly opened bottle by thermogravimetric analysis (Lab 7), and 0.8% by a KF method (Labs 9 and 13). None of the laboratories took the moisture content into consideration by adjusting the initial ribose content of the stock solution, although Lab 10 dried the ribose before weighing it. Information was not gathered on whether the ribose was weighed and prepared from a newly opened bottle, or whether the bottle had been previously opened and stored for a period of time.

In converting ribose to PRP content on a per gram basis, a conversion factor (g PRP/g ribose) is required, which requires knowledge of the cation complexed to the anionic saccharide, the two most common being sodium and calcium. The 1st IS and candidate standards when reconstituted in 1 ml water contain 10 mM and 19 mM NaCl (for both candidates), respectively. There are

approximately 13 mM Hib PRP saccharide repeating unit following reconstitution, and the PRP is in the sodium coordinated form. All participants used water as a diluent for the ribose standard curve, according to the instructions. Eight labs used conversions factors on a per gram basis, ranging from 2.439 to 2.5 g PRP repeating unit/g ribose. Labs 2 and 8, used formula weights for the PRP repeating unit of 368.14 and 368.21 g/mol for the sodium form of the repeating unit to convert from mol PRP to g repeating unit PRP. The converted values supplied by the labs were used for the ribose and other assays. Conversion factors are quoted in the bottom of the method table (Tables 3-5). Lab 12 did not supply a conversion factor and the most common factor of 2.448 was used.

Phosphorus (P) determination was carried out by 9 laboratories (Table 4): 6 labs used the Chen method (Chen *et al.*, 1956) which is also the Ph. Eur. monograph method (Ph. Eur. 20518). Two labs used the Ames modified method (Ames, 1966), and Lab 1 used inductively coupled plasma-atomic emission spectroscopy (ICP-AES), as described by Swartz *et al.* (2000). Labs 3 and 8 participated in the collaborative study for establishment of the 1st IS.

Six of the labs used sodium or potassium phosphate salt forms of phosphorous standard; Labs 2 and 8 used the sodium salt of ribose-5-phosphate which, it should be noted, contained moisture contents of 9 and 10.5%, respectively. Lab 1 used an accredited liquid standard of 1000 µg/ml phosphorous dihydrogen ammonium salt for ICP-AES. All standards were diluted in water. Conversion factors ranged from 11.9 to 12.0 g PRP/g phosphorous. Only Lab 3 corrected for moisture content of the standard.

The **HPAEC-PAD assay** was performed by 8 laboratories (Table 5), 4 of which took part in the previous collaborative study. Seven used the alkaline hydrolysis method (Tsai et al., 1994) and two used the acid hydrolysis method (Bardotti *et. al.*, 2000), with Lab 2 performing both. Four laboratories (Labs 7, 8, 10 and 11) used the WHO 1st IS for Hib PRP as a standard, Lab 2 used an in-house PRP PS standard, Lab 4 used Hib-TT bulk conjugate, and Lab 6 used the monosaccharide, D-ribitol.

Labs 4, 6, 7 and 10 participated in the previous collaborative study and performed alkaline hydrolysis (Labs 4, 7 and 10) at around room temperature for 12 to 20 h, as according to Tsai *et al.* (1994), while Lab 6 performed the acid hydrolysis method. The new laboratories all performed polysaccharide depolymerization at higher temperatures for shorter periods of time, using 3 h at 55 °C, or 2 h at 90 or 100 °C. Labs 8 and 10 appeared to have optimized their elution conditions to allow for relatively earlier elution of the PRP peak, and shorter run times. The longer run times and later elution used by other labs may be more appropriate for samples with neutral saccharide-containing formulations.

^{1}H-NMR was performed by one laboratory for a qualitative, diagnostic purpose only which used TSP as a reference and compared the spectra of the samples with that of the 1st PRP IS (Table 6).

Stability study

To determine the stability of the candidate standards, three studies are currently underway at Lab 7: real-time stability, accelerated degradation and stability of the reconstituted material. The real-time stability at -20ºC, using a -70ºC baseline sample, is being carried out on samples stored at -20ºC for 6 mo, 1 yr, 2.5 yr, 5 yr and 10 yr, and transferred to -20ºC at each timepoint.

The accelerated degradation study of the candidate standards (at temperatures of 4, 20, 37 and 56ºC), using a -20ºC baseline sample, is being carried out on samples from 1, 2, 4, 6 and 12 mo,

which are transferred to -20ºC at each timepoint. For the real-time and accelerated degradation study, samples are evaluated within 1 week of their timepoint.

In addition, the stability of the candidate standards reconstituted in water or 0. 9 % $^{w}/_{v}$ NaCl held at 4ºC for 0, 1, 2, 3 and 4 weeks (reconstituted, liquid) and of reconstituted sample held at -20ºC for 1, 3, 6, 9, 12, 18, 24 and 30 months (reconstituted, frozen) is being determined, using the -20ºC lyophilized sample as baseline. The reconstituted, liquid samples are transferred to -20°C at their timepoints and tested after the final timepoint, while the reconstituted, frozen samples are tested at their timepoints.

The methods used for analysis of the stability samples include the **ribose assay** for PRP content, according to Table 3 (Lab 7), **molecular sizing** to determine the % elution by a K_D value determined in the main peak of the -20°C sample and **pH determination**. Prior to analysis, stability samples (ampoules) from the real-time and accelerated degradation study were reconstituted with 1 ml of Milli-Q A10 ultrapure water at room temperature (22 ^{o}C ± 3°C) and stored at 4°C for up to 1 week during the duration of the analysis. It was assumed that each ampoule was filled with 5.0 mg PRP for the purposes of this study.

The molecular sizing was carried out using a Dionex HPLC DX-600 system with a Wyatt Optilab rEX refractometer. The columns used were Tosoh Bioscience TSK6000PWXL plus a TSK5000PWXL in series with a PWXL guard column. The sample was loaded onto the column using an AS-50 autosampler. One hundred (100) µl of the reconstituted sample, containing 50 µg saccharide was injected, and then eluted with PBS 'A' (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 171 mM NaCl, 3.4 mM KCl, pH 7.3) buffer at a flow rate of 0.25 ml/min, column oven temp 30°C. Column calibration markers for the void volume and total column volume were salmon DNA (Sigma D-1626) and tyrosine (Sigma T-3754), respectively. Additional markers for determining system suitability were thyroglobulin (T-9145), bovine serum albumin, monomer (A-1900) and carbonic anhydrase (C-7025), all from Sigma. The data were analysed for % of material eluting at a K_D of 0.45 using the refractive index signal and Chromeleon ver 6.80 software.

The pH of the samples was determined to ± 0.1 units using a Metrohm 827 pH lab meter calibrated with pH 7.0, 4.0 and 10.0 standard buffer solutions (Fisher Scientific).

Results

Statistical analysis

The Hib PRP polysaccharide contents were determined for the two candidate replacement standards, as well as for the 1st WHO IS, to ensure continuity between the 1st IS and its replacement 2nd IS. All individual assay results, together with mean estimates for each ampoule set are given in Appendix B Tables B1, B2 and B3 for ribose, phosphorus and HPAEC-PAD assays, respectively. Results for sample C, the meningococcal group Y polysaccharide, which served as a negative control, are presented as reported by the participants. Mean estimates are also shown in histogram form in Figures 1, 2, 3 and 4 for samples A (1st WHO IS, code 02/208), B (Candidate 1, 12/218), D (Candidate 2, 12/306) and E (a Hib-CRM_{197} bulk conjugate), respectively, with the laboratory code indicated in each box. The mean estimates for each ampoule set were combined using SAS PROC MIXED (SAS Institute Inc., Cary, NC, USA) with random factor laboratory to give final mean estimates for each sample and method, shown in Table 7.

For the current IS and both candidate standards, the highest mean estimate was obtained in the HPAEC-PAD assays and the lowest mean estimate was obtained in the phosphorus assays (5-7%

lower than HPAEC-PAD), but no significant differences between assay methods were observed. For all assay methods, lower variability was observed for the current IS when compared to the candidate standards, as indicated by the standard errors in Table 7.

Much higher PRP contents were determined for Sample E, the Hib-CRM_{197} bulk conjugate, using the phosphorous assay than with the ribose and HPAEC-PAD assays, and it can be concluded by this result that sample E contains phosphorous, possibly as part of a buffering compound. It is possible that Lab 2 dialyzed or diluted the sample adequately to eliminate the non-PRP phosphorous.

As the ribose assay was to be used to assign values to the candidate standards, expanded uncertainties were determined using the data shown in Table 8. Uncertainty due to the moisture content of the ribose standards was determined from the CoA data reported by participating laboratories (Table 3), using the maximum value of 0.4% and assuming a rectangular distribution from 0% to 0.4%. Uncertainty due to the purity of the ribose standards was similarly determined using the minimum value of 99%. Typical uncertainty due to weighing of the ribose standard was determined from calibration data for the balance used at NIBSC.

Expanded uncertainties calculated using a coverage factor of k=2.23 (Student's t statistic for 95% confidence [2-tailed] on 10 degrees of freedom) are shown in Figure 5. The result for the current IS, 4.989 ± 0.140, is consistent with its assigned value of 4.933 ± 0.267, and this will allow continuity between the 1st and proposed 2nd IS. The lower uncertainty determined for the 1st IS in this study is due to the lower level of variability between laboratories (inter-laboratory CV of 2.1% compared to 4.2% in the previous study), a greater number of laboratories performing the ribose assay (n=11 compared to n=7 in the previous study) and a lower estimated uncertainty due to variation in the weighing of ribose standards.

Method Analysis

As demonstrated by the low variability between lab estimates for sample A, there was very little assay-related variation. The conversion factors quoted in the ribose assay varied by 2%, however, and this was not considered as an experimental source of uncertainty, but has contributed to variation in the determined unitage.

The phosphorous analysis from the different labs gave a wider spread of PRP values, which is apparent from the histograms (Figures 1 to 3). It is striking that the values determined by ICP-OES using a certified standard are much lower, but without further study, and use of a common standard, it could not be concluded whether this difference was method or standard-related. The two labs performing the Ames modified method used ribose-phosphate standards, which contained high moisture content, which were not taken into account, and which could theoretically lead to an overestimate of P (and PRP). Lab 8's values were, in fact, higher than those determined from the other labs, but this was not borne out in the results of Lab 2.

The HPAEC-PAD results gave a lower standard mean error that the phosphorous assay, and a lower error in this collaborative study than the previous, and this is likely to be due to the use of a common standard, the 1st WHO IS, by four participants. Although a lower value was determined by Lab 6, using a ribitol standard for the PS samples, determinations of the PRP content in the Hib conjugate were close to the measured mean. The use of the higher temperature, shorter incubation time during alkaline hydrolysis by Labs 2, 6, 8, and 11 did not result in an overall trend. Lab 2 also performed both an alkaline and acid hydrolysis and measured the PRP

repeating unit and ribitol peaks, respectively, using the same standard. On average, only slightly higher results were obtained with the acid hydrolysis method, including for the bulk conjugate.

The ^{1}H-NMR method was used by Lab 11 for the purposes of verification of the identification of the samples, rather than for quantitation. Samples A, B and D passed the test for identification, as the spectrum was similar to those obtained with the Hib PRP PS (1st IS) reference solution.

The meningococcal group Y polysaccharide, sample D, gave only background absorbance in the ribose assay, signals which were below the limits of detection in the phosphorous assays, no peak at the expected elution time for PRP or ribitol in the HPAEC-PAD assay, and was identified as having a distinctly different spectrum in ^{1}H-NMR.

Stability

The PRP content of the two candidate standards did not change when stored at up to 12 mo at -20°C (designated storage condition) when compared with a -70°C sample (Figure 6). When stored at accelerated degradation conditions, the PRP content of Candidate 2 had comparable PRP content to the -20°C baseline. The content of Candidate 1 showed some variability, probably inherent to the assay, as there was no temperature-dependent trend.

The molecular size of the candidate standards is a more sensitive measure of PS stability than ribose or other quantitative assays based on degradative methods which rely on PS depolymerization to their repeating unit. Molecular sizing results for the candidate standards stored at elevated temperatures for up to 12 months are summarized in Table 9. Stability samples of Candidate 1 showed a slightly more progressive decrease in PS size than did samples of Candidate 2 (Figure 7). Samples of Candidate 1 stored at 20, 37 and 56°C showed a decrease in the percent of sample eluting at K_D 0.45 from the TSK 6000-5000PWXL column series at 1 mo compared to the -20 or 4°C sample. While the 20°C sample did not appreciably change beyond this, and had a parallel progression with the -20 and 4°C samples, logarithmic changes occurred in the 37 and 56°C samples up to 12 mo (Figure 7A), with 67 and 12% eluting by K_D 0.45 respectively, compared with 82% of the baseline sample.

Candidate 2 was of a slightly smaller size than Candidate 1 at T0, with 81% eluting at T0, compared with 100% of Candidate 1 (Figure 7B). Relative to the -20 °C baseline sample, there were no changes in Candidate 2 stored at up to 20 °C. At 37°C, a slight decrease in % eluting by the specified K_D was seen after 2 months, but this was in parallel to the baseline sample up to 12 mo. At 56°C, there was a noticeable decrease to 32%.

Molecular sizing results expressed relative to the -20°C sample at each time point were used to fit an Arrhenius equation relating degradation rate to absolute temperature (Kirkwood, 1977) and hence predict the degradation rates when stored at -20°C. This analysis gave predicted losses per year of 0.016% for Candidate 1 and 0.001% for Candidate 2. Both candidate standards were appreciably larger in size and chain-length than the 1st WHO Hib PS standard (02/208) (Figure 8).

The stability of the reconstituted samples held at 4°C was confirmed up to 4 weeks. The reconstituted stability of the standard kept at -20 °C for up to 3 mo showed little difference between the candidate standards. Both showed a 5-6% decrease in the % eluting by the specified K_D between 1 and 3 mo. It was not possibly at this early time-point to comment on the effect of storage in dilute saline compared with water, but it did not improve the stability.

The pH of freshly reconstituted samples was 7 to 7.5 for Candidate 1, and 6.7 to 7.2 for Candidate 2. There was no real change in the pH of the real-time stability samples.

Discussion

The absence of a biological potency assay for Hib conjugate vaccines makes the quantification (in SI units) of total and free PRP in the vaccine a critical release test for both manufacturers and national control authorities. Therefore, the presence of a reference preparation containing a determined amount of PRP should facilitate calibration of various methods and in-house references to quantify the PRP content of the bulk saccharide, bulk conjugate and final fills of the vaccine. Since its establishment in 2005, the demand for the 1st WHO IS for Hib PRP (NIBSC code 02/208) continued to increase. As only 932 ampoules were prepared, NIBSC had to impose a restriction on the number of ampoules issued to each customer in order not to exhaust the stock before the preparation of a replacement standard. The stock of the 1st WHO IS has reached a very low level (< 40 ampoules remaining).

Two preparations of Hib capsular polysaccharide (PRP) were characterized and found to be suitable candidates for a replacement of the 1st WHO IS. The finished products, 12/218 and 12/306 (labelled as Preparations B and D or Candidates 1 and 2, respectively, for the collaborative study), were fully characterized at NIBSC and found to comply with WHO recommendations for reference preparations with regard to precision of the fill, residual moisture content and oxygen head space. The two candidate standards were calibrated for the PRP content using the ribose assay and also evaluated for use in several other assays, including phosphorous and HPAEC-PAD.

As the candidate standards are calibrated in SI units, a single reference traceable method needed to be used, with assignment of uncertainty derived from the collaborative study data (WHO recommendations, 2004). The ribose assay was chosen for determination of the PRP content of the candidate standards, as for the 1st WHO IS.

In terms of PRP content, Candidate 2 had slightly more PRP per ampoule (4.904 mg PRP/ampoule) than Candidate 1 (4.618 mg PRP/ampoule). Candidate 2 (12/306) was slightly more stable than Candidate 1 (12/218) in real-time and accelerated degradation studies up to 12 months. Both candidates were appreciably larger in size and chain length than the 1st WHO Hib PS standard (02/208). SEC/MALLS determination will be performed to obtain a more accurate size.

Proposal

Based on the results obtained from the ribose assays performed by 11 participants in this collaborative study and from the stability study performed by lab 7, we propose that Candidate 2 (NIBSC code 12/306) is established as the 2nd WHO International Standard for PRP with a content of 4.904 ± 0.185 mg/ampoule (expanded uncertainty calculated using a coverage factor of 2.23 which corresponds to an approximate 95% level of confidence).
While the content has been assigned based on the ribose assays, the proposed standard is potentially suitable for use in the quantitation of PRP content by other assays.

Participants' comments on report

The participants were sent a draft report and asked to check their details, that their methods and results had been reported correctly, if they agreed with the recommendations and finally for any additional comments. Ten laboratories responded and agreed with the recommendations and no one had any additional comments, except for minor revisions.

Acknowledgements

We are very grateful to the National Institutes for Food & Drug Control (China) and to Serum Institute of India (India) for donating the two candidate materials and to the Hib PS working party for participating in the collaborative study. The authors also acknowledge Paul Jefferson and the staff in the Centre for Biological Reference Materials (CBRM) at NIBSC for the production fills, and Dr Paul Matejtschuk, Kiran Malik, Chinwe Duru and Ernest Ezeajughi in the Standardization Science Division at NIBSC for performing the trial fills, moisture content and providing advice on formulation of materials.

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Table 3. Details of the ribose assay conditions used by participants in the collaborative study

Laboratory	**1**	**2**	**3**	**4**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
# independent assays on each set	3	2 or 4	2	1	1	4	3, 1 set tested	3	1	1	2, 1 set tested
Standard & references											
Standard Source Cat number	D-Ribose Sigma-Aldrich R1757	D-Ribose Sigma R 7500	Ribose Merck 1.07605.0050	D-Ribose Sigma Aldrich R7500	D-Ribose Sigma R7500	D-Ribose ACROS 13236	D-Ribose Sigma R7500	D-Ribose Sigma R7500	D-Ribose Sigma Aldrich R7500	Ribose Merck 107605	D-Ribose Sigma R7500
Moisture content	0	0.40% (CoA)	0.2% (CoA)	0.40% (CoA)	0.1% (CoA) 0.43% TGA	0.2% (CoA)	0.77 % by KF	0%, Dried	0.1 % (CoA)	0.2% (CoA)	0.79% by KF
Purity (CoA)	100%	>99%	> 99%	>99%	>99%	≥99.5%	>99%	>99%	99%		99%
Other references	no			PRP-TT bulk	Ist IS PRP	Ist IS PRP	no	PRP	no	no	no
Standard curve range	2.5 - 10 µg	5 to 60 nmol	1.25 - 40 µg/ml	1.25 – 25 µg/ml	5-25 ug/ml	5 to 60 nmol	5-25 µg/ml	0.2-10 µg/ml	5-50 µg/ml	2.5-25 µg/ml	5 – 25 µg/ml
OD range	0.182-1.545	0.103-1.228	0.042-1.104	0.067- 1.411	0.12-1.4	0.110-1.250	0.13 – 0.667	0.015-0.373	0.042-1.803	0.075-0.923	0.102-0.433
# dilutions, #levels	1, 4	2, 5	1 , 6	1, 6	1, 4	1, 5	1, 5	3, 7	2, 6	2, 6	1, 5
Final reagent conc.											
Orcinol	31.97 mM		43.95 mM	16.75 mM	33.5 mM	15.07 mM	43.97 mM	33.51 mM	35.18 mM	33.5 mM	43.97 mM
$FeCl_3$	1.12 mM		2.89 mM	1.17 mM	0.88 mM	1.295 mM	2.89 mM	0.881 mM	0.83 mM	0.881 mM	2.89 mM
HCl, % w/v	22.42 %		28.90 %	23.49 %	17 %	25.20 %	28.91 %	17.6 %	16.57 %	17.53 %	28.90 %
Ethanol, % v/v	5.82 %		6.25 %	3.17 %	5 %	1.37 %	6.25 %	4.57 %	5 %	4.76 %	5.93 %
Incubation											
Method Incubation	Boiling water bath	Boiling water bath	Water bath	Boiling liquid bath	Oil bath	Boiling water bath	Boiling liquid bath	Water bath	Boiling water bath	Boiling water bath	Boiling liquid bath
Temp, time		90ºC, 20 min	80ºC, 15 min	100ºC, 20 min	100ºC, 20 min	90ºC, 20 min	5 min	95ºC, 20 min	90ºC, 40 min	20 min	100ºC, 5 min
Spectrophotometer All used 669 or 670 nm	Perkin Elmer Lambda 35	Shimadzu UV2450; Agilent Cary 60	Thermo Electron Genesys10	Perkin Elmer Lambda 35	Perkin Elmer Lambda 800	Shimadzu UV2450	Spectronic Genesys2	Ultrospec 5300, SWIFT II software	Molecular Devices Spectramax-M4	Jasco V630; Uvikon 930	Shimadzu UV2450
Conversion factor g PRP/g ribose	2.488	368.14 g/mol	2.5	2.488	2.448	368.21 g/mol	2.439	2.45	2.45		2.439

Table 4. Details of the phosphorus assay conditions used by participants in the collaborative study

Laboratory	1	2	3	4	5	8	9	11	12
Number assays/set	1	1	2 (same curve)	1	1	4	3 (1 set)	1	4 (1 set)
Standard & references									
Standard	Etalon PlasmaCal ICP/ICPMS-Phosphorus dihydrogen ammonium, SCP Science	D-ribose-5-phosphate disodium salt dehydrate Sigma 83875	Potassium dihydrogen phosphate Applichem A3620,0500	Potassium dihydrogen phosphate Merck 1.04873.0250	Potassium phosphate monobasic – Fisher P286	D-ribose-5-phosphate disodium salt dihydrate Sigma 83875	Potassium dihydrogen phosphate monobasic Sigma-Aldrich P0662	Sodium phosphate monobasic monohydrate, Sigma-Aldrich S9638	Potassium dihydrogen phosphate VWR 26 936
Purity	100%	100%	99.70%	99.90%	100	99	99%	99.60%	100%
Moisture content	liquid	9% (CoA)	0.2% (CoA)	<0.1% (CoA)	<0.1% (CoA)	10.5% (CoA)	0.10% (CoA)	not given	<0.1% (CoA)
Standard curve range	0.6-1.6µg/ml	5 - 40 nmoles	1-9 µg/ml	2.5-10 µg/ml	0.2- 2 µg/ml	5 - 40 nmoles	0.1-2.4 µg/ml	0.155-1.24 µg/ml	1-8 µg/ml
# levels	5	5	5	3	5	5	7	5	4
OD range		0.108-0.887	0.124-1.043	0.268-1.118	0.042-0.428	0.124-0.985	0.02-0.506	0.075-0.581	0.111-0.878
Sample details									
# replicates, #dilutns	1, 1	3, 2	3, 1	2, 1	2, 1	2, 1	2, 1	2, 1	2, 1
Method	ICP-AES	Ames	Chen	Chen	Chen	Ames	Chen	Chen	Chen
Incubation									
Method Incubation		Furnace and water bath	Bunsen apparatus (15-20 min)	Dry block and boiling liquid bath	Dry block	Furnace and water bath	Water bath	Oil bath	Dry block
Temp of incubation		600ºC, 99ºC	37ºC 90 min,	160ºC 4 h/ 160ºC 4 h; 39ºC 90 min	120-200ºC	600ºC, 98ºC, 37ºC	130ºC, 1 h, 180º, 1 h, 250º, 30 min, 37º, 2 h	200ºC, 60 min; 37ºC 60 min	100-105ºC, then 250ºC for 20 min
Spectrophotometer	Jobin Yvon Activa M	Shimadzu, UV - 2450	ThermoElectron Genesys 10UV7	Perkin-Elmer Lambda 35	Beckman Coulter DU-800	Shimadzu, UV - 2450	ThermoFisher Spectronic Genesys2	Molecular devices SpectraMax-M4	Jasco V630 Spectro UV
Wavelength	213.618 nm	820 nm	820 nm	820 nm	825 nm	820 nm	825 nm	820 nm	825 nm
Conversion factor (g PRP/gP)	11.900	11.89	11.73	12.04	11.905	368.21 g/mol	11.905	11.9	11.9

Table 5. Details of the HPAEC-PAD assay conditions used by participants in the collaborative study

Laboratory	2		4	6	7	8	9	10	11
	Hydrolysis method								
	HCl	NaOH							
Number of independent assays on each set	1	1	1	1	1	1, but 2 dilutions of each sample	1	3	1
Standard & references									
Standard	In-house PRP		In-House Hib-TT bulk	Adonitol, Sigma 02240	Ist WHO IS PRP	Ist WHO IS PRP	1st WHO IS PRP	Ist WHO IS PRP	Ist WHO IS PRP
Standard curve	0.225-6µg/ml		5-20 ug PRP/ml	0.75-1.05µg/ml	0.5-27 µg/ml	0.225-12.5µg/ml	3.125-50µg/ml	10-50µg/ml	1-15µg/ml
# levels	5		4	5	5	7	5	5	6
Internal spike					G-1-P, 4 µg/ml			G-1-P 0.05 mM	
Hydrolysis	6 M HCl	2M NaOH	0.09 M NaOH	0.3 M HCl	0.1 M NaOH	0.2 M NaOH	0.3 M NaOH	0.3 M NaOH	0.02 M NaOH
Incubation	2 h, 100ºC	3 h at 55ºC	12 h, 21ºC, then 4ºC	2 h, 100ºC	12 h, 23ºC, then 4ºC	2 h, 90ºC	12 h, 20-25ºC	20 h, 25ºC	3 h, 55ºC
Sample prep details	0.22µm filtration	0.22µm filtration		0.22 µm filtration		0.22 µm filtration		protein removed after hydrolysis	
Equipment									
Make/ Model	Dionex IC3000		Dionex ICS 3000	Dionex DX500	Dionex ICS3000	Dionex ICS 3000, 5000	Thermo ICS5000	Dionex ICS5000	Thermo IS5000
Electrode	Disposable gold electrode			Ag/AgCl	pH-Ag/AgCl gold working	pH/Ag/AgCl	Ag/AgCl	AgCl	pH-Ag/AgCl
Waveform	Quadruple	Quadruple			Quadruple	Quadruple			
Column (Carbopac)	Carbopac MA1	Carbopac PA10	PA10	MA1	PA-10	PA1	PA10	PA10	PA-10
Guard column	Carbopac MA1	Carbopac PA10	AminoTrap	Carbopac MA1	PA10, Amino Trap	Carbopac PA1	PA10	Carbopac PA1	Carbopac PA-10
Column and autosampler temperature	30ºC (5ºC)	30ºC (5ºC)	30ºC (4ºC)	30ºC (4ºC)	23ºC (4ºC)	25ºC	25ºC (4ºC)	25ºC	20-25ºC (15ºC)
Mobile phase	MQW + 1 M NaOH	MQW + 0.5M NaOAc + NaOH	32mM NaOH, 100mM NaOAc	1 M NaOH	1 M NaOH	200mM NaOH, 500mM NaOAc	28mM NaOH, 100mM NaAOAc	25mM NaOH 150mM NaOAc	50mM NaOH, 125mM NAOAc
Regeneration/wash	1 M NaOH		200mM NaOH	1 M NaOH	1 M NaOH	2 M NaOH	280mM NaOH, 1M NaOAc		
Flow rate, Run Time	0.4ml/min, 70 min	1.0ml/min, 20.10 min	1ml/min, 25 min	0.4ml/min,40 min	1ml/min, 30 min	21 min	1.2ml/min, 50 min	15 min	1ml/min, 20 min

Elution of G-1-P PRP/Ribitol	PRP, ~ 21 min	PRP, 12 min	PRP, 20 min	Ribitol, 15.6 min	G-1-P, 13min; PRP, 23 min	PRP, 7.7 min	Ribitol, 16.9 min	G-1-P, 6 min; PRP, 8 min	PRP, 11 min
Conversion factor				2.419 g PRP/g ribitol					

Table 6. Details of the NMR assay conditions used by participants in the collaborative study

Laboratory	**11**
Standard & references	
Standard	1st PRP IS (02/208)
Other references	None
Sample details	
Blank/Buffer	D_2O + 0.02 % TSP
Volume of sample	5 mg /0.6 ml D_2O+0.02 % TSP
# dilutions of A-D used	Single
Sample Drying	
Method	Freeze drying
Temp	30℃
Spectrometer	500.1324 MHz
Probe	TXI 5 mm
Temp	300 K
Software	Topspin 3.0
Data points	32 K
Spectral Window	8012.820 Hz

Table 7. Summary of results (PRP content in mg/ampoule)

Sample	Method	Estimate	LCL	UCL	SE	n
A	Ribose	4.989	4.911	5.068	0.035 (0.70%)	11
	Phosphorus	4.805	4.501	5.109	0.132 (2.75%)	9
	HPAEC-PAD	5.080	4.904	5.256	0.076 (1.50%)	9
B	Ribose	4.618	4.474	4.761	0.064 (1.40%)	11
	Phosphorus	4.418	4.051	4.786	0.159 (3.61%)	9
	HPAEC-PAD	4.710	4.364	5.056	0.150 (3.18%)	9
D	Ribose	4.904	4.760	5.049	0.065 (1.32%)	11
	Phosphorus	4.588	4.192	4.984	0.172 (3.74%)	9
	HPAEC-PAD	4.940	4.700	5.180	0.104 (2.10%)	9
E	Ribose	1.553	1.494	1.612	0.026 (1.70%)	11
	Phosphorus	3.753	2.720	4.786	0.422 (11.25%)	7
	HPAEC-PAD	1.433	1.352	1.514	0.034 (2.39%)	8

LCL Lower 95% confidence limit
UCL Upper 95% confidence limit
SE Standard error
n Number of labs

Table 8. Uncertainty estimation for ribose assays

Sample	Relative standard uncertainties			
	A	B	D	E
Standard error (collaborative study)	0.70%	1.40%	1.32%	1.70%
Homogeneity of filling weight	0.06%	0.07%	0.12%	n/a
Moisture content of ribose standard	0.12%	0.12%	0.12%	0.12%
Purity of ribose standard	0.29%	0.29%	0.29%	0.29%
Weighing of ribose standard	1.00%	1.00%	1.00%	1.00%
Combined	1.26%	1.75%	1.69%	2.00%

Sample	Estimates with expanded uncertainty			
	A	B	D	E
Mean estimate	4.989	4.618	4.904	1.553
Combined uncertainty	0.063	0.081	0.083	0.031
Expanded uncertainty (k=2.23)	4.849 - 5.130	4.438 - 4.797	4.720 - 5.089	1.484 - 1.622
Number of labs	11	11	11	11

Table 9. Molecular size elution of stability samples of candidate standards

Storage Temp (ºC)	% Eluting by K_D 0.45									
	Candidate 1 (12/218)					Candidate 2 (12/306)				
	Storage Time (months)					Storage Time (months)				
	1	2	4	6	12	1	2	4	6	12
-70				81.9	84.2				81.9	80.6
-20	100.0	83.7	99.4	79.8, 81.6	84.3	81.3	83.0	85.0	81.8, 81.6	73.4,76.9
4	100.0	81.5	87.2	76.7	83.3	79.8	80.7	85.9	81.1	78.5
20	81.4	84.8	88.3	78.8	79.0	75.8	80.4	84.2	83.5	76.6
37	85.0	79.0	71.8	67.2	61.5	80.2	77.4	83.9	76.8	68.4
56	55.9	40.8	28.1	15.7	5.6	69.1	62.1	65.6	46.7	31.9

Accelerated degradation and real-time stability samples of candidate standards were run on a TSK 6000PWX: + 5000 PWXL column series. K_D values were calculated using peak elution time of RI signals of Salmon DNA, tyrosine and candidate standards.

Figure 1. Results (PRP content in mg/ampoule) for sample A

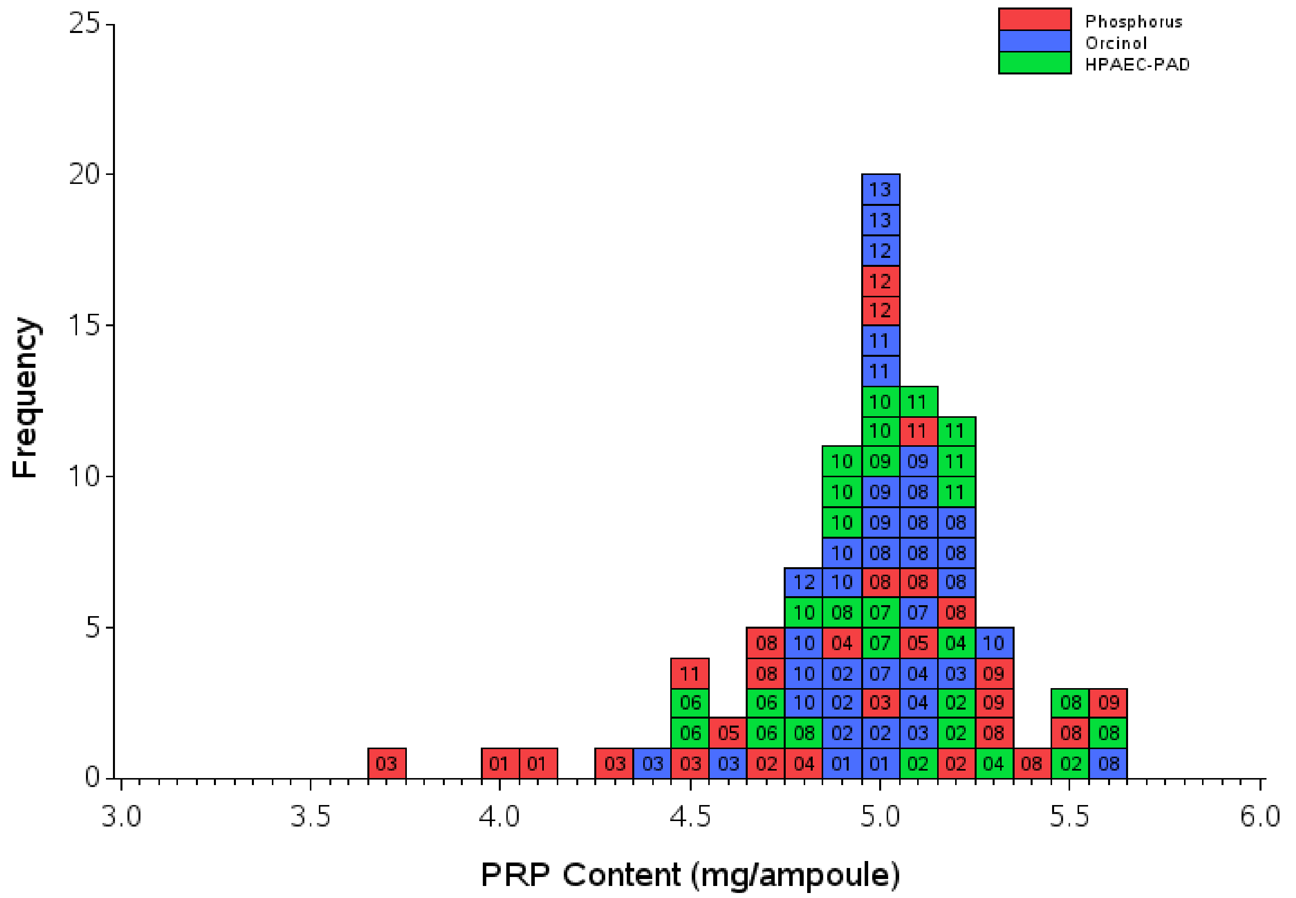


Figure 2. Results (PRP content in mg/ampoule) for sample B

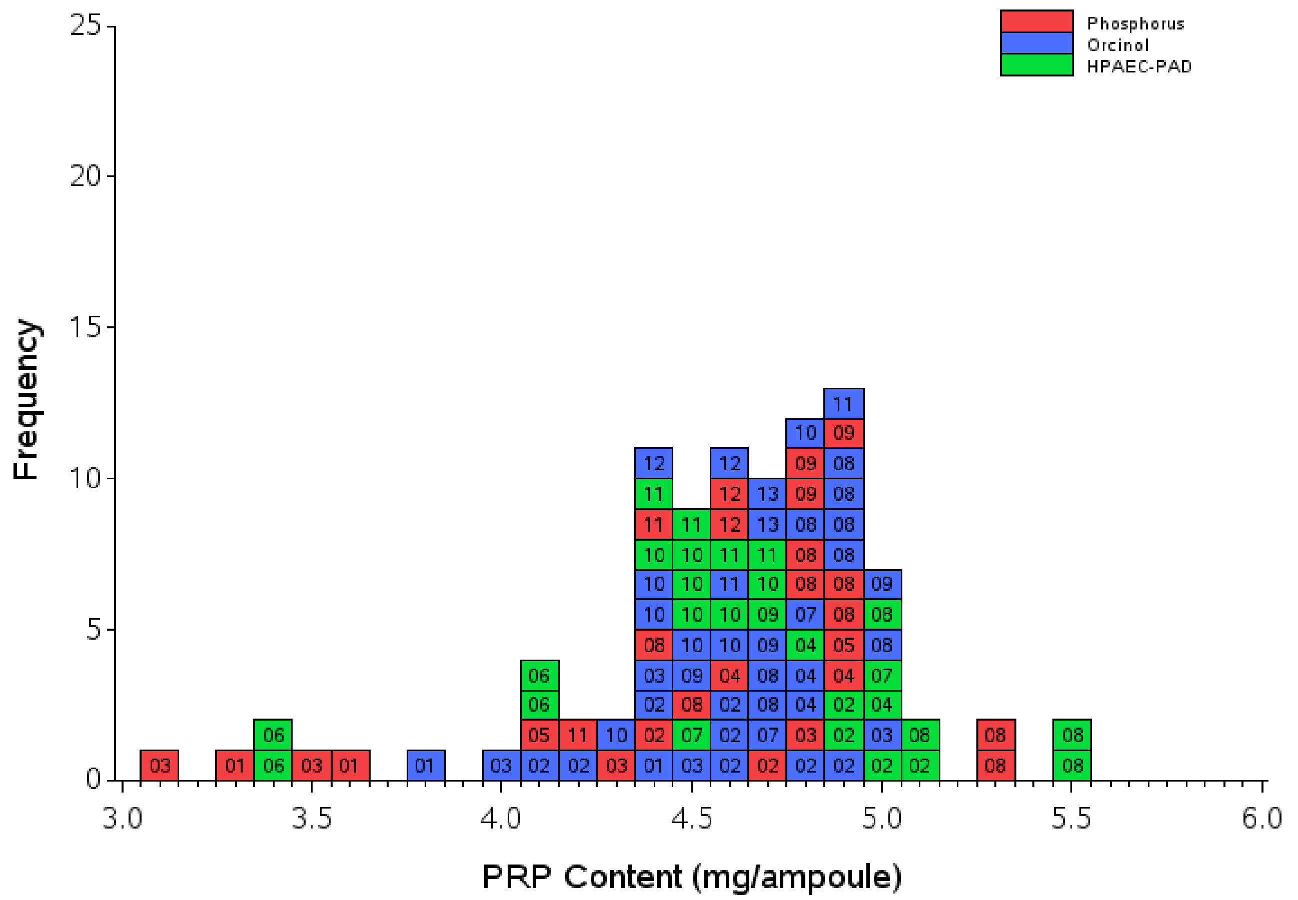


Figure 3. Results (PRP content in mg/ampoule) for sample D

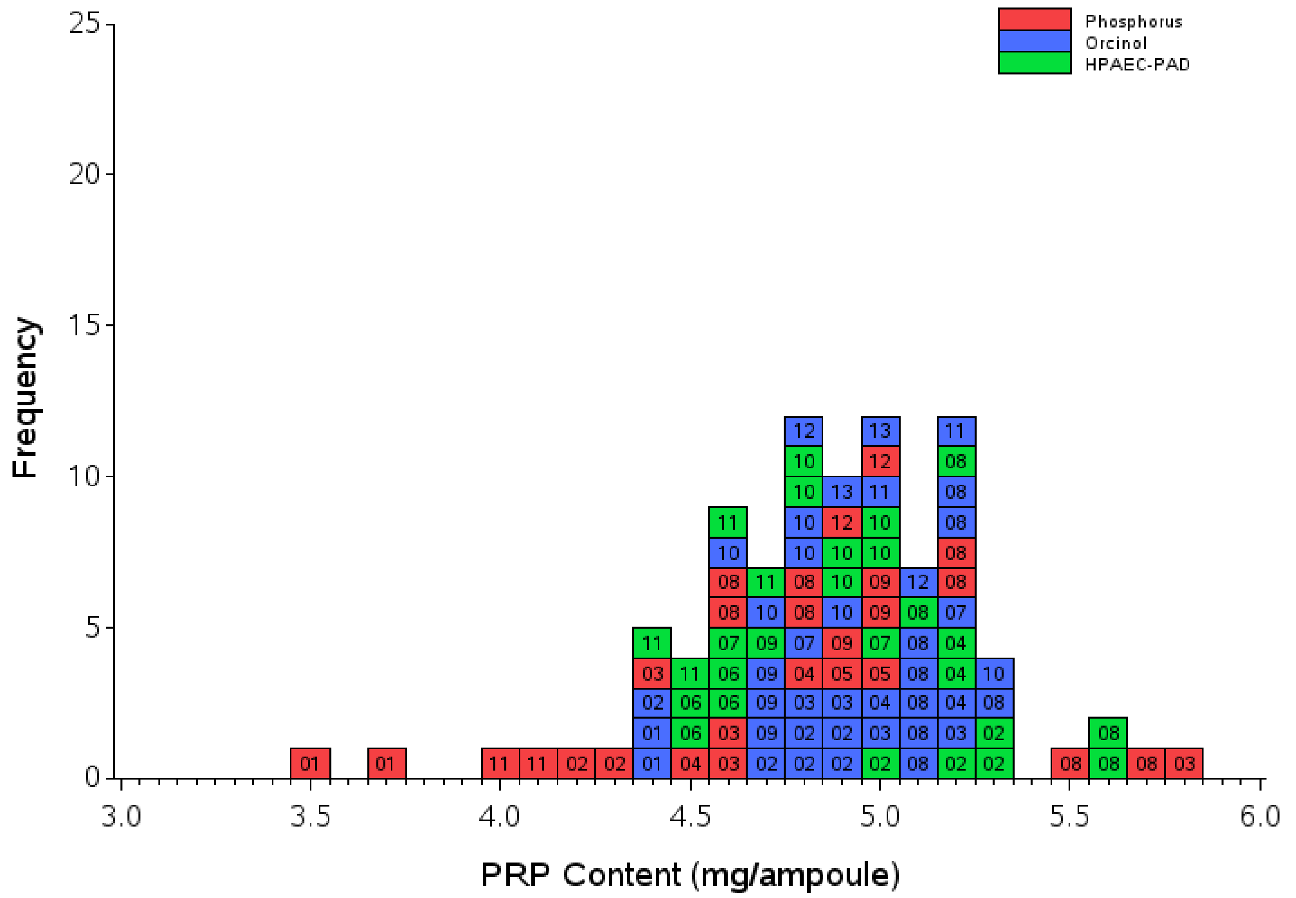


Figure 4. Results (PRP content in mg/ampoule) for sample E

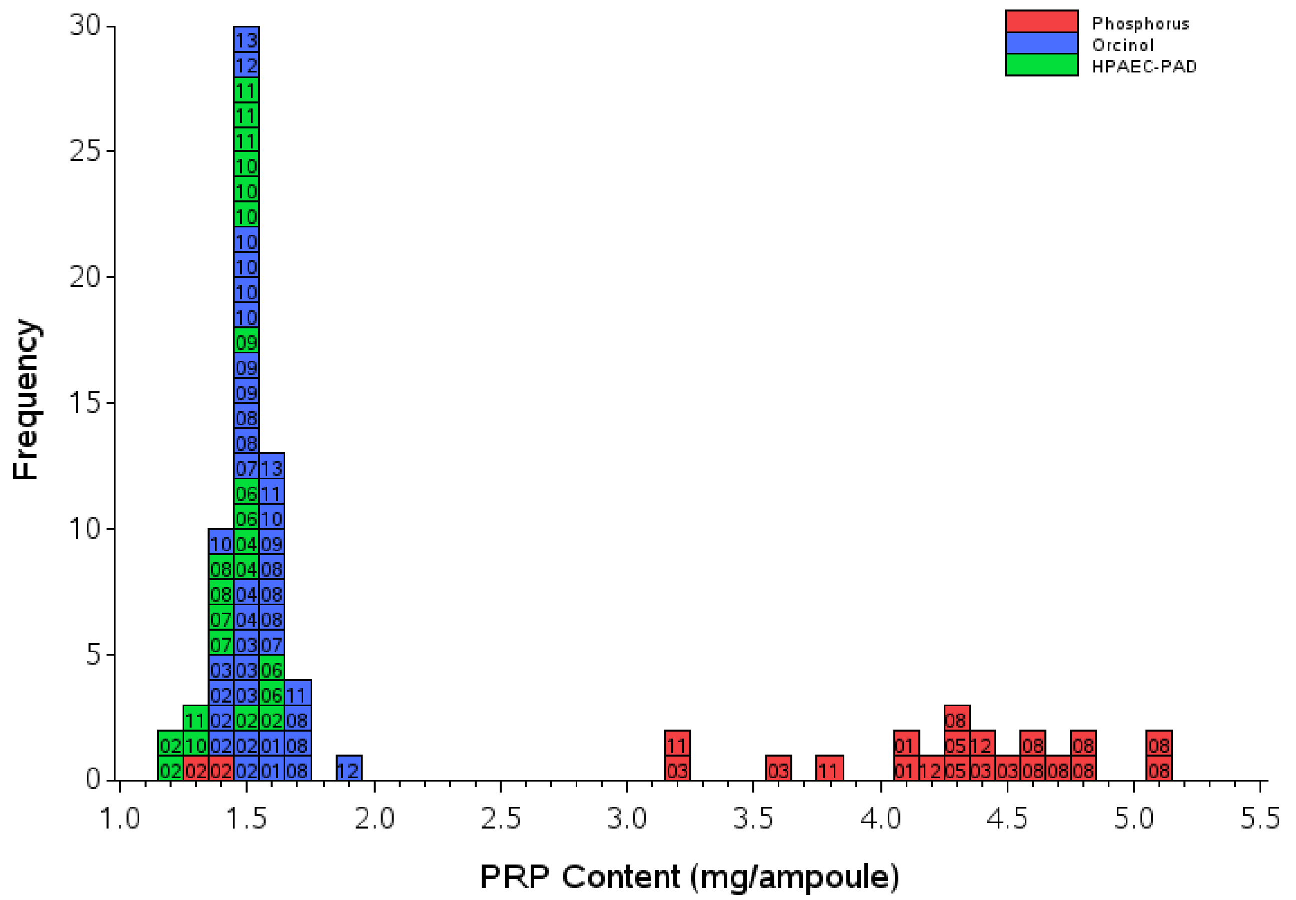


Figure 5. Mean estimates with expanded uncertainties from ribose assays

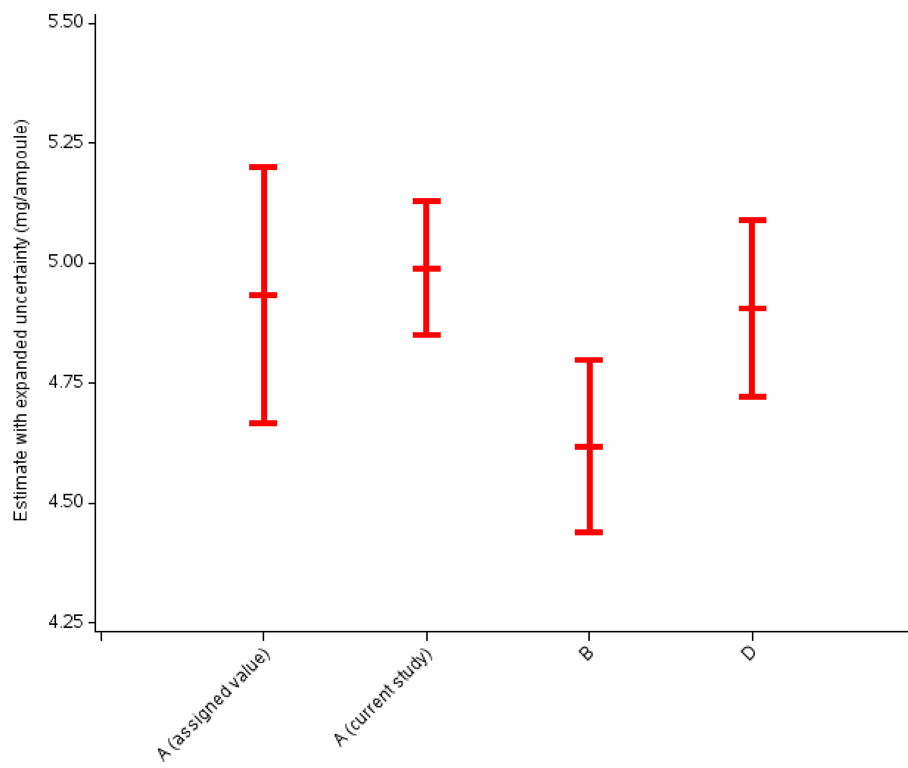
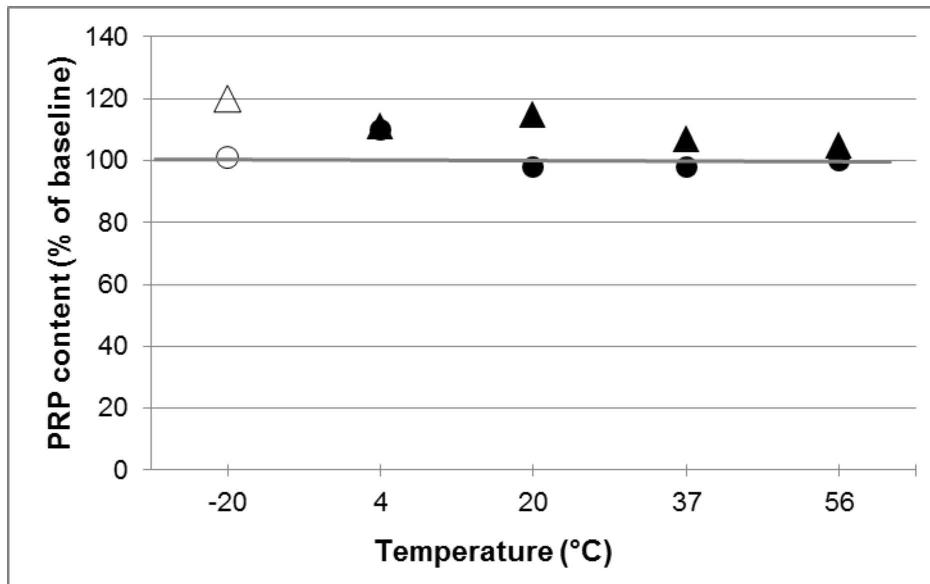
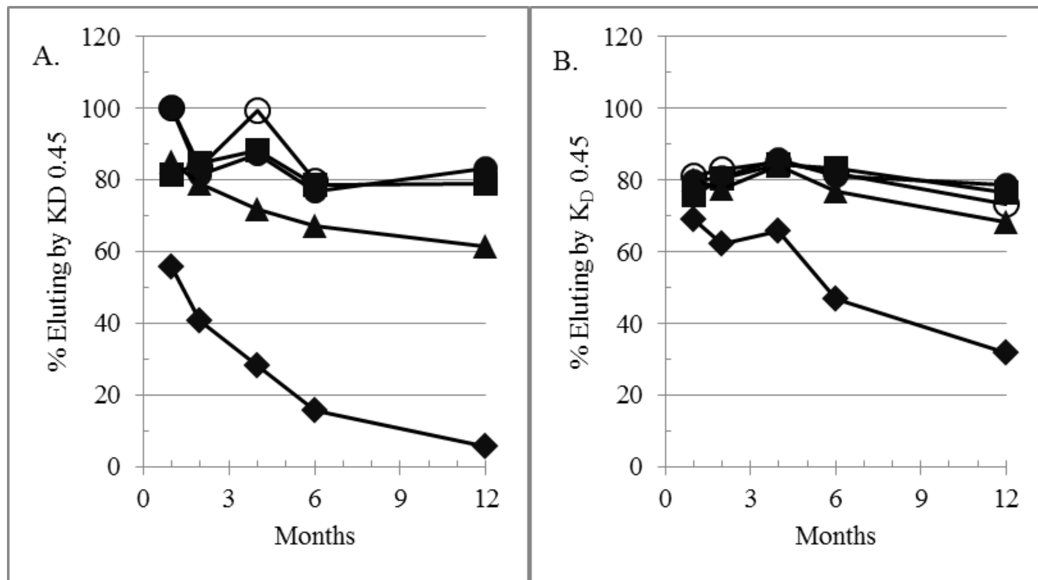


Figure 6. PRP content of candidate standards after 12 months incubation at different temperatures



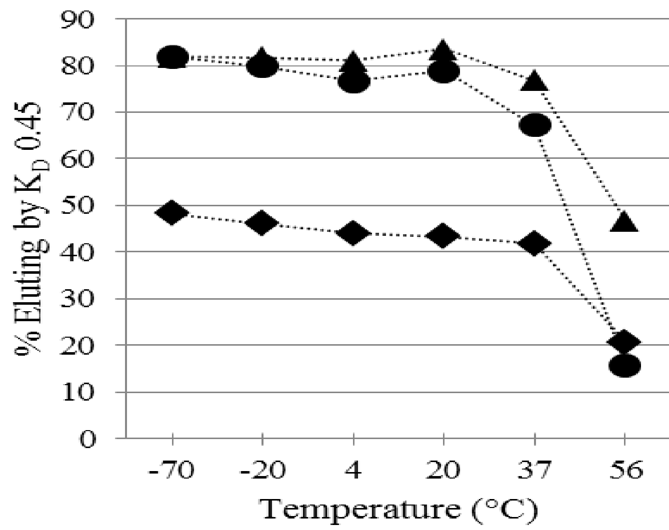
Real-time (open symbols) and accelerated degradation (closed symbols) for Candidate 1, 12/218 (❍, ●) and Candidate 2, 12/306 (△,▲) candidate standard are shown relative to baseline controls. The PRP content was determined using the ribose assay, using the 1st WHO International Standard as the quantitative standard.

Figure 7. Molecular sizing elution by a specified distribution coefficient (K_D) for the candidate standards A) 12/218 and B) 12/306



Accelerated degradation samples stored at -20°C (❍), 4°C (●), 20°C (■), 37°C (▲) and 56°C (◆) were analyzed at timepoints up to 12 months using a TSK 6000-5000PWXL column series.

Figure 8. Stability of the molecular size of the candidate standards compared with the 1st WHO IS



Data from 6 months stability samples for 02/218 (●) and 02/306 (▲) compared with 5 month data (accelerated degradation) and 6 mo (real-time) from 02/208, the 1st WHO Hib PS standard (◆).

Appendix A. List of Participants involved in the collaborative study forming the *Hib PRP IS Working Group*

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Appendix B1. Results (PRP content in mg/ampoule) from ribose assays

Sample	Lab	Individual Assay Estimates								Mean Estimates	
		Ampoule Set 1				Ampoule Set 2					
		Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4	Ampoule Set 1	Ampoule Set 2
A	01	4.935				5.044				4.935	5.044
	02	4.935				4.979				4.912	4.926
		4.888				4.872					
	03	5.145	5.191			4.378	4.552			5.168	4.465
	04	5.128				5.068				5.128	5.068
	07	5.084				5.004				5.084	5.004
	08	5.111	5.228	5.148	5.006	5.166	5.582	5.235	5.085	5.123	5.267
	09	5.083	4.978	5.015						5.025	
	10	4.891	5.265	4.771		4.806	4.799	4.905		4.976	4.837
	11	5.001				5.032				5.001	5.032
	12	5.009				4.796				5.009	4.796
	13	5.002				4.976				5.002	4.976
B	01	4.430				3.846				4.430	3.846
	02	4.213				4.142				4.472	4.622
		4.419				4.636					
		4.620				4.839					
		4.637				4.869					
	03	4.350	4.048			4.474	4.991			4.199	4.733
	04	4.804				4.757				4.804	4.757
	07	4.840				4.730				4.840	4.730
	08	4.837	4.729	4.926	4.850	4.884	4.998	4.877	4.691	4.836	4.863
	09	4.972	4.530	4.715						4.739	
	10	4.555	4.787	4.538		4.362	4.323	4.394		4.627	4.360
	11	4.635				4.905				4.635	4.905
	12	4.639				4.433				4.639	4.433

	13	4.741				4.718				4.741	4.718
C	01	< LOQ	< LOQ	< LOQ		< LOQ	< LOQ	< LOQ		< LOQ	< LOQ
	02	BQL				BQL				BQL	BQL
		BQL				BQL					
	03	0.013	0.020	0.019	0.019	0.032	0.051	0.047	0.048	0.0178	0.0445
	04	< 0.026	< 0.029			< 0.065	< 0.072			< 0.0275	< 0.0685
	07	0.071	0.054			0.173	0.132			0.0625	0.1525
	08	Nil				Nil				Nil	Nil
	09	0.025	0.004	0.008		0.060	0.011	0.020		0.0123	0.0303
	10	0.065				0.078				0.065	0.078
	11	Below Std Range				Below Std Range				Below Std Range	Below Std Range
	12	< 0.154				< 0.154				< 0.154	< 0.154
	13	0.079								0.079	
D	01	4.421				4.398				4.421	4.398
	02	4.770				4.767				4.745	4.758
		4.399				4.748					
		4.894									
		4.917									
	03	4.987	5.153			4.837	4.946			5.070	4.892
	04	5.178				4.970				5.178	4.970
	07	5.184				4.759				5.184	4.759
	08	5.084	5.121	5.136	5.073	5.104	5.320	5.236	5.200	5.104	5.215
	09	4.701	4.744	4.652						4.699	
	10	4.841	5.266	4.696		4.573	4.814	4.857		4.934	4.748
	11	5.179				4.996				5.179	4.996
	12	4.845				5.143				4.845	5.143
	13	4.980				4.903				4.980	4.903
E	01	1.566				1.609				1.566	1.609
	02	1.417				1.528				1.434	1.435
		1.450				1.356					

						1.420					
	03	1.480	1.369			1.507	1.496			1.424	1.501
	04	1.455				1.497				1.455	1.497
	07	1.597				1.538				1.597	1.538
	08	1.516	1.656	1.573	1.508	1.640	1.682	1.563	1.697	1.563	1.646
	09	1.543	1.522	1.585						1.550	
	10	1.391	1.489	1.460		1.475	1.536	1.554		1.447	1.522
	11	1.669				1.599				1.669	1.599
	12	1.528				1.912				1.528	1.912
	13	1.573				1.546				1.573	1.546

Appendix B2. Results (PRP content in mg/ampoule) from phosphorus assays

Sample	Lab	Individual Assay Estimates								Mean Estimates	
		Ampoule Set 1				Ampoule Set 2				Ampoule Set 1	Ampoule Set 2
		Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4		
A	01	3.987				4.086				3.987	4.086
	02	5.228				4.687				5.228	4.687
	03	4.266	4.458			3.740	4.962			4.362	4.351
	04	4.783				4.889				4.783	4.889
	05	4.583				5.076				4.583	5.076
	08	4.723	4.971	5.136	5.359	5.480	4.738	5.157	5.328	5.047	5.176
	09	5.625	5.274	5.337						5.412	
	11	4.533				5.076				4.533	5.076
	12	4.958				5.025				4.958	5.025
B	01	3.590				3.292				3.590	3.292
	02	4.410				4.692				4.410	4.692
	03	3.490	3.146			4.336	4.770			3.318	4.553
	04	4.942				4.627				4.942	4.627
	05	4.922				4.132				4.922	4.132
	08	4.539	4.778	4.947	4.941	5.326	4.384	5.321	4.811	4.801	4.961
	09	4.836	4.842	4.941						4.873	
	11	4.211				4.393				4.211	4.393
	12	4.568				4.605				4.568	4.605
C	01	< LOD	< LOD	< LOD		< LOD	< LOD	< LOD		< LOD	< LOD
	02	BQL				BQL				BQL	BQL
	03	0.012	0.011			0.060	0.022			0.0115	0.041
	04	N / A				< 0.379				N / A	< 0.379
	05	0.007				0.009				0.007	0.009
	08	Nil				Nil				Nil	Nil
	09	0.007	No test	No test						0.007	
	11	Below Detection Limit				Below Detection Limit				BDL	BDL

	12	< 0.095				< 0.095				< 0.095	< 0.095
D	01	3.669				3.530				3.669	3.530
	02	4.314				4.199				4.314	4.199
	03	4.641	5.797			4.408	4.577			5.219	4.493
	04	4.497				4.810				4.497	4.810
	05	5.037				4.883				5.037	4.883
	08	4.599	4.750	5.192	5.681	5.497	4.586	4.779	5.181	5.056	5.011
	09	4.950	4.949	5.033						4.977	
	11	3.975				4.070				3.975	4.070
	12	4.905				5.023				4.905	5.023
E	01	4.106				4.135				4.106	4.135
	02	1.448				1.293				1.448	1.293
	03	3.234	3.596			4.472	4.442			3.415	4.457
	04	.				.					
	05	4.327				4.292				4.327	4.292
	08	4.615	4.800	4.558	4.751	5.084	5.102	4.718	4.271	4.681	4.794
	11	3.831				3.167				3.831	3.167
	12	4.220				4.376				4.220	4.376

Appendix B3. Results (PRP content in mg/ampoule) from HPAEC-PAD assays

Sample	Lab	Individual Assay Estimates								Mean Estimates	
		Ampoule Set 1				Ampoule Set 2				Ampoule Set 1	Ampoule Set 2
		Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4		
A	02a	5.470				5.215				5.470	5.215
	02b	5.113				5.248				5.113	5.248
	04	5.226				5.277				5.226	5.277
	06	4.695				4.525				4.677	4.532
		4.659				4.538					
	07	5.027				5.011				5.027	5.011
	08	4.899				5.470				4.863	5.545
		4.826				5.619					
	09	4.995								4.995	
	10	5.010	4.900	4.970		4.870	4.920	4.840		4.960	4.877
	11	5.076				5.215				5.141	5.229
		5.206				5.243					
B	02a	4.946				5.019				4.946	5.019
	02b	4.934				5.143				4.934	5.143
	04	4.822				5.004				4.822	5.004
	06	3.352				4.066				3.370	4.078
		3.388				4.090					
	07	4.971				4.497				4.971	4.497
	08	5.060				5.514				5.047	5.487
		5.034				5.460					
	09	4.654								4.654	
	10	4.620	4.520	4.460		4.650	4.510	4.390		4.533	4.517
	11	4.659				4.517				4.610	4.482
		4.561				4.447					
C	02a	BQL				BQL				BQL	BQL
	02b	BQL				BQL				BQL	BQL

	04	< 0.006				< 0.006				< 0.006	< 0.006
	06	0.000				0.000				0.000	0.000
		0.000				0.000				0.000	0.000
	07	0.000				0.000				0.000	0.000
	08	Nil				Nil				Nil	Nil
		Nil				Nil				Nil	Nil
	09	Not detected								Not detected	
	10	No signal				No signal				No signal	No signal
	11	No Peak				No Peak				No Peak	No Peak
D	02a	5.183				5.336				5.183	5.336
	02b	4.954				5.260				4.954	5.260
	04	5.172				5.228				5.172	5.228
	06	4.501				4.568				4.501	4.559
		4.501				4.550					
	07	4.567				5.024				4.567	5.024
	08	5.180				5.589				5.138	5.585
		5.096				5.580					
	09	4.707								4.707	
	10	5.030	4.850	4.780		5.020	4.840	4.910		4.887	4.923
	11	4.569				4.391				4.654	4.461
		4.740				4.530					
E	02a	1.570				1.505				1.570	1.505
	02b	1.245				1.224				1.245	1.224
	04	1.532				1.520				1.532	1.520
	06	1.574				1.498				1.550	1.541
		1.526				1.584					
	07	1.371				1.400				1.371	1.400
	08					1.360					1.357
						1.354					
	09	1.455								1.455	
	10	1.310	.	.		1.530	1.540	1.450		1.310	1.507

	11	1.472 1.338				1.453 1.494				1.405	1.474

Results from Lab 02a are from acid hydrolysis, and from 02b are from basic hydrolysis.

WHO International Standard
WHO 2nd International Standard for Haemophilus influenzae polysaccharide Polyribosyl Ribitol Phosphate (PRP)
NIBSC code:
Instructions for use
(Version 2.00, Dated 24/07/2014)

Not for in vitro diagnostic use

1. INTENDED USE

The freeze-dried preparation of H. influenzae b (Hib) capsular polysaccharide PRP (polyribosyl ribitol phosphate; 5-D-ribitol-(1 → 1)-β-D-ribose-3-phosphate), provided by Serum Institute of India (SII) was prepared in ampoules (2013) at the Centre for Biological Reference Materials (CBRM, NIBSC) and coded 12/306. A collaborative study was carried out on this material by 13 laboratories in 2013 to determine the PRP content in SI units based on the ribose assay, and to evaluate its suitability for use as a standard for PRP quantification assays (including ribose, phosphorus and HPAEC-PAD assays) for Hib conjugate vaccines. In 2014, on the basis of the collaborative study, it was established as the second International Standard for PRP for potential use in phosphorus, ribose and HPAEC-PAD assays for quantification of PRP.

Critical issues on the actual testing methods are provided in the WHO/BS/2014.xxxx document and should be considered in the calibration of the secondary standards. NIBSC, Potters Bar, UK is the custodian and distributor of this material.

2. CAUTION

This preparation is not for administration to humans.

Not human or bovine source material

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

Based on the ribose assay carried out by 11 partcipanting laboratories in the collaborative study, the Second International Standard for Hib capsular polysaccharide PRP (ampoules coded 12/306) has a PRP content of 4.904 ± 0.185 mg/ampoule (expanded uncertainty calculated using a coverage factor of 2.23 which corresponds to an approximate 95% level of confidence) .

4. CONTENTS

Country of origin of biological material: United Kingdom.

Each ampoule contains the freeze-dried powder of 2 ml of PRP in 0.56 mg/ml NaCl. Each ampoule contains about 6.0 mg of dry material as estimated by weighing after freeze drying, with a moisture content of about 1.45%.

5. STORAGE

Ampoules should be stored at or below -20ºC.

Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

Din Ampoule

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.

Re-suspend the contents of the ampoule in 1ml of distilled water. The reconstituted material should be aliquoted and frozen at or below -20oC. The Standard can be used directly as a reference in the physicho-chemical assays or for calibrating of secondary standards.

8. STABILITY (Add or amend as necessary)

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label. It is the policy of WHO not to assign an expiry date to their International Reference materials when stored at the recommended storage temperature (-20ºC). Real time stability is ongoing and results of an accelerated degradation study carried out at NIBSC on the PRP standard stored at: 4, 20, 37 and 56ºC for 1, 2, 4, 6 and 12 months showed a predicted degradation rate of 0.016% per year when stored at -20ºC.

In addition, the stability of the standard reconstituted in water or 0.09 % w/v NaCl and stored at 4ºC for 0, 1, 2, 3 and 4 weeks, or at -20ºC for 1, 3, 6, 9, 12, 18, 24 and 30 months (reconstituted, frozen) is being determined, using the -20ºC lyophilized sample as baseline.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

MR Holliday & C Jones. WHO/NIBSC-co-sponsored informal workshop on the use of physico-chemical methods for the characterization of Haemophilus influenzae type b conjugate vaccines. 1999. Biologicals, 27: 51-53.

WHO/BS/05.2018. F Mawas, B Bolgiano, D Belgrave, D Crane, P Rigsby & MJ Corbel. International collaborative study to evaluate a candidate international standard for Haemophilus influenzae type b capsular polysaccharide.

F Mawas, B Bolgiano, P Rigsby, D Crane, D Belgrave & MJ. Corbel. Evaluation of the saccharide content and stability of the first WHO International Standard for Haemophilus influenzae b capsular polysaccharide. 2007. Biologicals, 35: 235 - 245.

10. ACKNOWLEDGEMENTS

We are grateful to Serum Institute of India for donating the PRP material.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET (Add or amend as necessary)

Physical and Chemical properties			
Physical appearance: Please complete		Corrosive:	Select...
Stable:	Select...	Oxidising:	Select...
Hygroscopic:	Select...	*Irritant:*	*Select...*
Flammable:	Select...	*Handling:See caution, Section 2*	
Other (specify):	Please complete		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 6.0 mg
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable**.** **Attached:** No Please add vet cert numbers separated by a space

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.