THE DEVELOPMENT OF PHYSICO-CHEMICAL QUALITY CONTROL METHODS FOR *Haemophilus influenzae* type b VACCINE PRODUCTION

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Master of Science

by

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Declaration

THE DEVELOPMENT OF PHYSICO-CHEMICAL QUALITY CONTROL METHODS FOR *Haemophilus influenzae* type b VACCINE PRODUCTION

I, Heinrich Behr, hereby declare the following:

1. That the above–titled thesis is my own work, both in concept and execution, apart from the normal guidance of my supervisor;

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3. That no part of this work has been, is being, or is to be submitted for another degree at this or any other university;

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Abstract

The development of a low cost *Haemophilus influenzae* type b (Hib) manufacturing platform at The Biovac Institute (TBI) required analytical method development in parallel with the production process development. Technology transfer enabled TBI to develop Hib vaccine production which could lead to the development of vaccine manufacturing capacity in sub-Saharan Africa. Initial studies were conducted in the Research and Development (R&D) department from where the process was transferred to the Good Manufacturing Process (GMP) environments of the Production and Quality Control departments respectively. Scaling of the development process to a process commercially viable required the development of additional quality control test methods.

The quality control of Hib is performed by characterisation of the manufactured batch using physico-chemical analysis. The data generated are compared against that of a successful clinical trial batch. Animal based models for the potency and safety tests of Hib are ineffective.

Chromatographic methods of analysis are often used in the pharmaceutical and biotechnological industry. Gas chromatography with flame ionisation detection (GC-FID) is a conventional technique used for the analysis of volatile analytes. The analysis of process residuals ethanol and ethylene glycol were performed using headspace or direct injection GC-FID analysis. Ethylene glycol, a non-volatile solvent, was chemically dried after which it was derivatised with a trimethylsilylating reagent. In addition, a method was developed to determine polyribosylribitolphosphate. Samples were dried by means of lyophilisation and then subjected to methanolysis to yield methyl glycosides. A trimethylsilylating reagent was used to volatilise the analyte and analysis was performed using GC-FID with direct injection.

The use of internal standards throughout the sample preparation processes minimised errors due to sample handling, processing or injector reproducibility. Analytical method validation parameters were applied to the developed methods.

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2. the Biovac Institute for use of their facilities and material;
3. my family, Janine, Emelda and Bruno for all the support.

For you dad….

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Abbreviations

BSTFA	N, O-bis (trimethylsilyl) trifluoroacetamide
DNA	deoxyribonucleic acid
DOC	deoxycholic acid
DPT	diphtheria, pertussis, tetanus
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl
ELISA	enzyme-linked immunoabsorbant assays
ESI-MS	Electrospray Mass Spectroscopy
GC	Gas Chromatography
GC-FID	Gas Chromatography with Flame Ionisation Detection
HCl	hydrochloric acid
Hib	*Haemophilus influenzae* type b
HMDS	hexamethyldisilazane
HPAEC-PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonisation
ICH Q2 R1	International Conference on Harmonisation Quarter 2 Revision 1
IQ	Installation Qualification
LLOQ	Lower Limit of Quantitation
LOD	Limit of Detection
LOQ	Limit of Quantitation
MWCO	Molecular Weight Cut-off
NMR	nuclear magnetic resonance
OQ	Operational Qualification
PDE	Permitted Daily Exposure
PQ	Performance Qualification
PRP	polyribosylribitolphosphate
PRP-OMPC	polyribosylribitolphosphate conjugated to meningococcal outer-membrane protein complex
PRP-TT	polyribosylribitolphosphate conjugate to tetanus toxoid
QC	Quality Control
R	Correlation coefficient
R & D	Research and Development
RID	Refractive Index Detector

RNA	ribonucleic acid
% RSD	Percentage Relative Standard Deviation
SEC-HPLC	Size Exclusion High Performance Liquid Chromatography
TBI	The Biovac Institute
TFA	trifluoroacetic acid
TMCS	trimethylchlorosilane
TRS	Technical Report Series
TT	tetanus toxoid
ULOQ	Upper Limit of Quantitation
URS	User Requirement Specification
USP	United States Pharmacopeia
UV	Ultraviolet
WHO	World Health Organisation

Chapter 1

Quality Control of *Haemophilus influenzae* type b (Hib) vaccine

The quality control of Hib conjugate vaccine is performed using physico-chemical test methods. Data generated are compared to specifications compiled from a successful clinical trial batch. Production of the Hib conjugate vaccine varies and can differ in not only process but also polysaccharide size, protein carrier, coupling or conjugation chemistry and final formulation. Quality control of process residuals as well as the quantification of polyribosylribitolphosphate (PRP) by chromatography will be evaluated.

1.1 Background

Hib is a gram-negative coccobacillus encapsulated in the polysaccharide, PRP. The disease spreads by means of person-to-person contact through the exchange of nasopharyngeal excretions. It is responsible for diseases such as meningitis, epiglottitis, arthritis, cellulitis, pneumonia, osteomyelitis and pericarditis. Hib is the leading cause of non-epidemic bacterial meningitis in children under 5 years.[1] It was listed in 2010 as the cause of 3 million cases of serious disease and the deaths of 386 000 children across the world.[2] The World Health Organisation (WHO) recommended the inclusion of a Hib vaccine in infant immunization programs in 1997 in countries where resources were available and where the incidence of the disease was high.[3] In 2006 the WHO recommended that Hib vaccine should be included in all routine infant vaccination programs. The routine administration of Hib vaccine in the United States led to a decrease in invasive Hib diseases by between 85-89% from 1983 to 1995.[2]

The immune system recognizes antigens which triggers either antibody production against a disease or lymphocytes production. Antibody and cell mediated immune responses can act independently or in combination against disease.[4] Antibody-antigen reaction is specific and is described as a lock and key interaction. Antigens can be divided into T-cell-independent and T-cell-dependent. T-cell-dependent antigens require the T-lymphocytes to stimulate antibody production by B-lymphocytes. T-cell-independent antigens stimulate antibody production without the aid of T-lymphocytes.[5] Hib vaccines consisted initially of only the capsular polysaccharide. The immune response was as per that for T-cell-independent antigens and was weak in infants. Polysaccharide vaccines do not provide long-term immune responses. The polysaccharide conjugated to a protein carrier enhanced responses in children < 2 years and long-term immunity by follow-up or booster vaccinations could be achieved.[6]

The Hib vaccine manufacturers have different production approaches. The vaccines produced can differ not only in process, but also polysaccharide size, protein carrier, coupling or conjugation chemistry and final formulation.

Effective Hib conjugate vaccines include:

- PRP-D, PRP conjugated with diphtheria toxoid (no longer licensed);
- PRP-CRM, PRP conjugated with a mutated diphtheria toxoid (CRM 197);
- PRP-OMP, PRP conjugated to a meningococcal outer-membrane protein;
- PRP-T, PRP conjugated to a tetanus toxoid.[3]

1.2 Quality Control

Traditional vaccine quality control approaches lot release per batch. Each batch of vaccine produced is considered as unique. These release tests often rely on potency and safety tests in animals. Animal tests are expensive, have a large amount of variance and have ethical considerations. Hib vaccine does not have an animal model to base its batch release on. Release of the vaccine is based on a combination of physico-chemical and immuno–chemical tests. The lot release approach follows closely that of the more modern and humane "consistency approach" in vaccine lot release. This approach relates all release data generated to that of a successful clinic trial batch. The test results obtained when testing for lot release are compared to specifications which are generated from data obtained from a successful clinic trial. Quality assurance such as consistency in manufacturing processes, monitoring by in–process tests as well as quality control of raw materials and or intermediates enables the reproducible production of vaccine. Good manufacturing processes (GMP) strengthen the quality assurance of vaccine production processes. This holistic approach to manufacturing vaccines decreases the probability of manufacturing a significantly different lot which may not be effective in the clinic.

Hib vaccine manufacturing can be divided into different stages:

- Fermentation
- Polysaccharide purification
- Polysaccharide activation
- Protein carrier modification
- Conjugation

The WHO Technical Report Series (TRS) 897 recommends purified polysaccharide characterisation by: [1]

- identifying if the correct antigen was manufactured;
- determining the size of the polysaccharide by size exclusion chromatography and evaluating consistency of the distribution coefficient between manufactured batches;
- performing quantitative assays for ribose and phosphorus in order to determine the composition of the polysaccharide. Ribose content should be ≥ 32 % and phosphorus 6.8 % - 9 % of polysaccharide determined on dry weight.

It recommends the characterisation of bulk conjugate by: [1]

- determining the molecular size distribution;
- the PRP-protein ratio;
- residual reactive functional groups;
- residual reagents;
- total PRP as well as conjugated and unbound/free PRP content.

Safety tests such as sterility, endotoxin content and specific toxicity of the carrier protein are evaluated as well. Potencies of Hib vaccines are directly related to the amount of conjugated polysaccharide. The free polysaccharide content is expressed as a percentage of the total and conjugated content can be inferred. Vaccines successful in clinical trials had unbound polysaccharide levels of < 10% up to 40% of the total PRP content.[1]

The Biovac Institute (TBI) is a public private partnership with the aim to produce low cost vaccines for sub-Saharan Africa. The manufacturing of a Hib vaccine formed part of a technology transfer from an international vaccine partner and was one of the strategies to develop vaccine production capacity in South Africa. The manufacturing process was developed on a pilot scale in the Research and Development department and then transferred to the Production department to upscale for commercial use. There was a need to develop quality control test methods using available equipment.

Quality control methods capable of evaluating removal of residual solvents were needed as it was too early in the vaccine development process to illustrate removal by means of process validation.[1] The control methods could either be performed on the bulk conjugate or during the production process at the individual stages.

1.3 Aim and Objectives

The aim of this study is to apply typical physicochemical test methods to the quality control of Hib conjugate vaccines manufacturing.

The objectives for this study are the development of methods to:

- determine the concentration of residual ethanol in purified polyribosylribitolphosphate,
- determine the concentration of residual ethylene glycol in modified polyribosylribitolphosphate
- and quantify the amount of polyribosylribitolphosphate.

In addition to develop an alternative method for quantifying the PRP content. The quantification of total and free PRP is both a lot release test and a stability indicating assay. The use of non–specific methods of analysis has the risk of interference. Methods of quantification that are specific for an analyte limits this risk. The formulation of Hib antigen as a part of a multivalent vaccine was also one of the long term objectives of TBI. The development of an assay that could in future be applied to Hib antigen quantification as part of a multivalent vaccine was needed.

Chromatographic techniques using gas chromatography will be applied to determine the quantity of residual ethanol and ethylene glycol as well as to quantify the amount of PRP in the Hib vaccine. Gas chromatographs with flame ionisation detection (GC-FID) are conventional laboratory equipment and have been used extensively in the pharmaceutical industry.

The abovementioned tests will be assessed using current validation practices.

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Chapter 2

Analytical Method Validation

Analytical method validation is the documented process of providing scientific evidence that an analytical technique is suitable for the purpose it is intended. This chapter provides an overview of industry standards with regards to validation parameters.

2.1 Introduction

Validation data, through specific laboratory investigations, demonstrate that the performance of a method is suitable and reliable for the analytical applications intended.[1] Method validation ensures that the analytical results generated are within an acceptable uncertainty level. [2] Validated analytical test methods are but a part of an integrated quality assurance process that ensures generation of reliable, accurate results.

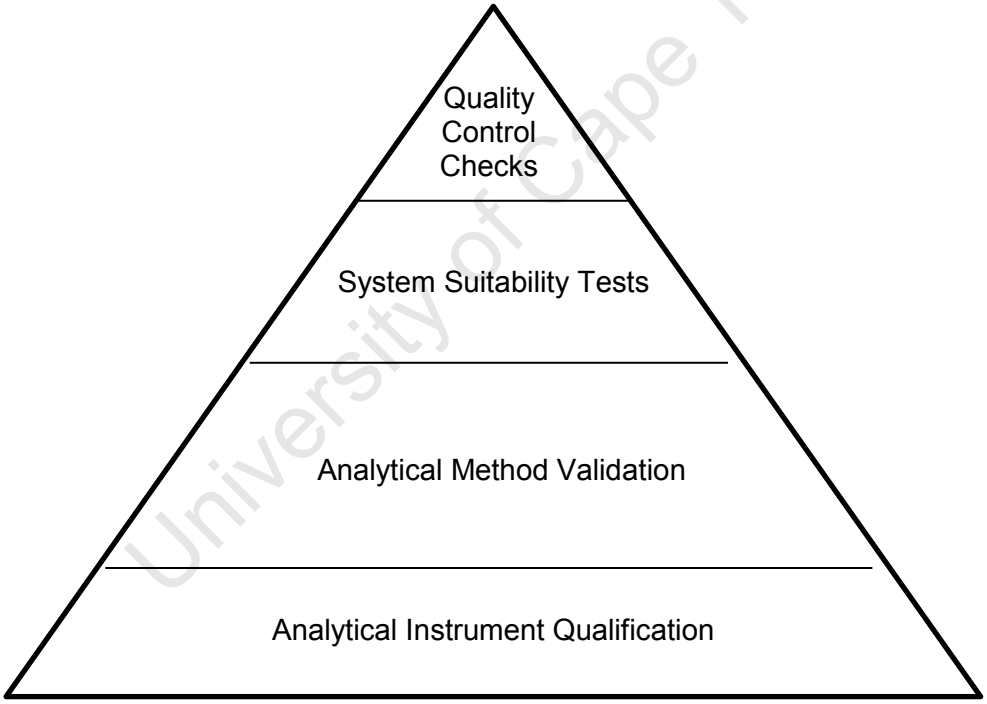

Figure 2.1 Data Quality triangle [3]

The quality assurance of the typical data generation process is illustrated by the Data Quality triangle (Figure 2.1). The quality assurance of data is not limited to method validation, but encompasses multiple facets. Some are inherent to the test method such as system suitability tests whereas others are equipment related such as calibration and functionality or operational tests. Equipment must be able to operate at the required specification be it quantitation levels or other operating parameters.

Equipment related quality assurance begins with User Requirement Specifications (URS), Installation Qualifications (IQ) and Operational Qualifications (OQ). A URS documents the specific technical requirements of an equipment item by the user. Factors such as application, detection limits, site preparation, certification requirements, qualification requirements and training requirements are documented. Approval by all stakeholders ensures the purchasing of equipment items fit for purpose. IQ protocols documents the physical installation process. Execution of the protocol during installation verifies parameters such as readiness of the area for the item, verifies that the correct item is installed and that all deliverables required from the supplier at installation are met. OQ protocols continue the process by verifying that the item delivered is in a functional condition. IQ and OQ protocols are executed during the commissioning stages of an equipment item while Performance Qualification (PQ) monitors equipment performance for the remainder of its lifecycle. Quality assurance during routine analysis after method validation is maintained by system suitability tests while equipment is maintained by and is maintained through routine calibration and performance testing or qualifications.

New test methods, modified existing test methods as well as test methods being applied to different concentration ranges and sample matrices needs to be validated. Figure 2.2 illustrates the typical life cycle of an analytical method.[3]

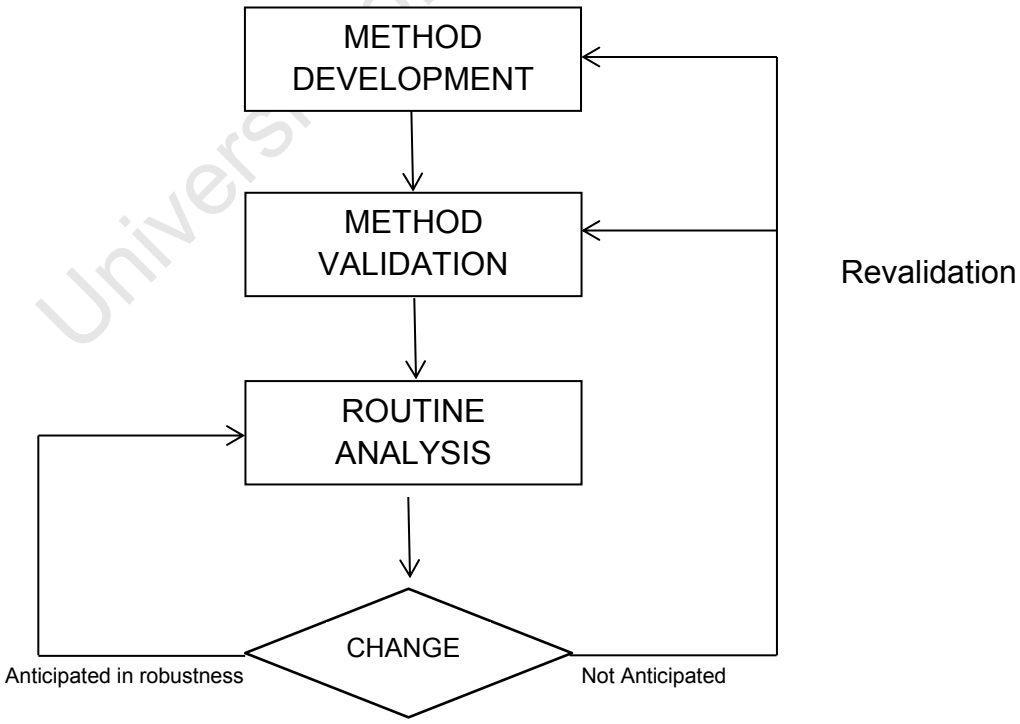

Figure 2.2 The analytical method life cycle from development phase through analytical validation to routine analysis [3]

Method validation follows the method development process. It is a documented process designed around the specific analyte defined prior to the experimental work. A validation protocol is generated which includes procedures and acceptance criteria for all characteristics. Result are documented in a validation report which also contains the final decision deeming the method validated or not.[4] The analytical test method is used in the routine environment after successful validation, but requires re-validation if any critical parameters not previously assessed change. This includes but is not limited to change in linear range, sample matrix and instrument model.

The International Conference on Harmonisation (ICH) categorises analytical procedures for validation into four sections: [5]

- Identification tests;
- Quantitative tests for impurities' content;
- Limit tests for control of impurities;
- Quantitative tests of the active moiety in samples of drug substances or drug product or other selected component(s) in the drug product.

The validation criteria are based on the classification of the analytical test methods above. Identification tests for example require only proof of specificity, whereas assays and impurity quantification tests require proof of most of the validation parameters.

2.2 Validation Parameters

2.2.1 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.[5] See Figure 2.3 for an illustration of linearity in an analytical test method.

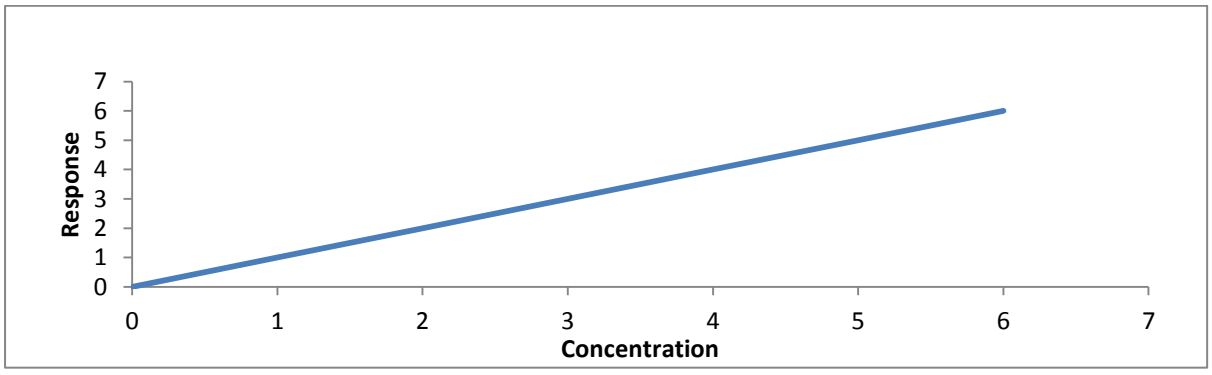

Figure 2.3 Linearity

A calibration curve is constructed using analytes of known concentrations. A minimum of 5 concentrations are required.[5] These are plotted through linear regression against the response obtained. The y-intercept of the calibration curve should approach zero. An intercept with a high value indicates a high response for the blank. It is possible to compensate for this by doing a blank subtraction, thus shifting the calibration curve parallel towards zero, see Figure 2.4. A blank containing all components of the sample matrix, besides the analyte, is analysed before constructing a calibration curve. Blank response is subtracted from the responses of the calibration standards.

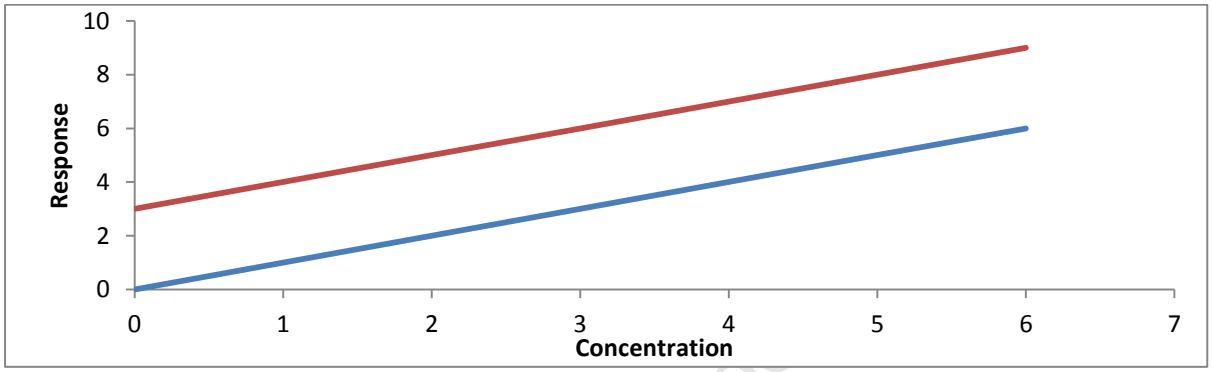

Figure 2.4 Blank subtraction

A y-intercept with a negative value indicates interference or saturation of the response.[1] A shift in the linear range where the method is used is required. See Figure 2.5.

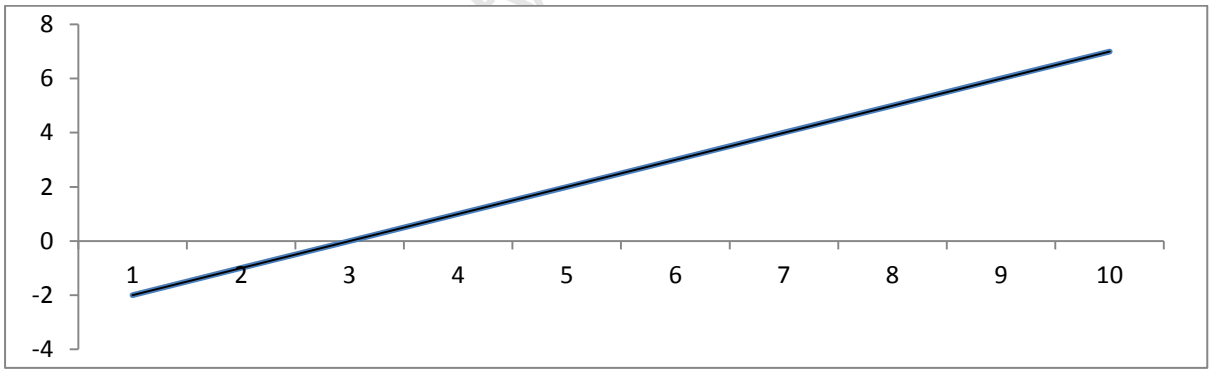

Figure 2.5 Negative y-intercept

The calibration curve cannot be used for concentrations lower than 3.0. This would result in negative responses being generated. A shift in the linear range to concentrations where negative responses are not generated is required. The United States Pharmacopeia (USP) provides guidance as to which concentration ranges should be considered, for different types of tests, when establishing linearity: [6]

- Assay of a substance: 80% to 120% of the test concentration.
- Impurity determination: 50% to 120% of the acceptance criterion. E.g. an acceptance criterion of 1.0% would require a linear response from 0.5% to 1.2%.
- Content uniformity: 70% to 130% of the test concentration
- Dissolution testing: ±20% over the specified range.

Calibration curves should be designed using standards that include the entire concentration range to be analysed. The standards should be evenly spaced across the linear range and should include a blank. A correlation coefficient (R) approaching 1.0 is acceptable as it indicates closeness or fit of the data points to the line. The R-value should have an absolute value that is statistically significant at a 95% confidence level. A R-value of more than 0.90 is statistically significant for a calibration plot consisting of 5 data points. The closer the R-value to 1.0, the lower the amount of uncertainty when using the plot to calculate unknown concentrations.[7]

2.2.2 Precision

The precision of an analytical test method is the measure of the degree of repeatability of an analytical method under normal operation. Precision is the level of agreement between individual test results, when applying the test method to multiple samplings of a homogeneous sample.[6] This is expressed as percentage relative standard deviation (% RSD) for a statistically significant number of samples. The ICH defines precision as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.[5]

It can be divided into repeatability and intermediate precision. Repeatability is a measure of how reliable the method is to give the same result for the same concentration of analyte. This is done by the same analyst on the same equipment over a short period of time. Intermediate precision is a study done between different laboratories using the samplings of the same sample. The study can also be performed in the same laboratory using different pieces of equipment and different analysts.

The ICH recommends repeatability to be either evaluated on six determinations of 100% of the test concentration or nine analyses over the range. Three replicates at the low, middle and high end of the range.[5]

2.2.3 Specificity

Specificity of the analytical test method has to be studied in order to show that there is no interference by components other than the analyte on the response. Components in the sample matrix other than the analyte should not suppress or enhance the response generated by the analytical procedure. Specificity and accuracy interact closely. Analysis of a "placebo" containing all sample components except for the analyte in question should yield no or very little response. A response generated would be a reflection of the blank of the method. Assuming that this would be the same for all analyses, analysing a blank before analysing the sample and then subtracting its response from all subsequent analyses, should remove the effect.

A placebo in most cases does not contain all components present in the sample matrix excluding the analyte. Impurities and degradants derived from the analyte would not be present. In order to take this into account a spike recovery experiment is more suitable. A typical spike recovery experiment is conducted as follows:

1. Analysis of a sample of known concentration.
2. Analysis of a known concentration of analyte (typically a reference standard)
3. "Spiking" a sample as analysed in 1 with the same concentration analysed in 2.

The response of the combined sample in 3 should be the same or close to the sum of the responses obtained for 1 and 2.

2.2.4 Accuracy

The accuracy of an analytical procedure is the closeness of test results obtained by the procedure to the true value.[6]

The accuracy can be tested by using different concentrations of reference standards. A known concentration of reference standard is analysed using the analytical procedure. The difference between what is yielded by means of the test method and the actual concentration is evaluated. Accuracy is assessed by performing analysis in triplicate over 3 concentrations in the low, middle and upper regions of the range.

The accuracy of the method can also be determined by comparison to a different validated test method for the analyte or it can be inferred after specificity, precision and linearity were determined.[5]

2.2.5 Limit of Detection

The limit of detection is the least amount of analyte that can be reliably detected, but not necessarily quantified.[6] Analyses of serial dilutions of a known concentration of analyte is performed. A distinct difference between the blank analysis and that of the sample must be obtained. This could be a colour difference in the case of titrations or the presence of a peak in a chromatogram in chromatography. A signal-to-noise ratio of 2:1 is a requirement for instrumental analyses.[6]

2.2.6 Limit of Quantitation

The limit of quantitation comprises of both Upper Limit of Quantitation (ULOQ) and Lower Limit of Quantitation (LLOQ). These are the highest and lowest concentrations at which the analyte can be quantified with accuracy and precision. See Figure 2.6 for an illustration.

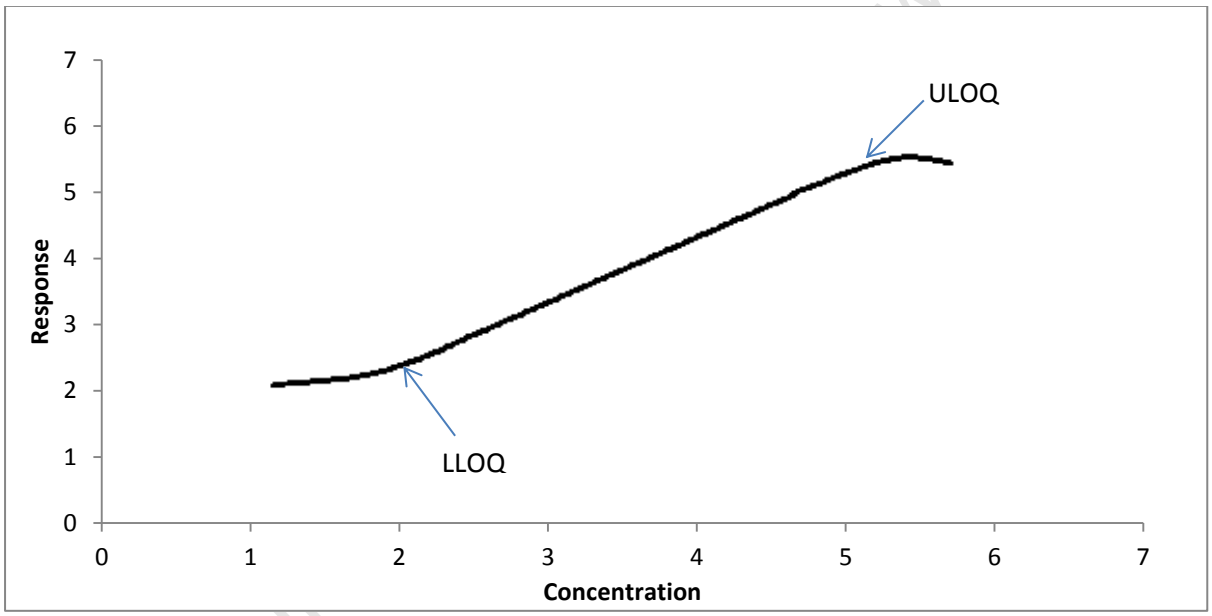

Figure 2.6 Limits of Quantitation

A signal to noise ratio of 10:1 is acceptable for the LLOQ.[6]

2.2.7 Range

The quantification range is the concentration range between the LLOQ and ULOQ that can be reliably quantified with accuracy and precision through the use of a concentration-response relationship. The validation parameters are all interrelated and in some way affect each other. Figure 2.7 illustrates the relationship between the range, LLOQ, ULOQ and linearity.

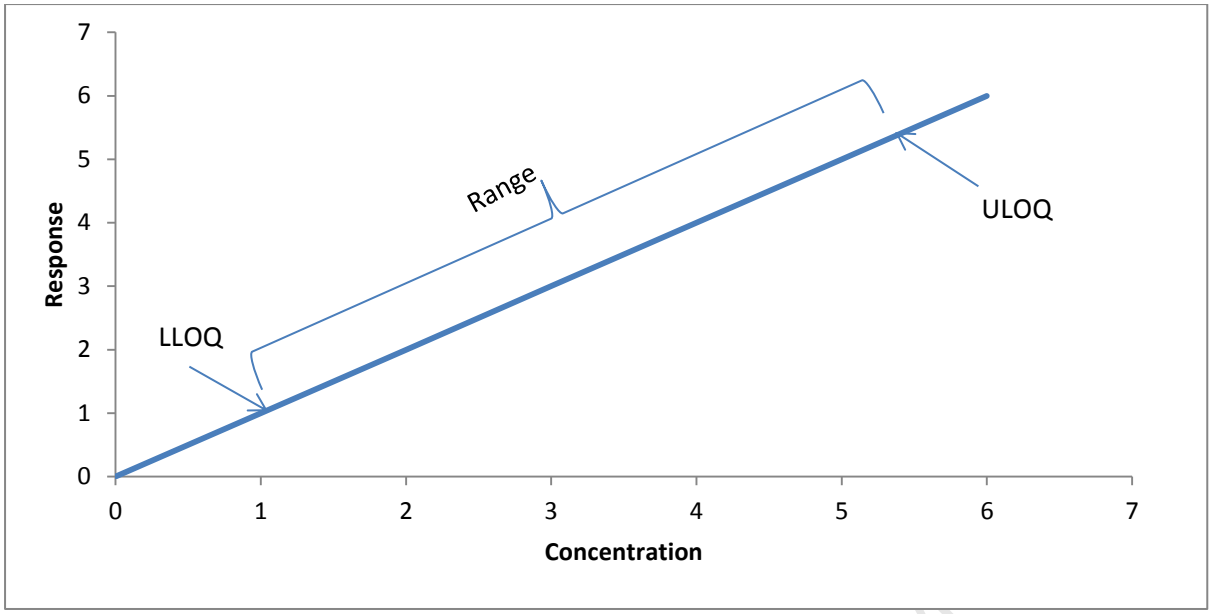

Figure 2.7 Range

2.2.8 Robustness

The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small but deliberate variations in its parameters. This is assessed during the development phase of the analytical procedure.[8] Changes made can vary e.g. incubation time and or temperature, slightly different volumes of reagents. Establishing this upfront during method development also gives an indication of which parameters are critical and cannot be changed.

2.3 Conclusion

The assessment of a method using qualified personnel, equipment and well defined protocols is an essential tool in assuring quality of test methods in a good manufacturing practice (GMP) facility. The assumption that a test method is acceptable or fit for purpose based on a linear response is invalid. Quantitative tests should at a minimum evaluate linearity, precision, specificity and accuracy. Linearity can be evaluated by serial dilutions of a known reference concentration and precision by multiple analyses of the same sample. Spike-recovery experiments evaluate sample matrix effects on the analysis of the analyte. Accuracy can be inferred after linearity, precision and specificity were confirmed. Qualitative tests do not need such a comprehensive validation assessment, but limit of detection and specificity should be evaluated. Subsequent chapters will evaluate test methods using the validation strategies applied to quantitative tests as listed above.

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Chapter 3

Development and assessment of an assay to determine residual ethanol in batches of polyribosylribitolphosphate (PRP)

Ethanol is used during the purification process of PRP. It is used to elute a salt, formed between the negatively charged PRP and a positively charged quaternary ammonium species, from a chromatographic column. Excess ethanol is removed from the purified PRP concentrate further downstream in the process by means of diafiltration. This assay determines the concentration of residual ethanol in the purified PRP concentrate.

3.1 Background

3.1.1 *Haemophilus influenzae* type b (Hib) Purification Process

After fermentation of Hib, the bacteria are heat inactivated. The capsular polysaccharide/ PRP are separated from the bacterial cells and subjected to a purification process.

The purification strategy involves the addition of the positively charged quaternary ammonium species, hexadecyltrimethylammonium bromide or cetyltrimethyl-ammonium bromide (CTAB). The positively charged CTA forms a water-insoluble salt with the negatively charged PRP. The CTA: PRP salt precipitates out of the fermentation broth and is filtered/ centrifuged out of the solution with the help of a diatomaceous earth filtration aid (Celite).

This Celite slurry/ paste is then loaded onto a chromatographic column and washed with copious amounts of aqueous solutions in order to elute excess media and water soluble impurities.

Elution of the CTA: PRP salt is performed by stepwise increasing the ethanol concentration to a final concentration of ~ 60%. Fractions containing PRP are identified by a modification of a colorimetric assay for ribose. The ethanol: product fractions are collected and combined. Addition of sodium chloride (NaCl) to the solution causes the displacement of CTA by sodium which renders an alcohol insoluble Na: PRP salt. CTA - Cl stays in the alcohol solution. The polysaccharide gets concentrated and purified further by means of diafiltration against a phosphate buffer solution. See Figure 3.1 for a process flow diagram of the purification.

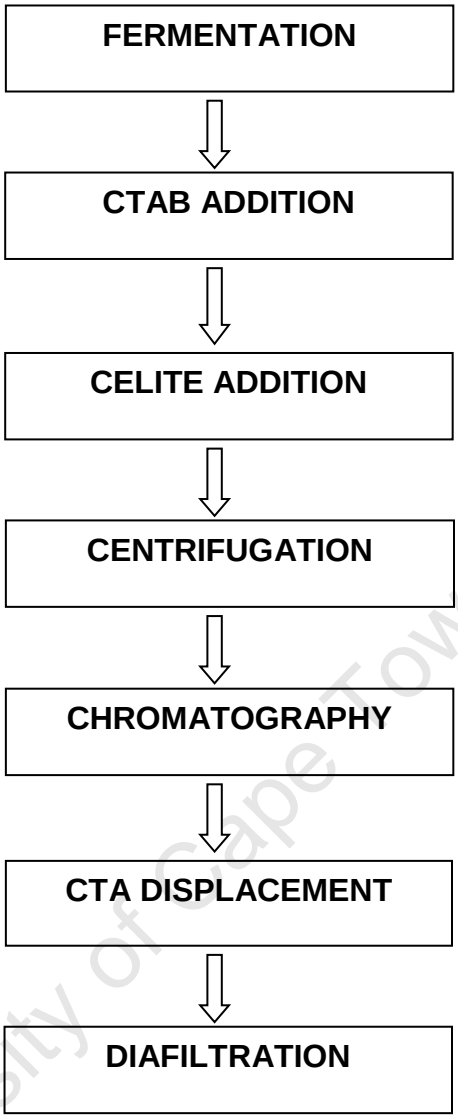

Figure 3.1 Purification process flow diagram

The aqueous purified PRP solution is frozen and stored at temperatures below 0 °C. Residual ethanol has exposure limits in pharmaceutical and biological products. This study evaluates ethanol content in bulk purified polysaccharide.

3.1.2 Analyte

Ethanol, also known as ethyl alcohol (chemical formula C_2H_6O), is a clear, colourless, flammable liquid that is miscible with water and most other organic solvents. The melting point is – 114.1 °C and boiling point 78.15 °C. Overexposure can lead to a variety of adverse reactions for example irritation of eyes, nose, skin, headache nausea, vomiting, liver damage, anaemia, reproductive and teratogenic effects.[1] The abovementioned effects necessitates the limitation of ethanol intake.

3.1.3 Residual Ethanol Specification

Ethanol is classified as a Class 3 solvent as per USP 34 <467>, Residual Solvents. These solvents are deemed as less toxic in short-term studies and negative in genotoxicity studies. Pharmaceuticals containing amounts that would lead to a daily intake of ≤ 5000 ppm or 0.5% for maximum dosage < 10 grams are acceptable without any justification.[2]

The International Conference on Harmonisation (ICH) Harmonised Tripartite Guideline recommends the use of Class 3 solvents for manufacturing of drug products. Ethanol is classified similarly to the USP with a Permitted Daily Exposure (PDE) of 50 mg or less per day.[3]

3.1.4 Method of Analysis of Ethanol

Class 3 residual solvents are generally considered as non-toxic and a non-specific method of quantification may be used.[1, 2, 3] Loss on Drying (LOD), a gravimetric analysis, is acceptable. The method involves subjecting an accurately weighed mass of sample to a specified temperature (typically in an oven) for a given time period. The mass before and after the exposure to heat is measured. The difference is expressed as a percentage of the original mass. The same method of analysis applies to samples where the drying occurs either over a moisture absorbent chemical or under a vacuum. A major disadvantage of this technique is the relatively large quantity of sample that is required. Other similar techniques include thermal analysis and thermogravimetric analysis. This method of analysis is however not applicable or viable for the purified PRP as it is in an aqueous solution.

Spectrophotometric techniques, making use of kits where ethanol reduces dichromate to a blue chromic colour, are available.[4] The sample absorbance is measured on a spectrophotometer at the absorbance maximum, 580 nm. A detection range of 0.04% to 4% is listed. A kit making use of an enzyme-catalysed kinetic reaction claims to be a more sensitive method with a detection limit of 0.0008% to 0.1%.[5]

The conventional method of analysis for residual solvents and organic volatile impurities is gas chromatography (GC). Gas chromatography with flame ionisation detection (GC-FID) is an extremely sensitive and robust technique. It has a wide linear detection range and is reliable.[6] Methods of sample introduction using GC-FID include but are not limited to static headspace, purge and trap extraction, multiple headspace and direct injection. Capillary columns are used resulting in excellent resolution. Columns can be either wide bore or narrow with different lengths, stationary phases and film thickness. The response of the

analyte in the sample is compared to that of a standard of known concentration. The concentration of the analyte in the sample matrix can be calculated provided the concentration fall within the linear detection range of the chromatographic test method.

Direct injection is the oldest GC sample introduction technique and is simple and reliable. The sample is dissolved in a high boiling point solvent which elutes much later than that of the analyte minimizing or preventing interference. Direct injection from the autosampler vial with an autosampler or even manually with a syringe is done directly into the heated injection port. Non-volatile components of the sample matrix are also injected. Column efficiency and lifespan as well as detector sensitivity can become compromised. The non-volatile components or high boiling sample matrix could result in carry over and interfere with subsequent analysis. This non-volatile component of the sample matrix could also remain in the split/splitless inlet liner of the GC. This technique also has a lower detection limit compared to the other GC sample introduction techniques.[6]

Headspace analysis has an advantage over direct injection in that only the volatile analytes in the sample matrix are injected. This sample introduction technique extends the lifetime of the columns and prevents contamination of the injector. [7]

Purge and trap (Dynamic Headspace) makes use of an extraction process whereby all solutes are extracted from the sample and trapped in an adsorbent trap. The sample is thermally desorbed from the trap and injected into the GC. Volatile organic compounds, with poor water solubility and boiling points lower than 200 °C, are concentrated. Volatile organic compounds soluble in water can be extracted this way as well, but the limit of quantification would be higher than the aforementioned water insoluble compounds. Purging efficiency is lower and higher temperatures and longer purging is required. An inert gas is bubbled through the aqueous sample matrix which leads to the liberation of the volatile organic compounds. The compounds are in turn adsorbed to the trap which is composed of multiple layers of different types of sorbent material.[8] Different traps exist for different types of compounds. Traps have a finite lifecycle and replacement is needed after time. Purge and trap techniques are not readily automated and repeated cleaning of glassware is required. It is however more sensitive than static headspace injection.[9] See Figure 3.2 for a diagram of a dynamic headspace device coupled to a gas chromatograph.

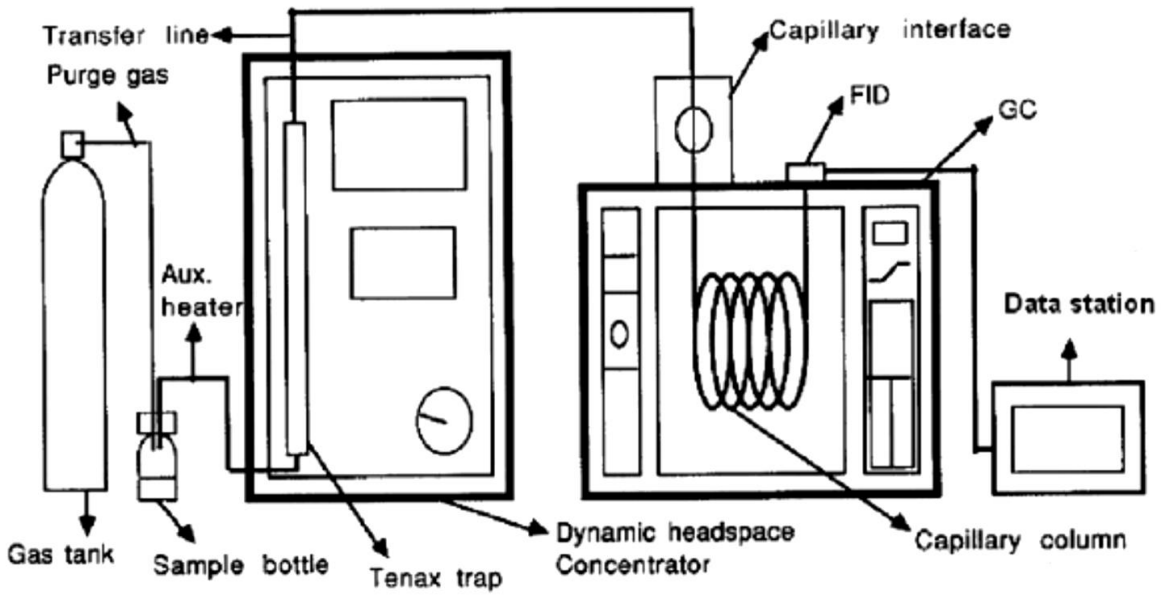

Figure 3.2 Schematic diagram of dynamic headspace device coupled to a gas chromatograph.[6]

Purge and trap or dynamic headspace analysis has lower precision compared to static headspace analysis.[6]

Static headspace has relatively simple method development and can be automated to accommodate a large number of samples. Interference caused by samples containing highly volatile analytes, which can interfere with the analysis of analytes with different volatilities, is decreased or prevented. Static headspace methods have a wide use where the sample is dissolved in a liquid matrix.[9] The technique has some drawbacks that have to be managed e.g. reproducibility, matrix effects and sensitivity for samples with low water solubility.[6] Figure 3.3 illustrates a typical static headspace sample vial.[10]

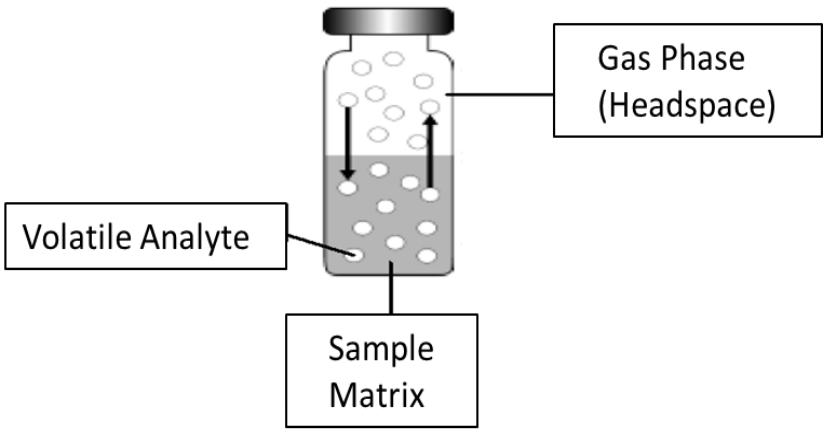

Figure 3.3 Static headspace vial [10]

This method was initially used to measure the solubility of anaesthetics and for the analysis of gases, alcohols and solvents in biological samples.[11] The vial is subjected to a constant temperature until the volatile analyte have equilibrated between the liquid and gas phases. Optimum sensitivity is achieved when the distribution coefficient has a small value. The distribution coefficient is related to the concentration of the analyte in the gas or liquid phase by the equation:

$K = \text{Concentration}_{Liquid}/\text{Concentration}_{Gas}$ ($K = C_L/C_G$).

The following equation describes the equilibrium: $C^0{}_1 X V_L = C_L V_L + C_G V_G$

$C^0{}_1$ = analyte concentration in the liquid phase before equilibrium
C_L and C_G = equilibrium concentrations
V_L and V_G = volumes in the liquid and gas phases.[11]

This can be affected by means of adjusting the pH, "salting out" or increasing the thermostating temperature.[9] Increasing the temperature of the vial would result in a decrease in solubility of the solvent in solution leading to a higher concentration of it in the gaseous phase.[11] The headspace of the vial would then contain the analyte in a gaseous phase which is relatively clean and readily available for injection via a gastight syringe or autosampler.

Static headspace with GC-FID will be used to determine the ethanol content in PRP. The analyte is easily dissolved in water and use of an internal standard will improve reproducibility. Multiple samples can be incubated simultaneously allowing for a quicker process time.

3.2 Experimental

3.2.1 Test Samples

- Purified PRP batch # P67, The Biovac Institute.
- Ethanol, Merck, CAS number 64-17-5
- Iso-propanol, Merck, CAS number 67-63-0

3.2.2 Reagents

- Sodium sulfate, Merck, CAS number 7757-82-6

3.2.3 Equipment

- Agilent 7890 Gas chromatograph
- CTC PAL autosampler
- Reacti-vap/ Reacti-therm sample evaporation unit, Thermo Scientific
- Eppendorf pipettes, 200 µl, 1000 µl
- Sartorius CP225 D 5 – place balance

3.2.4 Column Selection

Column selection was based on polarity of the analyte, lifetime of the column as well as analysis time. The J&W Scientific DB 624 column with length 30 m, diameter 0.53 mm and film thickness 3.0 µm was used. The column is mid polar and has a temperature limit of 250 °C with a longer lifetime compared to more polar columns. It has a polyethylene glycol stationary phase and the diameter can accommodate the high carrier gas flows typically used in headspace analysis. The stationary phase film is thick to minimize interaction of the column wall with the analyte.

3.2.5 Method Parameters

<u>Headspace unit</u>

- Incubation temperature: 80 °C
- Incubation Time: 300 seconds
- Syringe temperature: 50 °C
- Injection volume: 2000 µl

<u>Gas chromatograph</u>

Front Inlet:

- Heater : 250 °C
- Mode: Split
- Split ratio: The split ratio for the higher concentration evaluation was 35:1 and that of the lower 10:1
- Total flow: 39 ml/minute
- Septum purge flow: 3 ml/ minute

Flow parameters

- Flow rate: 1 ml/minute
- Carrier gas: nitrogen

Oven

- Equilibration time: 3 minutes
- Oven Program:
  50 °C hold for 5 minutes
  10 °C/ minute to 200 °C hold for 5 minutes
  Run Time 25 minutes

Detector unit

- Hydrogen: 200 ml/minute
- Nitrogen :300 ml/minute
- Air : 400 ml/minute

The oven temperature program and column flow was determined by using a resolution solution of ethanol and the internal standard, iso-propanol. The parameters yielding optimum peak separation, shape and retention were selected.

3.2.6 Sample Preparation

A volume of 1.0 ml of the sample to be analysed was added to a headspace vial containing 2.0 g of anhydrous sodium sulfate and 1.0 ml of the internal standard solution. Additional 3.0 ml purified water was added before sealing.

3.2.7 Internal Standard Selection

Iso-propanol was chosen as the internal standard. It has a boiling point of 82.5 °C and is miscible with water and alcohol. The response of iso-propanol is comparable to that of ethanol at similar concentrations. Resolution between the ethanol and iso-propanol peaks in the chromatogram is more than the USP calculated resolution factor of 2.0. See Figure 3.4 for the internal standard chromatogram.

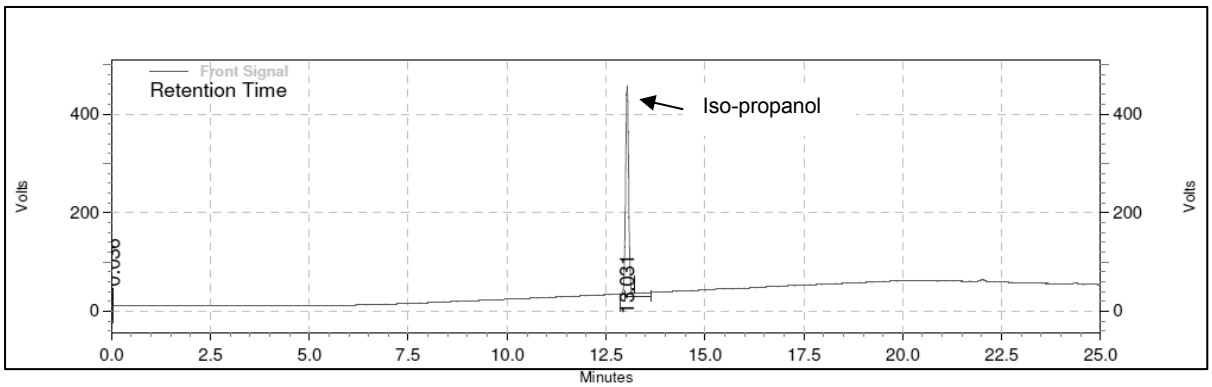

Figure 3.4 Chromatogram of iso-propanol

The retention time of iso-propanol relative to that of ethanol is 1.07. See Figure 3.5 for a chromatogram of ethanol and iso-propanol.

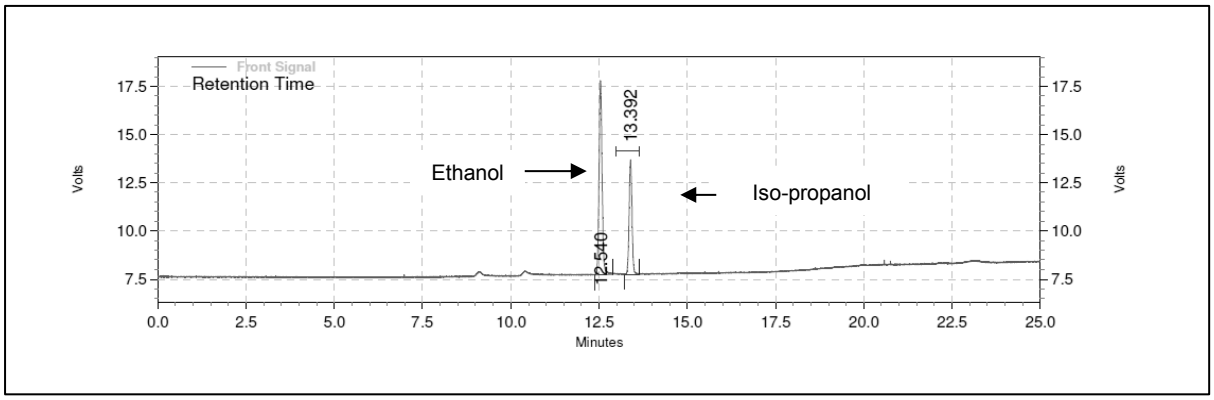

Figure 3.5 Chromatogram of iso-propanol, ethanol resolution solution

3.2.8 Sample Incubation Temperature

In order to determine the temperature at which the maximum amount of analyte is available in the gaseous phase of the headspace of the vial, incubation temperature was investigated. A sample solution containing 10 ppm of ethanol and 10 ppm of iso-propanol was prepared. 5.0 ml of the solution as well as 1.0 g of anhydrous sodium sulfate were placed in headspace vials and analysed using different incubation temperatures. Incubation time was set at 5 minutes. See Figure 3.6 for a plot of the effect of incubation temperature on response.

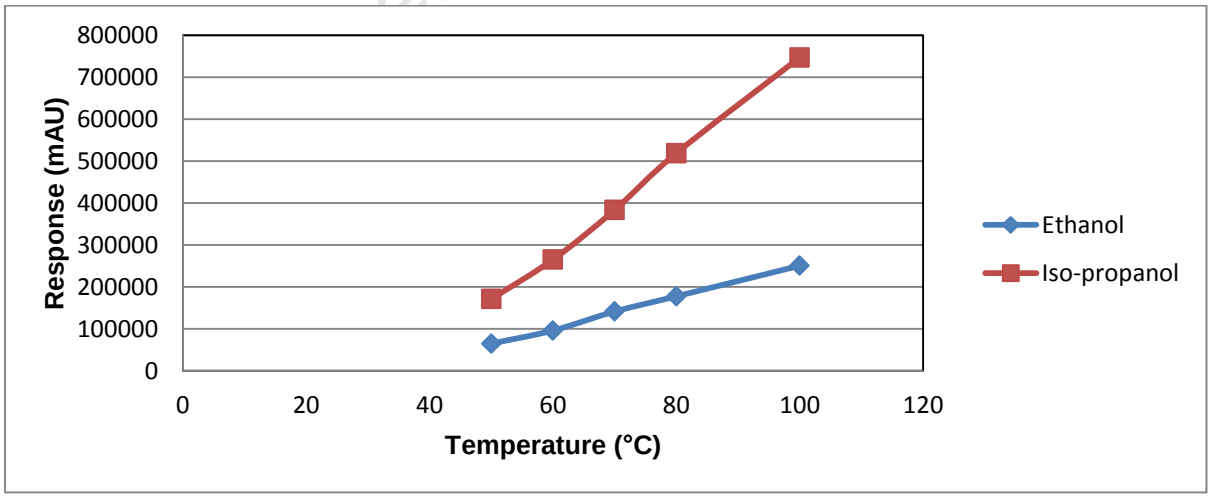

Figure 3.6 Incubation temperature vs. response

An increase in response is directly proportional to an increase in incubation temperature. This is due to an increase in the concentration of the volatile components in the headspace

of the vial as a result of the increased temperature. A temperature below the boiling point of water, but well above that of the volatile components was selected. This prolongs the column life by limiting the amount of water vapour and water soluble impurities being introduced.

3.2.9 Sample Incubation Time

The effect of incubation time at a constant temperature was investigated. The minimum amount of time needed to incubate a sample providing maximum availability can be determined. A sample solution containing 10 ppm of ethanol and 10 ppm of iso-propanol was prepared. 5.0 ml of the solution as well as 1.0 g of sodium sulfate anhydrous were placed in headspace vials and analysed using different incubation times. Incubation temperature was set at 70 °C. See Figure 3.7 for a plot of the effect of incubation time on response.

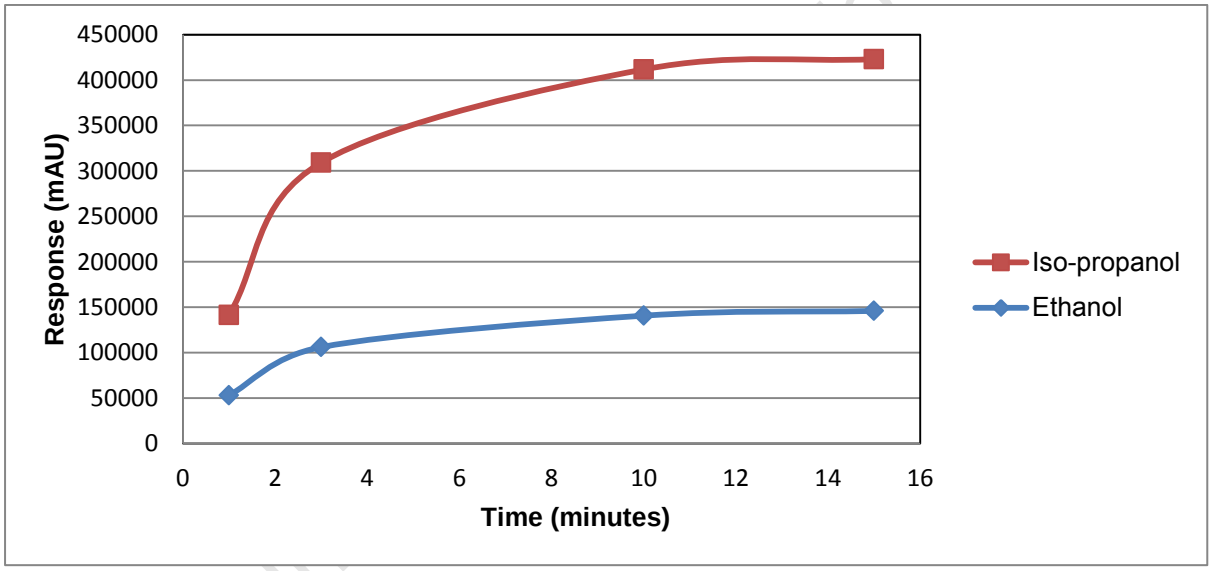

Figure 3.7 Incubation time vs. response

The ethanol and iso-propanol response increased with an increase in time, but started to plateau at about 5 minutes. This can be attributed to equilibrium being reached between the sample and the headspace of the vial. There was no significant change in response when increasing the incubation time for ethanol. Optimum incubation time was 5 minutes as maximum response for a relatively little amount of time was achieved.

3.2.10 Salt Addition

Salt concentration in the headspace vial was varied. This was done using both sodium chloride and sodium sulfate. The effect of salt concentration on both ethanol and iso-propanol response was investigated. A sample solution containing 10 ppm of ethanol and 10 ppm of iso-propanol was prepared. In separate vials to 5.0 ml of the solution different masses of sodium sulfate and sodium chloride were added. Incubation time was set at 5 minutes and incubation temperature at 70 °C. See Figure 3.8 for a plot of the effect of salt concentration and type on response.

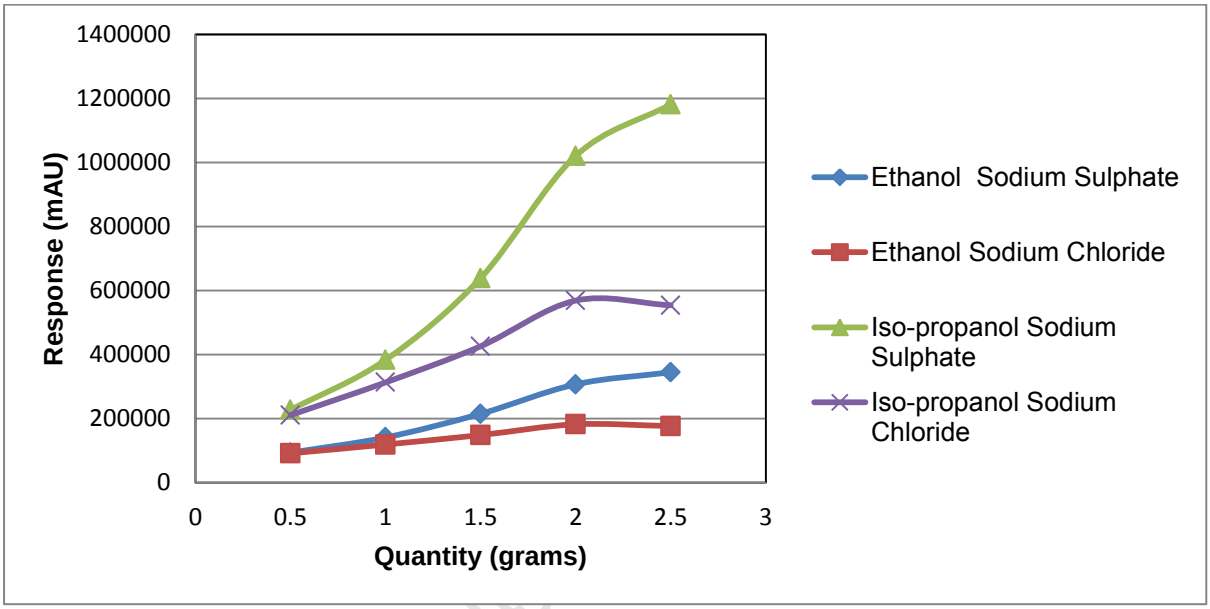

Figure 3.8 Salt concentration vs response

An increase in salt concentration elicits an increase in sample response. Sodium sulfate addition results in a higher response when compared to that of sodium chloride at a similar concentration. Sodium sulfate has a greater “salting out” effect on the two alcohols compared to sodium chloride. There is a strong intermolecular interaction between water and the salt. This interaction is affected by ionic radius and thus electrostatic field.[12] The greater effect of the sulfate ion is consistent with the predictions of the Hofmeister series.[13]

3.3 Results and Discussion

In order to assess the suitability of the method to determine ethanol content in bulk purified PRP, validation parameters linearity and range, specificity, precision and accuracy were evaluated.

3.3.1 Linearity and Range

The linearity was assessed by making up solutions of ethanol in purified water of different concentrations ranging from 50 ppm to 1000 ppm with an internal standard concentration of 500 ppm. The range was determined from the linear portion of the graph.

Table 3.1: Results obtained for the linearity and range study 50 ppm to 5000 ppm

Ethanol concentration (ppm)	Ethanol (mAU)	Internal Standard (mAU)	Internal Standard Corrected Response
50	397634	2937361	0.135371
100	719991	2856853	0.252022
500	3380004	2772061	1.219311
1000	7050994	2667975	2.642826
2000	15064630	2855055	5.276476
5000	36086085	2887189	12.49869

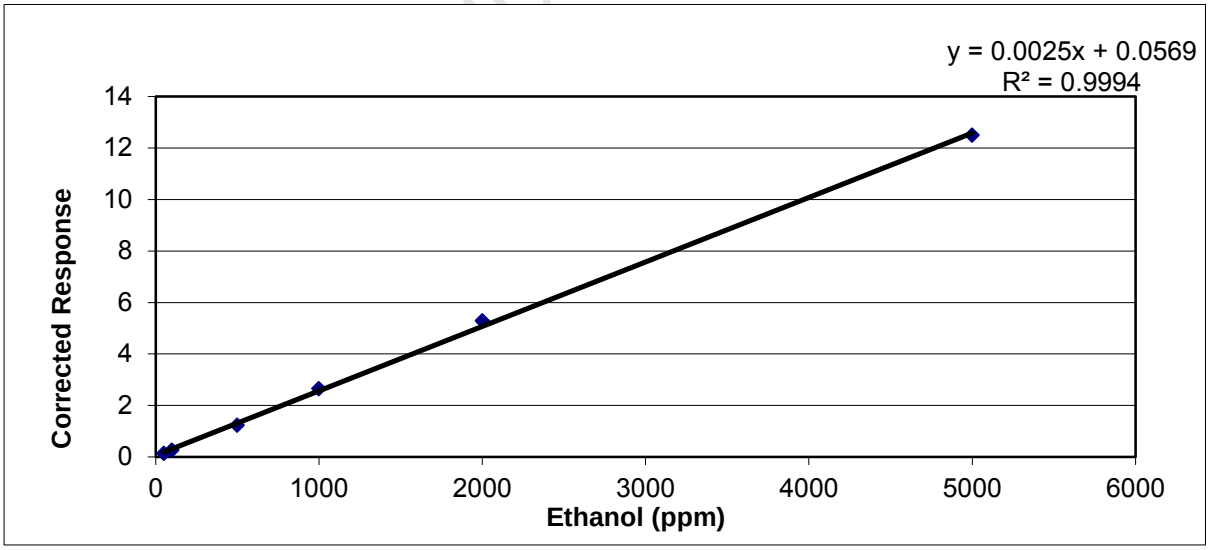

Figure 3.9 Ethanol response vs. concentration

The range was found to be linear throughout with a correlation coefficient of 0.9994 from a concentration of 50 ppm to 5000 ppm.

In order to accommodate for results in the lower concentration ranges, linearity was re-assessed between 0.25 ppm and 10 ppm with an internal standard concentration of 15 ppm.

Table 3.2: Results obtained for the linearity and range study 0.25 ppm to 10 ppm

Ethanol concentration (ppm)	Ethanol (mAU)	Internal Standard (mAU)	Internal Standard Corrected Response
0.25	33139	1814861	0.0183
0.5	60979	1935891	0.0315
1	107605	1786153	0.0602
2.5	254838	1724642	0.1478
5	510530	1725103	0.2959
10	986460	1626684	0.6064

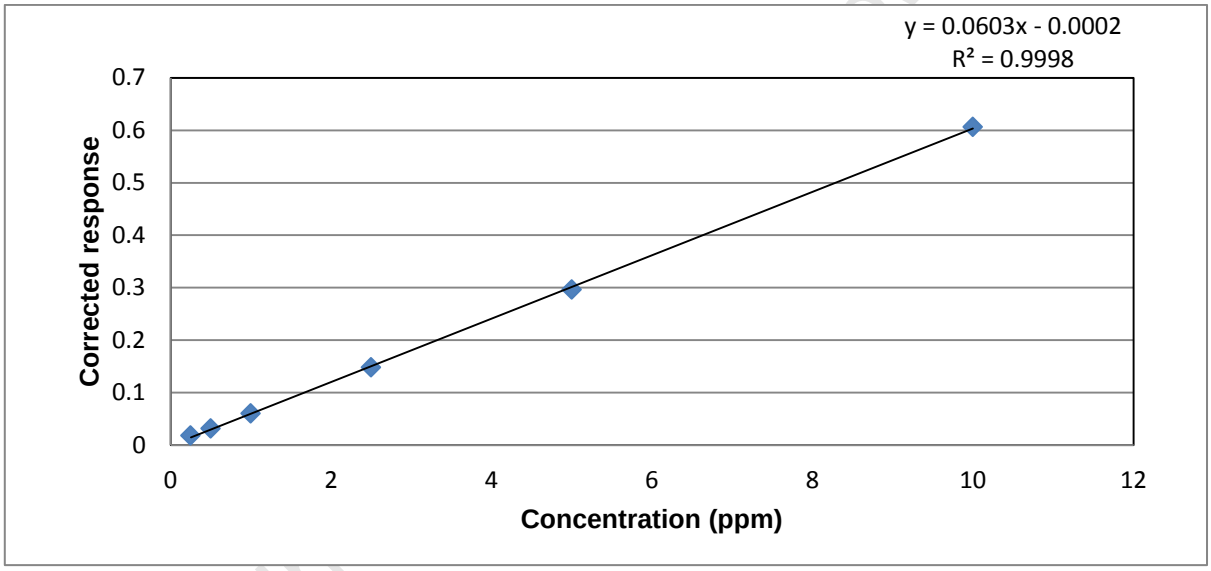

Figure 3.10 Ethanol response vs. concentration

The range was found to be linear with a correlation coefficient of 0.9998 from a concentration of 0.25 ppm to 10 ppm. The correlation coefficients of both data sets are close to 1 indicating a good fit of the data points to the regression line. The y-intercepts for both concentration ranges approaches zero. The effect on results generated would have an insignificant impact.

3.3.2 Specificity

Specificity was evaluated by testing a specific volume of polysaccharide solution spiked with a standard of a known concentration of ethanol (250 ppm). The same volumes of polysaccharide and standard were tested independently and the ethanol value of the spiked polysaccharide compared to the sum of the individual polysaccharide and standard solutions. The internal standard concentration was 500 ppm.

Table 3.3: Results obtained for specificity study

Sample	**Ethanol (mAU)**	**Internal Standard (mAU)**	**Corrected**	**Calculated Concentration (ppm)**	**% Recovery**
PRP + Std	1671704	2619021	0.64	233.24	**96.57**
PRP + H_2O	0	2971596	0	0	
Std + H_2O	1856976	2998373	0.62	225.24	

The recovery was calculated using the equation below:

% Recovery = [(PRP + H_2O) + (Std + H_2O)]/ (PRP + Std) X 100

The results indicate that the other components present in the polysaccharide do not interfere with the ethanol assay. All sample matrix effects can be eliminated based on this. There is no reference made by the ICH Guideline Q2 (R1), Validation of Analytical Procedures: Text and Methodology as to what the acceptance criteria for specificity should be. The acceptance criterion for accuracy at is 90 to 110% of the actual amount and will be applied in this study.

3.3.3 Precision

Precision was assessed by analysing an ethanol concentration six times. The % RSD was calculated and reported. Standard solutions with concentrations within the two different linear ranges were prepared and analysed. Concentrations of 100 ppm and 5.0 ppm were assessed with internal standard concentrations of 500 ppm and 15 ppm respectively.

Table 3.4: Results obtained for the precision study using a 100 ppm solution

Concentration (ppm)	Ethanol (mAU)	Internal Standard (mAU)	Corrected Response
100	716526	2845385	0.25
100	762032	2919793	0.26
100	727647	2736371	0.27
100	749210	2950442	0.25
100	730142	2884549	0.25
100	727561	2714912	0.27
Average	735519.7	2841908.7	0.26
Standard Deviation	16765.0	96893.8	0.010
% RSD	2.3	3.4	3.846 %

Table 3.5: Results obtained for the precision study using a 5.0 ppm solution

Concentration (ppm)	Ethanol (mAU)	Internal Standard (mAU)	Corrected Response
5.0	543135	1864098	0.29
5.0	523973	1761900	0.30
5.0	478214	1591590	0.30
5.0	506628	1741606	0.29
5.0	517491	1727667	0.30
5.0	493740	1663755	0.30
Average	510530.2	1725103	0.30
Standard Deviation	22950.86	92240.83	0.005
% RSD	4.495495	5.346976	1.667

Repeated analysis of the same sample yielded a low % RSD for both data sets. The 100 ppm solution had a % RSD of 3.85 % and the 5.0 ppm solution 1.67 %. The variances that can be expected when using this test method in these concentration ranges are acceptable.

3.3.4 Accuracy

Accuracy was assessed by the triplicate analyses of 3 different concentrations from the lower, middle and upper region of the linear range. The average, standard deviation and % RSD of each sample set was determined.

50 ppm, 500 ppm and 1000 ppm ethanol in purified water were analysed. The result of the 500 ppm solution was treated as the "actual" concentration. The internal standard concentration was kept constant at 500 ppm.

Table 3.6: Results obtained for accuracy study for the 50 ppm to 5000 ppm linear range

Concentration (ppm)	**Ethanol (mAU's)**	**Internal Standard (mAU's)**	**Corrected Response**
50	362566	2834692	0.127903
	380684	2924594	0.130166
	374837	2891780	0.129622
Average	372695.7	2883689	0.12923
Standard deviation	9246.862	45493.9	0.001181
% RSD	2.481076	1.577629	0.914081
500	3827307	3048885	1.255314
	3758255	3029023	1.240748
	2789331	2270133	1.228708
Average	3458298	2782680	1.24159
Standard deviation	580370	443990.1	0.013323
% RSD	16.78196	15.95548	1.073035
1000	7325346	2915809	2.512286
	6584379	2663942	2.471668
	6395010	2583855	2.474988
Average	6768245	2721202	2.486314
Standard deviation	491666.9	173226.4	0.022554
% RSD	7.264318	6.365806	0.907115

Accuracy was re-assessed in a lower concentration range as in the case of linearity before using concentrations 1.0 ppm, 2.5 ppm and 5.0 ppm. The internal standard concentration was kept constant at 15 ppm.

Table 3.7: Results obtained for accuracy study for the 0.25 ppm to 10 ppm linear range

Concentration (ppm)	**Ethanol (mAU's)**	**Internal Standard (mAU's)**	**Corrected Response**
1.0	105474	1802760	0.058507
	109237	1759730	0.062076
	108103	1795969	0.060192
Average	107604.7	1786153	0.060258
Standard deviation	1930.361	23133.54	0.001785
% RSD	1.793938	1.29516	2.962986
2.5	249561	1715461	0.145478
	260934	1763487	0.147965
	254020	1694979	0.149866
Average	254838.3	1724642	0.147769
Standard deviation	5730.492	35164.74	0.002201
% RSD	2.248677	2.038959	1.48937
5.0	506628	1741606	0.290897
	517491	1727667	0.299532
	493740	1663755	0.296762
Average	505953	1711009	0.29573
Standard deviation	11889.87885	41512.68	0.004409
% RSD	2.349996709	2.42621	1.490843

The recovery was calculated using the equation: % Recovery = (Actual/ Theoretical) X 100% The response obtained for the 500 ppm concentration was used to calculate the 50 ppm and 1000 ppm recovery.

50 ppm = [0.12923/ (1.24159/10)]*100 = 104.1 %

1000 ppm = [2.486314/(1.24159*2)]*100 = 100.1 %

The response obtained for the 2.5 ppm concentration was used to calculate the 1.0 ppm and 5.0 ppm recovery.

1.0 ppm = [0.060258/ (0.147769/2.5)]*100 = 101.9%

5.0 ppm = [0.29573/ (0.147769*2)]*100 = 100.1%

The amount of variance, of the triplicate analyses of the different solutions, was relatively low. The data obtained for the accuracy study at the higher concentration, yielded the lowest % RSD for the 50 ppm and 1000 ppm solutions with values of 0.91%. The triplicate analysis of the 500 ppm solution yielded a %RSD of 1.07%. Recoveries for the 50 ppm and 1000 ppm solutions, when using the response of the 500 ppm solution as the reference, were calculated as 104.1% and 100.1%.

The data obtained for the analysis of the lower concentrations had the lowest %RSD at the 2.5 ppm and 5.0 ppm concentrations with values of 1.49 % for both. The highest %RSD of 2.96 % was obtained for the analysis of the 1.0 ppm solution. Recoveries for the 1.0 ppm and 5.0 ppm solutions, when using the response of the 2.5 ppm solution as the reference, were calculated as 101.9% and 100.1%.

The recoveries for both the higher and lower concentrations were within acceptable limits. The data generated also confirms the precision of the test method as triplicate analysis over the lower, middle and upper region of the linear range were performed.

3.4 Conclusion

The determination of the ethanol content in bulk purified PRP using GC-FID analysis was evaluated. Ethanol is classified as a Class 3 solvent and there are limitations on daily intake.[2,3] The samples were analysed using GC-FID, static headspace with iso-propanol as an internal standard. Sodium sulfate was used to increase the availability of analyte in the gaseous phase. The incubation temperature and time used for the headspace oven were 80 °C and 5 minutes respectively. Chromatography was performed using a mid polar capillary column with polyethylene glycol as the stationary phase. Nitrogen was used as the carrier gas and the oven temperature was governed by a temperature program. In order to determine the suitability of the method, the validation parameters linearity and range, specificity, precision and accuracy were evaluated. Robustness was assessed by evaluating incubation time and temperature as well as salt concentration.

The linearity of the 50 ppm to 5000 ppm range had a R^2 value of 0.9994 and the 0.25 ppm to 10 ppm range had a R^2 value of 0.9998. The recovery for the specificity study was determined as 96.88%, the precision study of the 100 ppm solution yielded a %RSD of 3.85% and that of the 5.0 ppm study 1.67%. The recovery of the 50 ppm solution was determined as 104.1%, the 1000 ppm as 100.1%, the 1.0 ppm 101.9% and the 5.0 ppm solution as 100.1%. The developed method meets all validation acceptance criteria for both the 50 ppm to 5000 ppm and 0.25 ppm to 10 ppm concentration ranges. The retention times of both the ethanol and iso-propanol peaks in the chromatographs were less than 15 minutes. The peaks were well separated in the chromatographs and peak shapes were good. Baseline separation with a resolution between the peaks of more than 2.0 as well as a tailing factor of less than 2.0 was met. Sample preparation was minimal and total analysis time was relatively short. The method was shown to be robust and can be implemented in a laboratory for routine analysis of ethanol in purified PRP.

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Chapter 4

Development and assessment of an assay to determine residual ethylene glycol in oxidized polyribosylribitolphosphate (PRP)

The PRP polysaccharide is treated with sodium periodate to prepare the activated PRP intermediate which is subsequently conjugated to the protein carrier to form the Hib conjugate vaccine. The controlled periodate oxidation serves to both depolymerise the PRP polysaccharide to a size that is required for conjugation, as well as generate terminal reactive aldehyde groups required for the conjugation reaction. The sodium periodate oxidation reaction is quenched by the addition of excess ethylene glycol and the activated PRP recovered after diafiltration. This assay determines the concentration of residual ethylene glycol in the activated PRP.

4.1 Background

4.1.1 PRP Oxidation/ Modification Process

After purification of the polysaccharide of *Haemophilus influenzae* type b (Hib), the long chain length polysaccharide needs to be size reduced and activated prior to conjugation with a protein. Sodium periodate is used to cleave the polysaccharide to form shorter oligosaccharides having reactive aldehyde groups. The reaction takes place under controlled temperature for a set time. The oligosaccharide terminates in reactive aldehyde functional groups at both terminals of the chain (Figure 4.1).

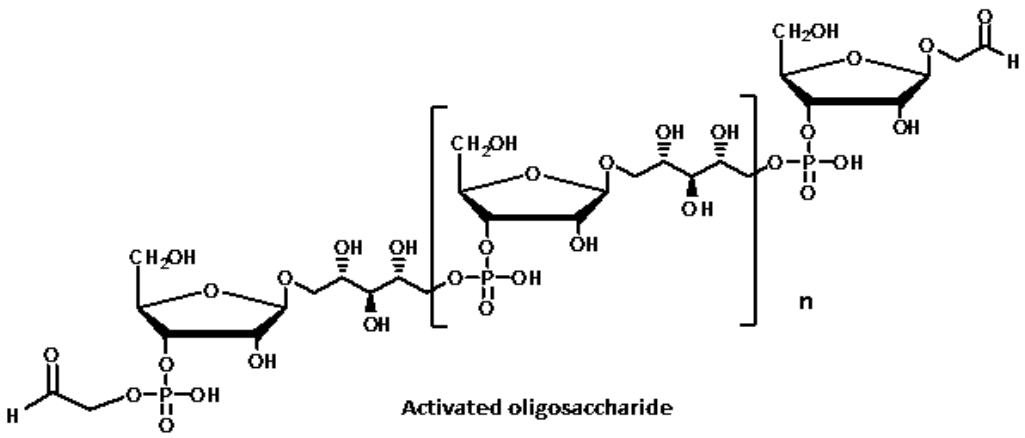

Figure 4.1 Structure of activated PRP

Formation of the oligosaccharide is monitored by size exclusion chromatography (SEC-HPLC) using refractive index (RID) as the means of detection. Molecular weight markers are used to calculate a distribution coefficient of the analyte.[1] The size distribution of the molecule is determined by means of retention time. Chromatograms of the molecular weight markers, PRP and activated PRP are shown in Figures 4.2, 4.3 and 4.4.

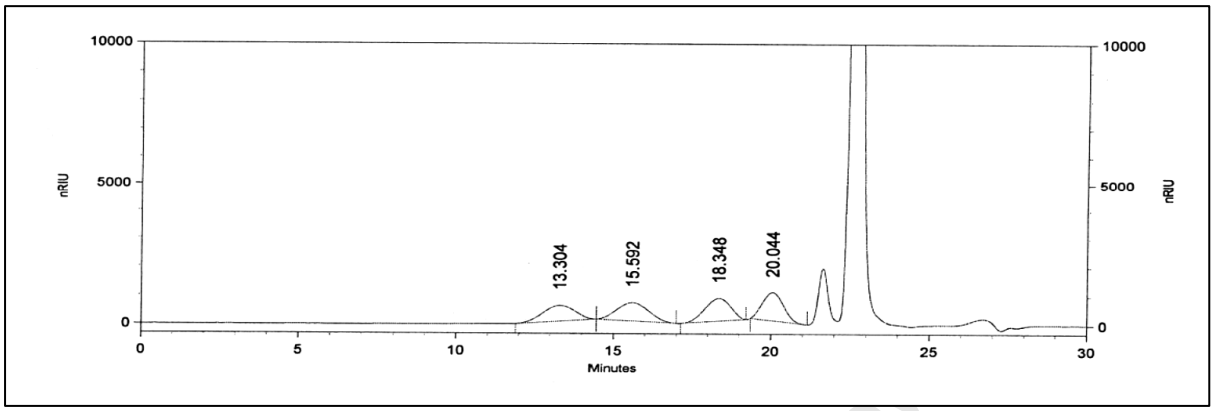

Figure 4.2 Chromatogram of molecular weight markers

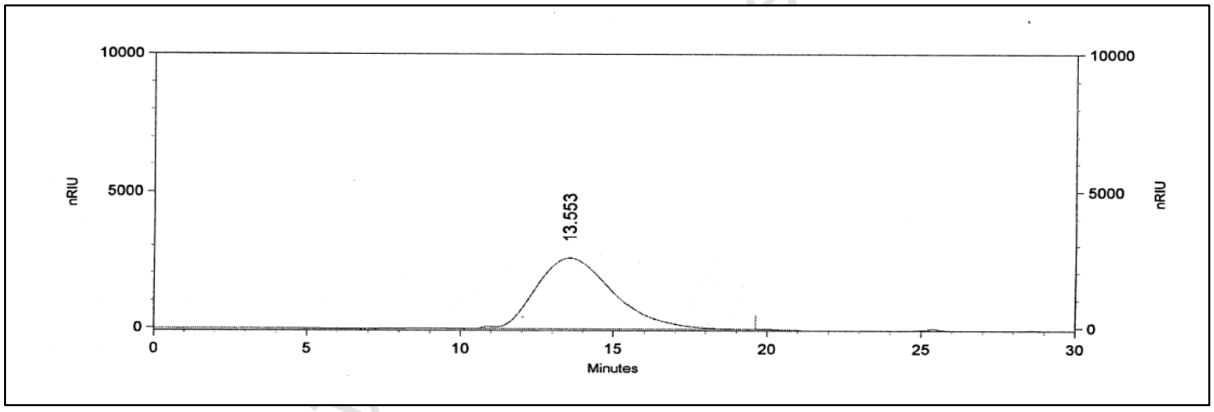

Figure 4.3 Chromatogram of PRP

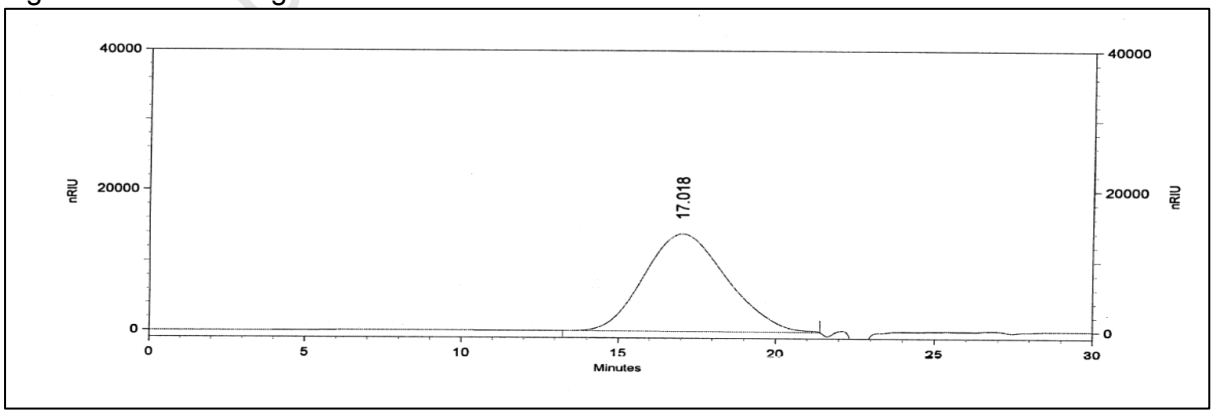

Figure 4.4 Chromatogram of activated PRP

Size exclusion chromatography is a chromatographic technique that can be applied to analytes with a high molecular weight. Analytes are separated in a sample matrix based on size. Size exclusion columns are packed with particles with a specific pore size. The packing forms a mesh through which the analyte must migrate or travel. Lower molecular weight molecules travel a longer path as they diffuse through both the spaces between the individual silica beads and the pores within the silica beads. They are subsequently retained longer. Molecules with a larger molecular weight penetrate the pores of the silica to a lesser extent and elute relatively early These molecules are of a size larger than that which the pores in the beads permits to enter, are excluded and travels only between the silica beads with no or little retention. There is an indirect correlation between elution time and molecular weight. The smaller the molecule the later it elutes whereas the larger the molecule the earlier it elutes.[2] The long chain purified PRP has a large hydrodynamic size and elutes at ~ 13.6 minutes whereas the shorter chain length activated PRP elutes at ~ 17 minutes.

The addition of ethylene glycol consumes or quenches the excess sodium periodate in the reaction mixture stopping the oxidation. Tangential flow diafiltration is used to remove excess reagents like sodium periodate and ethylene glycol as well as short chain oligosaccharides that are not included in the fraction that is conjugated. This filtration technique has the fluid pumped tangentially to the filtration membrane. Additional pressure is applied perpendicularly to the membrane. The result is a filtration process whereby the molecules too large to pass through the membrane are being swept along with the flow of the fluid. Blockage of the filter is prevented or delayed with this approach as opposed to "normal" filtration processes. A reservoir with a buffer solution replaces the filtrate substituting undesired components and salts with the buffer solution. After several passes, the amount of buffer used to replace the filtrate is reduced and this serves as a mechanism to concentrate the activated PRP. This chapter evaluates residual ethylene glycol content in the activated PRP intermediate.

4.1.2 Analyte: Ethylene Glycol

Ethylene glycol, also known as 1, 2-ethanediol (chemical formula $C_2H_6O_2$), is a clear, viscous liquid with a sweet taste that is very hygroscopic and able to absorb twice its weight in water at 100% humidity. See Figure 4.1 for the molecular structure. It is miscible with water and most other organic solvents. The melting point is – 13 °C and bp $_{760}$ of 197.6 °C.[3]

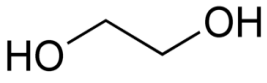

Figure 4.5 Molecular structure of ethylene glycol

Ethylene glycol is commonly used in anti-freeze solutions in the cooling systems of motor vehicles. Due to its misleading sweet taste it is often ingested. The initial effects are those of alcohol consumption thus accidental fatality due to overexposure by ingestion is common. Ethylene glycol poisoning can be fatal with the initial effect being suppression of the central nervous system followed by nausea and vomiting.[3]

Depending on the exposure as well as time before treatment, victims can lapse into a coma with renal and cardiopulmonary failure within 24 to 72 hours. The formation of glycolic, glyoxalic and oxalic acid by ethylene glycol metabolism results in metabolic acidosis.[4] Treatments include pumping the stomach after ingestion, activated carbon treatment, sodium bicarbonate intravenously to reverse acidosis and haemodialysis to remove ethylene glycol metabolites from the bloodstream.[5]

4.1.3 Residual Ethylene Glycol Specification

Ethylene glycol is classified as a Class 2 solvent in the United States Pharmacopeia (USP) 34 <467> Residual Solvents. These are solvents that need to be limited in use due to their inherent toxicity. It has a daily permitted intake limit of less than 620 ppm or 6.2 mg/day.[6]

The International Conference on Harmonisation (ICH) Harmonised Tripartite Guideline classifies ethylene glycol similarly to the USP with a Permitted Daily Exposure (PDE) of 6.2 mg per day.[7] The above limits are applicable to pharmaceutical products where the daily intake of the product does not exceed 10 grams.

There is no specification available for the presence of ethylene glycol in Hib vaccines. The World Health Organization (WHO) Technical Report Series (TRS) 897 Annex 1, Recommendations for the production and control of *Haemophilus influenzae* type b conjugate vaccines, states that residual active functional groups should be shown to be removed after final processing by means of validated test methods. Residual reagents and reaction by-products should be removed during the conjugate purification process.[1] There is however no mention of specific process residuals such as those used for the oxidation or PRP purification steps. Manufacturers differ in the processes used to produce a Hib vaccine. This includes a variety of PRP chain lengths, protein carriers, conjugation chemistry and purification strategies. It is thus difficult to set specifications that are vaccine and process specific and the WHO TRS 897 contains recommendations that are common to all manufacturers' irrespective of manufacturing process, coupling chemistry or protein carrier used.

4.1.4 Methods of Analysis of Ethylene Glycol

Class 2 solvents are solvents to be limited in use during the production of pharmaceutical products. Ethylene glycol is non-volatile and readily miscible with water rendering residual solvent determination by headspace analysis not a viable analytical technique. The high boiling point of 197.6 °C as well as its relatively high viscosity also limits direct injection onto a GC column.

High Performance Liquid Chromatography (HPLC) coupled with a refractive index detector can be used for the quantitative analysis of ethylene glycol. The direction and wave velocity of light changes when passing from one solution into another. Snell's law of refraction expresses the relationship between the incident angle and the angle of refraction. Wavelength of the incident light as well as the density of the medium affects the refractive index. Factors such as composition, temperature and pressure in turn affect the density of a solution or medium. Refractive index detection in modern refractive index detectors is more stable as temperature control for both the reference cell and sample flow cell are very accurate. The reference cell takes a "reference" of the mobile phase after equilibration. This is achieved by "opening" the reference cell to the flow of mobile phase and then closing after stabilisation. The refractive index of the contents of the flow cell is compared to that of the mobile phase "reference". Any changes in the composition of the contents of the flow cell would be recorded. The reference cell thus acts as a system blank and for this reason chromatography must be isocratic. Reversed phase chromatography using an octadecylsilyl column with water as the mobile phase and a refractive index detector has reported detection limits for ethylene glycol of 4 mg/l.[8, 9] Environmental temperature fluctuations have a negative influence on the baseline. Separation is a function of the column as gradient elution is not an option.

HPLC using ultraviolet (UV) detection can be used by derivatising ethylene glycol using benzoyl chloride. The reaction takes place in an alkaline solution and is terminated by the addition of glycine. Detection limits has been shown to be 10 mg/l with a linear range of 20 to 2000 mg/l. Cost of solvents used as well as sample preparation time are limitations to the method.[10] The reaction of ethylene glycol with benzoyl chloride with the subsequent extraction of benzoyl esters into a nonpolar solvent yields responses with low detection limits using liquid chromatography with either UV detection or electrospray mass spectroscopy (ESI-MS). A limit of detection (LOD), for UV detection at 237 nm, of 1 mg/l and limit of quantitation (LOQ) of 2 mg/l is listed. The LOD for ESI-MS is listed as 10 – 25 µg/l with a LOQ of 20 – 50 µg/l.[11]

The oxidation-reduction reactions of ethylene glycol with sodium periodate yields formaldehyde and iodate. The colorimetric determination of formaldehyde using acetyl acetone is well known and is described in the European Pharmacopeia as a limit test. The reaction of formaldehyde with methylbenzothiazolone hydrazine hydrochloride followed by the addition of ferric chloride-sulphamic acid yields a UV active compound with absorbance maximum at 628 nm. A calibration curve containing 1.25 µg/ml to 10.0 µg/ml can be constructed and ethylene glycol can be indirectly quantified by the quantification of the amount of formaldehyde.[12]

The direct injection of ethylene glycol on to gas chromatographic columns leads to tailing peaks and relatively low sensitivity. Derivatisation of ethylene glycol to a volatile compound improves the use of gas chromatography using a flame ionisation detector with direct injection. Derivatisation can eliminate or minimize tailing and improve sensitivity.

The derivatisation of ethylene glycol with phenylboronic acid produces a cyclic phenylboronate ester. It has a linear response over the range of 250 mg/l to 5000 mg/l with coefficient of variation less than 2.5%. The detection limit is reported as 10 mg/l and recovery as 96%.The derivatisation process is rapid.[13] Peak shape is however compromised and baseline resolution between peaks can be affected.

Ethylene glycol volatilisation by derivatisation with N, O-bis (trimethylsilyl) trifluoroacetamide: trimethylchlorosilane (BSTFA: TMCS) has a detection limit of 5 ppm.[4] Peak shape and baseline separation of both the analyte and internal standard are acceptable. The addition of the internal standard, 3-bromo-1-propanol, to the sample at the beginning of the derivatisation process ensures that it undergoes all transfers and reactions the analyte does. The acetonitrile used to dissolve the internal standard serves as a means to precipitate protein as well.[4] The PRP sample is in a buffer solution necessitating moisture removal in order to prevent interference with the derivatisation reagent. The addition of the mixture of 78: 20: 2 2, 2-dimethoxypropane: N, N-dimethylformamide: acetic acid facilitates this. The reaction between 2, 2- dimethoxypropane and water forms methanol and acetone using glacial acetic acid as a catalyst. The N, N-dimethylformamide “traps” the ethylene glycol preventing evaporation when drying the other solvents, methanol, acetone and acetonitrile off the sample. Flushing with nitrogen while heating on a heating block makes this a rapid process.[4]

The use of BSTFA (Figure 4.6), instead of BSA yields a highly volatile fluorinated by-product after donation of the TMS group. The advantage is that lower molecular weight analytes can be derivatised without the possibility of interference when analysed.

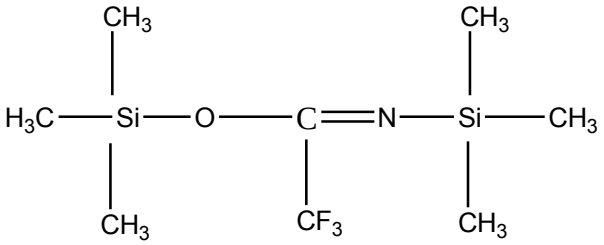

Figure 4.6 Structure of BSTFA[14]

The BSTFA by-product is very volatile and elutes early. The presence of fluorine atoms causes less flame ionisation detector fouling due to silica deposits. TMCS acts as a catalyst driving a faster reaction.[14]

A nucleophilic attack by the electrons on the hydroxyl group of the analyte on the silicon atom of the silylating reagent causes the leaving group, which in this case a fluorinated group, to leave. This results in the formation of a trialkylsilyl derivative (Figure 4.7).

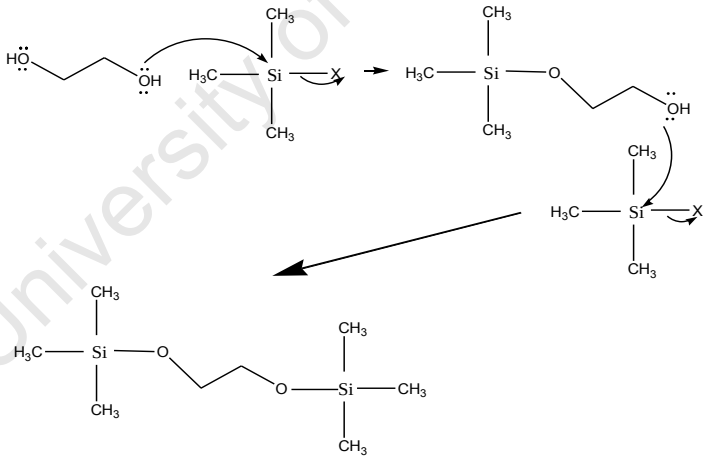

Figure 4.7 Reaction mechanism of silylating reaction

A limit of detection less than 10 mg/l, with recovery of 91% and intraassay coefficient of variation ≤ 2.8% is reported. Derivatisation is rapid and does not require incubation.[4] Ethylene glycol determination in activated PRP will be based on this procedure and the method will be evaluated in this chapter.

4.2 Experimental

4.2.1 Test Samples

- Activated PRP batch # X070908, The Biovac Institute.
- Ethylene glycol, Sigma Aldrich, CAS number 107-21-1

4.2.2 Reagents

- Acetic acid, Merck, CAS number 64-19-7
- Acetonitrile, Merck, CAS number 75-05-8
- 3–Bromo-1-propanol, Sigma Aldrich, CAS number 627-18-9
- BSTFA:TMCS reagent, Supelco
- 2,2-Dimethoxypropane, Sigma Aldrich, CAS number 77-76-9
- N,N-Dimethylformamide, Sigma Aldrich, CAS number 68-12-2

4.2.3 Equipment

- Agilent 7890 Gas chromatograph
- CTC PAL autosampler
- Reacti-vap/ Reacti-therm sample evaporation unit, Thermo Scientific
- Eppendorf pipettes, 200 µl, 1000 µl
- Reacti vials, Thermo Scientific

4.2.4 Sample Preparation

The internal standard 3-bromo-1-propanol was prepared by dissolving/ mixing 31.4 µl with acetonitrile in a 100 ml volumetric flask. This equates to a concentration of 0.5 ppm.

Samples were prepared by adding 200 µl of the internal standard solution to 100 µl of the sample. The solution was vortexed and to 100 µl of the supernatant added 500 µl of a mixture of 7.8: 2.0: 0.2 2, 2-dimethoxypropane: N, N-dimethylformamide: acetic acid. The solution was allowed to stand for 5 minutes at room temperature. Excess solvent and moisture was removed by drying on a heating block at 60 °C while flushing with nitrogen. To a volume of < 50 µl, 100 µl of the BSTFA: TMCS reagent was added and the solution transferred to an autosampler vial.

4.2.5 Instrument Parameters

Gas chromatograph

Front Inlet:

- Heater : 250 °C
- Mode: Split
- Split ratio: 20 : 1
- Total flow: 24 ml/minute
- Septum purge flow: 3 ml/ minute

Flow parameters:

- Flow rate: 1 ml/minute
- Carrier gas nitrogen

Oven:

- Equilibration time: 3 minutes
- Oven Program:
  90 °C hold for 3 minutes
  15 °C/ minute to 170 °C hold for 5 minutes
  20 °C/ minute to 220 °C hold for 4.167 minutes
  Run Time 20 minutes

Detector unit:

- Hydrogen 200 ml/minute
- Nitrogen 300 ml/minute
- Air 400 ml/minute

Column

J&W Scientific, HP 5 column, 30 metres X 0.320 millimetres X 0.25 micrometres.

4.2.6 GC-FID Analysis of Ethylene Glycol

See Figure 4.6 for a typical chromatogram of the derivatised ethylene glycol and internal standard.

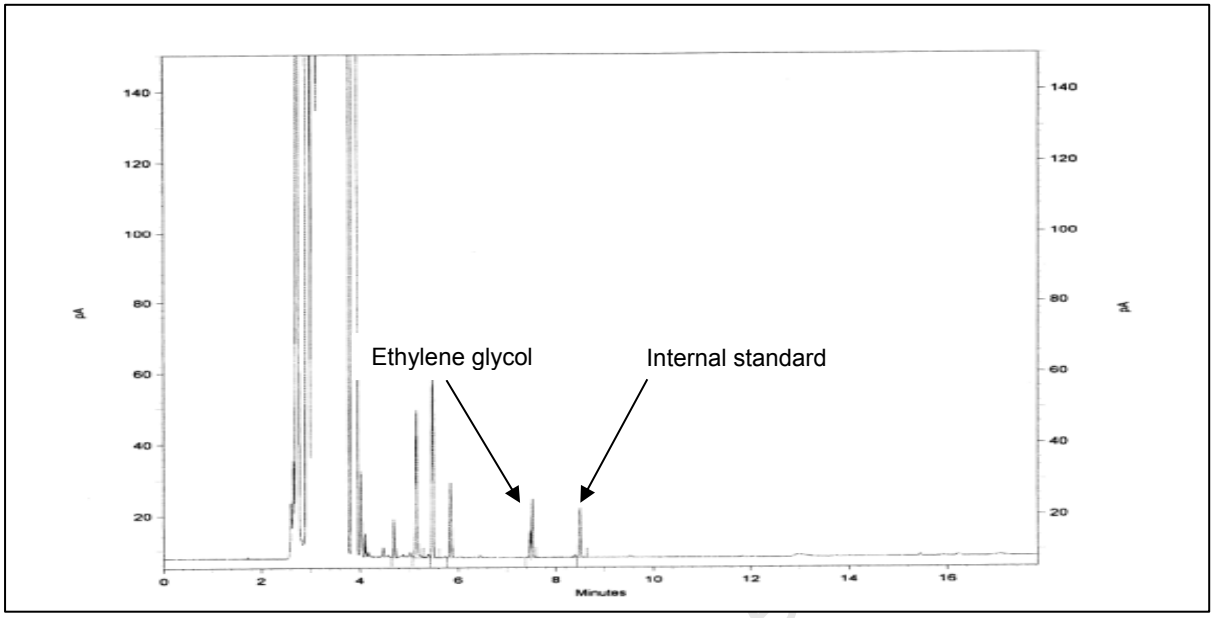

Figure 4.8 Chromatogram of ethylene glycol and the 3-bromo-1-propanol internal standard

Ethylene glycol elutes at a retention time of ± 7.6 minutes and 3-bromo-1-propanol at ± 8.2 minutes giving a relative retention time of 1.09 to the main analyte. The elution time is relatively short with good baseline separation between peaks. The analyte and internal standard have tailing factors of less than 2 indicating peaks approaching a normal or Gaussian shape. Solvent peaks elute early and do not interfere with that of the analyte and internal standard.

4.3 RESULTS AND DISCUSSION

The suitability of the method to quantify residual ethylene glycol in activated PRP was evaluated using current validation practices. Parameters assessed include linearity and range, specificity, precision and accuracy.

4.3.1 Linearity and Range

The linearity was assessed by making up solutions of ethylene glycol in purified water of different concentrations ranging from 5 ppm to 500 ppm. The range was determined from the linear portion of the graph.

Table 4.1: Results obtained for Linearity and Range study

Ethylene Glycol concentration (ppm)	Response (mAU)	I/S response (mAU)	Internal Standard Corrected Response
5	721154	273083	2.64
50	1275804	275532	4.63
100	1845864	217729	8.48
250	5601834	259564	21.58
500	10929265	261329	41.82

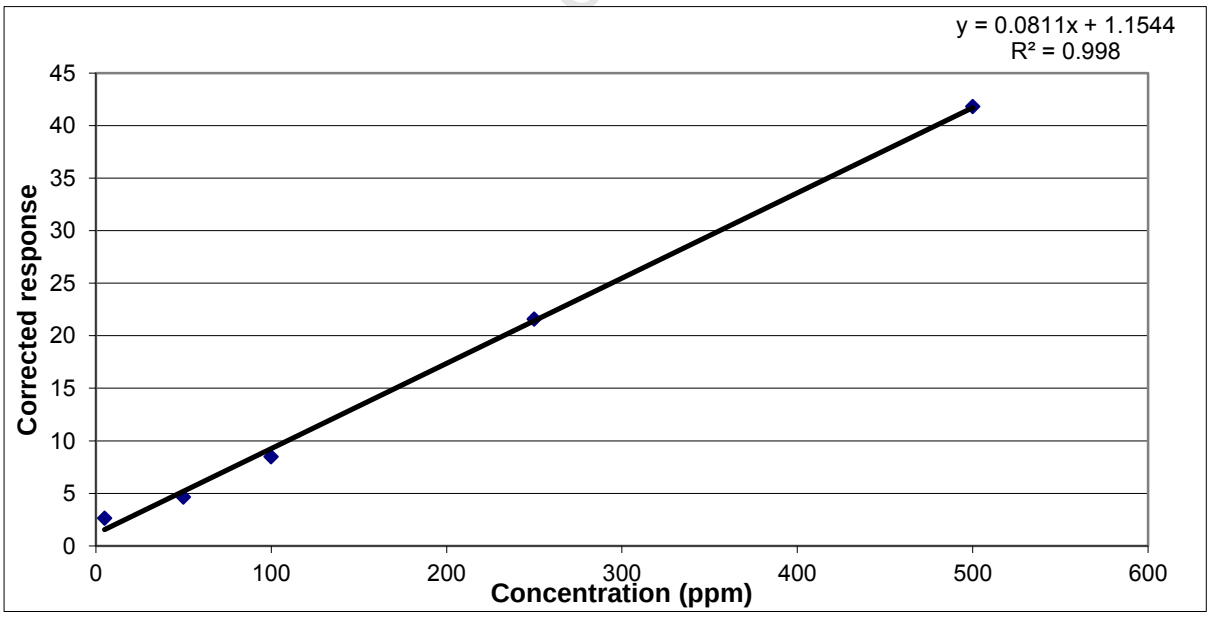

Figure 4.9 Ethylene glycol response vs. concentration

The range was found to be linear with a correlation coefficient of 0.998 from a concentration of 5 ppm to 500 ppm. The correlation coefficient of the data set is close to 1 indicating data

points close to the regression line. The y-intercept for the graph is positive resulting in a positive response for a 0 ppm solution. The response obtained is however negligible and would not have a significant impact on generated results.

4.3.2 Specificity

Specificity was evaluated by testing a specific volume of PRP polysaccharide spiked with a standard of a known concentration of ethylene glycol (125 ppm). The same volumes of polysaccharide and standard were tested independently and the ethylene glycol value of the" spiked' polysaccharide compared to the sum of the individual polysaccharide and standard solutions.

Table 4.2 Results obtained for specificity study

Sample	Response (mAU)	I/S response (mAU)	Corrected Response	Calculated Concentration (ppm)	% Recovery
PRP + Std	2542832	245079	10.38	113.76	**103.14**
PRP + H_2O	0	221776	0	0	
Std + H_2O	2427918	227521	10.67	117.33	

% Recovery = [(PRP + H_2O) + (Std + H_2O)]/ (PRP + Std) X 100

The results indicate that the other components present in the polysaccharide do not interfere with the ethylene glycol assay. All sample matrix effects can be eliminated based on this. The acceptance criterion for accuracy is 90 to 110% of the actual amount and will be applied to the specificity study.

4.3.3 Precision

Precision was assessed by analysing an ethylene glycol concentration six times. The % RSD was calculated and reported. A standard solution with concentration within the linear range was prepared and analysed. A concentration of 500 ppm was assessed.

Table 4.3: Results obtained for the precision study

Concentration (ppm)	Ethylene Glycol (mAU)	I/S response(mAU)	Corrected Response
500	9567152	225245	42.4744
500	9951651	247633	40.1871
500	11679844	265508	43.9906
500	9320429	222286	41.9299
500	9705991	226403	42.8704
500	9567152	225245	42.4744
Average	10045013	237415	42.29048
Standard Deviation	18653.98	18653.98	1.397736
% RSD	0.185704	7.857119	3.305084

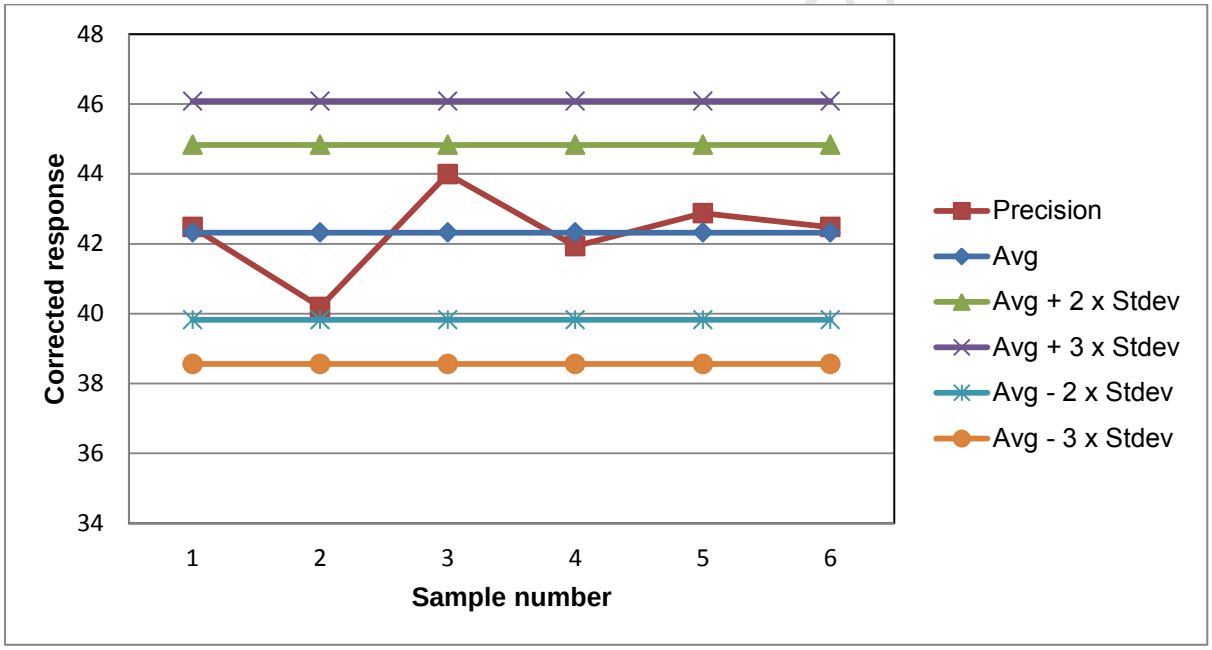

Figure 4.10 Ethylene glycol precision

Repeated analyses of the same sample yielded a low % RSD. Using the average and standard deviation of the analysis of the six samples enabled the calculation of upper and lower control limits. The minimum number of analyses required for plotting a statistical process control chart is 10. The value of a control chart is to monitor trends in the production processes to enable proactive instead of reactive interventions. Alert limits indicates when a process is on route to go out of control, whereas action limits indicates when a process is out of control and steps needs to be taken to bring it back to a state of control. Limits for a control chart is calculated from data gathered over a period over which different batches of

the same product are manufactured using the same technique, equipment and type of raw materials. The control limits of the control chart were calculated from the data generated from the precision study as routine production of the Hib vaccine at the Biovac Institute has not commenced.

4.3.4 Accuracy

Accuracy was assessed by the triplicate analyses of 3 different concentrations from the lower, middle and upper region of the linear range. The average, standard deviation and % RSD of each sample set was determined. 50 ppm, 100 ppm and 500 ppm ethylene glycol in purified water were analysed. The result of the 100 ppm solution was treated as the "actual" concentration.

Table 4.4: Results obtained for accuracy study

Concentration (ppm)	Ethylene Glycol (mAU's)	Internal Standard (mAU's)	Corrected
50	705582	497413	1.42
	686187	456344	1.50
	622361	432373	1.44
Average	671376.67	462043.33	1.45
Standard deviation	43542.43	32892.43	0.042
% RSD	6.49	7.12	2.90
100	1233602	428655	2.88
	1307814	441176	2.96
	1526812	494774	3.09
Average	1356076	454868.33	2.98
Standard deviation	152446.52	35121.80	0.106
% RSD	11.24	7.72	3.56
500	7365828	497911	14.79
	7633122	490779	15.55
	7579484	507431	14.94
Average	7526144.67	498707	15.09
Standard deviation	141404.86	8354.49	0.40
% RSD	1.88	1.68	2.67

The response obtained for the 100 ppm concentration was used to calculate the 50 ppm and 500 ppm % Recovery.

% Recovery = (Actual/ Theoretical) X 100%

50 ppm = [(1.45 X 2)/ 2.98] X 100
= 97.32 %

500 ppm = [(15.09/5)/2.98] X 100
= 101.28 %

The amount of variance, of the triplicate analyses of the different solutions, was relatively low. The triplicate analysis of the 50 ppm solution yielded a %RSD of 2.90% that of the 100 ppm solution was 3.56 % while that of the 500 ppm solution was 2.67%. The recoveries for the 50 ppm and 500 ppm solution, using the 100 ppm solution as reference, were 97.32% and 101.28% respectively. The recoveries obtained for the accuracy study were within acceptable levels.

4.4 Conclusion

Ethylene glycol is classified as a Class 2 solvent and it is recommended by the USP to limit use in production processes.[5] The WHO TRS 897 requires evidence of removal of residual solvents by either process validation or validated quality control test methods. Ethylene glycol was analysed using 3–bromo–1–propanol as an internal standard. The internal standard was added prior to sample processing ensuring exposure to the same conditions as the analyte. Moisture was removed by using a mixture of 2, 2-dimethoxypropane: N, N-dimethylformamide: acetic acid where the reaction with water produces the volatile solvents methanol and acetone. The sample was subjected to heat under a constant flow of nitrogen on a Reacti–vap/Reacti-therm unit. The derivatisation with the BSTFA: TMCS reagent was quick and injection on the HP5 capillary column, installed in a GC-FID, yielded two distinct peaks for the analyte and internal standard.

The analysis of ethylene glycol was linear across a range of 5 ppm to 500 ppm with a R^2 value of 0.998. Specificity was established with a recovery of 97.3%. The % RSD of the precision study of a 500 ppm solution was 3.31%. The accuracy study had recoveries for the 50 ppm solution of 97.32% and for the 500 ppm solution 101.28%.

The method meets all validation acceptance criteria for the linear range. The retention times of ethylene glycol and 3–bromo–1–propanol are less than 10 minutes resulting a relatively short analysis. This length of analysis together with the use of an autosampler enables multiple sample analysis. The method can be implemented in a laboratory for the routine analysis of ethylene glycol in activated PRP.

4.5 References

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Chapter 5

Development and assessment of an assay to determine polyribosylribitolphosphate (PRP)

The capsular polysaccharide of *Haemophilus influenzae* type b (Hib) is conjugated to the carrier protein tetanus toxoid for vaccine production at The Biovac Institute. A selective and sensitive method is needed to quantify the Hib polysaccharide antigen content. Conjugated polysaccharide alone is of immunological importance as potency is directly related to the amount of polysaccharide bound to the protein carrier. Colorimetric methods currently employed are non-specific and have the risk of being affected by other contributing components. The assay to be investigated is based on a gas chromatography with mass selective detector (GC-MS) method applied to pneumococcal conjugate vaccines which determines the PRP content and can be applied to both conjugated and "free" PRP.

5.1 Background

The quantification of PRP in both conjugated and unconjugated form is a requirement for both vaccine release testing as well as vaccine stability studies. PRP that is covalently bound to the carrier protein is immunologically important. Hib conjugate vaccines should be tested for both conjugated and unconjugated PRP against specifications agreed to by the national authority which is based on the data from a successful clinical trial.[1] See Figure 5.1 for the relationship between total Hib saccharide content, conjugated and unconjugated saccharide content.

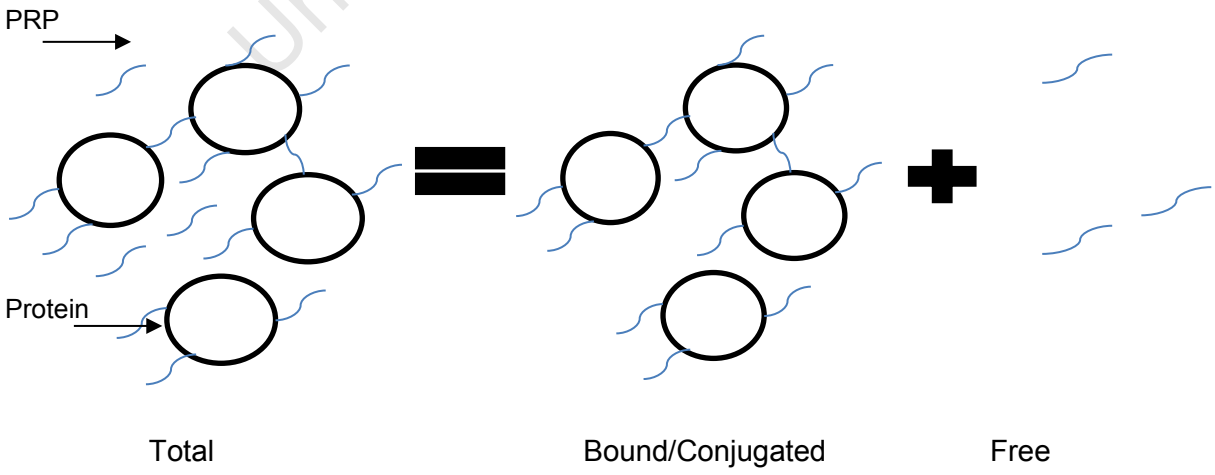

Figure 5.1 Total and free saccharide

The free saccharide content is reported as a percentage of the total saccharide. Vaccines with successful clinical trials contained unconjugated polysaccharide quantities of less than 10 % and up to 40 %.[1] Approved Hib vaccines consisting of PRP conjugated to a tetanus toxoid carrier protein have free PRP specifications of < 20%.[2]

Traditional vaccine potency and safety assays are performed in animals. Animal based models suffer from inherent variability and there is currently a lack of adequate biological assays for evaluating the potency or efficacy of Hib conjugated vaccines. Hib vaccine potency and efficacy were evaluated in mice and guinea pigs, but the results generated could not be correlated with the immune response of human infants. Animal assays are only needed during the vaccine development process in order to show the ability of the vaccine to induce a T-cell-dependent response. Lot release and quality control of Hib vaccines is done by the characterisation of the polysaccharide, carrier protein and conjugate by physico-chemical means as well as batch-to-batch consistency.[1] Quantification of the Hib antigen conjugated and unconjugated content, by means of physico-chemical methods, replaces traditional animal based potency assays.

Stability studies are done per product, presentation (vial/ syringe, volume, freeze-dried/liquid) and continue throughout the lifecycle of the product.[2,3] The stability of the amount of unconjugated polysaccharide is monitored over time at typical storage conditions. This should stay within a predefined acceptance criterion throughout the study. Hydrolysis of the phosphodiester bond between the PRP monomers results in an increase in the amount of free and or depolymerized PRP. In a multivalent vaccine, containing a Hib vaccine component, the rate of this hydrolysis is affected by temperature, adjuvant and divalent cation interaction.[4] The assay used to determine the amount of polysaccharide should be sensitive enough to determine changes in the amount of unconjugated polysaccharide.

5.1.1 The Conjugation Process

The PRP length, protein carrier and conjugation chemistry of Hib conjugate vaccine varies between manufacturers. This study is performed on a Hib vaccine where the PRP is conjugated to tetanus toxoid (TT).

The protein carrier has carboxyl groups which are activated by reacting with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) in the presence of adipic acid dihydrazide. The activated PRP which is treated with periodate is size-reduced and terminates in an aldehyde group on each terminus. Proximity of the electronegative oxygen atoms renders the carbon molecule of the aldehyde group electrophilic. Sodium cyanoborohydride acts as a

catalyst and a nucleophilic attack on the electrophilic PRP carbon takes place. A hydrazone derivative is formed which are stabilized after the conjugation process, to a stable acid hydrazide, with reduction by sodium borohydride. See Figure 5.2 for the conjugation process.

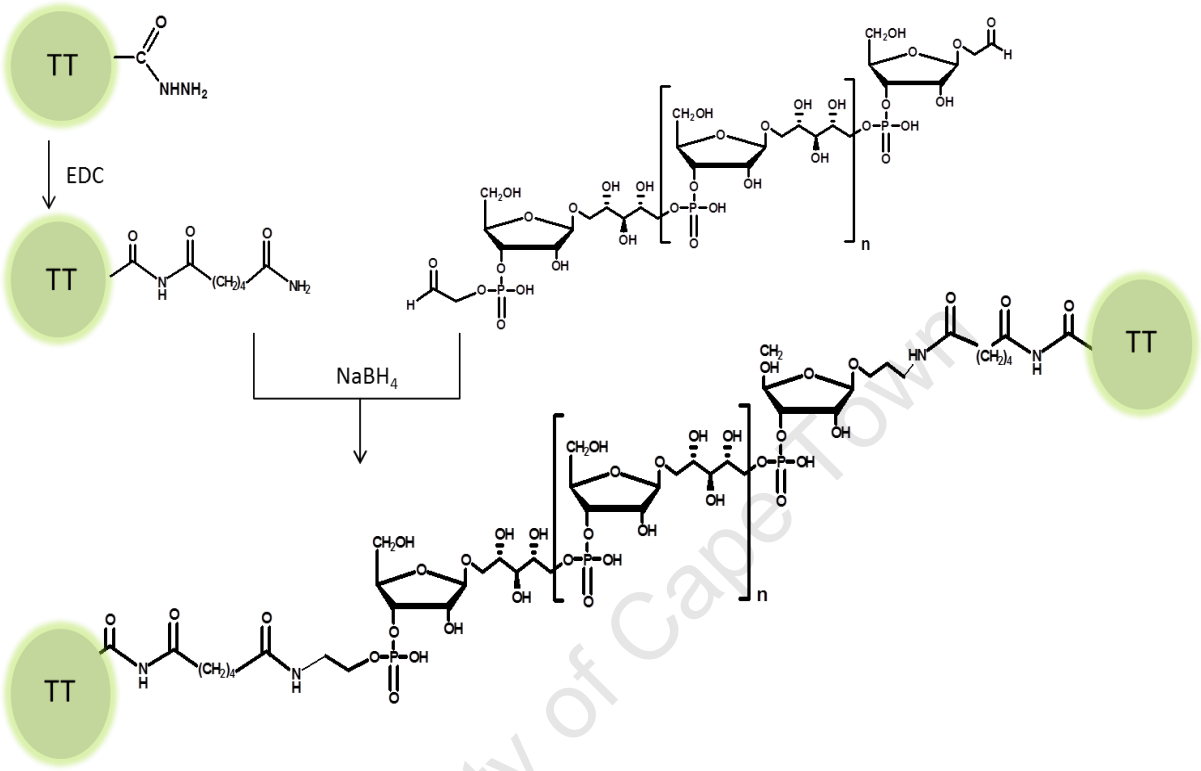

Figure 5.2 Conjugation of PRP with activated tetanus toxoid

The conjugation of the derivatized TT with the activated PRP yields a high molecular weight conjugate. Molecular size exclusion chromatography is used to monitor the conjugation reaction as well as the quality of the activated PRP and derivatized TT.[4,5,6]

5.1.2 PRP Specification

According to the World Health Organisation (WHO) Technical Report Series (TRS) 897, the amount of free PRP in conjugated bulk must be verified by validated test methods to ensure that it is within limits. These limits are based on batches proven to be clinically safe and efficacious. The total PRP content should be within ± 20% of the stated content. Limits of free PRP in conjugated bulk and total PRP in the final product must be agreed with the national control authority.[1] This required a method for PRP quantification and a separation technique separating free from conjugated PRP so that it can also be quantified. The free PRP is reported as a percentage of the total PRP.

5.1.3 PRP Quantification

The quantification of PRP can be performed directly by determining the concentration of ribitolribosephosphate subunits or by determining the concentration of ribitol, ribose or phosphate. The polysaccharide is characterised by the ribose and phosphorus content.

The Bial method or orcinol test is used to indirectly determine the amount of PRP. The orcinol assay determines the concentration of pentose and in this case D-ribose, an aldopentose. The WHO recommends a ribose content of not less than 32% of the polysaccharide weight on dry basis.[1] The polysaccharide is heated with a mineral acid, hydrochloric acid, containing ferric chloride and orcinol. The pentose forms a green coloured complex with the orcinol and ferric chloride. Detection is performed on a spectrophotometer at a wavelength of 670 nm. D-ribose is used to construct a calibration curve in order to determine concentration.[7] This method was evaluated at The Biovac Institute (TBI) and linearity was established between 2.0 µg/ml to 40.0 µg/ml with a % relative standard deviation (% RSD) of < 2.0%. Accuracy and specificity were determined between 90% to 100%. The presence of ribonucleic acid (RNA) increases the amount of ribose present in the sample. This method has limited application because PRP in multivalent vaccine formulations where RNA contamination by whole cell pertussis is present cannot be accurately quantified using this method.

PRP can be characterised by the phosphorus content. A phosphorus content of between 6.8% and 9% calculated on the polysaccharide dry weight is recommended.[1] The Chen method for the determination of phosphates begins with the mineralisation of samples using a mixture of sulfuric and perchloric acid which yields inorganic phosphate. This is performed on a heating block capable of temperatures of up to 250 °C. A phosphomolybdate complex is formed with ammonium molybdate which after reduction with ascorbic acid provides a blue colour. Absorbance can be measured on a spectrophotometer at 825 nm. In order to eliminate the contribution of inorganic phosphate in the sample matrix, “free” phosphate is determined in a similar manner. The mineralisation step is omitted. The amount of PRP can be calculated after the determination of the amount of organic phosphate.[8]

Organic phosphate = Total phosphate – Inorganic phosphate

Linearity was established in the quality control (QC) department of TBI between 7.5 µg/ml to 60.0 µg/ml with an intermediate precision % RSD of < 2.0%. Accuracy and specificity were determined between 90% to 100%. Phosphate measurement can alternatively be done as

per the Ames method where magnesium nitrate is used in conjunction with a high temperature oven to ash the sample. The colorimetric test follows the same route as the Chen method in that a complex with ammonium molybdate is reduced with ascorbic acid providing a blue colour. Absorbance is measured on a spectrophotometer at 820 nm. The free or inorganic phosphate is determined by omitting the ashing step.[9] The determination of phosphate by either the Chen or Ames method have to accommodate for inorganic phosphate in order to accurately determine the amount of PRP present. Formulations involving phosphate buffers can have elevated responses if the inorganic component is not accurately quantified.

Nuclear magnetic resonance (NMR) spectroscopy is used for the identification of the polysaccharide components of conjugate vaccines.[10] It is used to identify impurities, show degradation pathways as well as characterising intermediates during the manufacturing process. Quantitative analysis of the capsular polysaccharide: protein ratio of Hib using NMR has been evaluated, but not validated.[11] NMR is not routinely used in a commercial manufacturing site due to cost of equipment as well as operational costs. A high level of expertise is required to operate the instrument and interpret the data generated.[11,12] NMR does however not require the use of reference material as is the case with most other wet chemical, colorimetric or chromatographic analysis. Impurity isolation is not necessary and the analysis could be less time consuming than others.[13]

The interaction between Hib antibody and polysaccharide antigen enables the use of enzyme-linked immunoabsorbant assays (ELISA) for the qualitative analysis of Hib.[14] Quantitative ELISA analysis of Hib vaccines can also be performed. Concentrations between 0.04 µg/ml and 30 µg/ml purified polysaccharide are used to coat the chemically modified micro well plates. The coated plate is washed with a phosphate buffer solution and Tween 20 where after it is blocked with goat serum. Sera diluted with phosphate buffer and goat serum is added. Goat antihuman IgG coupled to peroxidase is added which bonds to the goat serum. The ELISA plate is incubated after the addition of a solution of o-phenylenediamine dihydrochloride in a citrate buffer mixed with hydrogen peroxide. 1M sulfuric acid stops the reaction and the absorbance is read at a wavelength of 492 nm. Intra-assay and inter-assay variation has Coefficient of Variation (% CV) of 1.1 % and 5.2 % respectively. Optimal coating of the ELISA plate is achieved at 1 µg/ml.[15] Direct coating of ELISA plates with polysaccharide have poor reproducibility and modification of the polysaccharide is required. The polysaccharide is often conjugated to a molecule more suited for binding to the ELISA plate. This alteration of the structure of the polysaccharide by covalent bonding is not consistent.[15] Calibrated reference material is expensive and not

always available. Hib vaccines consist of a heterogeneous mixture of conjugated and free polysaccharide of different lengths. Antigen antibody interaction of ELISA-based techniques can suffer interference due to size chain length variation of the polysaccharide.

The depolymerisation of the PRP polysaccharide followed by chromatographic analysis is selective and sensitive. Commercially available reference standards can be used to construct calibration curves for quantification of the analyte. Chromatography can be performed on native or derivatised analytes. Methods able to quantify nonderivatised carbohydrates at picomole levels are available.[16]

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) allows low level quantification of nonderivatised carbohydrates. Hydrolysis of the polysaccharide in Hib vaccine using base yields the ribitolribosephosphate subunit which can be directly quantified.[16] Base hydrolysis of Hib when in combination with diphtheria, pertussis and tetanus (DPT) leads to the elution of a peak coinciding with that of the Hib subunit. Hydrolysis using trifluoroacetic acid (TFA) releases ribitol which elutes with baseline separation from other peaks. A linear response of the ribitol standard from 0.15 µg/ml to 2.5 µg/ml as well as repeatability with %CV < 5% is achieved.[16]
TFA hydrolysis can be long and the alkaline mobile phase is relatively harsh on components. Running costs of the equipment are expensive. Baseline separation of peaks is not always achieved and column deterioration due to sample matrix effects is rapid.
Hydrolysis of covalently linked carbohydrates such as PRP-TT, results in the formation of monosaccharides. The monosaccharides are analysed to quantify the amount of polysaccharides. Conventional methods of hydrolysis make use of either an aqueous acid or base. In contrast the methanolysis of carbohydrates yields stable methyl glycosides of the constituent monosaccharides. The process causes less destruction of the carbohydrate when compared to aqueous acid hydrolysis.[17]

A quantitative analysis using gas chromatography with a mass selective detector has been applied to pneumococcal serotypes 1, 3, 4, 5, 6A, 6B, 7V, 9V, 14, 18C, 19A, 19F and 23F polysaccharides or conjugates. [18] Polysaccharides are subjected to anhydrous methanolysis with methanolic hydrochloric acid (HCl) and relatively stable methyl glycosides are formed. Derivatisation of the methyl glycosides by a silylating agent renders it volatile and the sample dissolved in a suitable solvent can be analysed on the GC. Gas chromatography provides good baseline separation or resolution; high efficiency or plate count and identification of peaks can be evaluated if combined with a mass selective detector. Pneumococcal vaccine serotypes 6A and 6B contain ribitol and have a phosphodiester bond. Methanolysis of

serotypes 6A and 6B result in the release of two forms of ribitol, a ring-form 1, 4 - anhydroribitol and ribitol.[18] Acid treatment of ribitol has been known to convert to 1, 4 - anhydroribitol.[19] Quantification of serotypes 6A and 6B are performed by using the response for both forms of ribitol.[18] Methanolysis has the advantage over aqueous acid hydrolysis in that it causes less destruction of carbohydrate.[17,20] The presence of moisture in methanolic HCl of more than 2% results in a decrease in methyl glycoside formation. Trimethylsilylation is also sensitive to the presence of moisture and concentrations of more than and equal to 0.2% v/v can result in a considerable loss of monosaccharide.[17] Silylating agents replaces the active hydrogen in the molecule effectively reducing polarity and hydrogen bonding. The silylating agent used is based on the procedure of Sweeley et al. A mixture of HMDS:TMCS:pyridine (3:1:9) reacts better than the individual silylating components.[20,21] See Figure 5.3 for the reaction mechanism. The nucleophilic attack on the silicon atom forms a reversible transition group. The reaction is pushed forward as a result of the basicity of the leaving group. The proton affinity of the leaving group enables the formation of the stable silyl derivative. [20,21]

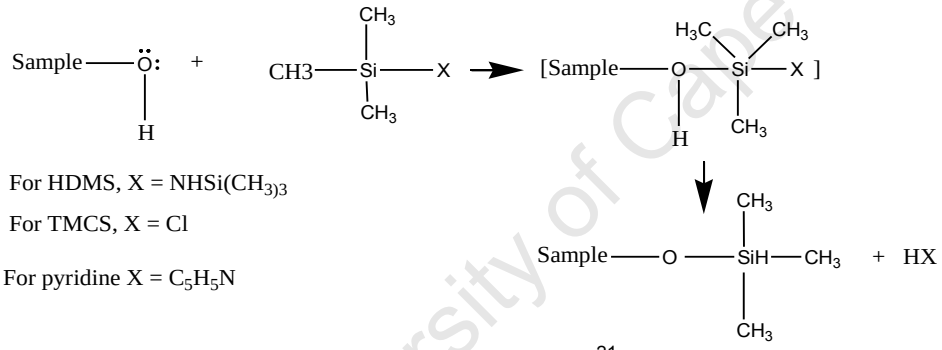

Figure 5.3 Derivatisation reaction mechanism [21]

5.1.4 Free Polysaccharide Separation

In order to quantify the amount of free saccharide, the saccharide conjugated to the carrier protein needs to be separated from the free.

PRP-OMPC (meningococcal outer-membrane protein complex high in molecular weight) is a conjugate Hib vaccine consisting of an oligosaccharide and a large protein. Quantification of the Hib vaccine was performed in a multivalent vaccine consisting of polio, pertussis, diphtheria, tetanus and hepatitis B. Passive adsorption of PRP to the aluminium adjuvant in combination vaccines was eliminated by using a dissolution buffer consisting of sodium hydroxide and sodium citrate. It was subjected to ultracentrifugation in order to separate the conjugated PRP from the unconjugated PRP. Ultracentrifugation was performed at 475 000 ± 10 000 x *g* at 10 °C ± 3 °C for 30 minutes. The supernatant containing the unconjugated

PRP was filtered through a molecular weight cut-off (MWCO) membrane microfilter of 10 000 MWCO in order to remove protein. PRP content was determined using HPAEC-PAD. A recovery bias of less than 5 % was reported when applied to PRP-OMPC in both monovalent and multivalent vaccines.[4]

A Hib vaccine consisting of PRP oligosaccharides conjugated to the carrier protein diphtheria CRM197 was analysed for total and free PRP content. The PRP content was evaluated in both monovalent and multivalent vaccine where the multivalent vaccine consisted of diphtheria, pertussis, tetanus (DPT). Analytical ultrafiltration was used to separate the conjugated PRP from the unconjugated. Ultrafiltration of the sample was performed using a cellulose membrane with a cut-off of 30 kDa. The filter was centrifuged for 30 minutes at 3000 rpm and washed with a saline solution. Recoveries between 80% and 110% for PRP, PRP conjugated to a protein carrier and also Hib as part of a combination vaccine was reported. This separation method was evaluated for oligosaccharide based conjugates [16]

PRP conjugated to tetanus toxoid carrier protein was analysed for total and free PRP content by HPAEC-PAD. The conjugated PRP was precipitated from the solution using deoxycholic acid (DOC) sodium salt. A volume of 100 µl of a 1% DOC solution is added to 1 ml sample, incubated in ice for 30 minutes. The pH is adjusted by adding 50 µl 1 M hydrochloric acid after which it is centrifuged. The supernatant is removed in order to analyse the free saccharide. A recovery of more than 90 % at a pH of 6.8 is listed for this procedure. Conjugate precipitation is listed as 100% while recovery of PRP 96%.[22]

5.1.5 Method of Analysis for PRP-TT

The Hib polysaccharide will be subjected to methanolysis in order to form stable methyl glycosides. Trimethylsilylating will then be performed rendering volatile complexes for analysis. A gas chromatography with flame ionisation detection (GC-FID) method based on that used for pneumococcal vaccine serotypes 6A and 6B will be evaluated for use in Hib quality control. Chromatography on a GC using capillary columns has the advantage of providing good separation and derivatized samples are relatively clean. Low level detection would enable analysis of Hib in either formulated bulk or as a final product. Determining the amount of PRP by means of quantifying ribitol has the added advantage of lacking interference by other vaccine components. Ribose and phosphate cannot accurately be determined in combination vaccines due to contributing factors like the presence of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). A GC-FID is a relatively common instrument in the laboratories of pharmaceutical and vaccine manufacturers. Free

saccharide separation will be done by DOC precipitation as it is a technique verified in the Research and Development department (R&D) of The Biovac Institute (TBI).

5.2 Experimental

5.2.1 Test Samples

- Purified PRP batch # P90, P41 The Biovac Institute.
- Conjugate batch # C170109, The Biovac Institute
- Ribitol, Sigma Aldrich, CAS number 488-81-3
- Myo-inositol, Sigma Aldrich, CAS number 87-89-8

5.2.2 Reagents

- n - Hexane, Merck, CAS number 110-54-3
- Methanol, Merck, CAS number 67-56-1
- 3 N Methanolic HCl, Sigma, CAS number 7647-01-0
- Sweeley reagent (HMDS:TMCS:pyridine, 3:1:9) , Supelco, CAS number 318974-69-5
- Deoxycholic acid sodium salt, Sigma Aldrich, CAS number 302-95-4

5.2.3 Equipment

- Agilent 7890 Gas chromatograph
- CTC PAL autosampler
- Reacti-vap/ Reacti-therm sample evaporation unit, Thermo Scientific
- Virtis bench top freeze drier
- Eppendorf pipettes, 200 µl, 1000 µl
- Reacti vials, Thermo Scientific

5.2.4 Instrument Parameters

<u>Gas chromatograph</u>

Front Inlet:

- Heater : 250 °C
- Mode:Split
- Split ratio: 5 : 1
- Total flow: 9 ml/minute
- Septum purge flow: 3 ml/ minute

Flow parameters:

- Flow rate: 1 ml/minute
- Carrier gas nitrogen

Oven:

- Equilibration time: 3 minutes
- Oven Program:
  50 °C hold for 3 minutes
  15 °C/ minute to 170 °C
  30 °C/ minute to 220 °C hold for 4.167 minutes
  Run Time 30 minute

Detector unit:

- Hydrogen 200 ml/minute
- Nitrogen 300 ml/minute
- Air 400 ml/minute

Column

J&W Scientific, HP 5 column, 30 metres X 0.320 millimetres X 0.25 micrometres

5.2.5 Standard Preparation

Two sets of standards were prepared, a set ranging from 0.25 µg/ml to 30 µg/ml and a set ranging from 0.01 mg/ml to 1.0 mg/ml. A myo-inositol stock solution of 1 mg/ml was prepared in purified water. A further 1:10 dilution was made giving a working solution of concentration 100 µg/ml. The ribitol standard was prepared in a similar manner by making a stock solution of 1 mg/ml. A further 1:10 dilution was made to give a 100 µg/ml solution.
Standard solutions were prepared in volumetric flasks by using a micro pipette then adding the required volume of the ribitol solutions. The internal standard was added thereafter and the solution diluted to the correct volume with purified water.

5.2.6 Sample Preparation

Samples were prepared by diluting the PRP and conjugate samples to within concentration ranges of 0.5 µg/ml to 30 µg/ml and 0.01 mg/ml to 1.0 mg/ml ribitol. Sample concentrations were based on colorimetric assays as per the Bial method assessed by the R&D department at TBI. A volume of the internal standard, myo - inositol, stock solution was added to the solutions.

5.2.7 Freeze Drying

A volume of 1.0 ml of each sample to be analysed was added to separate Reacti-vials, sealed with parafilm and frozen. In order to remove moisture, the frozen samples were dried by lyophilisation. The water undergoes sublimation, thus moving from the solid phase to the gas phase. This gaseous moisture is removed via vacuum from the sample leaving the dehydrated sample.

5.2.8 Methanolysis

Methanolysis was performed on all samples containing polysaccharide. The polysaccharide was hydrolysed into monomers through methanolysis with 3 N methanolic HCl. This step took place on a heating block at 80 °C for 2 hours. Samples were dried on the Reacti-vap/Reacti-therm unit at 40 °C using instrument grade nitrogen. In order to remove excess hydrochloric acid (HCl) from the samples, 120 µl methanol was used to rinse the vials. The solvent was evaporated after the rinsing. This washing of the residue with methanol was repeated 3 times.

5.2.9 Derivatisation

Derivatisation of the analyte was performed by adding 200 µl Sweeley reagent and incubating at 80 °C on a heating block for 20 minutes. The silylating reagent was added in excess. The solvent was dried off using nitrogen gas on the Reacti-vap/Reacti-therm unit and the analyte was dissolved in 250 µl n-hexane prior to injection. See Figure 5.4 for the flow diagram of the sample preparation process. The ribitol standards were freeze dried and derivatized only. The methanolysis step was omitted.

Care was taken to minimize moisture throughout the methanolysis and derivatisation process. The nitrogen gas was subjected to drying over silicon crystals prior to contact with the sample. Sweeley reagent was stored in a sealed desiccator over silicon drying crystals. Methanolic HCl was capped and sealed with parafilm. Both reagents were stored in a fridge between 2 °C and 8 °C and protected from light.

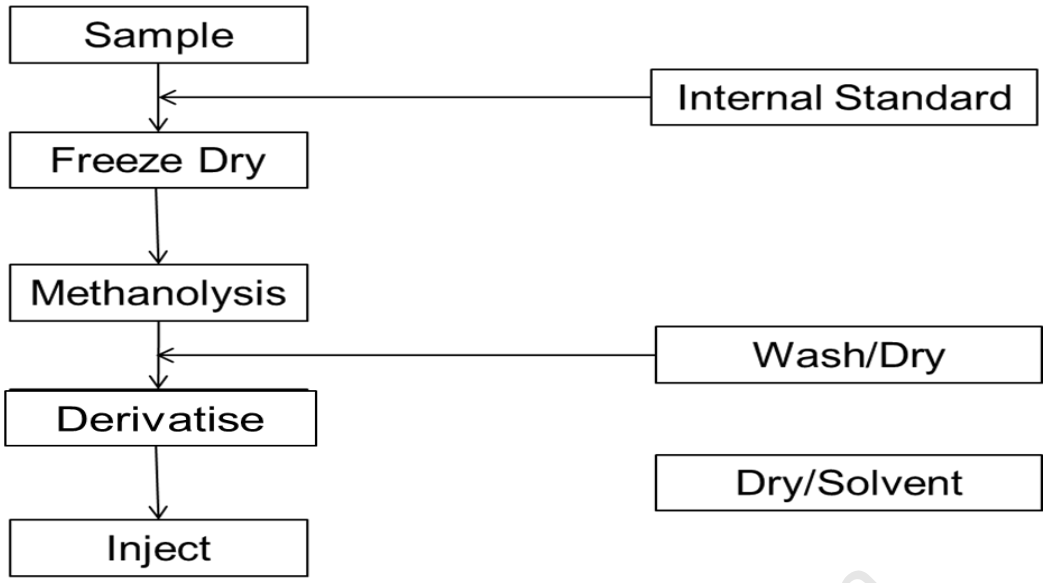

Figure 5.4 Flow diagram of sample preparation

5.2.10 Analysis

Analysis of a ribitol standard solution containing the myo–inositol internal standard yields distinct, well resolved peaks. Ribitol elutes at a retention time of ± 16 minutes and myo-inositol ± 26 minutes giving a relative retention time of 1.62 to the main analyte. The internal standard was added before freeze drying and was subjected to the same conditions as the analyte. Injector reproducibility as well as losses during sample preparation can be accounted for.

See Figure 5.5 for a typical chromatogram of the derivatised ribitol and internal standard.

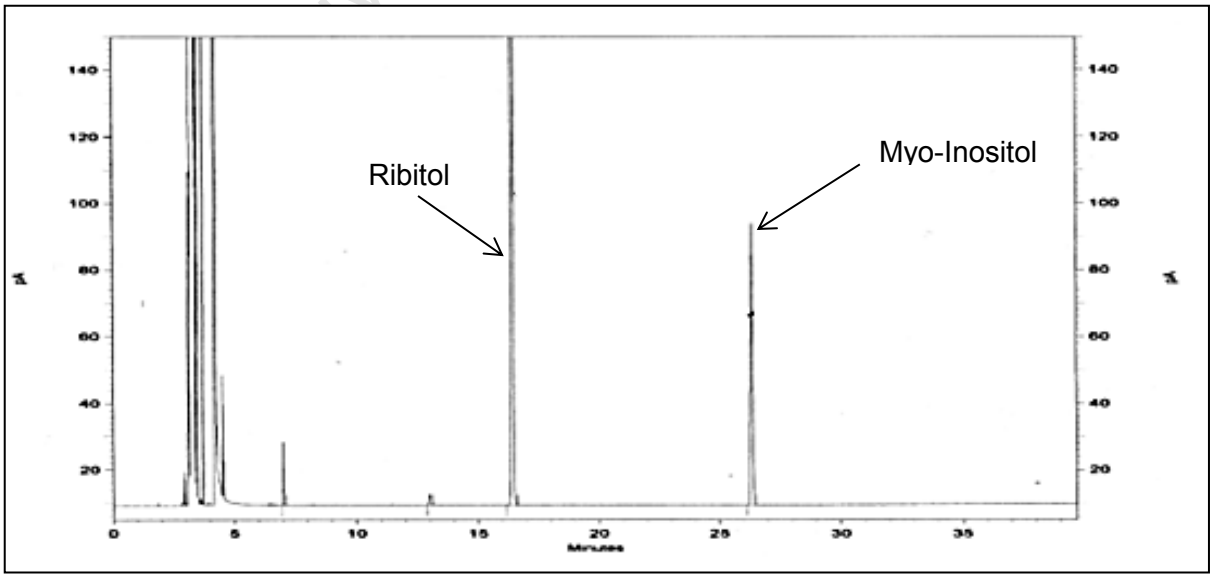

Figure 5.5 Chromatogram of ribitol and myo-inositol internal standard

The analyte and internal standard have tailing factors of less than 2 indicating peaks approaching a normal or Gaussian shape. Solvent peaks elute early and do not interfere with that of the analyte and internal standard. Methanolysis yields ribitol in a ring structure anhydro form as well. The anhydro form elutes earlier than the main peak with an elution time of ± 11 minutes and a RRT of 0.72. See Figure 5.6 for a chromatograph of the 1, 4 - anhydroribitol peak after methanolysis of a ribitol standard solution and Figure 5.7 after methanolysis of PRP.

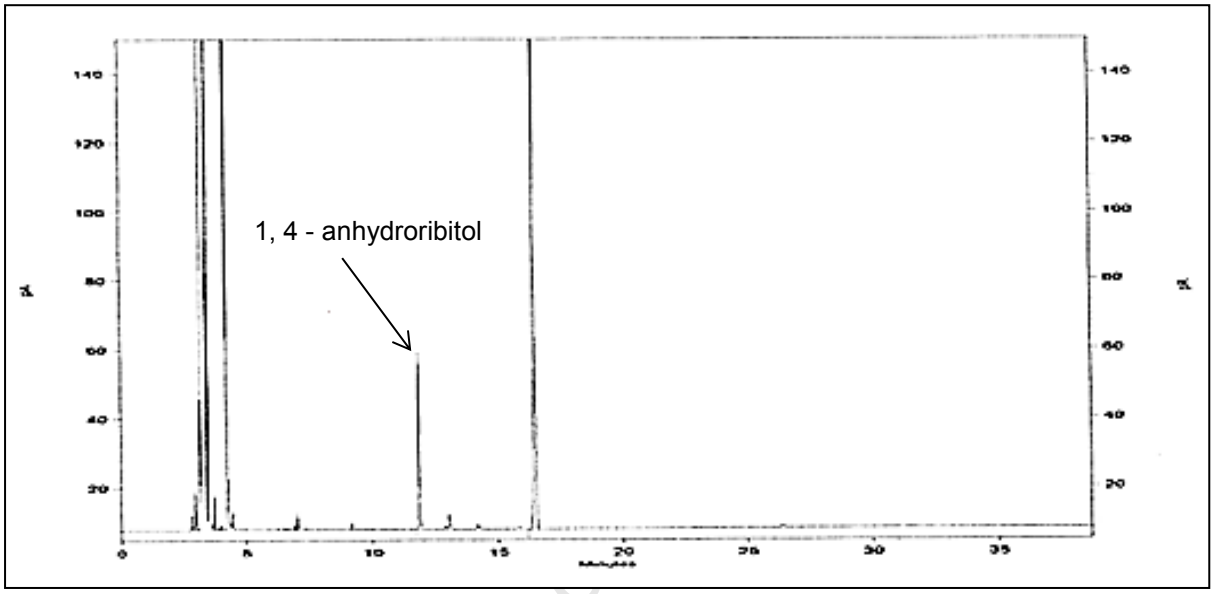

Figure 5.6 Chromatogram of 1, 4 -anhydroribitol after methanolysis of ribitol

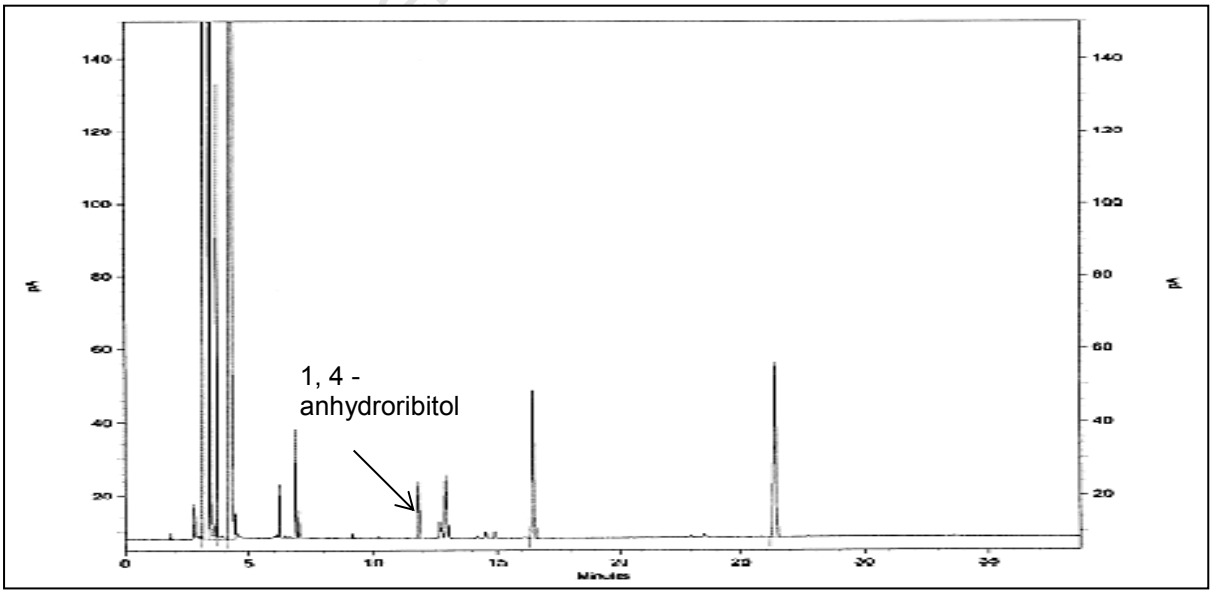

Figure 5.7 Chromatogram of derivatised PRP

Quantification of PRP was performed by combining the response for ribitol and 1, 4 - anhydroribitol. This was performed in accordance with the method of quantification for pneumococcal serotypes 6A and 6B.[20]

5.3 Results and Discussion

This study evaluates the development of a GC-FID method for the determination of PRP content using validation principles.

5.3.1 Linearity

The linearity was assessed by making up solutions of ribitol in purified water of different concentrations ranging from 0.25 µg/ml to 30 µg/ml with internal standard concentrations of 10.0 µg/ml.

Table 5.1 Results obtained for the 0.25 µg/ml to 30 µg/ml ribitol concentration linearity study

Ribitol Concentration (µg/ml)	Response (mAU)	I/S response (mAU)	Internal Standard Corrected Response
0.25	14639	595727	0.024573
0.5	33929	690419	0.049143
1.0	70129	699040	0.100322
5.0	323420	716601	0.451325
10.0	549775	769408	0.714543
20.0	1438191	751738	1.913155
30.0	2124414	715453	2.969327

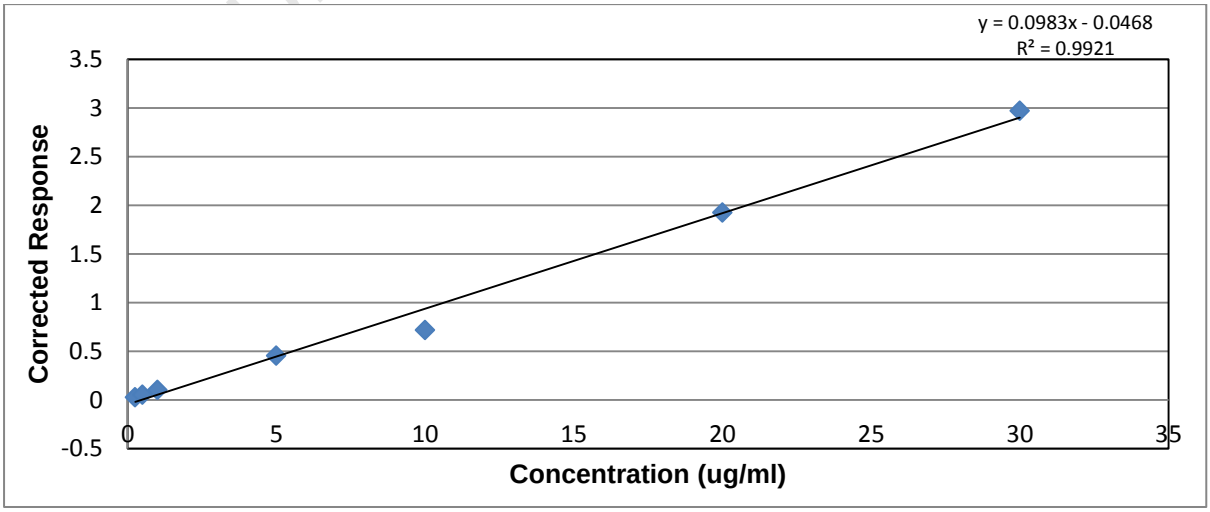

Figure 5.8 Calibration curve of lower concentration ribitol standards

The range was found to be linear with a correlation coefficient of 0.9921 from a concentration of 0.25 µg/ml to 30 µg/ml. The y-intercept for the graph was negative, but it approaches zero and will have a negligible effect on the data. A larger negative value would yield a large negative for the blank value. This is typical of a method not sensitive enough or interference by the blank on the response.

Linearity was also evaluated at a higher concentration of 0.01 mg/ml to 1 mg/ml with an internal standard concentration of 50 µg/ml.

Table 5.2 Results obtained for the higher ribitol concentration linearity study

Ribitol Concentration (mg/ml)	Response (mAU)	I/S response (mAU)	Internal Standard Corrected Response
0.01	554794	2909820	0.190663
0.05	2925459	3093531	0.945670
0.2	13911579	3464041	4.016000
0.5	32282380	3780446	8.539305
1	64178656	4232407	15.163631

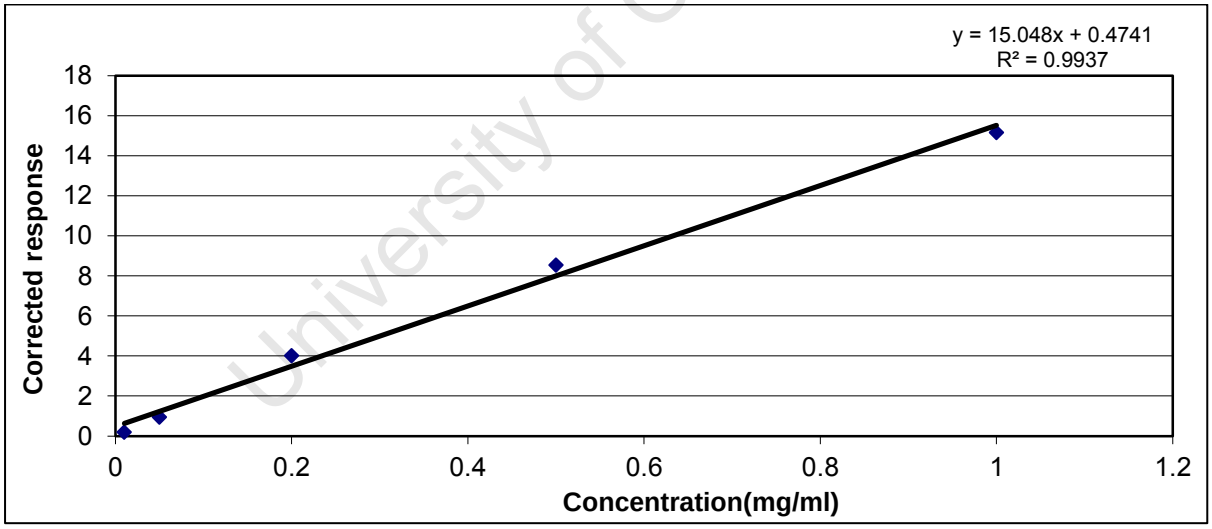

Figure 5.9 Calibration curve of higher ribitol standards concentration

The range was found to be linear with a correlation coefficient of 0.9937 from a concentration of 0.1 mg/ml to 1.0 mg/ml. The y-intercept for the graph was positive indicating that the blank would have a positive response. Blank subtraction of all data points will eliminate this and shift the graph through zero.

The experiment was repeated using PRP diluted in purified water of concentrations 0.9 µg/ml to 27.7 µg/ml and internal standard concentration of 5.0 µg/ml.

Table 5.3 Results obtained for the PRP linearity study

PRP Concentration (µg/ml)	Response (mAU)	I/S response (mAU)	Internal Standard Corrected Response
0.92	51733	398378	0.129859
2.31	78522	385074	0.203914
4.61	138139	391998	0.352397
6.92	117401	259757	0.451965
9.22	180750	280613	0.644126
13.83	292617	317313	0.922171
18.44	537010	430961	1.246076

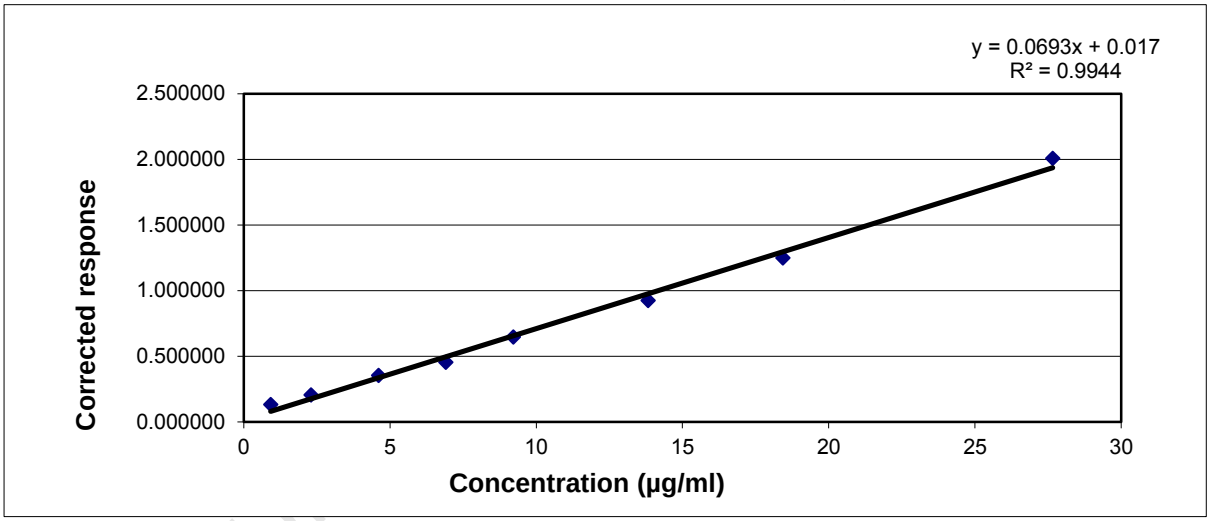

Figure 5.10 PRP response vs. concentration

The y-intercept in this case was positive and would lead to a positive result for a blank solution. Blank subtraction was needed in order to minimize this increased response.

5.3.2 Specificity

The specificity of the PRP assay was evaluated by testing a concentration of 6.8 µg/ml conjugate (PRP-TT) spiked with 5.9 µg/ml polysaccharide with internal standard concentration of 10.0 µg/ml. The conjugate and polysaccharide components of the spiked solution were also analysed separately. The ribitol response was compared to that of a 10 µg/ml ribitol standard containing a 10 µg/ml myo inositol internal standard.

Table 5.4 Results obtained for the conjugate polysaccharide spike recovery study

Sample	Response (mAU)	I/S response (mAU)	Corrected Response	Calculated Ribitol Concentration (µg/ml)
Conjugate	449844	1826912	0.246232	2.46
	404232	1597842	0.252986	2.53
	364285	1613992	0.225704	2.26
Average	406120.3	1679582	0.241641	2.42
Stdev	42810.75	127846.8	0.014209	0.14
%RSD	10.54139	7.611822	5.88004	5.79
Polysaccharide	413685	1528762	0.270601	2.71
	399090	1385649	0.288017	2.88
	366482	1453550	0.252129	2.52
Average	393085.7	1455987	0.270249	2.70
Stdev	24167.54	71587.62	0.017946	0.18
%RSD	6.14816	4.916776	6.640715	6.70
Spiked solution	434556	809125	0.537069	5.37
	844260	1572115	0.537022	5.37
	889972	1611625	0.55222	5.52
Average	722929.3	1330955	0.542104	5.42
Stdev	250782.3	452349.6	0.008761	0.087
%RSD	34.68974	33.98684	1.616155	1.61

The analysis of the first spike solution have a response for both ribitol and myo - inositol that is more or less half that of the remaining two analysis. The analyte response is corrected with that of the internal standard yielding a response similar to the second and third analysis. Polysaccharide content was calculated by multiplying the ribitol concentration by a factor of 2.45. The factor was determined from the molecular weight of ribitol and that of a single polysaccharide repeating unit.

The average corrected response for the 10 µg/ml ribitol standard was 1.01 and was used to calculate the response above.

$$\text{Sample concentration} = \frac{\text{(Ribitol standard concentration X Sample response)}}{\text{Ribitol standard response}}$$

The average PRP concentration of the conjugate sample was calculated as 5.93 µg/ml representing an 87.2% yield. The average concentration of the PRP sample was calculated as 6.62 µg/ml representing a 112.2 % yield. The spiked solution has a PRP concentration of 13.28 µg/ml. This corresponds to a 104.6 % yield when compared to the results based on the ribose assay. A comparison of the individual solutions and the spiked shows a recovery of 94.5 %. This was calculated as follows:

%Recovery = ([conjugate] + [PRP]) / [conjugate + PRP] X 100

Specificity was further assessed by spiking a 10 µg/ml ribitol standard with 6.8 µg/ml conjugate. The response for the spiked solution was compared against that of the individual components. The internal standard concentration was 10 µg/ml.

Table 5.5 Results obtained for the conjugate ribitol spike recovery study

Sample	**Response (mAU)**	**I/S response (mAU)**	**Corrected Response**	**Calculated Ribitol Concentration (µg/ml)**
Conjugate	407075	1462983	0.27825	2.7825
	390003	1451593	0.268672	2.6867
	374761	1392342	0.269159	2.6916
Average	390613	1435639	0.272027	2.7203
Stdev	16165.63	37926.6	0.005395	0.05395
%RSD	4.138529	2.641792	1.983152	1.983152
Ribitol Standard	1783255	1609151	1.108196	11.0896
	1722232	1575491	1.09314	10.9314
	1888996	1736716	1.087683	10.8768
Average	1798161	1640453	1.09634	10.9634
Stdev	84375.35	85048.34	0.010624	0.10624
%RSD	4.692313	5.184444	0.969085	0.969085
Spiked solution	1874099	1411143	1.328072	13.28072
	1903762	1452906	1.310313	13.10313
	1887077	1452119	1.299533	12.99533
Average	1888313	1438723	1.312639	13.12639
Stdev	14870.06	23887.93	0.014411	0.144107
%RSD	0.787478	1.660357	1.097839	1.097839

The results indicate that the other components present do not interfere with the assay. All sample matrix effects can be eliminated based on this.

5.3.3 Precision

Precision was assessed by the analyses of 6 aliquots of the same sample using the same analyst, reagents and equipment. Ribitol solutions of 5 µg/ml and 0.2 mg/ml and a PRP solution of 20 µg/ml were used. The internal standard concentrations were 10 µg/ml and 50 µg/ml myo–inositol for the ribitol solutions and 20 µg/ml for the PRP solution. The average, standard deviation and % RSD of each sample set was determined.

Table 5.6: Results obtained for the ribitol 5 µg/ml precision study

Concentration (µg/ml)	Ribitol (mAU)	I/S response (mAU)	Corrected Response
5.0	250481	544208	0.460267
5.0	326490	718808	0.454210
5.0	347004	774489	0.448043
5.0	278353	677601	0.410792
5.0	351628	680033	0.517075
5.0	348166	723302	0.481356
Average	317020.33	686406.83	0.461957
Stdev	42612.40	78113.04	0.035465
% RSD	13.44	11.38	7.677018

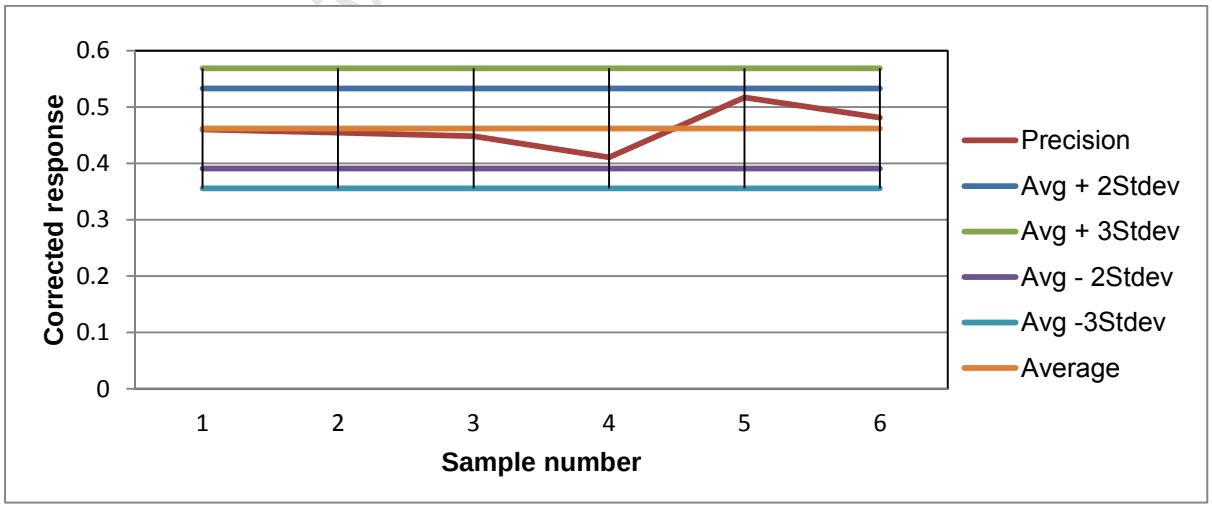

Figure 5.11 Ribitol precision 5.0 µg/ml

Repeated analysis of the same sample yielded a % RSD of < 8%.

Table 5.7: Results obtained for the ribitol 0.2 mg/ml precision study

Concentration (mg/ml)	Ribitol (mAU)	I/S response (mAU)	Corrected Response
0.2	16212481	3890924	4.166743
0.2	14882216	3451007	4.312427
0.2	11136087	2680798	4.154019
0.2	13791087	3357589	4.107438
0.2	15474688	3768828	4.105968
0.2	13529939	3304181	4.094794
Average	14171083	3408887.833	4.156898
Stdev	1796611.546	426004.6268	0.081476
% RSD	12.678012	12.496880	1.960018

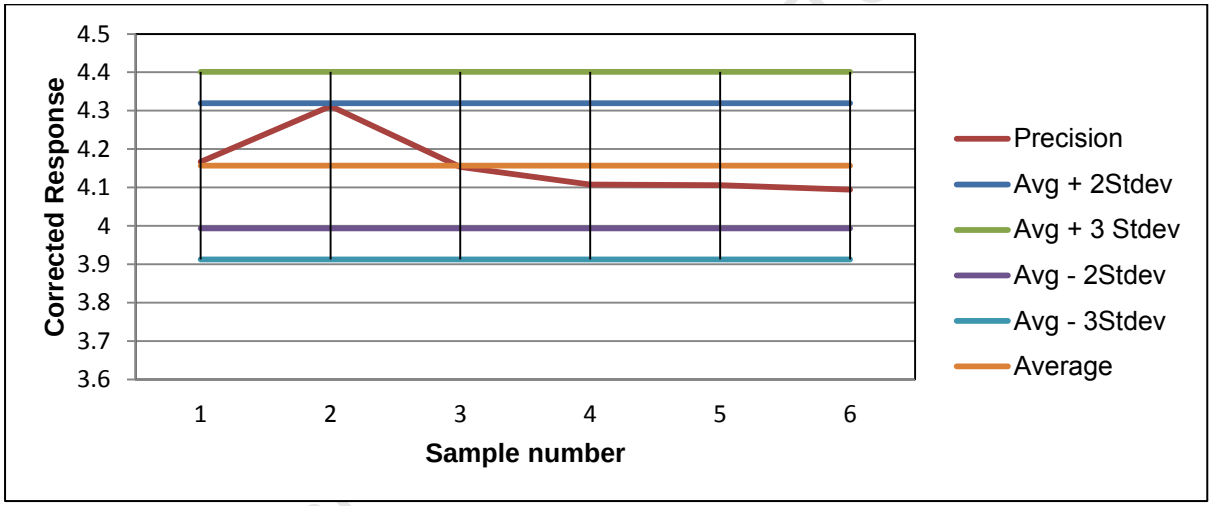

Figure 5.12 Ribitol precision 0.2 mg/ml

Repeated analysis of the same sample yielded a % RSD < 2.0%.

Table 5.8: Results obtained for the PRP precision study

Concentration PRP (µg/ml)	Total Ribitol (mAU)	I/S response (mAU)	Corrected Response
20	4013177	8024147	0.500138
20	3844254	7797555	0.493008
20	3495964	8389597	0.416702
20	4097227	8085140	0.50676
20	3962510	8413781	0.470955
20	120928	207006	0.584176
Average	3255677	6819538	0.49529
Stdev	1549996	3247818	0.05446
% RSD	47.60903	47.6252	10.99552

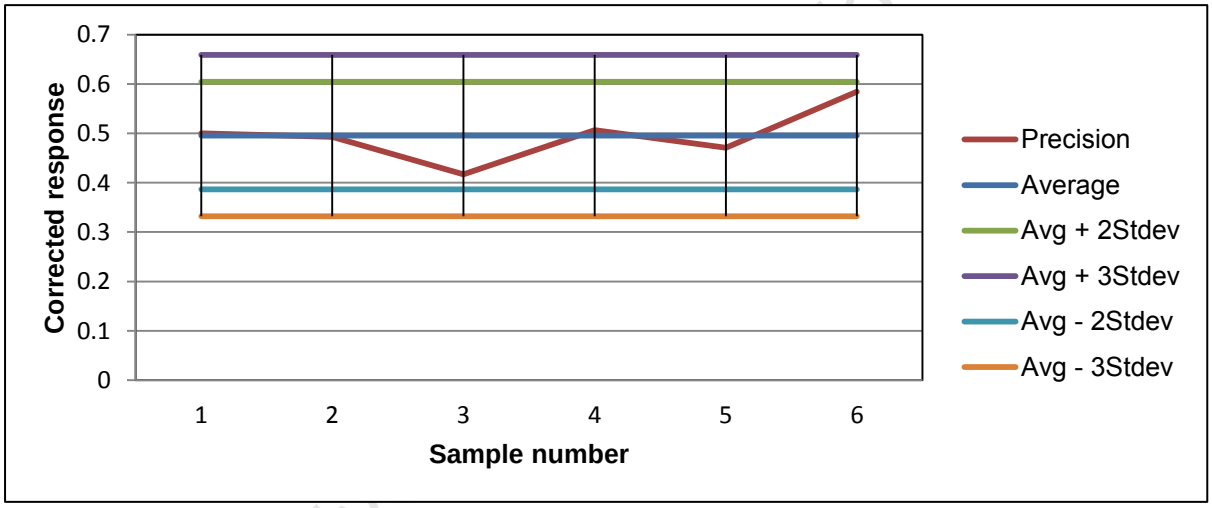

Figure 5.13 PRP precision study

Repeated analysis of the same sample yielded a % RSD < 11.

There was a significant difference in response between analysis of the sixth PRP sample and the rest. The response for the analyte, ribitol and the internal standard, myo–inositol was considerably less. The significance of the internal standard is illustrated here as the response for both decreased proportionally. The corrected response for the analyte however differs significantly from that of the other PRP samples. This value was discarded and removal from the % RSD calculation yielded a value of 7.66.

Statistical process control charts requires ten points for plotting action and alert limits. The precision study was performed in accordance with the ICH Q2 R1 guideline and only six replicates were analysed.[24] Control limits were calculated using the average and standard deviation of the six analyses.

5.3.4 Accuracy

Accuracy was assessed by the triplicate analyses of 3 different concentrations from the lower, middle and upper region of the linear range. The average, standard deviation and %RSD of each sample set was determined. 0.5 µg/ml, 5 µg/ml and 20 µg/ml ribitol in purified water were analysed. The internal standard concentration was 10.0 µg/ml. The result of the 5 µg/ml solution was treated as the "actual" concentration.

Table 5.9: Results obtained for accuracy study

Concentration (µg/ml)	**Ribitol (mAU's)**	**Internal Standard (mAU's)**	**Corrected**
0.5	35101	673608	0.052108942
	33634	636747	0.052821607
	33052	760903	0.043437863
Average	33929	690419.3333	0.049456137
Stdev	1055.873572	63762.40099	0.005224145
% RSD	3.112009113	9.235315107	10.56318923
5.0	345346	720948	0.479016517
	322645	779383	0.413974901
	321368	676903	0.474762263
Average	329786.3333	725744.6667	0.455917894
Stdev	13490.18541	51408.10839	0.036385927
% RSD	4.090583522	7.083497923	7.980806891
20.0	1350336	669744	2.016197234
	1442974	732930	1.968774644
	1521265	852542	1.784387162
Average	1438191.667	751738.6667	1.92311968
Stdev	85564.79296	92839.11706	0.122463297
% RSD	5.949470779	12.34991909	6.367949864

The response obtained for the 5 µg/ml concentration was used to calculate the 0.5 µg/ml and 20.0 µg/ml % Recovery.

% Recovery = (Actual/ Theoretical) X 100%

0.5 µg/ml = [(0.0495 X 10)/ 0.456] X 100
= 108.55 %

20.0 µg/ml = [(1.92/4)/0.456] X 100
= 105.26 %

The triplicate analysis of the 0.5 µg/ml solution yielded a %RSD of 10.56% that of the 5.0 µg/ml solution was 7.98 % while that of the 20 µg/ml solution was 6.37%. The %RSD's for the triplicate analysis of the three standards decreased with an increase in concentration. The values obtained for the lower concentrations correlates with those obtained for the precision study in section 5.3.3. The recoveries for the 0.5 µg/ml and 20.0 µg/ml solutions, using the 5.0 µg/ml solution as reference, were 108.55% and 105.26% respectively. The recoveries obtained for the accuracy study were within acceptable levels.

5.3.5 Robustness

In order to determine the effect of incubation time on the analyte a PRP sample was subjected to methanolysis over different time periods. Variation of incubation time during derivatisation was also evaluated. In addition the washing of the sample post methanolysis was investigated.

Derivatisation incubation time did not have a significant impact on the analyte response. Washing with methanol was a critical step as a reduction in the amount of methanol or the number of washing steps resulted in the formation of white precipitation. Yields of both the internal standard and ribitol were low.

Figure 5.13 illustrates the relationship between incubation time and response.

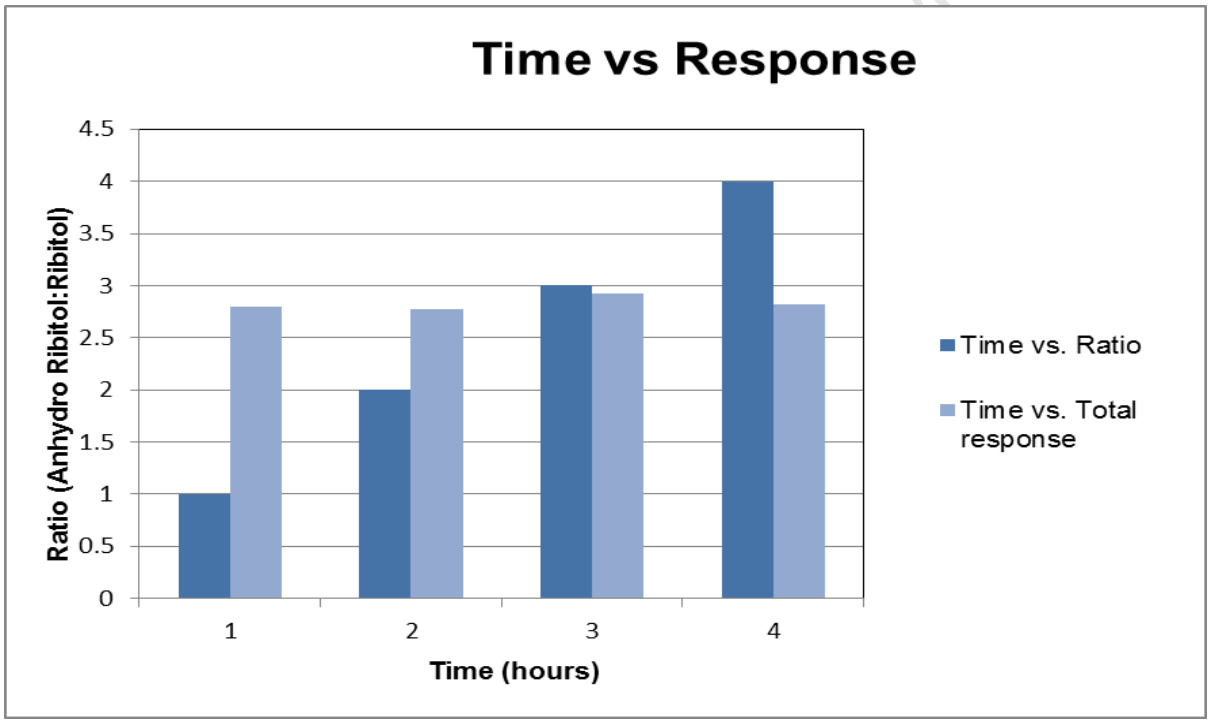

Figure 5.14 Relationship between response and incubation

Incubation temperature was kept constant at 80 °C throughout The sample analysed, PRP batch P41, was prepared at a concentration of 225 µg/ml P41 and 50 µg/ml myo-inositol. Increased incubation time resulted in an increase in the formation of 1, 4 -anhydroribitol. The total response for ribitol however remained more or less constant. This corresponds to reports in literature where acid treatment of ribitol resulted in conversion of ribitol to 1, 4 -anhydroribitol with time.[19, 20, 24]

5.3.6 Free Polysaccharide Quantification

The quantification of free PRP in Hib conjugate vaccine was established in the R&D department, at TBI, using colorimetric assays. DOC precipitation was used to separate conjugated from free PRP. The ability of the GC-FID method to detect free saccharide after DOC precipitation was evaluated. A solution of conjugate with concentration ± 80 µg/ml was spiked with 5.9 µg/ml polysaccharide. Precipitation using 1.0 ml of the solution was performed with a 10 µg/ml deoxycholic acid sodium salt (DOC) solution adjusted to pH 6.8 with 1M hydrochloric acid (HCl). A volume of 50 µl 1M HCl was added after 30 minute incubation at 2 °C – 8 °C. The supernatant was removed after centrifugation. To 1.0 ml of the supernatant added 1.0 ml of 10 µg/ml internal standard and analysed. Quantification was performed by comparison with a 10 µg/ml ribitol standard containing 10 µg/ml internal standard.

Table 5.10 Free saccharide quantification

Sample	**Response (mAU)**	**I/S response (mAU)**	**Corrected Response**	**Calculated Ribitol Concentration** (µg/ml)
Spiked	491854	1294671	0.3799066	3.799
	428796	1298281	0.3302798	3.303
Average	460325	1296476	0.3550932	3.551
Stdev	44588.74	2552.655	0.0350914	0.351
%RSD	9.686361	0.196892	9.8823101	9.882
Polysaccharide	431476	1572892	0.2743202	2.743
	444528	1503871	0.2955892	2.956
	440424	1537258	0.2864997	2.865
Average	438809.3	1538007	0.2854697	2.855
Stdev	6674.132	34516.6	0.0106719	0.107
%RSD	1.520964	2.244242	3.7383491	3.738

The % free saccharide for the conjugate was determined as 1.01% equating to 0.81 µg/ml ribitol.

Recovery was calculated by: $\frac{[\text{Polysaccharide}]}{[\text{Spiked}] - [\text{Free}]} \times 100 = 104.2\%$

The recovery for the free saccharide quantitation was 104.2%. This illustrates that the GC-FID method was able to detect the precipitated polysaccharide.

5.4 Conclusion

Methanolysis of the Hib polysaccharide using 3N methanolic HCL at 80 °C for 2 hours was sufficient to release ribitol and minimize the formation of 1, 4 -anhydroribitol. Myo-inositol was used as an internal standard as it is readily available, easily dissolved in water and was stable throughout the sample preparation process. The internal standard was added before freeze drying and was subjected to the same conditions as the analyte. Injector reproducibility as well as losses during sample preparation could be accounted for.

Washing of the sample after methanolysis and drying using pure, dry nitrogen was shown to be a critical step prior to derivatisation. The silylating agent reacts with excess methanol and moisture thus drying using moisture free nitrogen prevents loss in yield.

Methanolysis of the polysaccharide yielded the 1, 4 -anhydroribitol derivative in varying quantities and was accounted for by adding the response to that of the ribitol response. The 1, 4 -anhydroribitol derivative was identified by subjecting a ribitol standard to methanolysis. Subsequent analysis yielded an additional peak in the chromatogram of the ribitol standard with a relative retention time of ± 0.72 to the ribitol peak. The total response for the two peaks, in the chromatogram of the ribitol sample subjected to methanolysis, equalled that of the ribitol sample not subjected to methanolysis. Prolonged methanolysis of PRP resulted in an increase in response of this peak and a proportionate decrease in response for ribitol. This observation corresponds to that listed in literature. [19, 20, 24]

The analysis was linear from a lower concentration of 0.25 µg/ml to 30.0 µg/ml with a R^2 value of 0.9921 and at a higher concentration of 0.01 mg/ml to 1 mg/ml with a R^2 value of 0.9937. A linear curve was also constructed using concentrations of PRP ranging from 0.92 µg/ml to 18.4 µg/ml resulting in a R^2 value of 0.9944. Spike recovery experiments were conducted using both ribitol and PRP. Recoveries varied from 87.2% to 112.2%. Precision was determined using a 5 µg/ml and a 0.2 mg/ml ribitol solution. The % RSD's were 7.68% and 1.96% respectively. Precision was also evaluated using a 20 µg/ml PRP solution giving a % RSD of 11.0%.

Quantification of both total and free PRP is possible. The response for both the 1, 4 -anhydroribitol and ribitol were used. Sample preparation is relatively long as the freeze drying process is lengthy. Methanolysis and derivatisation takes a considerable amount of time as well. The analysis of PRP, using ribitol as a standard, by means of GC-FID is precise, accurate and specific. The use of an autosampler increased productivity by enabling

the analysis of multiple samples. The GC-FID is a conventional equipment item in the pharmaceutical industry and the expense of maintenance as well consumables are relatively low. Standards used to generate calibration curves are readily available. All the reagents used are commercially available removing the need for in-house preparation. The derivatisation reagent is commercially available in 1.0 ml glass ampoules which allows for a single use approach eliminating the possibility of moisture contamination due to storage.

The analysis is based on a method developed for the qualitative and quantitative analysis of pneumococcal vaccines using GC-MS. The use of mass spectroscopy enables the quantification of lower levels of analyte. The GC-FID analysis is however suitable for the analysis of Hib as a monovalent vaccine as ribitol concentrations as low as 0.25 µg/ml can be quantified. The analysis of Hib, as a component of a multivalent vaccine, using this technique, requires further studies.

5.5 References

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Chapter 6

Conclusions

Quantitative assays were developed for the quality control of the Hib manufacturing process. The chromatographic methods made use of a conventional gas chromatograph with flame ionisation detection (GC-FID) and using a CTC PAL combination autosampler/headspace sampler as the injector. Internal standards were used to accommodate for losses during sample preparation as well as that due to possible lack of injector reproducibility. The development was performed using current analytical method validation practices.

The headspace analysis of volatile residuals in pharmaceuticals using a GC-FID is a well-established technique. Residual ethanol analysis using iso-propanol as the internal standard were evaluated for application in bulk purified polyribosylribitolphosphate (PRP). Sodium sulfate was used to increase the availability of the analyte and internal standard in the headspace of the vial. Chromatograms generated yielded baseline separation with a United States Pharmacopoeial (USP) calculated resolution of more than 2.0 and peaks with little tailing or fronting. Analysis of the samples using a J&W Scientific DB 624 capillary column generated validation data within acceptable levels. The results generated were accurate, precise and specific for the analyte and can be implemented in routine quality control of bulk purified PRP.

A GC-FID direct injection method was developed to determine ethylene glycol in activated PRP. An internal standard, 3-bromo-1-propanol, was added prior to removal of moisture with a mixture of 2, 2-dimethoxypropane: N, N-dimethylformamide: acetic acid. The reaction between water and 2, 2-dimethoxypropane, while using acetic acid as the catalyst, resulted in the formation of methanol and acetone. N, N-dimethylformamide acted as a trap preventing the loss of ethylene glycol when drying. A commercially available drying and heating unit, React-vap/Reacti-therm unit, was used to reduce the volume of the samples through the evaporation of the volatile acetone and methanol components. Mild heating and dry nitrogen gas were used to aid evaporation. The derivatisation was performed using BSTFA: TMCS and samples were injected via the direct injection autosampler into the injection port. The sample preparation was relatively short and the analysis using a J&W Scientific HP5 capillary column was selective, precise and accurate.

The analysis of PRP was performed using methanolic hydrochloric acid to generate stable methyl glycosides through methanolysis. Derivatisation was performed using a trimethylsilylating reagent. Methanolysis and derivatisation were performed on the React-

vap/Reacti-therm unit. The test method was applied to the quantification of total and unconjugated Hib polysaccharide. Quantification was performed by taking into account the response for ribitol and 1, 4 –anhydroribitol. The linear range was established using ribitol standard solutions. Quantification of PRP was performed in both purified bulk and conjugate bulk. The analytical test procedure provided accurate and specific results. The ability to detect the polysaccharide after precipitation with deoxycholic acid was evaluated. A spike recovery experiment yielded accurate results. The application of this technique to the analysis of a multivalent vaccine should be evaluated in future. In addition, the method of analysis should be expanded by using a GC-MS. Detection limits of a GC-MS are lower and additional information regarding identity of peaks is possible.

An internal standard was used for each of the analyses. The substances were added prior to sample analysis and in some instances prior to sample manipulation. There are large variations in the responses of the internal standards. These variations are accompanied by similar fluctuations in analyte response. The purpose of the internal standard is illustrated here as losses due to poor injector reproducibility or sample manipulation can be accounted for. The calculation of the ratio between internal standard response and analyte response instead of using the individual responses is more accurate.