

**Determination of Molecular Size Distribution of Meningococcal
Polysaccharide Vaccine by Size Exclusion Chromatography Method**



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AUGUST, 2016

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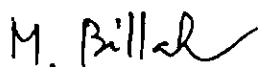
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DECLARATION

I hereby declare that this thesis is based on my original work except quotations and citations which have been duly acknowledged. The material here has not ever been submitted in a whole or in a part for a degree from Khulna University or any other institutions. This research work will not be published anywhere without the concern of supervisor.



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DEDICATED TO

MY BELOVED PARENTS

&

ALL THE LIGHT BEARERS IN MY LIFE

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The Author

ABSTRACT

Neisseria meningitidis, the leading cause of bacterial meningococcal disease has been considered as a serious public health threat worldwide due to its higher rate of morbidity and mortality. The capsular polysaccharides of these bacteria are potential vaccine candidates being efficacious to provide protection against meningococcal disease. Tetravalent vaccine is manufactured from distinct polysaccharides of serogroup A, C, W and Y that has been clinically proved to be a suitable vaccine against broad range of meningococcal infections. The molecular size distribution of polysaccharide vaccine is a critical parameter which is directly correlated with the immunogenicity. In the present study, the distribution coefficient value of meningococcal polysaccharide serogroup A, C, W and Y were evaluated by using a size exclusion chromatography method followed by a rapid and reliable quantitative rocket immunoelectrophoresis assay. The analyses were performed by high performance liquid chromatography system AKTA Purifier 100 on a XK 16/70 (700mm×16mm) column packed with cross linked Sepharose CL-4B for determination of molecular size distribution of eight different batches of Ingovax® ACWY, a meningococcal polysaccharide vaccine produced by Incepta Vaccine Ltd, Bangladesh. The calculated mean distribution coefficient and standard deviation values of serogroups A, C, W and Y were found to be 0.28 ± 0.14 , 0.20 ± 0.09 , 0.19 ± 0.11 and 0.14 ± 0.12 respectively and met the requirements of British Pharmacopeia. The results also included the calculation and comparative analysis of distribution coefficient value of each serogroup. The method was proved to be robust for determining the distribution coefficient values which is an obligatory requirement for vaccine lot release. In fine, the study provided the information on distribution coefficient values of the meningococcal polysaccharide vaccine whether they comply with the recommendation of British Pharmacopeia.

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CHAPTER ONE
INTRODUCTION

1.0 INTRODUCTION

Neisseria meningitidis is a fastidious, encapsulated, aerobic gram-negative diplococcus bacterium that exclusively infects humans. In 1887, Weichselbaum was the first to identify the meningococcus from the cerebrospinal fluid (CSF) of a patient with meningitis (Weichselbaum, 1887). Epidemics of meningococcal meningitis were first reported during the early nineteenth century in Geneva, Switzerland (Vieusseux, 1805), New Bedford, Massachusetts (Danielson and Mann, 1806) and in the early twentieth century in the African meningitis belt (Greenwood, 1999). At least 13 distinct meningococcal groups have been described so far on the basis of their immunological reactivity and structure of the capsule's polysaccharide. These serogroups are A, B, C, E-29, H, I, K, L, W-135, X, Y, Z, and Z'-29E. Only six serogroups (A, B, C, W-135, X, Y) cause life threatening disease. Serogroup identification is done by slide agglutination or polymerase chain reaction (PCR) assays, while other meningococcal typing is performed using monoclonal antibodies (mAbs), PCR, and DNA sequencing (Cohn and Harrison, 2013). Meningococcal disease has a high mortality rate and survivors are at high risk of having permanent sequelae like mental retardation, hearing loss, scars and amputations (Oostenbrink *et al.*, 2002; Scholten *et al.*, 1994). This infection occurs most commonly in children younger than 1 year, in those aged 15 to 25 years, and in the elderly persons. In 15 to 25 year olds, the annual incidence of infection is 3 in 100000. Over a 4 year period, the incidence of infection is 12 in 100000. Death occurs in 10% of appropriately treated patients, and permanent sequelae occur in another 15% (Soriano-Gabarro *et al.*, 2002). In most cases, serogroups A, C, W and Y cause meningitis and protective vaccines are available against these serogroups. The relative dispersion of each serogroup varies over time and geographical location. In African 'meningitis belt' the magnitude outbreaks was surveilled for serogroups A and C. Serogroup W135 was a menace for Hajj pilgrims in 2000 and 2001, respectively. In recent time, the epidemiological surveillance showed that serogroup Y meningococcal disease had rapidly increased in some parts of Europe (Lingappa *et al.*, 2003; Hahne, 2001; Broker *et al.*, 2012). Outside Africa, only Mongolia reported a large epidemic in the year of 1994-95. Large outbreaks of serogroup A historically occurred in China (Wang *et al.*, 1992), Nepal, India, and Russia but had recently been replaced by localized disease due to serogroups B and C. The incidence in Japan since World War II has fallen to very low levels, ~0.1 case per 100,000. Serogroups B and C are dominant now in Australia with a prolonged serogroup B epidemic in New Zealand occurring in the 1990s.

There is increasing evidence of serogroup W being associated with outbreaks of considerable size (Atlas *et al.*, 1997). In 2002, W was also emerged in Burkina Faso, striking 13,000 people and killing 1,500. The highest burden of meningococcal disease occurs in sub-Saharan Africa, which is known as the “Meningitis Belt”, an area that stretches from Senegal in the West to Ethiopia in the east, with an estimated total population of 300 million people (Goebel *et al.*, 1956). This hyper-endemic area is characterized by particular climate and social habits. During the dry season, between December and June, because of dust in winds and upper respiratory tract infections due to cold nights, the local immunity is diminished, increasing the risk of meningitis (Ajello *et al.*, 1984; Jennings, 1990; Artenstein *et al.*, 1970). At the same time, the transmission of *N. meningitidis* is favored by overcrowded housing at the family level and by large population displacements due to pilgrimages and traditional markets at the regional level. This conjunction of factors explains the large epidemics which occur during this season in the meningitis belt area (Peltola *et al.*, 1997). In 2006 and 2007, outbreaks of the disease occurred in the North of Ivory Coast and the southern region of Burkina Faso, Southern Sudan killing several children and adults. Meningococcal meningitis is not only important in Africa but also throughout the world (Fu *et al.*, 1995). It is considered an important disease not only for sub-saharan Africa but also for North America, UK, Ireland, Europe, South East Asia, the Middle East, and New Zealand.

In Bangladesh, a study was conducted at four hospitals major (250–350 beds), public, tertiary care hospitals from June 2003 to July 2005 to investigate the etiologies of bacterial meningitis. A total of 2,609 patients met the clinical case definition, and 766 had cerebrospinal fluid tested by latex agglutination, 16S rRNA gene sequencing, or real-time polymerase chain reaction for *N. meningitidis* A and C, culture results were noted from patient records. From the results, in total, 189 patients (24%) of those tested, representing all age groups, were diagnosed with bacterial meningitis; 136 (18%) had meningococcal infection. Case-fatality ratios were 10% for *N. meningitidis*. Bacterial meningitis from vaccine-preventable pathogens causes significant morbidity and mortality in Bangladesh in adults and children (Gurley *et al.*, 2009).

However, to reduce the chance of high mortality an efficacious polysaccharides vaccine of meningococcal disease has been clinically proved to be a suitable vaccine. The capsular polysaccharides of *N. meningitidis* are attractive vaccine candidates because they constitute the most highly conserved and most exposed bacterial-surface antigens. The use

of capsular polysaccharides as immune-prophylactic agents against human disease caused by encapsulated bacteria is poorly established. The capsular polysaccharides of the meningococcus are negatively charged and obtained in a high molecular-weight immunogenic form by precipitation (Romero and Outschoorn, 1994). In industrialized countries, meningococcal disease incidence varies by age, with infants <1 year at the greatest risk and, in some countries, a second peak in incidence observed among adolescents (Cohn *et al.*, 2010; Ramsay, *et al.*, 1997). However, cases occur in all age groups, making the development of vaccination strategies challenging. To prevent disease among young infants, protection needs to be achieved early in life. Adolescents also have relatively high rates of disease and are the most frequent carriers of *N. meningitidis* (Caugant *et al.*, 2009). Therefore, vaccine strategies need to include this age group as well. Vaccines that target adolescents should reduce nasopharyngeal carriage to impact transmission, creating herd protection. The wide range of meningococcal vaccines, in terms of serogroups included and age group targeted, allows countries to make vaccination decisions that would best address their specific disease epidemiology and prevention goals.

In this present study, a rapid and reproducible size exclusion chromatography method for determination of molecular size distribution of meningococcal polysaccharide (PS) vaccine was described. The experiments mainly focused on calculation and comparative analysis of distribution coefficient (K_D) value of each serogroup of eight different batches of meningococcal PS vaccines. In fact, this study was conducted for the estimation of the K_D values of the meningococcal PS vaccine whether they complied with the recommendations of British Pharmacopeia (BP).

CHAPTER TWO

BACKGROUND

2.0 BACKGROUND

2.1 Classifications of Meningococcal Serogroups

N. meningitidis can be classified into serogroups based on the immunologic reactivity of their capsular polysaccharides, which are the basis for currently licensed meningococcal vaccines (Giancetti *et al.*, 2015). Several different bacteria can cause meningitis. *N. meningitidis* is the one with the potential to cause large epidemics. There are 13 serogroups of *N. meningitidis* that have been identified, 6 of which (A, B, C, W, X and Y) can cause epidemics (Harrison *et al.*, 2009). Geographic distribution and epidemic potential differ according to serogroup. In most cases of meningococcal disease are caused by serogroups A and C, for which polysaccharide vaccines are effective, and serogroup B, which has a polysaccharide capsule that is poorly immunogenic in humans. The capsular polysaccharide is either a homopolymer or a heteropolymer consisting of monosaccharide, disaccharide, or trisaccharide repeating units. The main meningococcal capsular polysaccharides associated with invasive disease, except for serogroup A, are composed of sialic acid derivatives; the serogroup A capsule consists of repeating units of *N*-acetylmannosamine-1-phosphate. Meningococci are further classified on the basis of their class 1 outer-membrane proteins (serosubtype), class 2 or 3 outer-membrane proteins (serotype), and lipooligosaccharides (immunotype) (Shan *et al.*, 2015). Meningococci also have the capacity to exchange the genetic material responsible for capsule production and thereby switch from serogroup B to C or vice versa. Capsule switching may become an important mechanism of virulence with the widespread use of vaccines that provide serogroup-specific protection (Swartley *et al.*, 2015).

The purified meningococcal polysaccharide (ACWY) (PS) vaccine is derived from meningococci of serogroup A (*O*-acetylated repeating units of *N*-acetylmannosamine, linked with $1\alpha\rightarrow6$ phosphodiester bonds), C (*O*-acetylated repeating units of sialic acid, linked with $2\alpha\rightarrow9$ glycosidic bonds), W (*O*-acetylated alternating units of sialic acid and D-galactose, linked with $2\alpha\rightarrow6$ and $1\alpha\rightarrow4$ glycosidic bonds) and Y (*O*-acetylated alternating units of sialic acid and D-glucose, linked with $2\alpha\rightarrow6$ and $1\alpha\rightarrow4$ glycosidic bonds). The World Health Organization and British Pharmacopoeia requirements for *O*-acetyl content of purified polysaccharide for serogroup A and serogroup C are not less than 2 mmol and 1.5 mmol per gram. In case of Y and W polysaccharides the *O*-acetyl content are 0.3 mmol per gram (WHO & BP).

2.2 Microbiology of the Capsular Polysaccharides of *N. meningitidis*

Humans are the only natural reservoir of *N. meningitidis*, and the nasopharynx is the site from which meningococci are transmitted by aerosol or secretions to others. Meningococci overcome host defenses and attach to the microvillous surface of nonciliated columnar mucosal cells of the nasopharynx, where they multiply (i.e., colonize) (Shirley *et al.*, 2015). Pili are the major adhesins that may target the CD46 receptor, a membrane cofactor protein; subsequently, the opacity-associated proteins, Opa and Opc (Levy *et al.*, 2015) bind to CD66 (Gibson *et al.*, 2015) and heparan sulfate proteoglycan receptors, respectively. Binding stimulates engulfment of the meningococci by epithelial cells, which may then traverse the mucosal epithelium through phagocytic vacuoles. The survival of meningococci in the epithelial cells may be promoted by the IgA1 protease and by porB (Harcourt *et al.*, 2015). Five to 10 percent of adults are asymptomatic nasopharyngeal carriers of strains of *N. meningitidis*, (Nolan *et al.*, 2015) most of which are not pathogenic. In a small number of persons, *N. meningitidis* penetrates the mucosa and gains access to the bloodstream, causing systemic disease (Jozefczuk *et al.*, 2015). In most persons, however, carriage is an immunizing process, resulting in a systemic protective antibody response (Lalwani, *et al.*, 2015).

2.3 Pathogenesis of Bacterial Meningitis

Bacterial meningitis is the most common serious infection of the central nervous system. Infection of the subarachnoid space leads to cerebrospinal fluid (CSF) inflammation, meningeal irritation, and the clinical triad of headache, fever, and meningismus (Leib and Tauber, 1999). Bacterial meningitis is recognized as one of the top ten causes of infection-related death worldwide, and about 30–50% of the survivors sustains neurological sequelae. A key factor that contributes to this high mortality and morbidity is our incomplete understanding of the pathogenesis of this disease. For example, our current knowledge about the pathogenic mechanisms that contribute to central nervous system (CNS) complications and neuronal injury are largely derived from experimental models of meningitis, such as direct injection of bacteria into the cerebrospinal fluid (CSF) and bypassing of the blood–brain barrier (BBB). However, bacterial meningitis usually develops through several steps of bacteria–host interactions. These include mucosal colonization by the pathogen (usually upper respiratory or gastrointestinal tract), microbial invasion of the intravascular space, and multiplication resulting in bacteraemia. Bacteria then traverse the BBB, surviving and multiplying in the subarachnoid space to elicit

inflammatory responses from the host, and pathophysiological alterations such as pleocytosis and BBB disruption. Other routes of bacterial entry into the CNS, which occur less commonly, include spread from a contiguous source of infection (such as in cases of sinusitis or mastoiditis) and direct inoculation into the CSF (such as in cases of skull fracture or ventricular shunts). The molecular and cellular mechanisms that are involved in these sequential steps of bacteria–host interactions are unclear. Similarly, the downstream events that lead to cell death elicited by bacterial meningitis remain incompletely understood. Studies in experimental animals and humans have shown that two forms of neuronal injury are predominant after episodes of bacterial meningitis: necrotic cortical injury and apoptotic hippocampal injury, but the development of feasible therapeutic strategies to decrease neurological sequelae of bacterial meningitis remains a challenge (Kim, 2003).

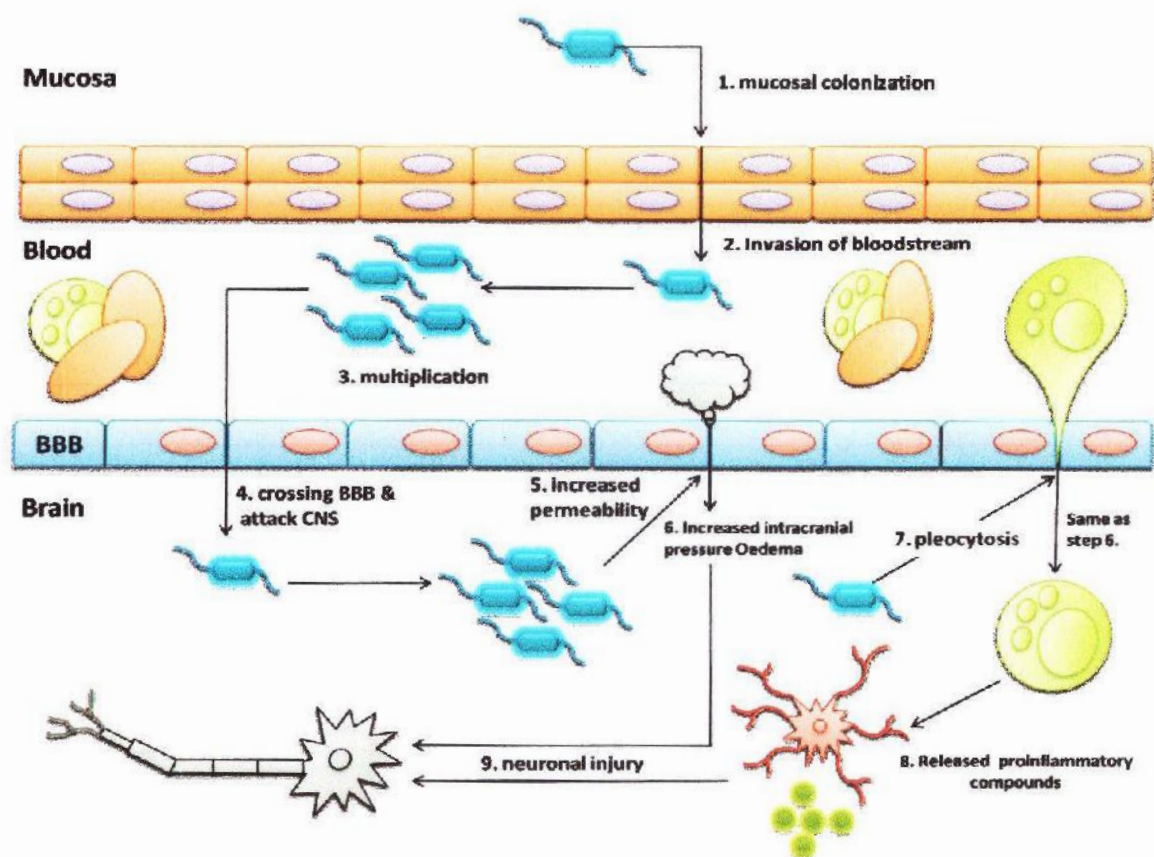


Figure 1: Pathogenesis of bacterial meningitis.

Figure 1 showed the bacteria-host interactions during the pathogenesis of bacterial meningitis. At first, mucosal colonization occurred in mucosa and transferred to invade the bloodstream. The bacteria then survive, multiply and crossing the blood brain barrier

(BBB). Invasion occurred in central nervous system and subsequently, bacteria induced an increased permeability of the BBB. Pleocytosis occurred and lead to oedema and increased intracranial pressure. Therefore, proinflammatory compounds released from infiltrated white blood cells and other host cells. And finally, this process lead to neuronal injury.

2.4 Meningococcal Tetravalent Polysaccharide Vaccines

There are several combinations of polysaccharide vaccines used globally, including bivalent (A, C), trivalent (A, C, W) and tetravalent (A, C, Y, W) vaccines. The first polysaccharide vaccines were developed at Walter Reed Army Institute and implemented in military recruits to prevent recurrent outbreaks among young soldiers (Gotschlich *et al.*, 1969). Polysaccharide meningococcal vaccines are immunogenic and safe. They are highly effective in closed populations of adults at high risk for disease including military recruits and household contacts of affected individuals (Gold and Artenstein, 1971; Biselli *et al.*, 1993; Greenwood *et al.*, 1978) and in outbreak control (Rosenstein *et al.*, 1998). They proved to be safe and effective in preventing group C disease in U.S. military recruits, and in controlling group A epidemics during mass campaigns in Africa. A bivalent PS vaccine against groups A and C is available worldwide. Since 2003, the W serogroup of *N. meningitidis* has emerged as a potential epidemic strain in countries of the meningitis belt (Mayer, *et al.*, 2000); prompting the development of trivalent A,C,W and the increased use of tetravalent A, C, W & Y PS vaccines (GSK and Sanofi-Pasteur). During the epidemic season in the African meningitis belt, vaccine from an international stockpile is made available to countries through the International Coordinating Group on Vaccine Provision for Epidemic Meningitis (ICG) set up in 1997 by WHO and its partners. However, PS vaccines are poorly immunogenic in young infants and children less than 2 years old, who are the major group at risk for these infections. In a recent study of a tetravalent PS vaccine in Saudi Arabia involving 423 children under 5 years of age, bactericidal antibody responses to group A and W, *N. meningitidis* were observed in about 90% and 60% 2 to 4 year old children, respectively, but only in 25% and 13% of 6 and 12 month old infants, respectively (Al-Mazrou, *et al.*, 2005).

The immunogenicity and clinical efficacy of the serogroups A and C meningococcal vaccines have been well established. The serogroup A polysaccharide induces antibody response among certain children as young as 3 months, although a response comparable with that occurring in adults is not achieved until age 4 to 5 years; the serogroup C

component is poorly immunogenic among recipients aged <18 to 24 months (Gold *et al.*, 1979; Peltola *et al.*, 1978). The serogroups A and C vaccines have demonstrated estimated clinical efficacies of >85% among school-aged children and adults and are useful in controlling outbreaks (Rosenstein *et al.*, 1998). Serogroups Y and W-135 polysaccharides are safe and immunogenic among adults and children aged >2 years (Griffiss *et al.*, 1982); although clinical protection has not been documented, vaccination with these polysaccharides induces production of bactericidal antibodies. The antibody responses to each of the four polysaccharides in the tetravalent vaccine are serogroup specific and independent. Persons whose spleens have been removed because of trauma or nonlymphoid tumors and persons who have inherited complement deficiencies have acceptable antibody responses to polysaccharide meningococcal vaccine (Beek, 2000). A study indicated that tetravalent polysaccharide vaccine substantially reduced the incidence of invasive meningococcal disease among patients with terminal complement deficiency compared with similar patients who were unvaccinated (Platonov *et al.*, 2003). Reduced clinical efficacy has not been demonstrated among persons who have received multiple doses of vaccine. However, recent serologic studies have reported that multiple doses of serogroup A and C polysaccharide vaccine might cause immunologic hypo-responsiveness (i.e., a reduced antibody response after subsequent challenge with the same polysaccharide antigen) to group A (Borrow *et al.*, 2001) and C (MacDonald *et al.*, 1998) polysaccharide. The clinical relevance of such hypo-responsiveness is still unclear.

However, among infants and children aged <5 years, measurable levels of antibodies against group A and C polysaccharides decreased substantially during the first 3 years after a single dose of vaccine; among healthy adults, antibody levels also decreased, but antibodies were still detectable <10 years after vaccine administration (Artenstein 1971; Lepow *et al.*, 1986, 1999). Similarly, although vaccine-induced clinical protection likely persists among school-aged children and adults for >3 years, the efficacy of the group A vaccine among children aged <5 years might decrease markedly within this period. In one study, efficacy among children aged <4 years at the time of vaccination declined from >90% to <10% within 3 years after vaccination; efficacy was 67% among children who were aged >4 years when vaccinated (Reingold *et al.*, 1985).

2.5 Size Exclusion Chromatography

The idea of molecular size based separation technique, also known as size exclusion chromatography (SEC) was first introduced by Synge and Tiselius (Synge, 1950). It is a widely used technique for determining the molecular size distribution (MSD) of polymeric materials (e.g. proteins, nucleic acids, polysaccharides) (Mori and Barth, 2013). Size-exclusion chromatography (SEC) is a popular method to separate biomolecules based on their size. Primarily, it is applied to the separation of biopolymers such as proteins and nucleic acids, i.e. water-soluble polymers. This system is also called gel filtration, typically with beads of dextran or agarose serving as gel matrix. Smaller molecules pass significantly slower through the column than larger molecules (Skoog *et al.*, 2011). Not to be mixed up with gel electrophoresis, there are big differences in terms of the separation principle. SEC does not require electric current and the sieving effect will not separate small molecules first. Proteins in solution, or other macromolecules, are applied to a column with a defined support medium. The behavior of the protein depends on its size and that of the pores in the medium. If the protein is small relative to the pore size, it will partition into the medium and emerge from the column after larger proteins. Besides a protein's size, this technique can also be used for protein purification, analysis of purity and study of interactions between proteins (Irvine, 2001). Chromatography column and media selection are indispensable for determination of molecular size distribution (MSD) due to the variability in column sizes and media specifications. In one study, McCauley and his co-authors suggested that Sepharose CL-2B rather than Sepharose showed better response for MSD of bacterial capsular polysaccharides in regard to performance (McCauley *et al.*, 1981). Other studies showed that SEC with Sepharose had been used for MSD analysis (Scheers and Dobbelaer, 1984; Newland, 1989) while Sepharose CL-4B or sepharose is a widely used for evaluation of MSD as guided by WHO and BP (WHO and BP).

Sepharose CL-4B is a well-proven cross-linked agarose gel filtration base matrix and is frequently used for coupling affinity ligands to the matrix. The matrix is not pre-activated and the user performs all steps in coupling. Some features of this resin are:

- 4% cross-linked agarose gel filtration media.
- Proven base matrix for coupling affinity ligands
- Higher degree of crosslinking improve matrix stability

Sepharose is available with 3 different agarose contents; 2, 4, and 6%, designated Sepharose 2B, Sepharose 4B and Sepharose 6B respectively, Both Sepharose and Sepharose CL have broad fractionation ranges which make them suitable for characterizing or cleaning-up samples containing components of diverse molecular weight.

Sepharose CL gels are cross-linked derivatives of Sepharose 2B, Sepharose 4B and Sepharose 6B. The cross-linked form of Sepharose is chemically and physically more resistant than Sepharose itself, offering the same selectivity with better flow characteristics. Cross-linked Sepharose gels are resistant to organic solvents and are thus the choices for separations in organic solvents (GE healthcare, 2006).

2.6 Molecular Size Distribution

The molecular size distribution is an important physico-chemical characteristic for monitoring both batch to batch consistency during the manufacturing steps and the stability of vaccine preparation (Clement *et al.*, 2013). The molecular size of the polysaccharide vaccine is an important parameter in establishing consistency of production and in studying stability during storage (Suker *et al.*, 2004). The size distribution specifications will be product-specific and should be consistent with lots shown to be immunogenic in clinical trials (Hsieh, 1999). The molecular size of purified polysaccharide provides an indication of the manufacturing consistency. The assay and an acceptable level of consistency should be in agreement with the national regulatory authority. The distribution coefficient (K_D) can be determined by measuring the molecular size distribution of the polysaccharide at the main peak of the elution curve obtained by a suitable chromatographic method. The K_D value and the mass distribution limits should be established and shown to be consistent lot-to-lot for a given product chromatographed on a specific column material. Gel filtration through Sepharose CL-4B or CL-2B (or similar) in a 0.2 molar buffer using either a refractive index detector, ultraviolet, or colorimetric assay for the detection of the polysaccharide; and high performance size-exclusion chromatography (HPSEC) either alone or in combination with light scattering and refractive index detectors (e.g. MALLS). The methodology and column used should be validated to demonstrate sufficient resolution in the appropriate molecular weight range (Bednar and Hennessey, 1993; Hunolstein *et al.*, 2003; Hsieh, 2000).

2.7 Rocket Immunelectrophoresis

Rocket immunelectrophoresis (also referred to as electroimmunoassay) is a simple, quick, and reproducible method for determining the concentration of a specific protein in a protein mixture. The method, originally introduced by Laurell (Laurell, 1966) involves a comparison of the sample of unknown concentration with a series of dilutions of a known concentration of the protein, and requires a monospecific antiserum against the protein under investigation. The electrophoresis of the antigen to be determined is carried out in a gel containing a comparatively lower concentration of the corresponding antibody. The test material and dilutions of a standard antigen used for calibration are introduced into different wells in the gel. During electrophoresis, migrating peak-shaped precipitation zones originating from the wells are developed. The front of the precipitate becomes stationary when the antigen is no longer in excess. For a given antibody concentration, the relationship between the distance travelled by the precipitate and the amount of antigen applied is linear. Rocket immunelectrophoresis is usually used for determination of total polysaccharide content in each serogroup (Walker, 1984).

CHAPTER THREE

MATERIALS

3.0 MATERIALS:

3.1 Materials Required:

In this experiment the materials has been chosen in such a way that is cost effective and serve the purpose well. Also the materials have been chosen according to the purpose, quality, price and availability. For example, Cross linked resin Sepharose CL-4B, Gel filtration molecular weight marker Blue Dextran 2000 and high performance column XK 16/70 were purchased from GE healthcare, the best in the business for size exclusion chromatography. Chemicals like Sodium Chloride (NaCl), Sodium hydroxide (NaOH) and Hydrochloric acid (HCl) were purchased from Applichem (Germany) whereas Ethanol, Sodium azide and Acetic acid were purchased from Merck (Germany). Filtration is a prime concern in the Pharmaceutical industry as it can hamper both-the quality of the product and analysis. 0.2 μ m syringe filter was purchased from Merck Millipore (Germany) and 0.2 μ m capsule filter was taken from Sartorius (Germany), two filtration giants.

Meningococcal polysaccharide standard and antiserum are also very important for the precision, reproducibility and accessibility of the analysis. *N. meningitidis* group A, C, Y, W Antiserum and Standard *N. meningitidis* group A, C, Y, W were purchased from Becton-Dickinson and Yuxi Walvax Biotechnology Ltd.

Here is the list of materials used for this experiment along with manufacturer, catalog and lot number:

Table I: List of materials along with manufacturer, catalog and lot number.

Sl. No.	Materials Name	Manufacturer	Lot Number	Catalogue Number
1	Sepharose CL 4B	GE Healthcare	4R016112	17-0150-01
2	Column: XK 16/70	GE Healthcare	4932493	28-9889-46
3	NaCl	Applichem	4R016112	A1371
4	Ethanol	Merck	K45600783-421	1009831000
5	Blue Dextran 2000	GE Healthcare	10056502	17-0360-01
6	Sodium Azide	Merck	1.06688.0100	K71290 455
8	Acetone	Sigma	0501591	

Sl. No.	Materials Name	Manufacturer	Lot Number	Catalogue Number
9	0.2µm syringe filter	Merck Millipore	R4SA42345	SLGP033RB
10	0.2µm Capsule Filter	Sartorius	538003503	
11	NaOH	Applichem	4U017025	A0991
12	Syringe	JMI	6016155006	
13	15 mL Centrifuge Tube	Kirgen	P10402	
14	Pipette 10mL	Eppendorf	D000041Q	
15	Pipette Controller	Falcon	403991581	
17	Agarose	Promega	000085707	V3121
18	Tris, free base	HiMedia	0000150896	MB029
20	Coomassie Brilliant Blue	Lobal Chemie	S44161409	6104-58-1
21	Hydrochloric acid	Applichem	4K015117	A6578,1000
22	Acetic acid	Merck	K40866663	
23	Glycerol	HiMedia	0000161007	RM081
24	<i>N. meningitidis</i> group A, C, Y, W Antiserum	Becton, Dickinson	4024238, 4135542, 4129884 & 4199800	222281, 222301, 222531 & 228811
25	Standard <i>N. meningitidis</i> group A, C, Y, W	Yuxi Walvax Biotechnology Ltd	A201506003, C201506004, Y201506001 & W135201403 002-1	
27	Micropipettes (100-1000 µl, 20-200 µl, 2-20 µl)	Eppendorf		
28	Microtips (100-1000 µl, 20-200 µl, 2-20 µl)	Eppendorf	D1586770	

3.2 Equipments Required:

In this study, AKTA Chromatography System (AKTA Purifier 100, GE Healthcare) was used for size exclusion chromatography analysis. It consists of a compact separation unit

and a personal computer running with Unicorn 5.31 control system. Refrigerator (2-8)⁰C and Fridge (-20)⁰C were used for initial sample and chromatography separated sample storage respectively both manufactured by Siemens (Germany). pH meter was purchased from Mettler Toledo and conductivity meter were supplied by Eutech whereas, Horizontal electrophoresis unit was from Biorad, USA.

Here is the list of equipments along with manufacturer and serial number:

Table II: List of equipments along with manufacturer and model.

Sl. No.	Equipments Name	Manufacturer	Model
1	AKTA Chromatography System	GE Healthcare	AKTA Purifier 100
2	-20 ⁰ C Fridge	Siemens	GS32NA23
3	2-8 ⁰ C Refrigerator	Siemens	KS38RA92/02
4	Peristaltic Pump	Watson Marlow	
5	pH Meter	Mettler Toledo	S220
6	Conductivity Meter	Eutech	CON700
7	Biosafety Cabinet	Fabtech Technologies	292/08/11
8	Horizontal Electrophoresis Unit	Biorad	PowerPac TM Basic
9	Vortex Mixer	Tarsons (Spinix)	1680

CHAPTER FOUR

METHODS

4.0 METHODS

4.1 Preparation of the Column

4.1.1 Washing of Sepharose CL-04B

Sepharose CL-4B was washed with purified water. The gel was stirred thoroughly and allowed to precipitate for an hour. The supernatant was removed along with the unwanted fine particles. This procedure was repeated three times and it was made sure that there were no fine particles suspended in it.

4.1.2 Column Packing

The stepwise column packing is described below:

- All materials were equilibrated to the temperature at which the chromatography will be performed.
- The gel was prepared in clean a glass beaker with freshly prepared 20% ethanol and the slurry concentration was calculated.
- A packing reservoir was mounted at the top of column and rinsed with purified water. Precautions were taken so that no air has been trapped under the net.
- The column outlet was closed with approximately one centimeter of eluent remaining in the column.
- The slurry was poured in a single operation with holding a glass rod against the wall of the column for minimizing the chance of air bubbles introduction.
- The reservoir cap was screwed tightly and connected it to the pump.
- The bottom outlet of the column was opened and the pump was operated at the packing linear flow rate 30 cm/h.
- The gel was packed until the gel bed reached a consolidate bed height.
- Then, the pump was stopped; the column outlet was closed and subsequently the packing connector and reservoir were disconnected.
- After that, the column was re-mounted from the stand. The rest of the column was filled with eluent to form an upward meniscus.
- The plunger was slowly pushed down the column so that the air above the net was removed.
- The position of bed surface on the column was marked; the pump was stopped, the column outlet was closed and the adaptor was adjusted to the bed surface and then the adaptor was pushed a distance of further 3-4 mm.

4.1.3 Performance Testing of Packed Column

Column efficiency testing plays a central role in the qualification and monitoring of packed bed performance. Even though it cannot be used as a single parameter to predict purity and recovery, it is a quick way to test the column and equipment performance before starting the purification process. This test can also be used in between runs to check for changes of the bed integrity. To check the quality of the column packing, an efficiency test should be performed to determine the theoretical plate number and peak asymmetry factor. The column performance test was done by injecting 200 μ l of 2% (v/v) acetone (Sigma) through the column using purified water as eluent. The recommended flow rate in this case was 15 cm/h. After finishing sample run the plate number is calculated by following formula:

$$N = 5.54 (V_R/W_h)^2 \times 1000/L \dots\dots\dots(i)$$

L = bed height (cm)

N = number of theoretical plates

V_R = peak retention volume

W_h = peak width at half height

The peak asymmetry factor (A_S) is calculated by:

$$A_S = b/a \dots\dots\dots(ii)$$

a = ascending part of the peak width at 10% of peak height

b = descending part of the peak width at 10% of peak height

The value has been denoted in figure 2. Unicorn software was used for column performance testing by generating chromatogram like figure 2.

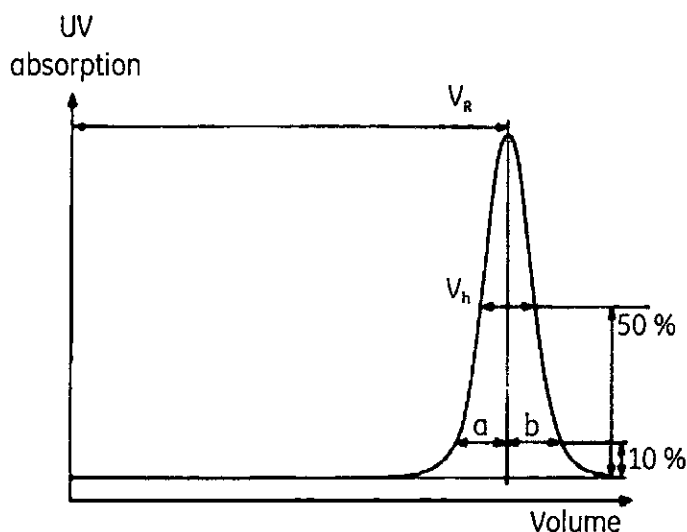


Figure 2: Column performance test evaluation

4.1.4 Calibration of the Column

The calibration of the column was conducted in steps sequentially as outlined below:

- Void volume (V_0) determination: The void volume (V_0) was determined with 0.2% (w/v) blue dextran 2000. Peak of the blue dextran 2000 was determined from the chromatogram of 206nm. The elution volume up to the first peak was considered as V_0 .
- Total bed volume (V_t) determination: The total bed volume (V_t) was determined with 0.5% sodium azide. Peak of the sodium azide was determined from the chromatogram of 260nm. The elution volume up to this peak was considered as V_t .
- 0.2% (w/v) blue dextran 2000 and 0.5% sodium azide were prepared in 0.2 M NaCl and filtered with 0.2 μ m syringe filter and kept in a sterile falcon.
- The elution profile of void volume (V_0) and total bed volume (V_t) were determined by ultraviolet absorption 206 nm or 260 nm.
- The distribution coefficient (K_D) value was determined by following formula:

$$K_D = (V_e - V_0) / (V_t - V_0) \dots \dots \dots (iii)$$

Here,

V_0 = void volume

V_t = total bed volume

V_e =elution volume

In this experiment, we have also calculated the recovered percentage of eluted polysaccharide before K_D value 0.50 by following formula:

% of recovered polysaccharide before K_D 0.5

$$= \frac{\text{Recovered polysaccharide of the group upto elution volume 80 mL}}{\text{Loaded polysaccharide of the group}} \times 100 \dots \dots \dots (iv)$$

The regulatory requirements for purified polysaccharides are, at least 65 per cent of group A, 75 per cent of group C, 80 per cent of group Y and 80 per cent of group W polysaccharide should be eluted before a distribution coefficient (K_D) of 0.50. In addition, the percentages eluted before this distribution coefficient were within the limits approved for the particular product (BP).

For a tetravalent vaccine (group A + group C + group Y + group W), the vaccine complied with the test if K_D for the principal peak was:

- not greater than 0.70 for group A and group C polysaccharide,
- not greater than 0.57 for group Y polysaccharide,
- not greater than 0.68 for group W polysaccharide.

4.2 Experimental Procedures

4.2.1 Sample Preparation

Ingovax® ACWY was manufactured by Incepta Vaccine Limited used in this study. It is a freeze-dried vaccine which contained $\geq 50 \mu\text{g}$ of each of the polysaccharide antigens from serogroups A, C, W and Y. Eight batches of Ingovax® ACWY vaccines were tested to determine the elution profile of each polysaccharide serogroups. From each batch, 40 vials containing $\geq 2.0 \text{ mg}$ of total PS were reconstituted in 2.5 ml of 0.2 M NaCl (pH 7.35, cond. 20.7 mS/cm) for analysis.

4.2.2 Chromatography Column Preparation

The high performance column XK16/70 (700mm $\times$ 16mm, porosity 10 μm) was used in this study. The column packed with 4% cross linked Sepharose CL-4B (Particle Size 45 μm -165 μm). These cross linked bead formed agarose matrix particles have an exclusion limit between 70 and 20,000 KDa. All the chromatography operation was conducted at temperature $23\pm 2^\circ\text{C}$.

4.2.3 Size Exclusion Chromatography (SEC)

SEC was performed for MSD analysis by AKTA purifier 100 consisting of gradient pump, multiple wavelength detection, conductivity and pH sensor. The Unicorn 5.31 software package was used for programming method run and data analysis. 200 μL of acetone was used for column performance testing and purified water was used as an eluent. By unicorn software the packed column theoretical plate number was calculated 7173 N/m and peak asymmetry factor was found 1.05 which fulfills the acceptance criteria of sample run for sepharose CL-4B packed column (Figure 3).

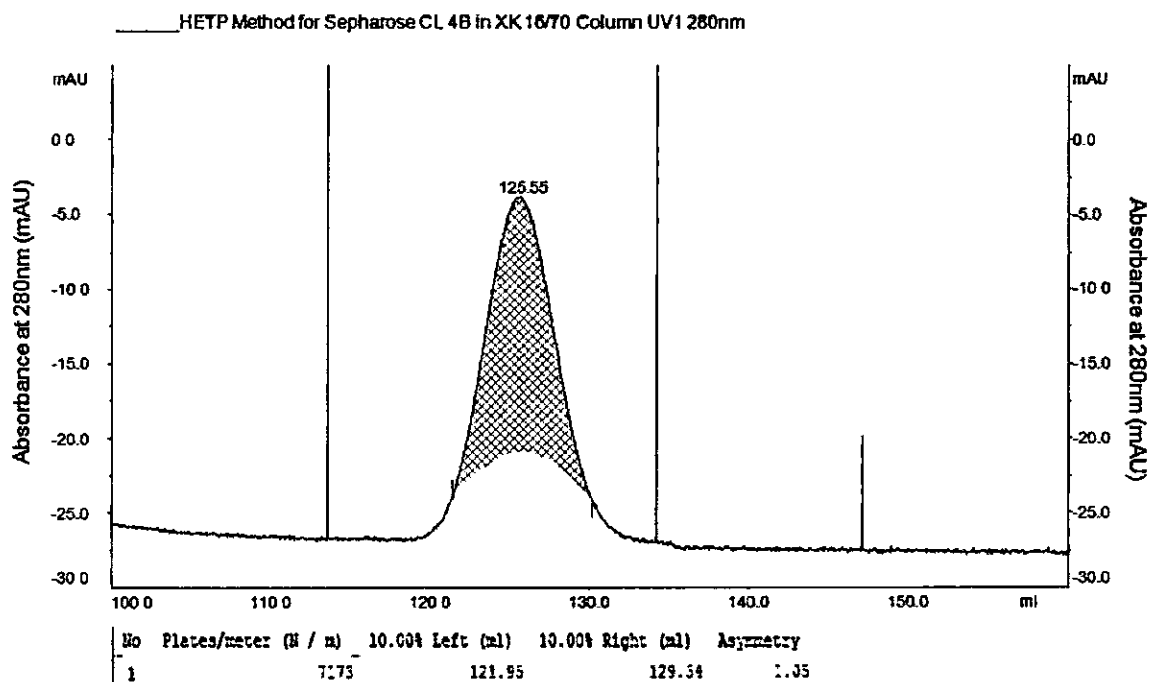


Figure 3: Performance testing of XK16/70 packed with Sepharose CL-4B column.

The column was equilibrated with 1.5 CV (187.5 mL) of 0.2 M NaCl (pH 7.39, cond. 21.2mS/cm, Temp. 25°C). The 2 mL sample loop was used for sample load. A flow rate of 0.3mL/min was considered. The unicorn programming and automatic fraction collector was applied to collect fixed volume of 2 mL fractions. The fractions were stored at -20°C until further test.

4.2.4 Determination of K_D Value

The K_D values for Ingovax® ACWY vaccines were calculated according to Acker's formula (Ackers, 1994; Alsop and Vlachogiannis, 1982):

$$K_D = (V_e - V_o) / (V_t - V_o) \dots \dots \dots (iii)$$

Where, V_o = void volume, V_t = total bed volume and V_e =elution volume

Here, V_o interprets the exterior part of the beads and determined by using a molecule which is larger than the exclusion limit of a particular matrix. Small molecule elutes at the end of the column and is determined by V_t . In this method, V_o and V_t were determined by 0.2% (w/v) blue dextran 2000, a polysaccharide covalently bonded with blue dye, MW 2000 KDa, and 0.5% (w/v) sodium azide (MW 65 Da) respectively. The absorbance for blue dextran 2000 and sodium azide were monitored at 206 nm and 260 nm respectively

(Figure 4). The elution volume (V_e) was calculated by estimating the principal peak of polysaccharides.

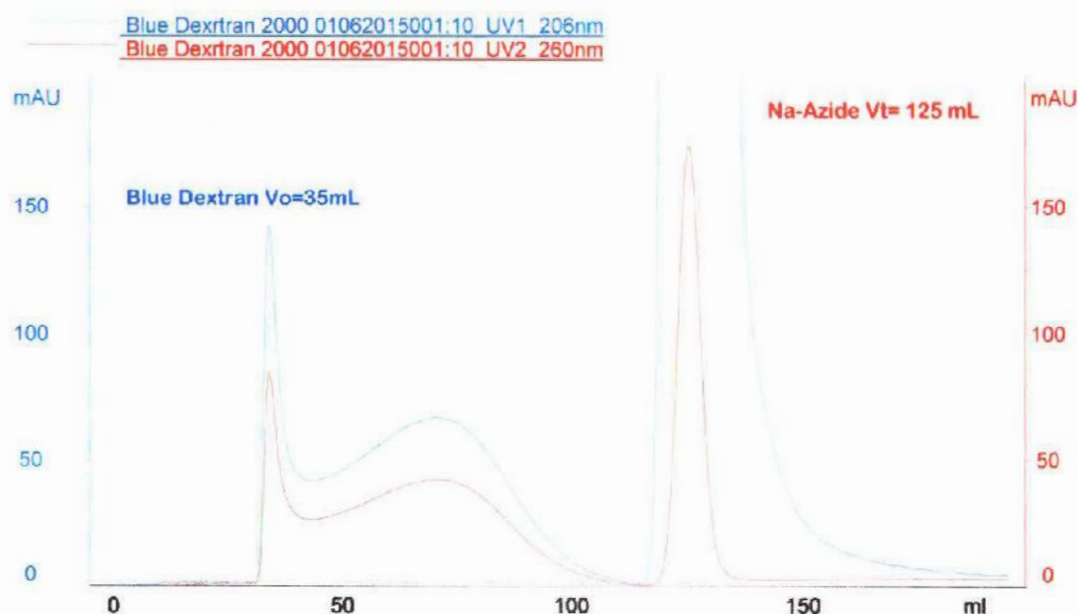


Figure 4: The elution profile of blue dextran 2000 and sodium azide. The absorbance was monitored at 206 nm for blue dextran 2000 and 260 nm for sodium azide.

4.2.5 Rocket immunoelectrophoresis

Rocket immunoelectrophoresis was used for determination of total polysaccharide content in each serogroup. The procedure is outlined below:

■ Preparation of Reagents

- *0.1M Tris buffer, pH 8.2:* 6.05 gm of tris free base dissolved in 500 ml of purified water and 0.575 gm of sodium azide added to the solution, then the pH was adjusted to 8.2 with the help of dilute HCl.
- *0.9% Sodium chloride solution:* 0.9 gm of sodium chloride dissolved in 100 ml of purified water and sonicated for 5 minutes for good mixing.
- *Staining solution:* 0.25 gm of Coomassie brilliant blue dissolved in 100 ml of purified water. Then in 125 ml of ethanol, 25 ml of acetic acid was added and mixed it properly.
- *De-staining solution:* 70 ml of acetic acid, 100 ml of ethanol and 40 ml of glycerol were mixed in 1000 ml of purified water.

- Reference standard solution was 100 µg/ml of polysaccharide in 0.1M Tris buffer, pH 8.2.

▪ **Test Procedure For Each Group (A, C, Y & W)**

In brief, agarose (1%) was melted in 20 ml of 0.1M Tris buffer, pH 8.2 by boiling in heating mantle. Liquid agarose was cooled to 50-55°C and 200 µl of *N. meningitidis* antiserum was added and mixed gently. The gel was immediately poured onto a gel tray placed on a horizontal surface. The gel was allowed to solidify and 3mm sample wells were made using gel-puncher. The gel was set in the electrophoresis unit. The reference standard solution was 100 µg/ml of polysaccharide in 0.1M Tris buffer, pH 8.2. The total amounts of samples were distributed in 8 different pools for our experiment suitability. 8µl of samples and standards were directly loaded to the well in duplicate. Electrophoretic run was conducted for 15 hr at 9 mA. The gel was stained using staining solution for 40 minutes using mini orbital shaker with gentle agitation. The staining solution is prepared by dissolving 0.25 gm of Coomassie brilliant blue in 100 ml of purified water and then added 125 ml of ethanol, 25 ml of acetic acid to it with gentle mixing. After that, the gel was de-stained using de-staining solution. The solution was changed after every 10 minutes for 30 minutes duration and let the gel dry at room temperature. The de-staining solution is prepared by mixing 70 ml of acetic acid, 100 ml of ethanol and 40 ml of glycerol in 1000 ml of purified water. After sufficient drying of the gel the rocket peak height was measured and a graph of concentration against peak height plotted. The polysaccharide content was calculated by following formula:

$$\text{Polysaccharide content} = \frac{\text{Sample height} \times \text{Average standard concentration}}{\text{Average standard height}}$$

..... (v)

CHAPTER FIVE

RESULTS

5.0 RESULTS

In this work, we determined K_D value of meningococcal polysaccharide serogroups A, C, W and Y in meningococcal PS vaccine. For this purpose, V_o and V_i were determined by blue dextran 2000 and sodium azide respectively. Figure 4 shows their corresponding values as 35 mL and 125 mL, respectively. The elution profile of individual eight batches of Ingovax® ACWY was considered for MSD analysis. The serogroups A, C, W and Y showed symmetric peak at absorbance 206 nm (Figure 5). The chromatogram indicated that the peak started from 35 mL in all eight batches (Figure 5, panel A-H). The fraction volumes were collected row-by-row in a 2 mL volume using automated fraction collector. The polysaccharide content of each serogroup was determined by rocket immunoelectrophoresis (see appendix). The principal peak was determined by estimating polysaccharide concentration of each fraction. The estimated individual polysaccharide content of each serogroup of eight batches was graphically represented in figure 6A to 6H. The principal peaks of each serogroup were accumulated in figure 7.

Table III: Main peak fraction volumes and corresponding K_D values of eight batches of Ingovax®ACWY vaccine (highest value denoted as bold italic and lowest value denoted as bold).

Batch no.	Serogroup-A		Serogroup-C		Serogroup-W		Serogroup-Y	
	Main Peak fraction, mL	K_D	Main Peak fraction, mL	K_D	Main Peak fraction, mL	K_D	Main Peak fraction, mL	K_D
15001	68	0.37	58	0.26	58	0.26	38	0.03
15002	74	0.43	62	0.30	62	0.30	62	0.30
15003	68	0.37	58	0.26	58	0.26	58	0.26
15004	38	0.03	38	0.03	38	0.03	38	0.03
15005	58	0.26	58	0.26	58	0.26	44	0.10
15006	46	0.12	46	0.12	46	0.12	46	0.12
15007	68	0.37	58	0.26	38	0.03	38	0.03
15008	60	0.28	48	0.14	60	0.28	60	0.28

At batch 15001, the principle peak of meningococcal serogroup A was observed at fraction C10 with V_e of 68 mL. For serogroup C, the principal peak was found at C5 (V_e =58 mL). In case of serogroups W and Y, the principal peak was at C5 (V_e =58mL) and B7 (V_e =38 mL) respectively. The K_D values for serogroups A, C, W and Y were found to be 0.37, 0.26, 0.26 and 0.03 respectively (Figure 7; Table III). However, the

principal peak for serogroup A was observed at fraction D1 ($V_e=74$ mL) at batch 15002. For serogroups (C, W and Y), the principal peak was found at the same fraction C7 ($V_e=62$ mL). The K_D values for serogroups C, W and Y were 0.30 while it was 0.43 for serogroup A at this batch. The principal peaks for serogroup A, C, W and Y for batch 15003 were (C10, C5, C5 & C5) respectively; for batch 15004 (B7, B7, B7 & B7) respectively; for batch 15005 (C5, C5, C5 & B10) respectively; for batch 15006 (B11, B11, B11 & B11) respectively; for batch 15007 (C10, C5, B7 & B7) respectively and for batch 15008 (C6, B12, C6 & C6) respectively. The highest K_D value was found at batch 15002 whereas, observed lowest at batch 15004. Therefore, the mean $K_D \pm SD$ of serogroups A, C, W and Y, derived from the analysis of eight different batches, corresponded to 0.28 ± 0.14 , 0.20 ± 0.09 , 0.19 ± 0.11 and 0.14 ± 0.12 respectively.

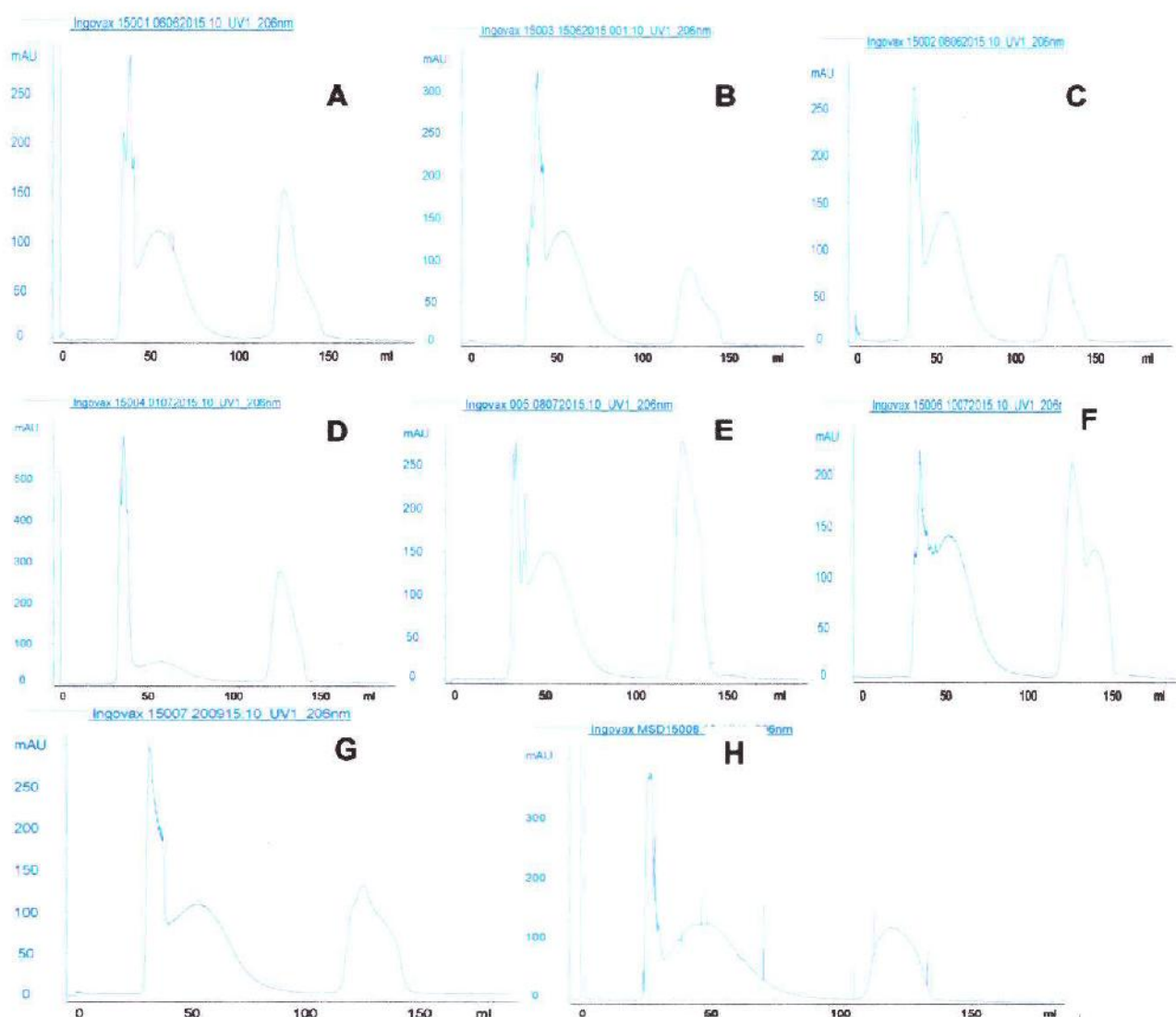


Figure 5: Elution profile of eight batches of Ingovax®ACWY vaccine. Chromatograms of (A) Batch 15001; (B) 15002; (C) 15003; (D) 15004; (E) 15005; (F) 15006; (G) 15007 and (H) 15008 are shown. The peak absorbance was monitored at 206 nm.

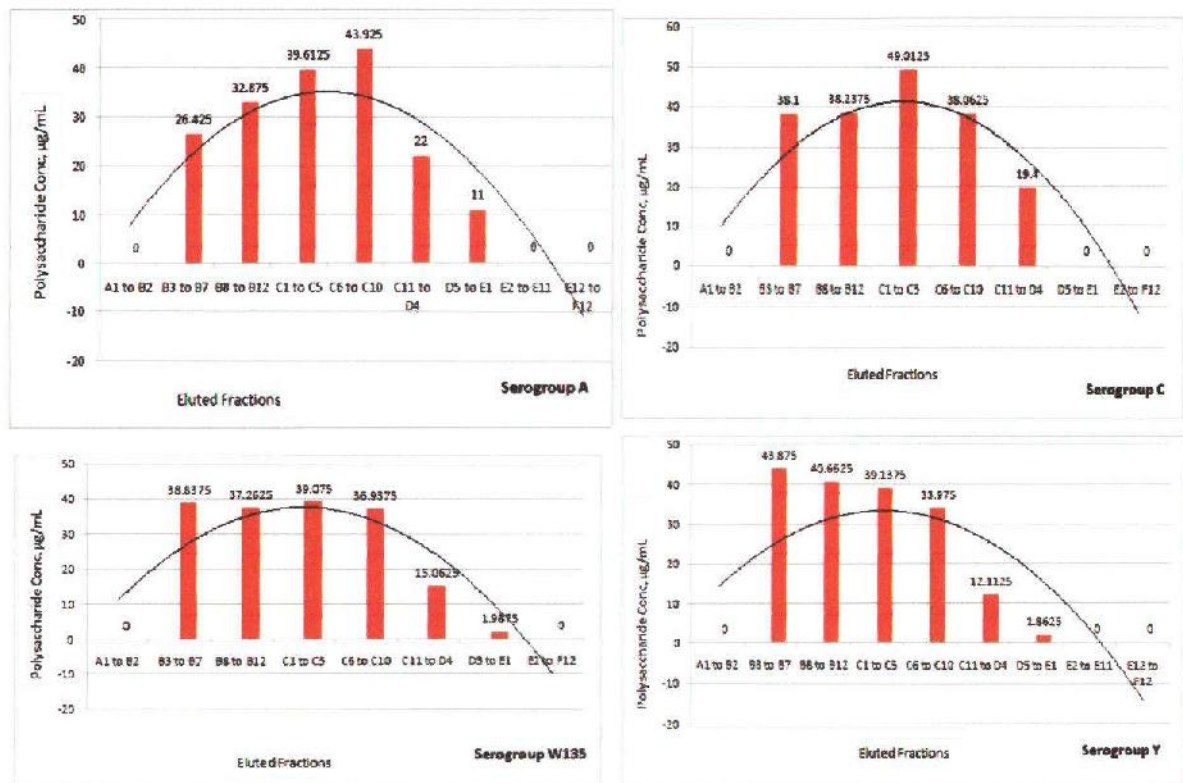


Figure 6A: Principal peaks determination of meningococcal serogroup A, C, W and Y polysaccharide content in eluted fractions for batch 15001.

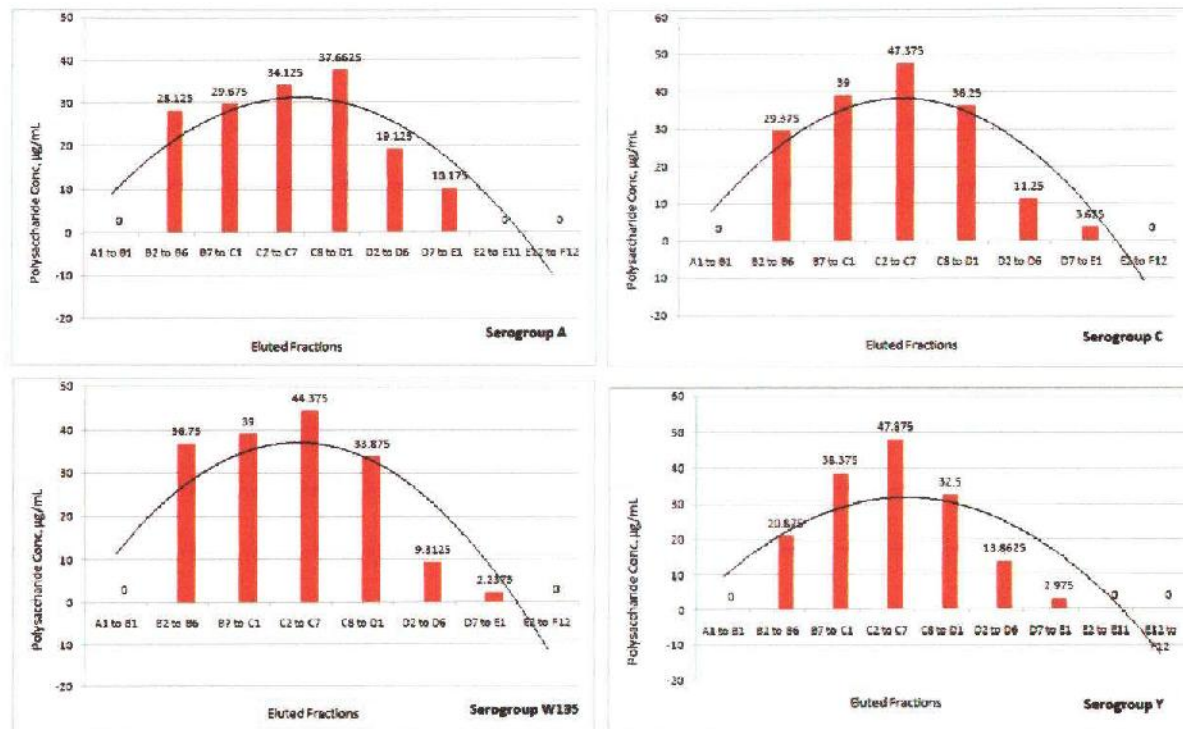


Figure 6B: Principal peaks determination of meningococcal serogroup A, C, W and Y polysaccharide content in eluted fractions for batch 15002.

In addition, the elution pattern of batch 15001, 15002, 15003, 15005, 15007 and 15008 were indistinguishable from each other. However, it was slightly different at batch 15004 and 15006. In the cases of batch 15001, 15003 and 15005, the principal peak appeared at fraction C5 ($V_e=58$ mL) for both serogroups C and W while the K_D value was found to be 0.26. Besides, for serogroup A the principal peak was seen at fraction C10 ($V_e=68$ mL) with K_D value of 0.37 at batch 15001 and 15003. Interestingly, all the principal peaks appeared at the same position for each serogroup at batch 15004 (B7, $V_e=38$ mL) and 15006 (B11, $V_e=46$ mL), though the elution patterns were found to be different. The K_D values were 0.03 for batch 15004 and 0.12 for batch 15006. The K_D values for serogroup W and Y were similar 0.03 for batch 15007 and for batch 15008 K_D values for serogroup A, W and Y were similar 0.28.

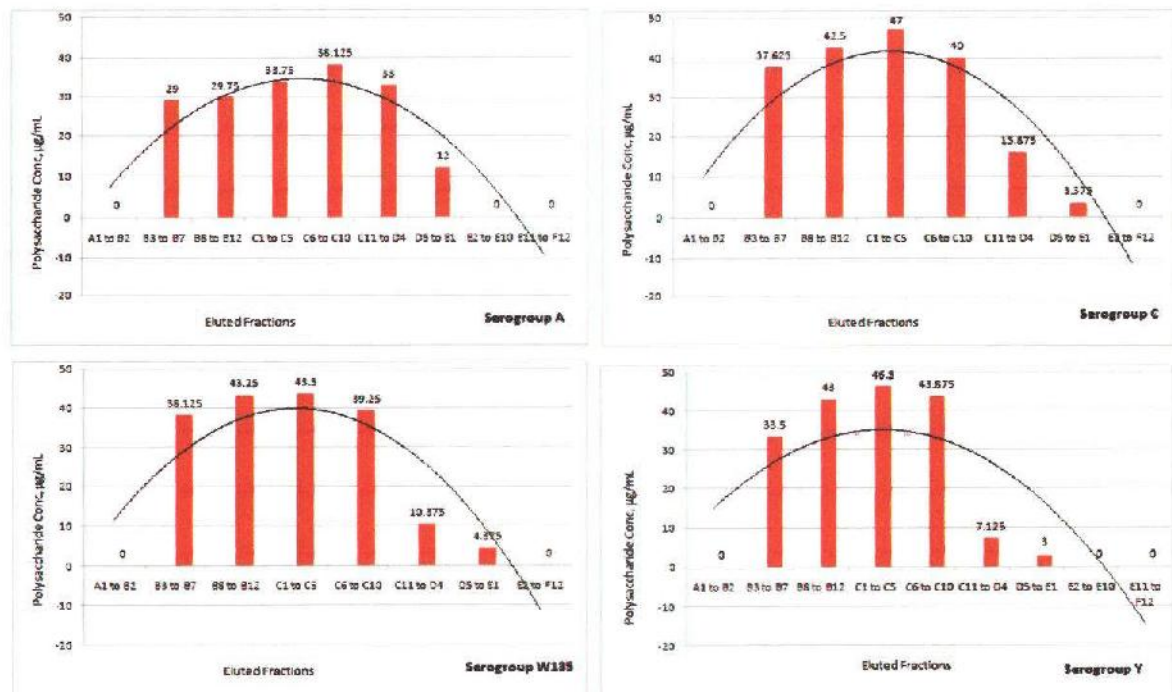


Figure 6C: Principal peaks determination of meningococcal serogroup A, C, W and Y polysaccharide content in eluted fractions for batch 15003.

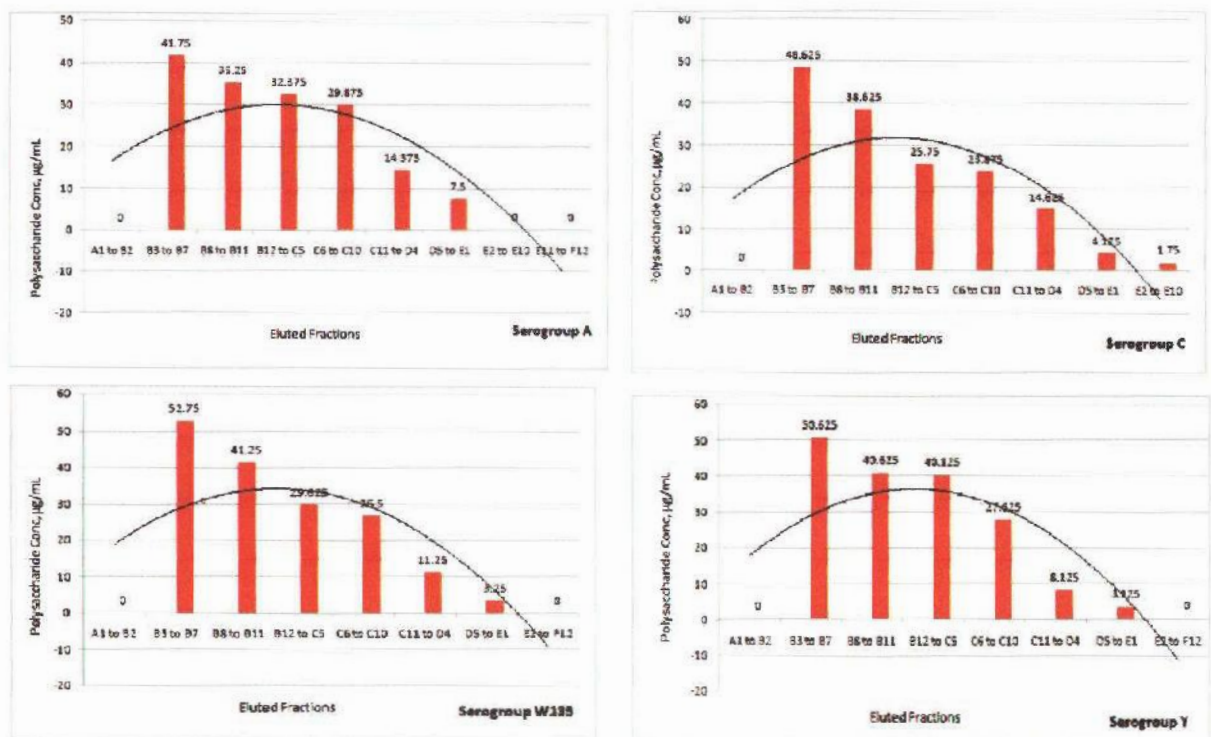


Figure 6D: Principal peaks determination of meningococcal serogroup A, C, W and Y polysaccharide content in eluted fractions for batch 15004.

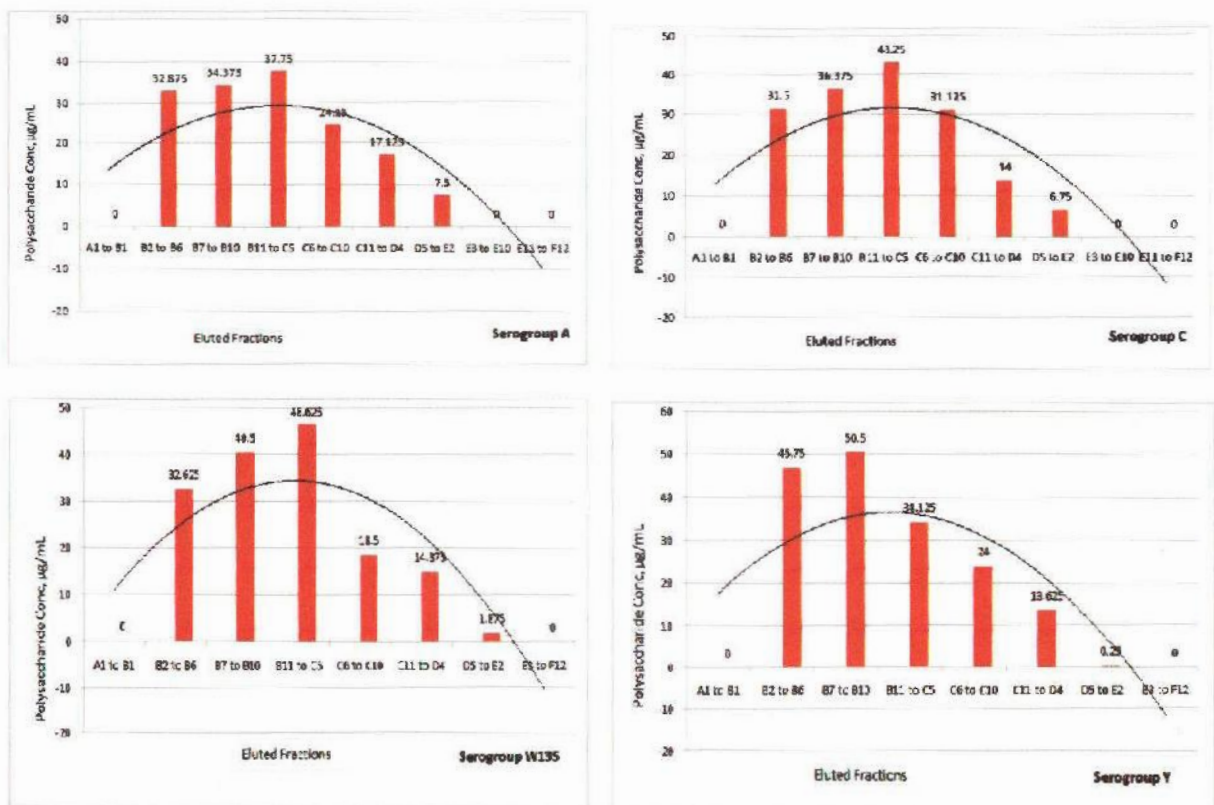


Figure 6E: Principal peaks determination of meningococcal serogroup A, C, W and Y polysaccharide content in eluted fractions for batch 15005.

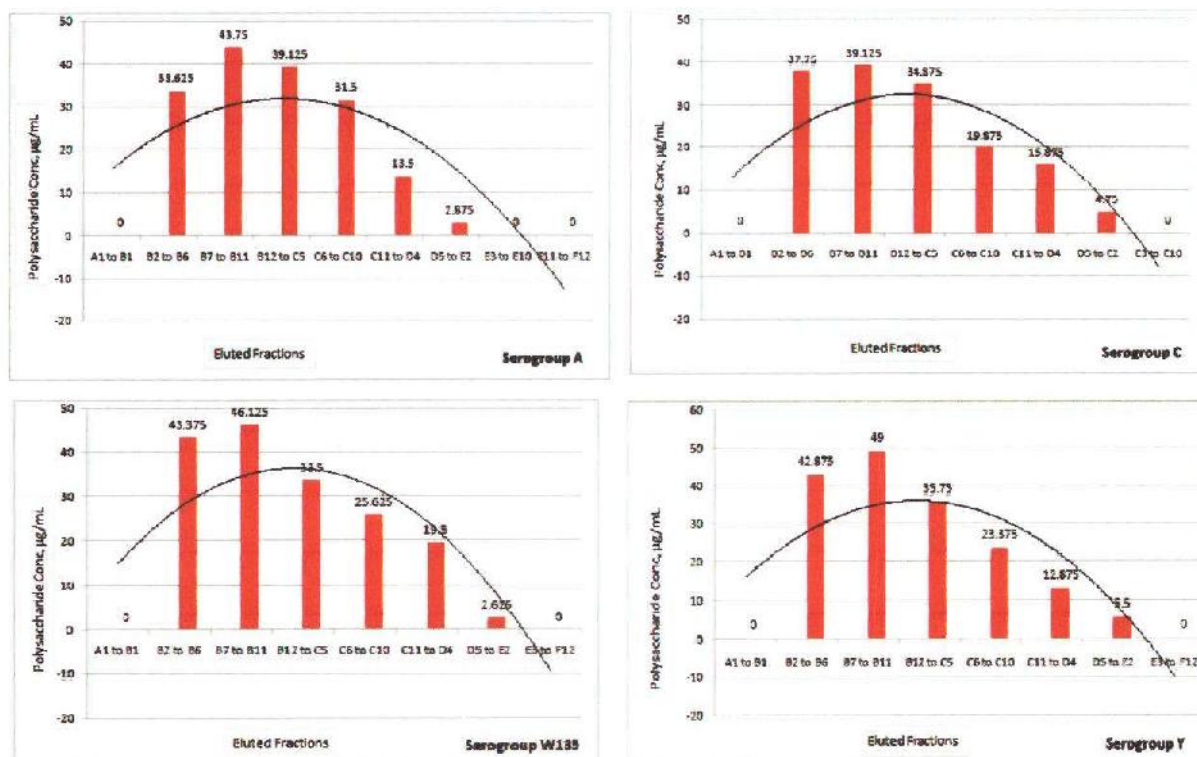


Figure 6F: Principal peaks determination of meningococcal serogroup A, C, W and Y polysaccharide content in eluted fractions for batch 15006.

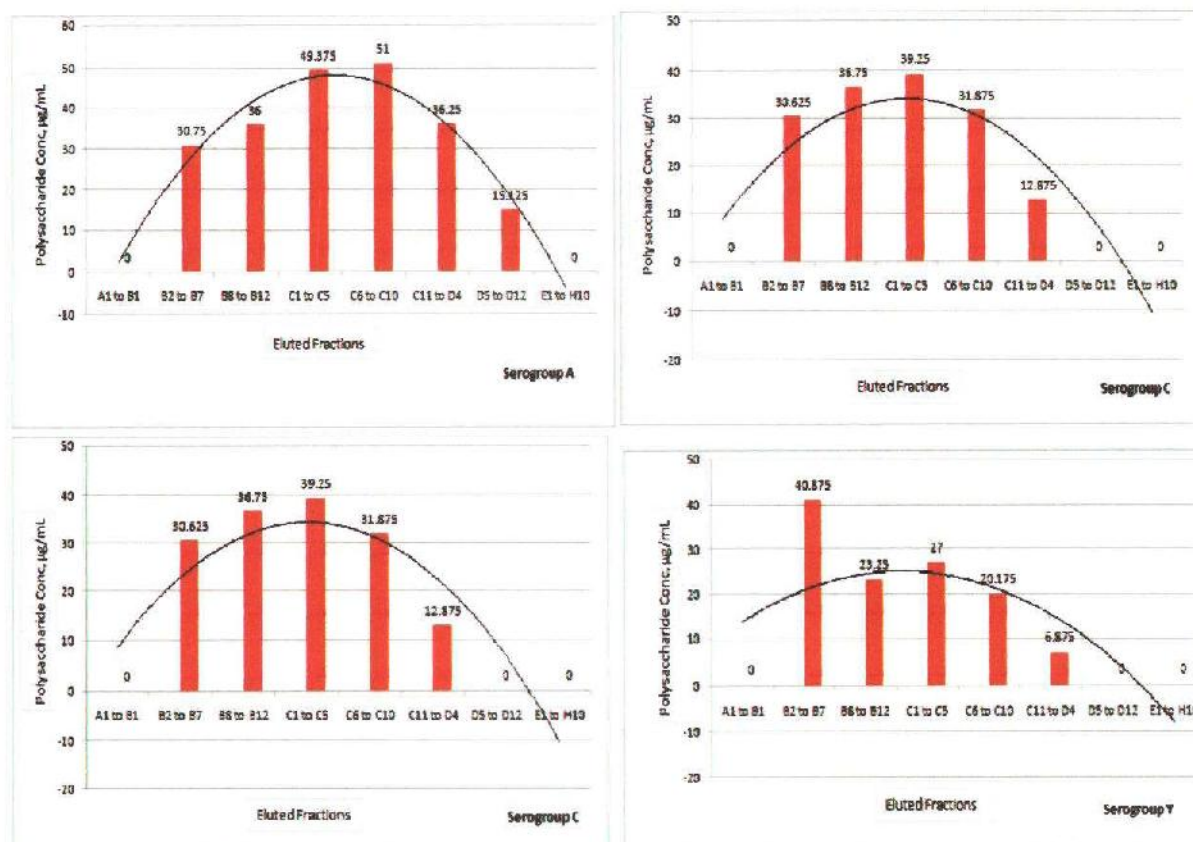


Figure 6G: Principal peaks determination of meningococcal serogroup A, C, W and Y polysaccharide content in eluted fractions for batch 15007.

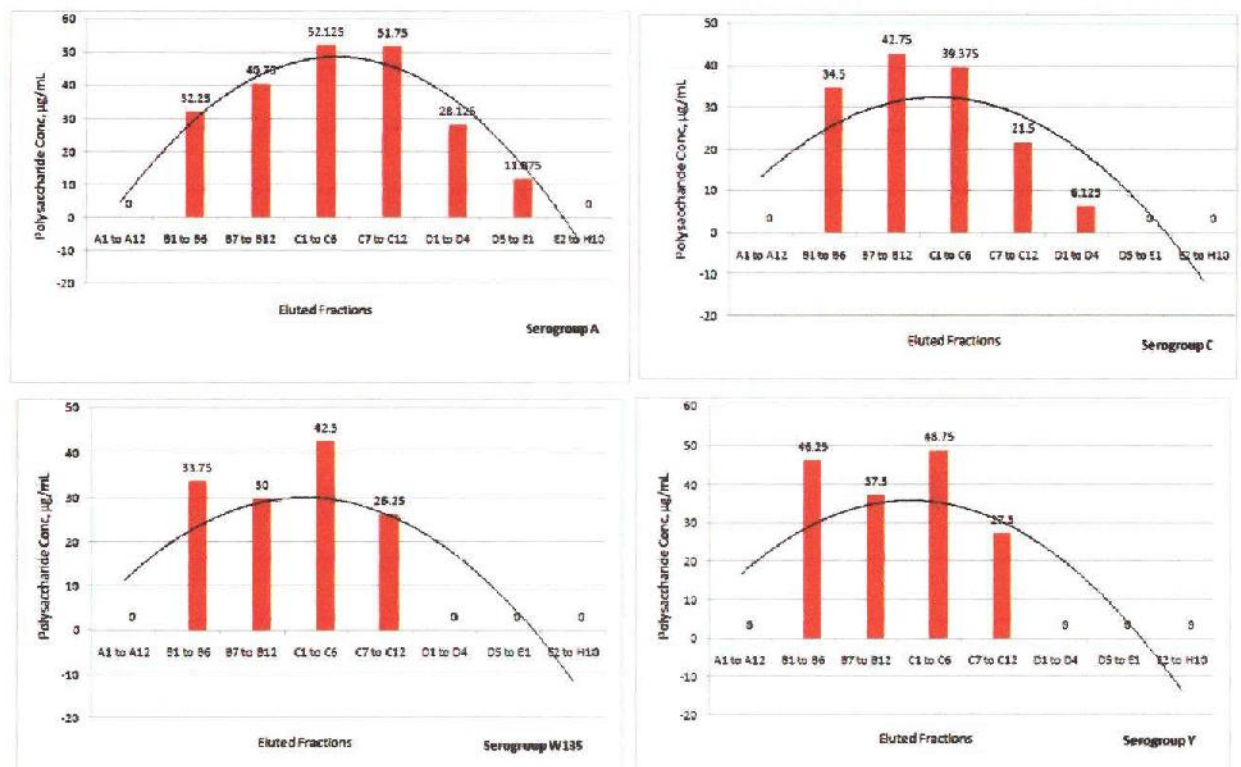


Figure 6H: Principal peaks determination of meningococcal serogroup A, C, W and Y polysaccharide content in eluted fractions for batch 15008.

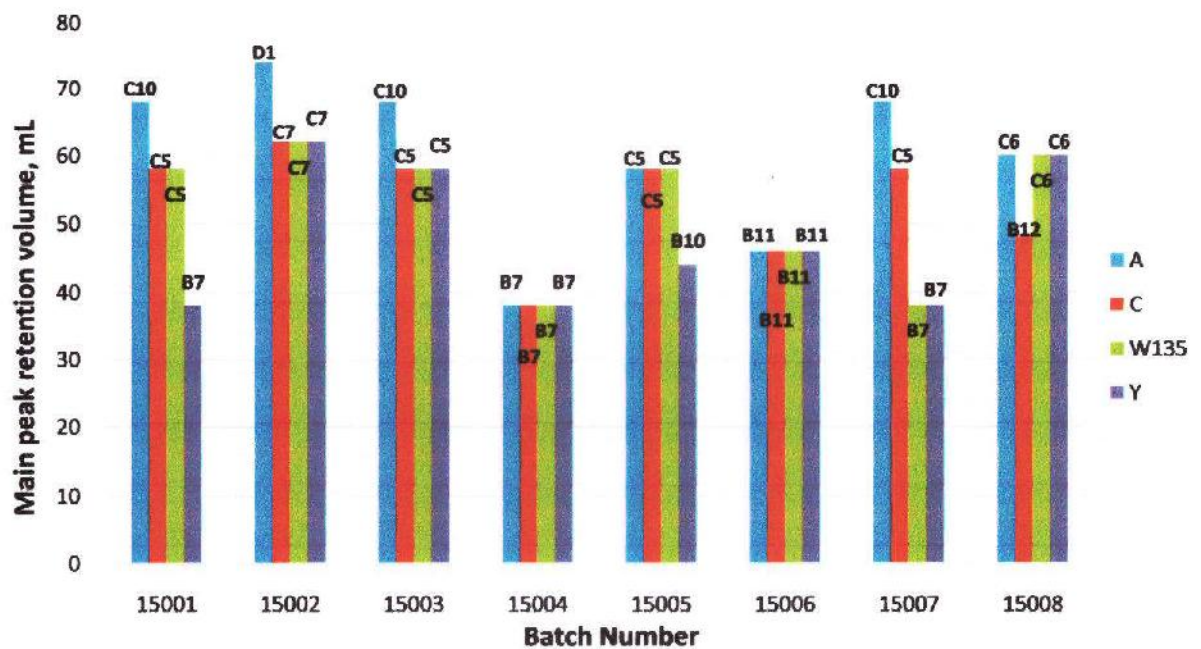


Figure 7: Principal peak fraction volume of eight batches of Ingovax®ACWY vaccine.

Additionally, we calculated the total recovery percentage of polysaccharides eluted before K_D value 0.5 for each serogroup of eight batches. The highest recovery of serogroup A was found at batch 15002 (98.56%). For serogroup C, Y and W the highest recovery was found at 97.31%, 93.75% and 98.19% of batch 15001, 15007 and 15008 respectively. The lowest recovery of serogroup A, C & W was found in Batch 15004 (80.75%, 84.42% & 80.53% respectively) and of serogroup Y in Batch 15007 (81.02%) (Table: IV). The mean $\pm$ SD recovery percentages of A, C, Y & W were 92.56 $\pm$ 7.62, 91.15 $\pm$ 5.30, 84.88 $\pm$ 4.39 and 88.71 $\pm$ 6.87 respectively. Each of the individual sero-specific polysaccharides for each batch comply the requirements of BP.

Table IV: The injected and recovered polysaccharide content of serogroups A, C, Y and W of eight batches of Ingovax®ACWY vaccine.

Batch no.	Serogroup-A		Serogroup-C		Serogroup-Y		Serogroup-W	
	Injected (μ g)	Recovered (% before K_D 0.5)	Injected (μ g)	Recovered (% before K_D 0.5)	Injected (μ g)	Recovered (% before K_D 0.5)	Injected (μ g)	Recovered (% before K_D 0.5)
15001	1921.73	98.37	1918.50	97.31	2121.49	81.16	2097.17	81.15
15002	1919.49	98.56	2067.17	97.07	2229.44	84.81	2054.43	96.23
15003	1975.04	97.12	2163.87	88.85	2084.93	86.73	2000.19	92.22
15004	2098.08	80.75	1924.00	84.42	2122.78	82.10	2075.58	80.53
15005	2028.51	84.00	1908.48	95.67	2146.75	81.88	2059.10	82.75
15006	2059.74	85.86	1941.50	85.66	2092.32	87.64	2070.50	86.96
15007	2208.00	98.18	1841.57	86.92	2360.00	81.02	2182.02	98.19
15008	2401.00	97.76	1829.70	93.27	2048.00	93.75	1734.34	91.68

CHAPTER SIX
DISCUSSION

6.0 DISCUSSION

The MSD is a key indicator for process consistency, confirming the vaccines immunogenicity and safety (Lei *et al.*, 2000; Lemercinier and Jones, 1996; Ho *et al.*, 2001). Therefore, a robust and reliable method for determination of MSD of meningococcal PS vaccine is crucial. Hunolstein and his co-authors (Hunolstein *et al.*, 2013) reported the experimental conditions for SEC on a tandem equipped PL Aquagel-OH 60 column (7.5 x 300 mm) and PL Aquagel-OH guard column (50 mm x 7.5 mm) for MSD analysis of meningococcal PS vaccine. In this study, we described a robust SEC method for MSD analysis of meningococcal PS vaccine on a XK 16/70 column packed with Sepharose CL-4B. However, Sepharose CL-2B showed better performance over Sepharose for MSD analysis (Sadao and Howard, 1999). In addition, MSD could also be determined by Sepharose CL-2B or Sepharose CL-4B as recommended by WHO (WHO, 1996). The present study is perhaps the first type of its kind on determination of MSD for meningococcal PS vaccine.

We identified K_D values of eight batches of Ingovax® ACWY vaccine and analysed the batch-to-batch fluctuations. The lower K_D value indicates the higher molecular size of polysaccharide which suggests the polysaccharide to be more immunogenic (Sadao and Howard, 1999; Arickx, 1979). In the present investigation, very little variance was observed amongst the batches. This indicates that the method was accurate and reproducible. Again, K_D values of individual serogroups of eight batches were compared where lowest values were found in all serogroups at batch 15004 and 15006 while the highest values were found at batch 15002. This can be explained from the fact that determination of absolute molecular size distribution is difficult as a result of polydispersity ranging from 5×10^4 to 2×10^5 Da (WHO). Therefore, some fluctuations in K_D values could be considered insignificant. Misplay of formulation and filling may affect the structural integrity and size of polysaccharides (Maiden, 2006) and hydrolysis reduces the molecular size of polysaccharide which may deteriorate the immunogenic capability (Suker *et al.*, 2004). In our study, the polysaccharides were stored in -20°C to reduce the possibility of such types of degradation.

According to the requirements of BP, the K_D value for the principal peak should not be greater than 0.70 for serogroup A & C; 0.57 for serogroup Y; 0.68 for serogroup W for meningococcal PS vaccine. In our study, the highest values of serogroup A, C, W and Y

were found to be 0.43, 0.30, 0.30 and 0.30 respectively in batch 15002. Therefore, the K_D values of each serogroups of eight batches of Ingovax® ACWY vaccine comply with the requirements of BP. Additionally, we calculated the total recovery percentage of polysaccharides for each serogroup of six batches. According to requirement of BP, at least 65% polysaccharide of serogroup A, 75% of serogroup C, 80% of serogroup W and 80% of serogroup Y should be eluted before K_D value 0.50. Our method shows the higher recovery percentage of polysaccharide than the specified requirements of BP. Overall results of this experiment showed that Ingovax® ACWY was highly immunogenic and could be served as a potential meningococcal PS vaccine for human use in compliance with WHO and BP.

CHAPTER SEVEN
CONCLUSION

7.0 CONCLUSION

Meningococcal polysaccharide vaccines are widely required for its preventive roles in conferring protection against meningococcal diseases. The mandatory requirements for development of successful vaccine candidates are their immunogenicity and safety. In this vaccine candidate, the immunogenicity depends on molecular size of PS which is determined by K_D value analysis. We identified and described the K_D values of eight different batches total 32 serogroups polysaccharides. The calculated K_D values successfully accomplished the regulatory requirement of BP and satisfy the consideration to be used as a vaccine for human use. Therefore, SEC with Sepharose CL-4B could be employed successfully for MSD analysis of polysaccharide vaccine. It could provide meaningful information in regard to reproducibility and accuracy of a method towards fulfilling the requirements of regulatory authority's approval of a vaccine for human use.

CHAPTER EIGHT

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REFERENCES

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APPENDIX:**Batch: 15001**

Well No.	Name of Standard	Rocket Height (mm)				*Concentration (µg)
		Group A	Group C	Group W-135	Group Y	
1	Std-0.5	11.09	-	-	6.72	0.05
2	Std-1	13.84	10.34	17.34	9.01	0.1
3	Std-2	16.73	16.05	19.09	11.31	0.2
4	Std-4	18.96	19.02	20.89	13.63	0.4
5	Std-8	21.54	22.60	22.46	15.26	0.8
Pool No.	Fractions	Rocket Height (mm)				
		Group A	Group C	Group W-135	Group Y	
1	A1-B2	0	0	0	0	
2	B3- B7	11.21	13.82	16.53	12.67	
3	B8-B11	13.97	13.87	15.86	11.74	
4	B12 - C5	16.80	17.78	16.63	11.30	
5	C6 - C10	18.63	13.81	15.72	9.81	
6	C11- D4	9.34	7.04	6.41	3.5	
7	D5 - E1	4.71	0	0.85	0.54	
8	E2 - E10	0	0	0	0	

Batch: 15002

Well No.	Name of Standard	Rocket Height (mm)				*Concentration (µg)
		Group A	Group C	Group W-135	Group Y	
1	Std-0.5	13.93	-	-	6.98	0.05
2	Std-1	16.69	10.13	16.87	9.99	0.1
3	Std-2	19.04	12.32	18.28	11.87	0.2
4	Std-4	22.17	13.87	19.98	14.61	0.4
5	Std-8	24.98	15.39	20.96	17.03	0.8
Pool No.	Fractions	Rocket Height (mm)				
		Group A	Group C	Group W-135	Group Y	
1	A1-B2	0	0	0	0	
2	B3- B7	14.09	8.13	14.93	6.63	
3	B8-B11	14.83	10.79	15.84	12.01	
4	B12 - C5	17.03	13.08	18.01	14.97	
5	C6 - C10	18.82	10.03	13.75	10.15	
6	C11- D4	9.56	3.12	3.78	4.33	
7	D5 - E1	5.09	1.01	0.91	0.93	
8	E2 - E10	0	0	0	0	

Batch: 15003

Well No.	Name of Standard	Rocket Height (mm)				*Concentration (µg)
		Group A	Group C	Group W-135	Group Y	
1	Std-0.5	13.065	-	-	7.12	0.05
2	Std-1	15.325	10.27	17.24	9.33	0.1
3	Std-2	17.896	13.38	18.60	11.47	0.2
4	Std-4	21.012	16.61	20.02	13.56	0.4
5	Std-8	23.731	20.04	21.87	15.98	0.8
Pool No.	Fractions	Rocket Height(mm)				
		Group A	Group C	Group W-135	Group Y	
1	A1-B2	0	0	0	0	
2	B3- B7	13.68	12.13	15.82	9.97	
3	B8-B11	14.01	13.69	17.97	12.78	
4	B12 - C5	15.87	15.13	18.04	13.81	
5	C6 - C10	17.92	12.87	16.32	13.02	
6	C11- D4	15.51	5.13	4.31	2.14	
7	D5 - E1	5.64	1.09	1.84	0.89	
8	E2 - E10	0	0	0	0	

Batch: 15004

Well No.	Name of Standard	Rocket Height (mm)				*Concentration (µg)
		Group A	Group C	Group W-135	Group Y	
1	Std-0.5	16.52	15.70	-	-	0.05
2	Std-1	17.99	18.83	17.96	10.78	0.1
3	Std-2	19.29	21.21	21.61	12.69	0.2
4	Std-4	22.04	25.85	24.21	14.93	0.4
5	Std-8	25.76	28.29	26.96	17.08	0.8
Pool No.	Fractions	Rocket Height (mm)				
		Group A	Group C	Group W-135	Group Y	
1	A1-B2	0	0	0	0	
2	B3- B7	21.92	27.61	25.54	14.99	
3	B8-B11	18.51	21.88	19.99	12.01	
4	B12 - C5	16.96	14.62	14.32	11.87	
5	C6 - C10	15.73	13.52	12.86	8.17	
6	C11- D4	7.55	8.26	5.64	2.43	
7	D5 - E1	3.94	2.35	1.58	0.91	
8	E2 - E10	0	1.01	0	0	

Batch: 15005

Well No.	Name of Standard	Rocket Height (mm)				*Concentration (µg)
		Group A	Group C	Group W-135	Group Y	
1	Std-0.5	12.87	8.88	-	-	0.05
2	Std-1	13.99	9.06	15.98	8.14	0.1
3	Std-2	15.76	11.01	18.13	9.52	0.2
4	Std-4	17.43	12.52	21.21	10.98	0.4
5	Std-8	19.01	14.07	24.05	12.21	0.8
Pool No.	Fractions	Rocket Height (mm)				
		Group A	Group C	Group W-135	Group Y	
1	A1-B2	0	0	0	0	
2	B3- B7	13.39	9.06	13.80	10.19	
3	B8-B11	14.01	10.41	17.18	11.00	
4	B12 - C5	15.42	12.39	19.76	7.43	
5	C6 - C10	9.90	8.92	7.85	5.23	
6	C11- D4	6.98	4.01	6.29	2.98	
7	D5 - E1	3.08	1.96	0.81	0.06	
8	E2 - E10	0	0	0	0	

Batch: 15006

Well No.	Name of Standard	Rocket Height (mm)				*Concentration (µg)
		Group A	Group C	Group W-135	Group Y	
1	Std-0.5	7.91	13.81	-	-	0.05
2	Std-1	10.01	15.65	11.60	16.81	0.1
3	Std-2	11.97	16.42	13.19	17.97	0.2
4	Std-4	13.76	18.63	15.36	19.29	0.4
5	Std-8	16.10	19.69	17.08	21.03	0.8
Pool No.	Fractions	Rocket Height (mm)				
		Group A	Group C	Group W-135	Group Y	
1	A1-B2	0	0	0	0	
2	B3- B7	10.4	16.41	13.23	17.20	
3	B8-B11	13.51	17.02	14.08	19.61	
4	B12 - C5	12.07	15.19	10.21	14.32	
5	C6 - C10	9.71	8.41	7.81	9.41	
6	C11- D4	4.15	6.91	5.94	5.18	
7	D5 - E1	0.89	2.09	0.81	2.19	
8	E2 - E10	0	0	0	0	

Batch: 15007

Well No.	Name of Standard	Rocket Height (mm)				*Concentration (µg)
		Group A	Group C	Group W-135	Group Y	
1	Std-0.5	7.91	13.81	-	-	0.05
2	Std-1	10.01	15.65	11.60	16.81	0.1
3	Std-2	11.97	16.42	13.19	17.97	0.2
4	Std-4	13.76	18.63	15.36	19.29	0.4
5	Std-8	16.10	19.69	17.08	21.03	0.8

Pool No.	Fractions	Rocket Height (mm)			
		Group A	Group C	Group W-135	Group Y
1	A1 to B1	0	0	0	0
2	B2 to B7	10.4	16.41	13.23	17.20
3	B8 to B12	13.51	17.02	14.08	19.61
4	C1 to C5	12.07	15.19	10.21	14.32
5	C6 to C10	9.71	8.41	7.81	9.41
6	C11 to D4	4.15	6.91	5.94	5.18
7	D5 to D12	0.89	2.09	0.81	2.19
8	E1 to H10	0	0	0	0

Batch: 15008

Well No.	Name of Standard	Rocket Height (mm)				*Concentration (µg)
		Group A	Group C	Group W-135	Group Y	
1	Std-0.5	7.91	13.81	-	-	0.05
2	Std-1	10.01	15.65	11.60	16.81	0.1
3	Std-2	11.97	16.42	13.19	17.97	0.2
4	Std-4	13.76	18.63	15.36	19.29	0.4
5	Std-8	16.10	19.69	17.08	21.03	0.8

Pool No.	Fractions	Rocket Height (mm)			
		Group A	Group C	Group W-135	Group Y
1	A1 to B1	0	0	0	0
2	B2 to B7	10.4	16.41	13.23	17.20
3	B8 to B12	13.51	17.02	14.08	19.61
4	C1 to C5	12.07	15.19	10.21	14.32
5	C6 to C10	9.71	8.41	7.81	9.41
6	C11 to D4	4.15	6.91	5.94	5.18
7	D5 to D12	0.89	2.09	0.81	2.19
8	E1 to H10	0	0	0	0

CALCULATION:

*Polysaccharide content/8 μ l=

(Sample height $\times$ Average standard concentration)/Average standard height (i)

*Polysaccharide content /sample pool =

[(Polysaccharide content /8 μ l) $\times$ Total volume of sample pool]/8..... (ii)

% of recovered polysaccharide before Kd 0.5

$$= \frac{\text{Recovered polysaccharide of the group upto elution volume 80 mL}}{\text{Loaded polysaccharide of the group}} \times 100$$

..... (iii)