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THE APPLICATION OF PHYSICOCHEMICAL METHODS FOR THE ANALYSIS OF SMALL AND COMPLEX PHARMACEUTICAL DRUGS

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To my parents and siblings for believing in me, this work is for you. Leka moso, MALEBO.
# SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>B₀</td>
<td>External (applied) magnetic field</td>
</tr>
<tr>
<td>B₁</td>
<td>Oscillating magnetic field</td>
</tr>
<tr>
<td>LCP</td>
<td>Left circularly polarised light</td>
</tr>
<tr>
<td>RCP</td>
<td>Right circularly polarised light</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>Ultra-violet/visible</td>
</tr>
<tr>
<td>GSC</td>
<td>Gas-solid chromatography</td>
</tr>
<tr>
<td>LSC</td>
<td>Liquid-solid chromatography</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas-liquid chromatography</td>
</tr>
<tr>
<td>LLC</td>
<td>Liquid-liquid chromatography</td>
</tr>
<tr>
<td>IEC</td>
<td>Ion-exchange chromatography</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>n</td>
<td>Refractive index of a medium</td>
</tr>
<tr>
<td>Vᵥ</td>
<td>Speed of light in a vacuum</td>
</tr>
<tr>
<td>V</td>
<td>Speed of light in a given medium</td>
</tr>
<tr>
<td>HPAEC-PAD</td>
<td>High performance anion-exchange chromatography-pulsed amperometric detection</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>Number of theoretical plates or number of chiral centres</td>
</tr>
<tr>
<td>D</td>
<td>Dextrotyal</td>
</tr>
<tr>
<td>L</td>
<td>Levo-rotary</td>
</tr>
<tr>
<td>R</td>
<td>Rectus</td>
</tr>
<tr>
<td>S</td>
<td>Sinister</td>
</tr>
<tr>
<td>TEAA</td>
<td>Triethylamine acetate</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
</tbody>
</table>
CONFERENCE PROCEEDINGS ARISING FROM PART OF THIS STUDY

2002: 36th Convention of the South African Chemical Institute, University of Port Elizabeth, Port Elizabeth, South Africa

ORAL: HPLC separation of racemates with NMR as a predictive tool in the selection of conditions for HPLC, Analytical Chemistry, Student Session
ABSTRACT

The application of physicochemical methods for the analysis of small and complex pharmaceutical drugs was investigated. The methods were applied to small chiral molecules and further extended to analysis of complex glycoconjugate vaccines.

Chiral NMR spectroscopy and HPLC were used to analyse and separate enantiomers of a racemic mixture. NMR was used firstly to predict conditions that were used in both chiral and achiral HPLC separations. NMR studies indicated chiral discrimination of enantiomers with hydroxypropyl-β-cyclodextrin (HP-β-cyclodextrin) as a chiral selector in a mixture of 50/50 methanol-d₄/D₂O.

These results were used for enantiomeric separation using HPLC with HP-β-cyclodextrin as a chiral selector. The enantiomers were separated when HP-β-cyclodextrin was used as chiral stationary phase, using methanol/water as a mobile phase. The selectivity and resolution of enantiomers was increased when methanol/triethylamine acetate (TEAA) buffer was used as a mobile phase.

The second study involved the application of optical spectroscopy, NMR spectroscopy and HPLC-SEC to investigate the thermal stability of the meningococcal A-CRM₁₉₇ glycoconjugate vaccine for group A meningitis currently in development. The samples were incubated at 4°C, RT, 37°C and 55°C for five weeks and the hydrodynamic size, conformational changes of the protein and the molecular integrity of the saccharide were analysed to monitor the stability of the conjugate vaccine.

The results obtained showed an increase in the hydrodynamic size, change in conformation of protein caused by unfolding of the protein and a loss in saccharide content form the conjugate at 37°C and 55°C. The thermal stability of the CRM₁₉₇ carrier protein was found to be similar to the stability of other CRM₁₉₇ conjugate vaccines.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS i
ABBREVIATIONS AND SYMBOLS ii
ABSTRACT v
TABLE OF CONTENTS vi

CHAPTER 1

GENERAL INTRODUCTION

1.1. INTRODUCTION 1
1.2. AIM AND OBJECTIVES OF THE STUDY 2

CHAPTER 2

PHYSICOCHEMICAL METHODS

2.1. PHYSICOCHEMICAL METHODS 4
2.1.1. SPECTROSCOPIC METHODS 4
   2.1.1.1. Introduction 4
   2.1.1.1. Properties of the Electromagnetic (EM) Radiation 4
   2.1.1.2. Quantitative Description of Interaction of EM and Matter 5
   2.1.1.2. Fluorescence Spectroscopy 5
      2.1.1.2.1. Principles of Fluorescence Spectroscopy 6
      2.1.1.2.2. Fluorescence Intensity 7
      2.1.1.2.3. Instrumentation 8
      2.1.1.2.4. Applications 9
   2.1.1.3. Circular Dichroism (CD) Spectroscopy 10
2.1.1.3.1. Theory of Circular Dichroism 11
2.1.1.3.2. Instrumentation 13
2.1.1.3.3. Applications 13
2.1.1.4. Nuclear Magnetic Resonance (NMR) Spectroscopy 15
  2.1.1.4.1. Introduction 15
  2.1.1.4.2. Basic NMR Principles 16
    2.1.1.4.2.1. Relaxation Processes 17
    2.1.1.4.2.2. Chemical Shift 17
    2.1.1.4.2.3. Coupling Constant 18
  2.1.1.4.3. Basic NMR Spectrometer 18
  2.1.1.4.4. Applications 19

2.1.2. CHROMATOGRAPHY 20
  2.1.2.1. Introduction 20
  2.1.2.2. Theoretical Aspects of Chromatography 21
    2.1.2.2.1. Basis of Separation Process 21
    2.1.2.2.2. Theoretical Plate Model 23
    2.1.2.2.3. Peak Resolution 23
  2.1.2.3. Basic HPLC Instrumentation 24
    2.1.2.3.1. Mobile Phase 24
    2.1.2.3.2. Injector 25
    2.1.2.3.3. Pump 25
    2.1.2.3.4. Columns 25
    2.1.2.3.5. Detectors 25
      2.1.2.3.5.1. UV detectors 26
      2.1.2.3.5.2. Refractive Index (RI) Detector 27
  2.1.2.4. Chiral High Performance Liquid Chromatography 27
    2.1.2.4.1. Principles of Chiral Separation 28
    2.1.2.4.2. Methods of Chiral Separation 28
    2.1.2.4.3. Cyclodextrins 29
      2.1.2.4.3.1. Structure and Physicochemical Properties of Cyclodextrins 30
      2.1.2.4.3.2. Toxicity 31
      2.1.2.4.3.3. Formation of Cyclodextrin Inclusion Complexes 31
      2.1.2.4.3.4. Applications of Cyclodextrins 32
2.1.2.5. Size Exclusion Chromatography (SEC) 33
  2.1.2.5.1. Theory and Principles 33
  2.1.2.5.2. Column Packings 35
  2.1.2.5.3. Applications 36

REFERENCES

CHAPTER 3

HPLC SEPARATION OF ENANTIOMERS USING CYCLODEXTRIN AS A CHIRAL SELECTOR: THE USE OF NMR AS A PREDICTIVE TOOL FOR HPLC CONDITIONS

3.1. INTRODUCTION 37

3.1.1. THE IMPORTANCE OF CHIRALITY IN BIOLOGICAL SYSTEMS 37

3.1.2. CHIRALITY 38
  3.1.2.1. Enantiomers and Diastereoisomers 39
  3.1.2.2. Nomenclature of Stereoisomers 40

3.2. EXPERIMENTAL 43

3.2.1. MATERIALS 43

3.2.2. NMR EXPERIMENTS 43
  3.2.2.1. Reagents and Chemicals 43
  3.2.2.2. Experiments 43

3.2.3. HPLC EXPERIMENTS 44
  3.2.3.1. Reagents and Chemicals 44
  3.2.3.2. Chromatographic Conditions using the Chiral Mobile Phase 44
  3.2.3.3. Chromatographic Conditions using the Chiral Stationary Phase 45

3.3. RESULTS AND DISCUSSION 46

3.3.1. RESULTS 46
  3.3.1.1. NMR Results 46
3.3.1.1.1. Effect of β-cyclodextrin 46
3.3.1.1.2. Solvent Effect 47
3.3.1.2. HPLC Results obtained using a Chiral Mobile Phase 48
  3.3.1.2.1. Effect of β-cyclodextrin 49
  3.3.1.2.2. Effect of the Mobile Phase 49
  3.3.1.2.3. Effect of Buffer in the Mobile Phase 50
3.3.1.3. HPLC Results obtained using a Chiral Stationary Phase 51
  3.3.1.3.1. Effect of the Mobile Phase 51
  3.3.1.3.2. Effect of Buffer in the Mobile Phase 54

3.3.2. DISCUSSION 56
  3.3.2.1. Effect of the Type β-cyclodextrin 56
  3.3.2.2. The Effect of the Type and Amount of Organic Solvent 58
  3.3.2.3. The Effect of Buffer in the Mobile Phase 59

3.4. CONCLUSION 60

3.5. FUTURE WORK AND RECOMMENDATIONS 62

REFERENCES

CHAPTER 4

EVALUATING THE STABILITY OF MENINGOCOCCAL A- CRM197 GLYCOCONJUGATE VACCINE BY USE OF SPECTROSCOPIC AND CHROMATOGRAPHIC METHODS

4.1. MENINGOCOCCAL DISEASE, THERAPY AND PREVENTION 63
4.1.1. HISTORY OF MENINGITIS 63
4.1.2. MENINGOCOCCAL DISEASE 63
  4.1.2.1. Clinical Features 64
    4.1.2.1.1. Asymptomatic Nasopharyngeal Carriage Meningitis 64
4.1.2.1.2. Meningitis 64
4.1.2.1.3. Meningococcaemia 65
4.1.2.1.4. Metastatic Manifestation 65
4.1.2.2. Diagnosis 65

4.1.3. CAUSES OF MENINGOCOCCAL DISEASE 65
   4.1.3.1. Meningococcus 65
   4.1.3.2. Transmission of Meningococcus 67

4.1.4. EPIDEMIOLOGY 68

4.1.5. THERAPY AND PREVENTION 70
   4.1.5.1. Antibiotic Therapy 70
   4.1.5.2. Prevention 71
      4.1.5.2.1. Chemoprophylaxis 71
      4.1.5.2.2. Vaccination 72
         4.1.5.2.2.1. Early Vaccines 72
         4.1.5.2.2.2. Capsular Polysaccharide Vaccines 72

4.2. MENINGOCOCCAL GLYCOCONJUGATE VACCINES 75

4.2.1. INTRODUCTION 75

4.2.2. IMMUNOGENICITY AND EFFICACY OF THE CONJUGATE VACCINES 76

4.2.3. CONJUGATION STRATEGIES 77
   4.2.3.1. Polysaccharide 78
   4.2.3.2. Carrier Protein 78
   4.2.3.3. Saccharide-Carrier Protein Conjugation 79
      4.2.3.3.1. Activation of Capsular Polysaccharide 80
         4.2.3.3.1.1. Random Activation (cross-linking) 80
         4.2.3.3.1.2. Selective Activation (non-cross linking) 80
      4.2.3.3.2. Activation of the Carrier Protein 81
   4.2.3.3.3. Conjugation Methods/Strategies 82

4.2.4. ANALYSIS OF CONJUGATES 82
   4.2.4.1. Characterisation of the Capsular Polysaccharide 83
   4.2.4.2. Characterisation of the Carrier Protein 84
   4.2.4.3. Characterisation of the Conjugate Vaccine 85
      4.2.4.3.1. Stability of the Conjugate 85
4.2.4.3.2. Unconjugated Carrier Protein 86
4.2.4.3.3. Protein-Saccharide Ratio 86
4.2.4.3.4. Covalent Linkage 86

4.2.5. MENINGOCOCCAL A-CRM₁₉₇ CONJUGATE VACCINE 87
4.2.5.1. Meningococcal A Capsular Polysaccharide 87
4.2.5.2. The Carrier Protein (CRM₁₉₇) 88
4.2.5.3. Preparation of the meningococcal A-CRM₁₉₇ Conjugate Vaccine 89

4.3. EXPERIMENTAL 91

4.3.1. MATERIALS 91

4.3.2. STABILITY SAMPLE TREATMENT 91

4.3.3. ANALYSIS OF THE SIZE OF THE CONJUGATE VACCINE 91
   4.3.3.1. HPLC-SEC using UV Detection 91
   4.3.3.2. HPLC-SEC coupled to a Refractive Index Detector 93

4.3.4. CONFORMATIONAL ANALYSIS OF THE PROTEIN 93
   4.3.4.1. Fluorescence Spectroscopy 93
   4.3.4.2. Circular Dichroism 93

4.3.5. SACCHARIDE ANALYSIS 94
   4.3.5.1. Total Organic Carbon (TOC) 94
   4.3.5.2. NMR Spectroscopy 94

4.4. RESULTS AND DISCUSSION 95

4.4.1. RESULTS 95
   4.4.1.1. Effect of Incubation Temperature on the Conjugate 95
     4.4.1.1.1. HPLC-SEC 95
   4.4.1.2. Effect of Incubation Temperature on Conformation of the Carrier Protein 97
     4.4.1.2.1. Fluorescence Spectroscopy 98
     4.4.1.2.2. Circular Dichroism Spectroscopy 99
   4.4.1.3. Saccharide Analysis 100
     4.4.1.3.1. NMR Spectroscopy 100
     4.4.1.3.2. Total Organic Carbon (TOC) 106
4.4.2. DISCUSSION

4.4.2.1. Molecular Size of the Men A-CRM$_{197}$ Conjugate Vaccine 108
4.4.2.2. Carrier Protein Moiety 109
4.4.2.3. Meningococcal A Capsular Polysaccharide 109

4.5. CONCLUSIONS 111

4.6. FUTURE STUDIES AND RECOMMENDATIONS 112

REFERENCES

CHAPTER 5

GENERAL CONCLUSIONS

5.1. CONCLUSIONS 113

APPENDICES
CHAPTER 1

GENERAL INTRODUCTION
1.1. INTRODUCTION

Physicochemical methods, including spectroscopy and chromatography, have been used in the pharmaceutical industry routinely for evaluation of drugs for drug research. They have been used for characterisation of pharmaceutically active substances and drug preparations to determine the properties of the drug molecule. These tests follow guidelines issued by Food and Drug Administration (FDA); which include structure, identity, impurities, heavy metal tests, level of active ingredient in the final product, verification of manufacturers' specification, in vitro studies etc. The methods can also be used to complement time-consuming and costly animal experiments.

So far these methods have been used for analysis of small molecules and biomolecules such as proteins, polymers and nucleic acids. However, their use has been extended for the analyses of complex pharmaceutical drugs such as glycoconjugates vaccines. Glycoconjugates are made of both protein and saccharide, it is therefore, important to determine the physicochemical properties of both components and of the whole complex. These techniques are now being used for analysis of glycoconjugate vaccines for meningitis.

They have provided, so far, information about the identity of the materials used in the vaccine, the stability of the conjugate and conformation of manufacturer's' consistency, for vaccines already on the market or undergoing clinical trials. Unlike biological assays, these parameters can be used to determine the structure of the vaccine, and also allow optimisation of formulation, shelf life and storage conditions of the vaccines.
1.2. AIM AND OBJECTIVES OF THE STUDY

Physicochemical methods have proved to be efficient and consistent for routine analysis of pharmaceutical drugs for decades. The study will focus on developing physicochemical methods, which will be used to analyse small chiral drugs and vaccines.

FIRST OBJECTIVE: ANALYSIS OF SMALL CHIRAL DRUGS

Current methods that have been used to analyse enantiomers include polarimetry, NMR spectroscopy and chromatography. The advantage of chromatography over other methods is that the enantiomers can be separated and quantified by gas chromatography (GC) or high performance liquid chromatography (HPLC). Chiral HPLC has, however, proven to be one of the best methods for direct separation and analysis of enantiomers. HPLC, with the use of chiral substances as additives to the stationary or mobile phase is commonly employed for separation of enantiomers in a racemic mixture. However, due to the vast range of approaches available, selecting the best conditions i.e. which stationary phase, mobile phase etc to use, is not easy.

The objective of the study was to develop an HPLC method of separating enantiomers of model compounds or drugs using a chiral selector in the mobile or stationary phase. These objectives will be evaluated using β-cyclodextrins as a chiral selector. The effect of the cyclodextrins in the mobile and stationary phase and the effect of mobile phases will be investigated. The conditions for HPLC separation will be established using NMR spectroscopy.
SECOND OBJECTIVE: ANALYSIS OF GLYCOCONJUGATE VACCINES

Glycoconjugate vaccines, unlike polysaccharide vaccines, are effective in protecting infants and induce immunological memory against bacterial pathogens such as *Haemophilus influenzae type b* (Hib), *Neisseria meningitidis* groups A and C (Men A and Men C) and *Streptococcus pneumoniae*. The vaccines are made of a polysaccharide moiety coupled to a carrier protein. Studies carried out on Hib glycoconjugate vaccines using animal models have shown that immunogenicity studies are poor predictions of protection in infants. Therefore physicochemical methods are needed to characterise and control glycoconjugate vaccines during their development, production and storage.

It is believed that the immunogenicity of glycoconjugate vaccines is compromised due to degradation of the polysaccharide chain, reduction of the saccharide chain length in the conjugate and changes in conformation of the carrier protein. Therefore, understanding the pathway by which glycoconjugate vaccines degrade will allow optimisation of formulation and shelf life of the vaccine to be established.

At the present moment, there are no methods of analysing vaccines, hence, the main objective of this study is to apply physicochemical methods to monitor and predict the stability and shelf life of glycoconjugate vaccines being developed against meningococcal A disease. This will be achieved by carrying out an accelerated study using the following approach:

- The samples will be incubated at 4°C, RT, 37°C and 55°C for five weeks.
- Physicochemical methods will then be used to analyse these samples to monitor changes in conformation of the carrier protein, CRM197, changes in the size of the meningococcal A capsular polysaccharide and changes in the hydrodynamic size of the glycoconjugate vaccine.

The results obtained will be used to predict the shelf life of the glycoconjugate vaccine over period of two years. The results obtained will be compared with physicochemical data obtained for licensed glycoconjugate vaccines.
CHAPTER 2

PHYSICOCHEMICAL METHODS
2.1. PHYSICOCHEMICAL METHODS

2.1.1. SPECTROSCOPIC METHODS

2.1.1.1. INTRODUCTION

Spectroscopy, by definition, is the interaction of electromagnetic radiation (EM) with a chemical species, and the data obtained from the atomic and molecular spectra can be used to obtain information regarding the molecular structure and the chemical properties of the molecule, atom or ion [1,2]. The electromagnetic spectrum is divided into different types of radiation such as X-ray, visible, ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), microwave, and radio-frequency radiation [3].

The nature of the radiation of the electromagnetic spectrum is the same, except for the frequency, wavelength and physical and chemical effects that they produce [4]. The spectral measurements may be classified as either absorption, in which radiation is absorbed as it passes through the medium or emission, in which case the radiation is emitted as the excited species relaxes to its original state [3]. Spectrochemical methods have provided scientists with effective methods of elucidating structural and chemical properties of organic, inorganic and biological species.

2.1.1.1.1. Properties of the EM Radiation

All radiation is characterised by a wavelength (\(\lambda\)), amplitude (\(a\)), frequency (\(\nu\)) and energy (\(E\)), and they are all related by the following equations [1,2,5,6];

\[ E = h\nu \]  \hspace{1cm} 2.1.1

where \(h = 6.626 \times 10^{-34}\) Js, Planck’s Constant
\[ c = \nu \lambda \]  

where \( c = 2.997 \times 10^8 \, \text{m/s} \), velocity of light in vacuum

These quantities can be used to determine energy transitions between energy levels, thus giving information on the properties of the molecules.

### 2.1.1.2. Quantitative Description of the Interaction of EM and Matter

The interaction of radiation with matter can be quantified using absorption laws, which have been derived by Lambert and Beer. These laws were combined to form Beer-Lambert's law shown in the equation below [7,8].

\[ \log_{10} \frac{I_0}{I} = \varepsilon b \]  

where \( I_0 \) and \( I \) are the intensities of the incident and transmitted light, respectively, \( l \) is the path length of the absorbing solution (cm), \( b \) is the concentration of the solution (mol/l), and \( \varepsilon \) is the molar extinction coefficient (1 mol\(^{-1}\) cm\(^{-1}\)) [7,8]. The above equation can be rewritten as follows:

\[ A = \varepsilon b \]

where \( A \) is the absorbance of the sample at a given wavelength. The absorbance of the solution can be measured using a spectrophotometer by passing radiation through the solution and measuring the intensity of the transmitted light, which is then converted to absorbance values.

### 2.1.1.2. FLUORESCENCE SPECTROSCOPY

Photoluminescence or luminescence is a process by which a chemical system undergoes excitation by electromagnetic radiation, and consequently reemits radiation, either of the same, or longer wavelengths [9-11]. Crookes found that certain minerals such as diamond and emeralds glow when subjected to cathode
rays in a vacuum tube [12]. These findings have led to the introduction of photoluminescence in science, especially chemistry. The two most common types of photoluminescence are fluorescence and phosphorescence, and the two can be differentiated by the rate at which the excited species return to the ground state. With fluorescence, the relaxation process occurs rapidly, at a rate of about $10^8$ sec$^{-1}$ [10]. Phosphorescence on the other hand usually persists for a period of time, from months down to second's [13]. Currently fluorescence spectroscopy is more widely used than photoluminescence in analytical chemistry due to its inherent sensitivity [9,14]. The technique is now considered as part of the analytical tools in chemistry and life sciences.

2.1.1.2.1. Principles of Fluorescence Spectroscopy

Fluorescence involves absorption of electromagnetic radiation from the ground state to the excited state followed by emission of a photon as the electron drops to the ground state [8]. The process usually involves $\pi \rightarrow \pi^*$ transition rather than $n \rightarrow \pi^*$ because the former possess shorter average lifetimes. In most cases the exciting radiation is in the ultraviolet (UV) region with the emitted radiation usually in the visible region [15]. The process is represented schematically by the Jablonski diagram, named after A Jablonski, who first demonstrated the process using energy diagrams (Figure 2.1.1) [16].

![Jablonski Diagram](image-url)

*Figure 2.1.1 Schematic representation of the Jablonski diagram*
The molecule is usually in the ground state at room temperature, but upon electromagnetic radiation, the molecule is excited from the ground vibrational level ($S_0$) to one of the vibrational levels in the first or second electronic excited state ($S_1$ or $S_2$) [1,6,9,15]. Following absorption not all molecules return to the ground state by fluorescence emission, other processes including internal conversion, vibrational relaxation, intersystem crossing, and phosphorescence usually takes place [6,9,15].

2.1.1.2.2. Fluorescence Intensity

From the Beer-Lambert law, the intensity of light absorbed is given by:

$$I = I_0 \left(1 - 10^{-db}\right) \tag{2.1.5}$$

where $I_0$ is the incident light intensity, $\varepsilon$ is the molar absorptivity, which depend on the material, wavelength of the incident light and the solvent, $l$ is the path length and $b$ is the molar concentration of the solution [11]. The intensity of fluorescence, $I_\varepsilon$ is given by:

$$I_\varepsilon = \varphi_I I_0 \left(1 - 10^{-db}\right) \tag{2.1.6}$$

where $\varphi_I$ is the quantum yield, which is defined as the ratio of the number of molecules that fluoresce to the total number of excited molecules [6,9,11,17]. It can be seen from the equation that $I_\varepsilon$ becomes constant when the product $\varepsilon b$ is large, as shown in equation 2.1.7 below.

$$I_\varepsilon = \varphi_I I_0 \tag{2.1.7}$$

For small $\varepsilon b$ ($\leq 0.01$), it can be shown that fluorescence intensity becomes

$$I_\varepsilon = 2.303 \varphi_I I_0 \varepsilon b \tag{2.1.8}$$
Thus, for low concentrations, the fluorescence intensity is directly proportional to the concentration and proportional to the intensity of the incident radiation. Several factors including molecular structure and chemical environment also affect the intensity of fluorescence [6,9,11,15,17].

2.1.1.2.3. Instrumentation

This chapter will only deal with the basic components of the instrument as further discussion of each component is covered in most analytical textbooks. Four essential components or elements of a fluorometer or spectrofluorometer can be identified from the diagram shown in Figure 2.1.2 and these include a light source, a fluorophore, wavelength filters (for fluorometers) or monochromators (for spectrofluorometers) and a detector connected to a readout device [1,11,14,15,17]. Compatibility of these four elements is essential for optimising fluorescence detection.

Figure 2.1.2. Schematic representation of a fluorometer/spectrofluorometer
The light source is usually a high-pressure mercury-vapour lamp, which produces an intense line spectrum or a xenon arc lamp, which produces a continuous spectrum over a range of about 250 nm upwards [15]. The light passes through the filter or monochromator to select the required wavelength for excitation, followed by the interaction of monochromatic light with the sample, which excite the fluorophores from the ground state to the excited state. The sample compartment in most fluorescence spectrometers contain polarisers, phosphoroscopes and thermostating equipment, the latter is used more often as fluorescence intensities are temperature dependent [11].

The second monochromator or filter is then used to remove the scattered emission light before the fluorescence is measured. The emission light is detected usually using a photomultiplier, which is connected at right angle to the incident light in order to avoid interference with the incident excited beam. Due to its high sensivity, the signals are amplified prior to detection with a photomultiplier, which is connected to a readout device. Fluorometers are generally more sensitive than spectrofluorometers because filters have a higher radiation throughput than monochromators [17].

2.1.1.2.4. Applications

Fluorescence spectroscopy has many applications in organic, inorganic and biological systems [10,15]. The technique is most widely used in biochemistry to give useful information regarding conformation and stability of proteins and other macromolecules [1]. These studies include protein folding, protein-ligand and structure of membrane proteins [18]. A variety of biological molecules contain naturally occurring or intrinsic fluorophores, including proteins, nucleic acids, cofactors, riboflavin and flavin adenine dinucleotide (FAD) [10,16].

The most highly fluorescent amino acid in proteins is tryptophan, which accounts for about 90% of the emission in proteins containing tryptophan residues. Tyrosine is the second most fluorescent amino acid, but its fluorescence is only relevant in proteins, which do not contain tryptophan residues [18]. Phenylalanine is fluorescent but the peaks are weak. Nucleic acids and nucleotides are in general not fluorescent, however, tRNA contains a base known as Y-base, which is highly fluorescent and
has an emission maximum near 470 nm [16]. Cofactors, such as NADH (reduced β-nicotinamide adenine dinucleotide) are another class of naturally occurring fluorophores, and many studies have been done on the properties of NADH upon binding to proteins. Frequently, fluorescence properties of natural fluorophores are not adequate for certain studies. Labelling these macromolecules with fluorophores, called extrinsic fluorescence, can enhance these properties; these have been used in proteins for studying protein structures and their environment [18].

2.1.1.3. CIRCULAR DICHROISM (CD) SPECTROSCOPY

Chiroptical methods include polarimetry, optical rotatory dispersion (ORD) and circular dichroism [19-21]. Polarimetry and ORD measures the direction and magnitude of the angle of rotation (\([\alpha]\)) of an incident beam of linearly polarised light upon transmission through a chiral medium. ORD measures the specific rotation as a function of wavelength and the sign of ORD curve reflects the configuration of the chromophore [22]. CD is the differential absorption (\(\Delta A = \Delta A_R - \Delta A_L\)) between the left and right circularly polarised light in the UV-Visible region and is measured as a function of wavelength (\(\lambda\)) [20,21,23].

Chiral or asymmetric molecules absorb left and right handed polarised light to a different extent and are thus considered to be "optically active" [21]. Biological molecules such as proteins and DNA are composed of optically active structures and the technique is particularly used widely for studying peptide and protein conformation [24]. In proteins, the CD spectra in the far ultraviolet region (180 to 250 nm) provide information on the conformation of the peptide backbone, whereas spectra in the near ultraviolet (250 to 350 nm), provide information on the aromatic side chains and disulfides [24,25]. The main applications of CD and ORD spectroscopy are to determine the structure and stability of many types of biological macromolecules such as carbohydrates, proteins, and metal-ligand complexes.
2.1.3.1. Theory of Circular Dichroism

Electromagnetic radiation is a complex wave form that can be considered to behave as two wave motions at right angles to each other. The radiation consists of the "e" component which oscillates in all directions perpendicular to the plane of propagation and thus produce unpolarised light. Linearly or plane polarised light on the other hand results when the "e" component is restricted to a plane perpendicular to the direction of propagation while its magnitude oscillates, this applies to the "m" component as well. Circularly polarised light is another form of polarisation where the magnitude of the oscillation is constant and the direction oscillates (Figure 2.1.3).

![Diagram of linearly and circularly polarised light](image)

*Figure 2.1.3. Schematic representation showing the electric component linearly polarised light (left) and right-handed circularly polarised light (right). Electronic vectors are shown below each as viewed along the axis of propagation from left of the diagram.*

The circular polarised light can be resolved into its two circularly polarised components ($E_R$ and $E_L$) by use of a prism and a retarder [26], but a thin birefringement crystal is used instead [23]. $E_R$ and $E_L$ represent the right and left circularly polarised waves, respectively, and in an ordinary medium the waves rotate at the same speed and the sum of the two waves results in a plane polarised light, $E_v$ vector (Figure 2.1.4 (A)) [27,28]. In an optically active medium the two components are absorbed to different extents resulting in a $E_v$ vector which follows an elliptical path, which makes an angle $\alpha$ with the direction of the incident light [19,28]. The medium is said to exhibit circular dichroism (CD) and the transmitted light become elliptically polarised.
Figure 2.1.4. Schematic representation showing the left and right circularly polarised light of the same magnitude (A), (B) shows the production of elliptically polarised light in CD and (C) shows the major and the minor axes of the ellipse which form an angle \( \Psi' \), the ellipticity [(tan\(^{-1}\) (b/a))] and \( \alpha \) correspond to the angle of rotation.

In CD the difference in absorbance (\( \Delta A \)) is used to calculate the dichroism of the sample

\[
\Delta A = \Delta A_R - \Delta A_L \tag{2.1.9}
\]

The CD spectra can also be expressed as molar extinction coefficients as shown in the equation below:

\[
\Delta \varepsilon = \varepsilon_R - \varepsilon_L \tag{2.1.10}
\]

with \( \varepsilon_R \) and \( \varepsilon_L \) being the molar extinction coefficients for the right and the left, respectively. The difference in molar extinction coefficient of the right and the left circularly polarised light can be related to molar circular dichroism as shown in equation 2.1.11 [19,21,27]

\[
[\theta] = 3300 \Delta \varepsilon \tag{2.1.11}
\]

where \([\theta]\) is the molar ellipticity and the units are degcm\(^2\)dmol\(^{-1}\).
2.1.1.3.2. Instrumentation

There are several instruments available for CD measurements but the most common instruments currently produced are the Jasco spectrometers [23]. A circular dichroism spectrometer shown in Figure 2.1.5 below is similar to an absorption spectrophotometer except for the difference in polarising elements and electro-optic modulator present in CD spectrometer [19,29]. The instrument consists of a high power incident light, produced in most cases by a xenon arc lamp with a temperature control to keep the lamp cool [19,23]. The instrument contains an electro-optic modulator, which is used to resolve the plane-polarised light into two circularly polarised lights (LCP and RCP) as mentioned in Section 2.1.1.3.1.

![Figure 2.1.5. Schematic representation of CD instrument. LCP and RCP are the left and right circularly polarised light beams, respectively.](image)

The sample then absorbs the LCP and RCP light to different extents and the differences is detected by a photocell with wavelengths ranging from 180 nm to 850 nm [22]. The instrument is purged with nitrogen to remove oxygen, thus reducing the production of ozone formed [19,26].

2.1.1.3.3. Applications

As mentioned in Section 2.1.1.3, the main application of CD is in the structural study and chiral analysis of biological molecules, in particular, proteins. The technique has been used extensively for the structural determination of a variety of proteins as well.
as the conformation and conformational changes of proteins under different conditions [20,23,24]. The transition is mainly due to absorption by amide bonds of the peptide backbone in the "far-UV" range, 180 to 250 nm (secondary structure) and the aromatic side chains in the "near-UV" range, 250 to 350 nm (tertiary structure) [24,30]. The main two electronic transitions of amide are the \( n \rightarrow \pi^* \) transition observed at 210 to 230 nm and the \( \pi \rightarrow \pi^* \) transition observed at 180 to 200 nm and these are shown in Figure 2.1.6 below [24].

**Figure 2.1.6.** Schematic diagram showing the directions of the \( n \rightarrow \pi^* \) magnetic dipole transition and \( \pi \rightarrow \pi^* \) electric dipole transition moment in the peptide group.

The main secondary structure of proteins are the \( \alpha \)-helix, \( \beta \)-sheet and random coil, which give characteristic CD spectra shown in Figure 2.1.7 and Table 2.1.1 [24,30,31]. The tertiary structure of proteins is obtained by absorption of the aromatic residues, namely, phenylalanine, tyrosine and tryptophan.

**Figure 2.1.7.** Circular dichroism spectra of pure secondary structures; \( \alpha \)-helix, \( \beta \)-sheet and random coil conformations.
Table 2.1.1. Table showing characteristic bands of α-helix, β-sheet and random coil conformations

<table>
<thead>
<tr>
<th>Conformation</th>
<th>Band</th>
<th>Transition</th>
<th>UV region (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>positive</td>
<td>π → π⁺</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>negative</td>
<td>π → π⁺</td>
<td>208</td>
</tr>
<tr>
<td>β-sheet</td>
<td>negative</td>
<td>n → π⁺</td>
<td>220</td>
</tr>
<tr>
<td>Random coil</td>
<td>positive</td>
<td>n → π⁺</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>negative</td>
<td>π → π⁺</td>
<td>216</td>
</tr>
</tbody>
</table>

CD can also be used to determine changes in the structure and conformation of proteins, and is thus an excellent method of following protein denaturation and stability.

2.1.1.4. NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

2.1.1.4.1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is the study of absorption of radio-frequency (RF) radiation by nuclei and is based on the magnetic properties of atomic nuclei [32]. The technique was first discovered by Bloch and Purcell, who were able to show that nuclei absorb electromagnetic radiation in a strong magnetic field [33,34]. The data obtained reveals useful information on molecular structure and chemical reactions. The development of multidimensional NMR techniques has resulted in a breakthrough in the determination of three dimensional (3D) structures of proteins, nucleic acid and carbohydrates. NMR is now applied in organic, inorganic and biochemistry in molecular structure elucidation [32].
2.1.1.4.2. Basic NMR Principles

In 1924, Pauli suggested that certain nuclei (e.g. $^1$H, $^{13}$C, $^{31}$P) have spin and magnetic properties, and that exposure to a magnetic field leads to splitting of their energy levels [32]. Only nuclei with spin quantum number $(I) \neq 0$ can absorb radio-frequency (RF) and they are said to have a magnetic moment, $\mu$, which in turn produces a dipolar magnetic field [35,36].

From quantum mechanics, it can be shown that a nucleus of spin $I$ will have $2I + 1$ possible orientations, that is a nucleus with spin $1/2$ will have two possible orientations with equal energy in the absence of an external magnetic field [36,37]. In the presence of $B_0$, there will be an interaction between $\mu$ and $B_0$ that generates a torque, that is, the Z-axis will process around the magnetic filed as shown in Figure 2.1.8 [36]. According to quantum mechanics, these interactions lead to different energy states of the spins.

![Figure 2.1.8. Precession of nuclei ($I = 1/2$) around the external magnetic field](image)

Upon application of radio-frequency radiation of a frequency that corresponds to the energy differences, these energy differences can be examined in a similar manner to optical spectroscopy. Modern NMR equipment irradiates sample with pulses, and the subsequent dynamics of the spins are monitored and converted into relaxation measurements (spin echo decay curves) or spectra.
2.1.1.4.2.1. Relaxation Processes

The relaxation of spins is governed by two relaxation time constants, $T_1$ and $T_2$ [32]. The spin-lattice relaxation ($T_1$) describes the exchange of excess spin energy with the surrounding (lattice) in the form of heat as the system returns to equilibrium [168]. The process is caused by the interaction of excited nuclei with small, local oscillating magnetic fields, which rotate at the same frequency as the Larmor frequency ($\omega_0$) [38,39]. $T_2$ is caused by static or slowly fluctuating magnetic field $B_0$ variations resulting in loss of phase coherence among groups of nuclei rotating in the transverse plane (x,y plane) [33,37]. The process begins immediately after absorption and is always less than $T_1$. The two relaxation times can be quantified using specific pulse sequences.

2.1.1.4.2.2. Chemical Shift ($\delta$)

Chemical shift ($\delta$) of nuclei is the amount of energy required for nuclear resonance and is observed when identical nuclei lie in different chemical environments and thus are non-equivalent in chemical terms. It is measured in ppm, relative to the reference compound, usually tetramethylsilane (TMS) [40]. Chemical shift is used to identify functional groups and as a result aid in determining the structure of the molecule (Figure 2.1.9).

![Figure 2.1.9. Chemical shifts of different $^1$H nuclei](image-url)
The two main factors that affect the observed chemical shift are diamagnetic effect and paramagnetic effect. Diamagnetic effect occurs due to shielding by electrons, which reduces the effective magnetic field around the nucleus under consideration [32]. This causes the resonance to occur at higher applied magnetic fields and the greater the electron density, the greater the shielding.

Paramagnetic effect occurs when the effective field at the nucleus is increased through induction by applied magnetic field associated with electrons moving in their orbits [41]. This leads to a deshielding of the nucleus and a shift to a lower magnetic field strength.

Chemical shifts of proton spectra of amino acids have been used to determine their behaviour in solution [42].

2.1.1.4.2.3. Coupling Constant (J)

Coupling constant (J) is another parameter that can be used to give additional structural information obtained from NMR spectroscopy [42]. It arises from the spin-spin coupling of neighbouring protons, and provides chemical information on the interaction of these protons. The spin-spin couplings give rise to a fine structure of the NMR resonances and their magnitude is expressed in hertz (Hz). Molecular structure of an unknown compound can be determined by careful analysis of chemical shifts and spin-spin coupling.

2.1.1.4.3. Basic NMR Spectrometer

The NMR spectrometer is basically a radio-frequency radiation and it consists of the following components: magnet, high frequency transmitter, receiver coil and the electronics (computer and a plotter) as shown in Figure 2.1.10. The magnet, which is normally a superconductor, produces a stable, strong and homogenous magnetic field with field strength \( B_0 \) [14,43]. The advantage of using a strong magnet is to increase the separation (resolution) between the signals as well as to enhance the intensity of the signal [44].
The sample cell, shown in blue, is placed in a probe, which is placed between the oscillating magnetic field, $B_1$, and excited via pulsation in the radio-frequency input circuit \cite{45}. The radio-frequency signal produced by the resonating nuclei is detected by means of a coil is Fourier transformed into a spectrum. The pulse is repeated several times to allow the signals to be identified from the background noise.

![Diagram of NMR spectrometer](image)

\textit{Figure 2.1.10. Schematic diagram of NMR spectrometer}

\subsection*{2.1.4.4. Applications}

NMR spectroscopy is often used as a complementary technique to other chemical analytical methods, such as mass spectrometry (MS), UV-Visible, fluorescence and infrared spectroscopy to confirm the chemical structure of new synthesised compounds. In some cases, the information provided by NMR spectroscopy can aid in selecting appropriate methods for conventional chemical analysis of small and complex pharmaceutical compounds. The technique is widely used in particular for molecular structure analysis of biological fluids, enantiomeric purity (using cyclodextrins), biomolecules such as polymers, proteins and nucleic acids \cite{46-51}.

The development of two dimensional (2D) NMR spectroscopy is very useful and is applied to elucidate the structure of complete molecules such as secondary structures of proteins, including $\alpha$-helix and $\beta$-sheets \cite{52}. The technique has been extended to the application of complex biomolecules such as glycoconjugates, which will be studied.
2.1.2. CHROMATOGRAPHY

2.1.2.1. INTRODUCTION

Chromatography is a physical method of separation, identification and determination of the components in a chemical mixture [53,54]. The technique was originally described by Tswett between 1903-1906, who used a chalk column to separate various plant pigments [54,55]. The separated species, such as chlorophylls and xanthophylls, were obtained as colored bands, hence the name chromatography, from the Greek chroma meaning "color" and graphein meaning "to write".

The basic definition of chromatography is the separation of substances based on their distribution between a mobile phase and a stationary phase. Components partition between two phases as the sample components are carried through the stationary phase by the mobile phase. SoluteS are thus separated due to differences in how they interact with the two phases. Individual species are retarded by stationary phase based on various interactions such as: surface adsorption, relative charge, relative solubility, size-exclusion etc. The chemical interaction between the sample in the mobile phase with the stationary phase determines the degree of migration and separation of components contained in the sample.

Chromatography is subdivided according to the mobile phase and the stationary phase used (Figure 2.2.11). An absorbing solid is used as stationary phase in gas-solid (GSC) and liquid-solid (LSC) chromatography while a liquid phase (coated on a solid phase) is used in gas-liquid (GLC) and liquid-liquid (LLC) chromatography. LSC can be further divided into four categories based on the type of interactions responsible for separation; partition chromatography, adsorption chromatography, ion exchange chromatography (IEC) and size exclusion chromatography (SEC).
<table>
<thead>
<tr>
<th>General Classification</th>
<th>mobile/stationary</th>
<th>Type of Equilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid chromatography</td>
<td>liquid:liquid</td>
<td>partition</td>
</tr>
<tr>
<td></td>
<td>liquid:solid</td>
<td>adsorption</td>
</tr>
<tr>
<td></td>
<td>liquid:solid</td>
<td>ion-exchange</td>
</tr>
<tr>
<td></td>
<td>liquid:solid</td>
<td>size exclusion</td>
</tr>
<tr>
<td>Gas chromatography</td>
<td>gas:liquid</td>
<td>partition</td>
</tr>
<tr>
<td></td>
<td>gas:solid</td>
<td>adsorption</td>
</tr>
<tr>
<td>Supercritical fluid</td>
<td>fluid:solid</td>
<td>partition</td>
</tr>
<tr>
<td>chromatography</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Figure 2.1.11. Classification of chromatographic methods*

### 2.1.2.2. THEORETICAL ASPECTS OF CHROMATOGRAPHY

The theory of chromatography falls into two categories, plate theory and rate theory (kinetic theory). Both theories provide useful tools for understanding how analytes are separated in a complex mixture, but only the plate theory will be discussed.

#### 2.1.2.2.1. Basis of Separation Process

All chromatographic separations are based upon differences in the extent to which solutes are partitioned between the mobile and the stationary phase:

\[
C_M \rightleftharpoons C_S \quad 2.1.12
\]

where \(C_S\) is the molar analyte concentration of the solute in the stationary phase and \(C_M\) is its concentration in the mobile phase.
The equilibrium involved can be quantitatively described by the chromatographic separation partition coefficient, \( K \), which is defined as:

\[
K = \frac{C_S}{C_M} \tag{2.1.13}
\]

The experimental chromatographic retention behaviour of an analyte may be described by three different terms: the retention time, the adjusted retention time and the retention factor. The retention time \( (t_R) \) is the time between the sample injection and the apex of the analyte peak (Figure 2.1.12).

![Figure 2.1.12. Schematic illustration of retention measurements](image)

The adjusted retention time \( (t'_{R}) \) is the difference between the retention time of the analyte of interest and that of another analyte which is not retained by the stationary phase \( (t_M) \), frequently referred to as the dead volume or void time (equation 2.1.14).

\[
\text{Adjusted retention time} \quad t'_{R} = t_{R} - t_{M} \tag{2.1.14}
\]

The capacity factor \( (k) \) is used to describe the migration rates of solutes on the column. It is defined as the product of the adjusted retention time divided by the retention time of the unretained analyte.

\[
\text{Capacity factor} \quad k = \frac{(t_{R} - t_{M})}{t_{M}} \tag{2.1.15}
\]
The separation between two bands is described by the selectivity factor ($\alpha$), the relative retention calculated for a particular pair of peaks.

$$\text{Selectivity factor} \quad \alpha = \frac{k_2}{k_1} \quad (k_2 > k_1) \quad 2.1.16$$

### 2.1.2.2.2. Theoretical Plate Model

The plate model supposes that the chromatographic column contains a large number of separate layers, called theoretical plates. Within each plate, the analyte is assumed to be equilibrated between the stationary and the mobile phases. The analyte thus move down the column by transfer of mobile phase from one plate to the next. These plates serve as a way of measuring column efficiency, that is, the number of plates, $N$, in the column, and the plate height, $H$. The two terms are related by the equation shown;

$$N = \frac{L}{H} \quad 2.1.17$$

where $L$ is the length of the column (cm). The efficiency of the column increases as the number of plates increase and the plate height become smaller. $N$ can be obtained from a chromatogram by measuring the retention time of a peak, $t_R$ and the width of the peak at the base, $W$, and thus can be written as;

$$N = 16 \left(\frac{t_R}{W}\right)^2 \quad 2.1.18$$

### 2.1.2.2.3. Peak Resolution

The resolution $R_s$ is defined as the separation between two peaks and it measures the ability of a column to separate two analytes. It can be expressed as;

$$R_s = 2[(t_R)_A - (t_R)_B] / [W_A + W_B] \quad 2.1.19$$

where ($t_R)_A$ and ($t_R)_B$ are the retention times of species A and B respectively; $W_A$ and $W_B$ are width of the peaks for species A and B, respectively.
The resolution can also be expressed in terms of the number of plates, the selectivity factor and the capacity factors of the two solutes:

$$R_s = \frac{1}{4} \left( \frac{(a-1)\alpha}{a} \right) \frac{k}{(k+1)} N^{1/2}$$  \hspace{1cm} 2.1.20

A complete resolution (baseline separation) is obtained when $R_s > 1.5$ [57].

2.1.2.3. BASIC HPLC INSTRUMENTATION

The basic instrument consists of a mobile phase reservoir, pump, injector, column, detector and recorder or data system, Figure 2.1.13. The mobile phase and the stationary phase depend on the mode of chromatography used, and therefore, varies in polarity of the solvent and the type of column material used.

Figure 2.1.13. Schematic diagram of liquid chromatogram system

2.1.2.3.1. Mobile Phase

The type or polarity of the mobile phase or eluent has a big effect on retention as it can either promote or suppress ionisation of the analyte molecules. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase.
2.1.2.3.2. Injector

The function of an injector is to inject the sample in the mobile phase. Modern instruments have an autosampler, which can inject a large number of samples and can be programmed and operates automatically.

2.1.2.3.3. Pump

A pump is used to deliver the mobile phase through the packed stationary phase beds by applying pressure, usually at a rate of about 1 ml/min. It needs to be controlled, and is both accurate and precise.

2.1.2.3.4. Columns

Different columns are used for different chromatographic method. The general requirements for a column are chemical stability; it must be able to withstand pressure. These factors facilitate equilibration and increases efficiency.

2.1.2.3.5. Detectors

A detector emits a response due to eluting sample compounds and subsequently produces a peak on the chromatogram. It is positioned after the column in order to detect the compounds as they elute. The basic detector requirements are fast response, high sensitivity, wide linear dynamic range, and should ideally be non-destructive. A universal detector will detect everything that passes though it, whereas a selective detector will detect only certain components. Sensitivity is the ratio of the output to the input, so a detector should have a large detector signal for a small amount of solute. A linear detector has a response that is directly proportional to the amount or concentration of solute. The linear range of the detector is that concentration range in which the proportionality is obeyed.
A variety of detectors can be used to measure various properties of solutes. The most commonly used detectors are fluorescence, UV/Vis, refractive index (RI) and electrochemical detectors (Table 2.1.2). Only the UV and RI detectors will be dealt with in details as they were used in this study.

Table 2.1.2. Types of detectors used in HPLC separations

<table>
<thead>
<tr>
<th>Detector type</th>
<th>Sensitivity</th>
<th>Selectivity</th>
<th>Range of application</th>
<th>Gradient elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-Vis</td>
<td>**</td>
<td>**</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>***</td>
<td>***</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>RI</td>
<td>*</td>
<td>*</td>
<td>**</td>
<td>No</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>***</td>
<td>***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* low/poor; ** moderate; *** high/good

2.1.2.3.5.1. UV Detectors

UV detection follows Beer-Lambert’s law, which has been discussed in detail in Section 2.1.1[61]. The detector measures the ability of a sample to absorb UV light and they are by far the most popular detectors in HPLC. The principle is that the mobile phase from the column is passed through a small cell held in the radiation beam of a UV/Visible photometer or spectrophotometer. The detectors are selective, sensitive, stable and are not sensitive to change in temperature and pressure. The mobile phase used should, however, absorb little or not at all, that is, it must have a high transmittance at the wavelength selected.

There are three different modes of UV/Vis detectors that are available; fixed wavelength, variable wavelength and diode array detectors [62]. The fixed wavelength detectors are most common and the simplest. The detector uses mercury lamp (emission at 254 nm) and zinc lamp (emission at 214 nm). A variable wavelength detector uses a wide range of wavelengths and it has the advantage over fixed wavelength in that it offers greater selectivity as one can use a wavelength where only the solute absorbs. Diode array detectors are the latest UV/Vis detectors...
and are more useful than conventional UV/Vis detectors. The detectors can detect intensity of the solute components over a wide range of wavelengths simultaneously, and is mostly used in method development.

2.1.2.3.5.2. Refractive Index (RI)

Refractive index (RI) of a medium is the ratio of the speed of electromagnetic wave in a vacuum to that in a give medium \( n = V_v / V \) [61,63]. Refractive index (RI) detector measures the ability of the sample molecules to bend or refract light, that is; it measures changes in the refractive index of the mobile phase due to dissolved analytes [64]. RI detectors are universal in nature, however, they are less sensitive compared to UV/Vis detectors due to its universal nature.

Another disadvantage of RI detectors is their sensitivity towards changes in mobile phase composition and changes in temperature, which can be controlled by thermostatting, and pressure [65], which makes it impossible to use gradient elution. The detector is mostly used to detect molecules which contain no UV absorbing chromophore, such as carbohydrates and lipids, and is used particularly in size exclusion chromatography [66]. The detector is now slowly being replaced by amperometric detection, which is more sensitive and specific for the analyte [67].

2.1.2.4. CHIRAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The concept of chirality is of major concern in the pharmaceutical industry as conventional methods used produce drugs as a racemate. Often one enantiomer has desired biological activity, while the other may exhibit no effect, or in other cases may be toxic. Therefore, separation of racemates into enantiomers is an essential procedure. Modern chiral analytical and synthetic procedures are now available to overcome the earlier problems encountered. The use of HPLC with chiral selectors to either the stationary or the mobile phase has, however, increased the possibilities of enantiomeric separations [68-70]. β-cyclodextrins have been used successfully for chiral separation by HPLC as it forms stable diastereomeric inclusion complexes and it has proven to be effective in the separation of many isomers [71,72].
2.1.2.4.1. Principle of Chiral Separation

Chiral separation of enantiomers is achieved by formation of a diastereoisomeric complex between the analyte and the chiral discriminator (chiral selector). Diastereoisomers, unlike enantiomers, differ in their physical and chemical properties. The diastereoisomers are formed by fixed positions in space called the "three point" contact system, which are stereoselective [73-75]. The model was first proposed by Dalgleish, and the concept states that a chiral molecule must be associated with another chiral moiety via these points [74,75].

The interaction "points" include, hydrogen-bonding interactions, electrostatic interactions, hydrophobic and charge transfer. In Figure 2.1.14, the enantiomer on the left has three contact (A, B, and C) which matches the corresponding sites (A', B', and C') of the chiral selector. The sites of the mirror image (right enantiomer) on the other hand does not match that of the chiral selector, thus chiral discrimination is achieved.

![Figure 2.1.14](image)

*Figure 2.1.14. Illustration of the "three" point contact model for enantioselectivity in intermolecular interactions (A, B, C)*

2.1.2.4.2. Methods of Chiral Separation

Separation of enantiomers can be achieved by two methods, the indirect and direct methods. The indirect separation involves the formation of a pair of diastereoisomers by derivatisation with a chiral derivatising agent (CDA) before analysis using achiral HPLC columns and mobile phases. This method can be time consuming and in some
cases lead to formation of unwanted products. To avoid this, one can use the direct chromatographic approach.

There are two ways of achieving direct separations, either by using chiral stationary phases (CSPs) or by chiral mobile phase additives (CMPAs) in normal- or reversed-phase chromatography. In the former, the chiral selector is chemically bound to the surface of the support material, and in the latter the chiral selector is added to the mobile phase and the separation is achieved using normal columns.

The use of chiral stationary phases is the fastest growing area of chiral separations. Pirkle introduced the first commercially available CSP for HPLC in 1981 [76], and today many chiral phases are commercially available and these include Pirkle-type and related CSPs (based on amino acid derivatives), derivatised cellulose [77,78], cyclodextrins [79] and proteins [80,81]. The use of CSPs, however, have drawbacks, including cost, flow rate, pH range, mobile phase restrictions and stationary phase stability.

In chiral mobile phase additives, there are three major types of additive based on ligand exchange (transition metal ion), ion pairs, and inclusion of the solute into a cavity (such as cyclodextrins) and they all rely on the formation of reversible complexes [82]. The use of chiral selectors as mobile phase additives with an achiral column offers advantages of flexibility, a wide range of possible additives, and often lower cost compared with equivalent chiral stationary phase. However, few attempts have so far been made to use such a system for resolution of enantiomers [83-87]. Therefore this study investigates further the use of cyclodextrins as chiral mobile phase additives (Chapter 3). The use of both methods is still preferred over other methods in industries such as the pharmaceutical industry.

2.1.2.4.3. Cyclodextrins

Cyclodextrins were first isolated in 1891 by Villiers as degradation products of starch by Bacillus macerans [88]. They were characterised as cyclic oligosaccharides in 1904 by Schardinger, hence the name Schardiner dextrins as reported in early literature [89]. It was not until 1938 that Freudenberg et al. reported that cyclodextrins
are constructed from α-(1,4)-linked glucose units; he also recognised that cyclodextrins could form inclusion compounds [89]. Cramer et al. systematically studied the formation of cyclodextrin-guest complexes, and in the process discovered that cyclodextrins have a catalytic action in some reactions [89]. These experiments laid the foundations for the chemistry of cyclodextrins.

2.1.2.4.3.1. Structure and Physicochemical Properties of Cyclodextrins

Cyclodextrins are cyclic (α-1,4)-linked oligosaccharides of α-D-glucopyranose containing a relatively hydrophobic cavity as a result of the electron-rich environment provided mostly by the glycosidic oxygen [90,91]. The outer surface of the cyclodextrin molecule is hydrophilic due to the presence of primary and secondary hydroxyl groups. Due to lack of free rotation about the bonds connecting the glucopyranose units, the cyclodextrins are not perfectly cylindrical molecules but are toroidal or cone shaped (Figure 2.1.15). The primary hydroxyl groups are located on the narrow side of the torus while the secondary hydroxyl groups are located on the wider edge. The most common cyclodextrins are α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin, which consists of six, seven or eight glucopyranose units, respectively.

![Chemical structure and toroidal shape of the α-, β- and γ-cyclodextrin](image)

*Figure 2.1.15. Chemical structure and toroidal shape of the α-, β- and γ-cyclodextrin*

Chemical and physical properties of the common cyclodextrins are given in Table 2.1.3 [92-95]. The solubility of cyclodextrins generally decreases in the presence of organic molecules, owing to complex formation. The properties of natural cyclodextrins can be optimised by substituting the hydroxyl groups to form cyclodextrin derivatives and the most common cyclodextrin derivatives are the alkylated cyclodextrins [90,96]. The melting points of α-, β- and γ-cyclodextrin have
been found to be between 240 and 265°C, which is consistent with their stable crystal lattice structure [97]. The glycosidic bonds of cyclodextrins are fairly stable in alkaline solutions, whereas they are hydrolytically cleaved by strong acids to give linear oligosaccharides [92,98].

Table 2.1.3. Some characteristics of α, β- and γ-cyclodextrin

<table>
<thead>
<tr>
<th></th>
<th>α</th>
<th>β</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of glycopyranose units</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>972</td>
<td>1135</td>
<td>1297</td>
</tr>
<tr>
<td>Central Cavity diameter (Å)</td>
<td>4.7-5.3</td>
<td>6.0-6.5</td>
<td>10.3-11.2</td>
</tr>
<tr>
<td>Water solubility at 25 °C (g/100ml)</td>
<td>14.5</td>
<td>1.85</td>
<td>23.2</td>
</tr>
</tbody>
</table>

2.1.2.4.3.2. Toxicity

The toxicity of the three common natural cyclodextrins and some of their derivatives has been reviewed extensively. These studies have shown that orally administered cyclodextrins are non-toxic due to lack of absorption from the gastrointestinal tract [89,99-101]. However, toxicological studies have shown that they are unsuited for parenteral (intravenous or intramuscular) administration.

2.1.2.4.3.3. Formation of Cyclodextrin Inclusion Complexes

The most remarkable property of cyclodextrins is their ability to form inclusion complexes with a variety of molecules, which can fit entirely or partially into the cyclodextrin cavity [92]. The hydrophobic cavity provides a hydrophobic microenvironment, into which suitable sized drug molecules may enter and be included [98]. A number of factors influence the formation and strength of interaction of the inclusion complex. In aqueous solution, the most significant factors are the so-called "hydrophobic effect" and hydrogen bonding.
The hydrophobic effect refers to inclusion of an apolar portion of the guest molecule into the cavity of the cyclodextrin molecule. Retention of the hydrophobic solute is largely dependent on the efficiency of the contact with the hydrophobic interior. The inclusion complex exists in equilibrium and can be expressed as shown in Figure 2.1.16 [93]. Stability measurements, equilibrium constants ($K_c$) or the distribution constants ($K_D$), have shown that the complex is dependent upon the concentration of the cyclodextrin, guest and water [102, 103].

![Figure 2.1.16. Schematic representation of cyclodextrin complex equilibrium](image)

(1:1 cyclodextrin:guest)

2.1.2.4.3.3.4. Applications of Cyclodextrins

Cyclodextrins have many applications in food, cosmetics, pharmaceutical industries and biomedical products and other industries [90, 92, 98, 104-107]. In the pharmaceutical industry, cyclodextrins have been used as complexing agents to increase the aqueous solubility of water insoluble drugs, and to increase their bioavailability and stability. In addition, cyclodextrins can be used to reduce or prevent gastro-intestinal (GI) irritation, reduce or eliminate unpleasant smells or tastes and prevent drug-drug or drug-additive interactions.

Cyclodextrins can also be used as chiral discriminators because their cavities are chiral [108]. The chiral discrimination is usually observed in NMR spectra due to shifts in proton signals of both the cyclodextrin and the guest molecule. The chiral "guest" molecules effectively form diastereomeric complexes which display different properties that allow the enantiomers of the guest to be distinguished, and thus result in a more efficient separation. The formation of an inclusion complex depends on a number of factors, including the size, shape and spatial geometry of the guest and the size of the cyclodextrin cavity. Guest molecules can interact through hydrogen
bonding or dipolar interactions with the functional groups on the rims of the cyclodextrin molecule and by van der Waals forces within the cavity.

2.1.2.5. SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Size exclusion chromatography (SEC), also referred to as gel permeation or gel filtration, is a type of chromatography where molecules are separated according to their hydrodynamic size or volume. The technique originated in 1959 at the Biochemical Institute by Porath and Flodin [109,110]. It involves the separation of solutes in order of decreasing in size or volume, that is, very large molecules elute much faster than small molecules. The advantage of this technique over other forms of chromatography is that solutes are eluted according to size, which provide a means of obtaining information on the molecular size distribution in a polymer fraction. Theory and applications of this technique are discussed.

2.1.2.5.1. Theory and Principles

As mentioned earlier, in SEC, molecules are separated according to differences in their sizes as they pass through the column, which is packed with a gel as shown in Figure 2.1.17. The sample to be analysed is applied at the top of the column and passes through the column gravitationally with the mobile phase. As the sample moves through the column, it passes through the porous beads [64].

![Figure 2.1.17. Schematic representation of the mechanism of size exclusion chromatography](image-url)
Small molecules get retained within the pores in the beads and move through the column slower than large molecules, which pass through the beads freely. Very large molecules will elute in the void volume \( V_o \), whereas very small molecule will elute at the total volume \( V_t \) of the column [111-114]. The passage of the solute molecule through the column is described by the distribution coefficient \( K_D \) which is expressed as follows:

\[
K_D = \frac{(V_o - V_M)}{V_S} \tag{2.1.21}
\]

where \( V_o \) is the elution volume of the solute, \( V_M \) is the volume of the mobile phase and \( V_S \) is the volume within the porous particles of the stationary phase.

The total volume of the SEC column can be represented by,

\[
V_t = V_o + V_s + V_g \tag{2.1.22}
\]

where \( V_g \) and \( V_o \) represents the volume occupied by the gel matrix and the void volume, respectively. For large molecules which are completely excluded, \( V_g = V_o \) and \( K_D = 0 \) [115]. On the other hand, small molecules which are retained in the gel are therefore capable of distributing themselves equally between the two phases, with \( K_D = 1 \) [112]. \( K_D \) values greater than one indicates that adsorption between the matrix and the solute takes place. \( K_D \) values are difficult to determine as \( V_s \) and \( V_g \) values are hard to measure, therefore an alternative expression for \( K_D \) is obtained [65,116];

\[
K_{av} = \frac{(V_o - V_o)}{(V_t - V_o)} \tag{2.1.23}
\]

where \( K_{av} \) represent average distribution coefficient and is easy to obtain; \( V_o \) can be read from the chromatogram, \( V_t \) can be calculated from column geometry, \( V_o \) is the retention volume of a completely excluded molecule such as blue dextran (molecular weight of about 2x10^6)
### 2.1.2.5.2. Column Packings

A SEC chromatographic system has the same components as the normal HPLC system (Figure 2.1.13). The column packings of SEC act as molecular sieve, and the pore size determines the size of the molecules to be excluded. There are several criteria that a SEC media should exhibit; the media should not exhibit adsorption properties, should be insoluble and stable in various buffer composition, should be sufficiently rigid to allow good flow rate and should not cause denaturation of the solute molecules. Unfortunately, such an ideal media is still not available, but there are several media, which have met some of these criteria to a greater or lesser extent.

A summary of some of these columns is shown in Table 2.1.4 and they are available commercially [117-120].

<table>
<thead>
<tr>
<th>Table 2.1.4. Summary of commercially available SEC column packings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gel Permeation (organic solvents)</strong></td>
</tr>
<tr>
<td>Polystyrene beads</td>
</tr>
<tr>
<td>Porager®, Styrage®</td>
</tr>
<tr>
<td>Bio-Beads®-S</td>
</tr>
<tr>
<td><strong>Porous silica or glass</strong></td>
</tr>
<tr>
<td>Porasil®, Bio-Glas®</td>
</tr>
<tr>
<td>Merkogel®-Si</td>
</tr>
<tr>
<td><strong>Gel Filtration (aqueous solvents)</strong></td>
</tr>
<tr>
<td>Semi-rigid (low molecular weight)</td>
</tr>
<tr>
<td>Sephadex®</td>
</tr>
<tr>
<td>Bio-Gel®-P</td>
</tr>
<tr>
<td>Non-rigid (high molecular weight)</td>
</tr>
<tr>
<td>Sepharose®</td>
</tr>
<tr>
<td>Bio-Gel®-A</td>
</tr>
</tbody>
</table>
These materials are available in a number of different pore sizes suitable for different for separations of compounds with different molecular weights, the finer the particle size, the greater the resolution and the slower the flow rate [121]. Polystyrene/divinylbenzene gels are most commonly used stationary phases; the materials are highly cross-linked, macroporous and spherical in nature.

Porous silica or glasses are rigid, therefore, they can be used at elevated temperatures. However, silica packings turn to adsorb the sample onto their surface, thus giving undesirable retention behaviour [117,118]. Sephadex® and Bio-Gel®-P are made from cross-linked dextran and polyacrylamide, respectively. The dextrans have been described in greater details by Porath and Flodin [110]. Sepharose® and Bio-Gel®-A on the other hand are composed of agarose (poly-galactopyranone) and are used to separate very high molecular weight molecules such as proteins, nucleic acids, viruses and polysaccharides.

2.1.2.5.3. Applications

Size exclusion chromatography is mostly used in separations of macromolecules including proteins, carbohydrates, lipids and nuclei acids [122,123]. The technique has mainly been used in fractionation, molecular weight determination and distribution and separation of proteins, glycopeptides and oligosaccharides [65,67,112,124].

The disadvantage of this method for saccharide analysis is long analysis time, which can be several hours. Due to this problem, conventional SEC is slowly being replaced by a better technique, high performance anion exchange chromatography coupled to pulsed amperometric detector (HPAEC-PAD), which is now being used as a better method for analysis of oligosaccharides.
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CHAPTER 3

HPLC SEPARATION OF ENANTIOMERS USING CYCLODEXTRIN AS A CHIRAL SELECTOR: THE USE OF NMR AS A PREDICTIVE TOOL FOR HPLC CONDITIONS
3.1. INTRODUCTION

3.1.1. IMPORTANCE OF CHIRALITY IN BIOLOGICAL SYSTEMS

The fundamental connection between chiral molecules and living matter has been appreciated by scientists since it was first observed by Pasteur in the 19th century [1,2]. It is now recognised that all of the crucial biopolymers associated with life are homochiral, i.e. the monomer protein units consist of the L-enantiomers of 18 optically active protein amino acids [3]. The monomer units of the nucleic acid polymers DNA and RNA and those of the biologically important polysaccharides are associated with D-enantiomers [2,4].

This homochiral characteristic of life has been a matter of intense investigation for many years, it has been argued that biomolecular replication and the existence and the origin of life itself would be impossible without both homochirality and the chiral purity of protein and nucleic acid biopolymers [5-7]. Proteins, nucleic acids, and polysaccharides possess chiral characteristic structures that are closely related to their functions [8]. Due to chirality, living organisms usually show different biological responses to one of a pair of enantiomers such as in drugs and pesticides.

For example, S-Limonene smells like lemons, while R-Limonene smells like oranges. A number of compounds currently in pharmaceutical development are chiral. Chirality offers a means of increasing the specificity of drug action by providing optimal spatial orientation of functionality in the drug. Chiral drugs produced by conventional synthetic organic chemistry contain a 50/50 (racemic) mixture of enantiomers. Frequently, one enantiomer has therapeutic activity, while the other may exhibit no effect. In other situations, the non-active enantiomer can inhibit the activity of the therapeutic form, or it may be toxic [8].

Thalidomide was used as a sedative in pregnant women to combat many of the symptoms associated with morning sickness [9]. One enantiomer displayed the desired sedative effect, whereas the other had teratogenic activity, where babies
were born dead and those who survived had birth defects such as deafness, blindness, disfigurements and many other internal disabilities [10,11].

In foods, the chirality of compounds may determine its nutritional value, sensory properties or biological activity. Table 3.1.1 lists the activities of some optical isomers. There is a need to accurately assess enantiomeric purity of pharmaceutical, agricultural or other chemical entities to minimise the potential for side effects.

Table 3.1.1. Activities of some stereoisomers

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drugs</td>
<td></td>
</tr>
<tr>
<td>Amphetamine</td>
<td>d-isomer is potent central nervous system stimulant, while l-isomer has little, if any, effect</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>l-isomer is 10 times more active as a vasoconstrictor than d-isomer</td>
</tr>
<tr>
<td>Propranolol</td>
<td>Racemic compound is used as drug; however only (S)-(−)-isomer has desired β-adrenergic blocking activity</td>
</tr>
<tr>
<td>Propoxyphene</td>
<td>α-1-isomer is antitussive; α-d-isomer is analgesic</td>
</tr>
</tbody>
</table>

3.1.2. CHIRALITY

Chiral objects or processes possess an intrinsic "handedness" in three-dimensional space, and are characterised by having nonsuperimposable mirror images, a familiar example of chiral objects are the left and hand [2,15]. Chiral molecules contain atoms forming non-planar structures by covalent bonding. Stereochemistry (from the Greek word stereos, meaning solid) refers to the chemistry in three dimensions [16]. It is the study of how the geometry affects the properties of molecules and how they react. Even remarkably small differences in molecular geometry can affect the reactivity of a molecule and the products of a reaction. Isomerism refers to the existence of two or more molecules having the same molecular formula but differ in the way the atoms are arranged (see Figure 3.1.1) [17].
Figure 3.1.1. Schematic representation of isomerism

Steroisomers are two molecules with the same structural formula but they have different three dimensional arrangements of atoms. There are two types of stereoisomers, namely geometrical isomers and optical isomers:

- **geometrical isomers**: two different arrangements around a bond with restricted motion (isomers containing either a double bond or a ring).
- **optical isomers**: different three-dimensional arrangements of atoms around one or more chiral carbon atoms.

### 3.1.2.1. ENANTIOMERS AND DIASTEREISOMERS

Although chiral molecules containing stereogenic centres are by far the most common, chiral molecules without a stereogenic centre exist and they include axial [18-22], planar [23,24] and helical [25] chiral molecules. The most common type of chirality is central chirality, and molecules with central chirality have an atom bonded tetrahedrally (sp³) to four different atoms (see Figure 3.1.2) [2,26]. Such molecules are non-superimposable mirror images of each other and are called stereoisomers or enantiomers.
Figure 3.1.2. Illustration of non-superimposable mirror images

Enantiomers of a compound have identical physical characteristics, such as, solubility, boiling and melting point, same reaction rates with ordinary reagents and yield the same absorption spectrum, except the direction in which they rotate plane polarised light (optical activity) [2,13,26,27]. If the plane-polarised light is rotated in a clockwise direction, the optical activity is denoted as positive (+) or dextrorotatory (d) and rotation in a counter-clockwise direction is denoted as negative (-) or levorotatory (l).

Diastereomerism results from molecules with two or more chiral centres, which give rise to sets or pairs of enantiomers (N chiral centres \( \rightarrow 2^N \) stereoisomers) [2]. It is the relationship between an enantiomer of one pair with the enantiomer of the other pair. Diastereoisomers, unlike enantiomers, have different physical properties and can therefore be identified/resolved using achiral methods e.g. NMR spectroscopy and chromatography [2,26].

3.1.2.2. NOMENCLATURE OF STEREOISOMERS

In order to distinguish between enantiomers, a set of rules has to be established. The oldest system for referring to the stereochemistry of molecules is the D-and L-system (dextro-rotatory and levo-rotatory, respectively); it is based on which direction the molecule rotates plane polarised light [2,28]. This system is limited in applicability, for example in the classification of carbohydrates, which can have multiple chiral
centres. An example of compounds upon which the system is based is shown in Figure 3.1.3.

![Figure 3.1.3. Naming enantiomers using the D- and L-naming system](image)

The D-configuration is given to the form which has the hydroxyl group of the chiral carbon on the right when viewed with a Fischer projection (longest carbon chain drawn vertically with the most oxidised end of the chain at the top) and the L-configuration is given when the hydroxyl group is on the left. The assignment of D- and L-configuration is determined by the orientation of the chiral centre which is most distant from the reference group (ketone or aldehyde group for sugars).

A more useful set of rules known as the Cahn-Ingold-Prelog system or the R- and S-system, where R and S refer to the Latin terms *rectus* (meaning right) and *sinister* (meaning left), respectively, was established by three chemists, R.S. Cahn, C.K. Ingold and V. Prelog [29,30]. Using this set of rules, a priority rating is given to each substituent on a chiral centre shown in Figure 3.1.4.

![Figure 3.1.4. Illustration of the "R"- and "S"-system](image)
Due to growing emphasis on the pharmacological properties of chiral compounds, the U.S. Food and Drug Administration (FDA) has issued a guideline that for chiral drugs, enantiomer of the drug should be studied separately for pharmacological activities and only its therapeutic active isomer should be marketed. Racemates can be marketed as well provided both enantiomers show similar therapeutic activities. Many analytical methods with suitable selectivity and sensitivity have been developed as result and they include HPLC, circular dichroism (CD) and NMR spectroscopy [14]. In particular HPLC has played an important role in determining and quantifying optical isomers. Separation of enantiomers using this technique is discussed in details in Chapter 2.
3.2. EXPERIMENTAL

3.2.1 MATERIALS

Chlorthalidone, Dansyl-DL-leucine, Dansyl-DL-methionine, Dansyl-DL-norleucine were purchased from Sigma Chemicals and 4,5,7-Trihydroxyflavanone was purchased from Aldrich Chemicals. The structures of the compounds are shown in Figure 3.2.1. Hydroxypropyl-β-cyclodextrin (HP-β-cyclodextrin) was obtained from Fluka Chemicals and sulfated-β-cyclodextrin (S-β-cyclodextrin, degree of substitution 13) was obtained from Aldrich Chemicals.

3.2.2. NMR EXPERIMENTS

3.2.2.1. REAGENTS AND CHEMICALS

D₂O (deuterated water) was obtained from GOSS Scientific Instruments Ltd, acetonitrile-d₃ was purchased from Aldrich Chemicals and methanol-d₄ was obtained from Cambridge Isotope Laboratories.

3.2.2.2. EXPERIMENTS

All spectra were recorded on a Bruker B-ACS-60 Ultrashield NMR spectrometer operating at ambient temperature. Experiments were carried out on various cyclodextrin/analyte mixtures at molar ratios of 10:1 (cyclodextrin:analyte). The samples (stock solution) were prepared by dissolving 3mg of analyte in either 50/50 MeOD/D₂O or 50/50 Acetonitrile-d₃/D₂O in a 3ml volumetric flask to give a solution with a concentration of 1mg/ml. Appropriate amounts of cyclodextrin (ten times the amount of analyte) were dissolved in 1ml of the stock solution. ¹H spectra of the compounds with and without the cyclodextrin were recorded.
3.2.3. HPLC EXPERIMENTS

3.2.3.1. REAGENTS AND CHEMICALS

TEA (Triethylamine) was purchased from Fisher Scientific International Company and glacial acetic acid and the water were supplied by BDH Laboratory Supplies. Acetonitrile and methanol were obtained from ROMIL Ltd. All solvents were HPLC grade and mobile phases were filtered through a 0.45 μm filter.

3.2.3.2. CHROMATOGRAPHIC CONDITIONS USING THE CHIRAL MOBILE PHASE

Table 3.2.1. Summary of the method and conditions using a chiral mobile phase

<table>
<thead>
<tr>
<th>Mobile phases</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) 50% water / 50% MeOH</td>
</tr>
<tr>
<td>(2) 50% water / 50% MeCN</td>
</tr>
<tr>
<td>(3) 80% water / 20% MeOH</td>
</tr>
<tr>
<td>(4) 80% water / 20% MeCN</td>
</tr>
<tr>
<td>(5) 50% TEAA pH 4 / 50% MeOH</td>
</tr>
<tr>
<td>(6) 50% TEAA pH 4 / 50% MeCN</td>
</tr>
</tbody>
</table>

Gradient profile: Isocratic
Flow rate: 1.0 ml/min
Injection volume: 5 μl
Detection: 254 nm
Analysis Time: 30 minutes
Sample preparation: 5mg of racemic mixture was dissolved in 50/50MeOH/water to give a solution with a concentration of 1mg/ml

# = each mobile phase contain either HP-β-cyclodextrin (0.14mM) or S-β-cyclodextrin (0.08mM).
* = Triethylamine acetate (TEAA) buffer

Preparation of the mobile phases is outlined in Appendix 1.1
3.2.3.3. CHROMATOGRAPHIC CONDITIONS USING THE CHIRAL STATIONARY PHASE

Table 3.2.2. Summary of the method and conditions using a chiral stationary phase

| HPLC system: | Agilent HPLC 1100 |
| HPLC column: | Nucleodex β-OH 200 x 4 mm i.d. |
| Column Temp: | 30°C |
| Mobile phases: | |
| (1) | 50% water /50% MeOH |
| (2) | 50% water / 50% MeCN |
| (3) | 80% water / 20% MeOH |
| (4) | 80% water / 20% MeCN |
| (5) | 50% TEAA* pH 4 / 50% MeOH |
| (6) | 50% TEAA pH 4 / 50% MeCN |

Gradient Profile: Isocratic
Flow rate: 0.7 ml/min
Injection volume: 5 μl, unless otherwise specified
Detection: 254 nm
Analysis time: 30 minutes
Sample preparation: 5mg of racemic mixture was dissolved in 50/50MeOH/water to give a solution with a concentration of 1mg/ml

* = Triethylamine acetate (TEAA) buffer

Figure 3.2.1. Chemical structures of the compounds studied; (a) Dansyl-DL-norleucine, (b) Dansyl-DL-leucine, (c) Dansyl-DL-methionine, (d) Flavanone, (e) Chlorthalidone
3.3. RESULTS AND DISCUSSION

3.3.1. RESULTS

3.3.1.1. NMR RESULTS

The $^1$H NMR spectra of all compounds were recorded in deuterated methanol/water (CD$_3$OD/D$_2$O) and deuterated acetonitrile/water (CD$_3$CN/D$_2$O) with HP-β-cyclodextrin and SB-β-cyclodextrin at a molar ratio of 10:1 (cyclodextrin:analyte) on a Bruker B-ACS-60 Ultrashield NMR spectrometer. NMR spectroscopy has been used in many studies for chiral discrimination of enantiomers. In this study the technique is used to study the interaction of β-cyclodextrin with enantiomers of a racemic mixture to form diastereoisomers, which can be separated by chiral HPLC. Two variables were investigated: the effect of cyclodextrin and solvent on the racemic mixture.

3.3.1.1.1. Effect of β-cyclodextrin

The compounds studied, with the exception of DL-methionine, showed evidence of enantiomeric interaction (resonance splitting) with HP-β-cyclodextrin. The spectra of DL-leucine are shown to illustrate the effect of cyclodextrin on the racemate mixture (Figure 3.3.1). The NMR spectrum of DL-leucine in the presence of HP-β-cyclodextrin shows resonance splitting between 0 and 1 ppm (two pairs of doublets). In contrast, there was no evidence for the formation of inclusion complexes of any of the compounds with the S-β-cyclodextrin.
Figure 3.3.1. $^1$H NMR (NHCHCH$_2$) spectra of DL-leucine in CD$_3$OD to show the effect of cyclodextrin (a) without cyclodextrin, (b) with HP-$\beta$-cyclodextrin, (c) with S-$\beta$-cyclodextrin

3.3.1.2. Solvent Effect

The effect of solvent is shown using HP-$\beta$-cyclodextrin in both methanol and acetonitrile. A summary of this effect is given in Table 3.3.1 and Figure 3.3.2 shows spectra of chlorthalidone to highlight this effect.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Splitting region (ppm) in CD$_3$OD/D$_2$O</th>
<th>Splitting region (ppm) in CD$_3$CN/D$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-norleucine</td>
<td>0.2 to 0.6 (2 triplets)</td>
<td>-</td>
</tr>
<tr>
<td>DL-leucine</td>
<td>0 to 1 (two pairs of doublets)</td>
<td>-</td>
</tr>
<tr>
<td>Flavanone</td>
<td>2.6 to 3.2, 5.8 to 7.0</td>
<td>0.5 to 1.1</td>
</tr>
<tr>
<td>Chlorthalidone</td>
<td>7.2 to 8.4</td>
<td>4.8 to 5.9</td>
</tr>
</tbody>
</table>

*no splitting observed
There is evidence of enantiomeric resolution (signal splitting) for DL-norleucine, DL-leucine, flavanone and chlorthalidone in CD$_3$OD/D$_2$O in the presence of HP-β-cyclodextrin as shown in Table 3.3.1. Flavanone and chlorthalidone shows some interaction with HP-β-cyclodextrin in both solvents.

![Figure 3.3.2. $^1$H NMR (NH) spectra of chlorthalidone in (a) CD$_3$OD and (b) CD$_3$CN, without cyclodextrin, (c) CD$_3$OD and (d) CD$_3$CN, with HP-β-cyclodextrin](image)

These findings indicated that chiral discrimination could be achieved in the presence HP-β-cyclodextrin and therefore HPLC was used to separate the enantiomers using both β-cyclodextrins in the mobile phase.

### 3.3.1.2. HPLC RESULTS OBTAINED USING A CHIRAL MOBILE PHASE

The effects of type of cyclodextrin, mobile phase, and buffer on the retention time and separation will be discussed. The chromatograms obtained are not shown, instead, the retention time of the analytes under these conditions are tabulated.
3.3.1.2.1. Effect of $\beta$-cyclodextrin

The concentration and type of cyclodextrin in the mobile has a big effect on the separation of enantiomers. $\beta$-cyclodextrins are known to form better complexes than $\alpha$- and $\gamma$-cyclodextrin. Modified $\beta$-cyclodextrins forms better complexes than native $\beta$-cyclodextrins as the polarity of the cyclodextrin cavity relative to the exterior is altered. Two modified cyclodextrins were used, hydroxypropyl-$\beta$-cyclodextrin (HP-$\beta$-cyclodextrin) and sulfated-$\beta$-cyclodextrin (S-$\beta$-cyclodextrin), where the hydroxyl groups at the rim are replaced by hydroxypropyl or sulfate groups, respectively.

There was no resolution of the enantiomers when either of the cyclodextrins were used (Table 3.3.2). The retention time for each compound was similar in both cases.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Eluent</th>
<th>$t_1$ (min)</th>
<th>$t_2$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Methionine</td>
<td>50/50MeOH/water</td>
<td>2.307</td>
<td>-</td>
</tr>
<tr>
<td>DL-Leucine</td>
<td>50/50MeOH/water</td>
<td>2.896</td>
<td>-</td>
</tr>
<tr>
<td>DL-Norleucine</td>
<td>50/50MeOH/water</td>
<td>3.071</td>
<td>-</td>
</tr>
<tr>
<td>Flavanone</td>
<td>50/50MeOH/water</td>
<td>4.339</td>
<td>3.931</td>
</tr>
<tr>
<td>Chlorthalidone</td>
<td>50/50MeOH/water</td>
<td>1.703</td>
<td>1.673</td>
</tr>
</tbody>
</table>

$t_1$ and $t_2$ is the retention time of the analyte with HP-$\beta$-cyclodextrin and S-$\beta$-cyclodextrin in the mobile phase, respectively, *the results are not available*

3.3.1.2.2. Effect of Mobile Phase

The type and amount of organic solvent has a large effect on the separation and resolution of enantiomers as it can either hinder or promote interaction between the analyte and the cyclodextrin. Two solvents, methanol and acetonitrile commonly used in reverse-phase chromatography, were studied. The effect of mobile phase on separation using HP-$\beta$-cyclodextrin in the mobile phase is shown in Table 3.3.3. There was no evidence of enantiomeric separation observed with either of the mobile phases. There is however, a decrease in retention time moving from ethanol to acetonitrile.
Table 3.3.3. Results to show the effect of mobile phase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Eluent</th>
<th>t₀ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Methionine</td>
<td>50/50MeOH/water</td>
<td>2.307</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/water</td>
<td>1.138</td>
</tr>
<tr>
<td>DL-Leucine</td>
<td>50/50MeOH/water</td>
<td>2.896</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/water</td>
<td>1.172</td>
</tr>
<tr>
<td>DL-Norleucine</td>
<td>50/50MeOH/water</td>
<td>3.071</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/water</td>
<td>1.255</td>
</tr>
<tr>
<td>Flavanone</td>
<td>50/50MeOH/water</td>
<td>4.339</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/water</td>
<td>1.812</td>
</tr>
<tr>
<td>Chlorothalidone</td>
<td>50/50MeOH/water</td>
<td>1.703</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/water</td>
<td>1.309</td>
</tr>
</tbody>
</table>

*ₜ₀ is the retention time of the analyte

3.3.1.2.3. Effect of Buffer (TEAA) in the Mobile Phase

The presence of buffer in the mobile phase increases the resolution and peak symmetry due to suppression of ionisation of the molecules. The effect of buffer was investigated and the results are shown in Table 3.3.4 below, only the results using methanol-buffer as a mobile phase with HP-β-cyclodextrin are reported.

Table 3.3.4. Results to show the effect of buffer

<table>
<thead>
<tr>
<th>Compound</th>
<th>Eluent</th>
<th>t₀ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Methionine</td>
<td>50/50MeOH/water</td>
<td>2.307</td>
</tr>
<tr>
<td></td>
<td>50/50MeOH/TEAA</td>
<td>11.859</td>
</tr>
<tr>
<td>DL-Leucine</td>
<td>50/50MeOH/water</td>
<td>2.896</td>
</tr>
<tr>
<td></td>
<td>50/50MeOH/TEAA</td>
<td>25.172</td>
</tr>
<tr>
<td>DL-Norleucine</td>
<td>50/50MeOH/water</td>
<td>3.071</td>
</tr>
<tr>
<td></td>
<td>50/50MeOH/TEAA</td>
<td>27.724</td>
</tr>
<tr>
<td>Flavanone</td>
<td>50/50MeOH/water</td>
<td>4.339</td>
</tr>
<tr>
<td></td>
<td>50/50MeOH/TEAA</td>
<td>5.665</td>
</tr>
<tr>
<td>Chlorothalidone</td>
<td>50/50MeOH/water</td>
<td>1.703</td>
</tr>
<tr>
<td></td>
<td>50/50MeOH/TEAA</td>
<td>1.911</td>
</tr>
</tbody>
</table>

*ₜ₀ is the retention time of the analyte
There was however, no evidence of separation or increase in resolution when the buffer was used instead of water. Instead there was an increase in retention time, with a longer retention time observed for DL-norleucine, DL-leucine and DL-methionine in 50/50 0.1% TEAA/methanol.

3.3.1.3. HPLC RESULTS OBTAINED USING A CHIRAL STATIONARY PHASE

The results from NMR spectroscopy showed resolution of the enantiomers when HP-β-cyclodextrin was used, however the complexes formed could not be separated by HPLC using a chiral mobile phase. The next step was to use a cyclodextrin stationary phase to try and separate the enantiomers.

A hydroxypropyl-β-cyclodextrin (HP-β-cyclodextrin) stationary phase was used to determine the effects of cyclodextrin concentration on chiral separation. The HP-β-cyclodextrin stationary phase was chosen instead of S-β-cyclodextrin for this study as it showed positive results in NMR experiments (Section 3.3.1). Capacity factor (k), selectivity (α) and resolution (Rs) data were obtained and used to determined the chiral resolution of each compound in each of the mobile phase. Selected chromatograms are shown to demonstrate the effects of mobile phase and buffer on separation.

3.3.1.3.1. Effect of Mobile Phase

The results shown in Table 3.3.5 and Figures 3.3.3 and 3.3.4 show that better separation of the enantiomers was achieved in methanol:water than in acetonitrile:water, based on the values obtained for the capacity factor, selectivity and resolution. There is no evidence of resolution for DL-methionine in both mobile phases. There is a decrease in resolution and selectivity moving from methanol to acetonitrile, however, the values increase when only 20% of acetonitrile is used (Figure 3.3.3) and these were comparable with values obtained using 50% methanol. There is greater selectivity when 20% methanol is used compared to selectivity values obtained using the same amount of acetonitrile in the mobile phase (Figure 3.3.5).
Table 3.3.5. Effects of mobile phase on separation and retention on the analyte

<table>
<thead>
<tr>
<th>Compound</th>
<th>Eluent</th>
<th>( t_m )</th>
<th>( t_{11} )</th>
<th>( t_{22} )</th>
<th>( k_1 )</th>
<th>( k_2 )</th>
<th>( R_s )</th>
<th>( \alpha )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Methionine</td>
<td>50/50MeCN/water</td>
<td>-</td>
<td>1.720*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20/80MeCN/water</td>
<td>-</td>
<td>7.650*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DL-Leucine</td>
<td>50/50MeOH/water</td>
<td>2.488</td>
<td>7.203</td>
<td>8.848</td>
<td>1.895</td>
<td>2.556</td>
<td>0.179</td>
<td>1.349</td>
</tr>
<tr>
<td></td>
<td>20/80MeCN/water</td>
<td>1.433</td>
<td>4.644</td>
<td>5.800</td>
<td>2.241</td>
<td>3.047</td>
<td>0.188</td>
<td>1.360</td>
</tr>
<tr>
<td>DL-Norleucine</td>
<td>50/50MeOH/water</td>
<td>1.076</td>
<td>4.461</td>
<td>5.619</td>
<td>1.615</td>
<td>2.294</td>
<td>0.174</td>
<td>1.420</td>
</tr>
<tr>
<td></td>
<td>20/80MeCN/water</td>
<td>-</td>
<td>9.588*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavanone</td>
<td>50/50MeOH/water</td>
<td>7.536</td>
<td>19.831</td>
<td>20.643</td>
<td>1.632</td>
<td>1.739</td>
<td>0.159</td>
<td>1.066</td>
</tr>
<tr>
<td></td>
<td>20/80MeCN/water</td>
<td>1.773</td>
<td>24.067</td>
<td>25.417</td>
<td>12.574</td>
<td>13.336</td>
<td>0.233</td>
<td>1.061</td>
</tr>
<tr>
<td>Chlorthalidone</td>
<td>50/50MeOH/water</td>
<td>1.773</td>
<td>3.996</td>
<td>4.690</td>
<td>1.254</td>
<td>1.645</td>
<td>0.155</td>
<td>1.312</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/water</td>
<td>1.167</td>
<td>2.114</td>
<td>2.229</td>
<td>0.811</td>
<td>0.910</td>
<td>0.119</td>
<td>1.121</td>
</tr>
</tbody>
</table>

*: retention time of the main peak, unresolved from the void \( t_m \), \( t_{11} \), and \( t_{22} \); retention of enantiomer 1 and 2 respectively, \( k_1 \) and \( k_2 \): capacity factor of enantiomer 1 and 2 respectively, \( R_s \): resolution, \( \alpha \): selectivity

Figure 3.3.3. Chromatograms of chlorthalidone in different mobile phases

Similarly, chlorthalidone enantiomers were resolved in both mobile phases. The resolution improved with the decrease in organic solvent in the mobile phase. Methanol gave best results with all the compounds. DL-methionine showed no enantiomer separation with either mobile phase.
The solutes are not retained, however, when acetonitrile:water was used as a mobile phase, and therefore no resolution of enantiomers was obtained. Acetonitrile displaces the analyte from the cyclodextrin cavity, thus resulting in low resolution. This was evident from the $R_s$ and $\alpha$ values for each compound as shown from Table 3.3.5.

The retention time is increased by decreasing the amount of acetonitrile in the mobile phase (i.e. reducing the solvent strength of the eluent). This effect can be seen with DL-leucine and flavanone, when partial separation was observed in 20/80 acetonitrile/water and no separation in 50/50 acetonitrile/water. The use of a lower amount of acetonitrile permits more interaction with the cyclodextrin stationary phase which results in better separation, as observed in Figure 3.3.4 below.

![Chromatograms of flavanone in different amount of organic solvent](image)

*Figure 3.3.4. Chromatograms of flavanone in different amount of organic solvent*
3.3.1.3.2. Effect of Buffer in the Mobile Phase

The use of 0.1% v/v triethylamine acetate (TEAA) buffer instead of water, resulted in sharper and more symmetrical peaks (Table 3.3.5 and Figures 3.3.5 and 3.3.6).

Table 3.3.5. Effects of buffer on separation and retention on the analyte

<table>
<thead>
<tr>
<th>Compound</th>
<th>Eluent</th>
<th>( t_m )</th>
<th>( t_1 )</th>
<th>( t_2 )</th>
<th>( k_1 )</th>
<th>( k_2 )</th>
<th>( R_s )</th>
<th>( \alpha )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Methionine</td>
<td>50/50MeCN/water</td>
<td>-</td>
<td>1.720*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/TEAA</td>
<td>1.717</td>
<td>2.180</td>
<td>2.373</td>
<td>0.269</td>
<td>0.382</td>
<td>0.069</td>
<td>1.417</td>
</tr>
<tr>
<td>DL-Leucine</td>
<td>50/50MeCN/water</td>
<td>-</td>
<td>2.047*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/TEAA</td>
<td>2.214</td>
<td>2.568</td>
<td>3.175</td>
<td>0.159</td>
<td>0.434</td>
<td>0.076</td>
<td>2.714</td>
</tr>
<tr>
<td>DL-Norleucine</td>
<td>50/50MeCN/water</td>
<td>-</td>
<td>1.515*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/TEAA</td>
<td>1.882</td>
<td>2.234</td>
<td>2.453</td>
<td>0.187</td>
<td>0.303</td>
<td>0.058</td>
<td>1.622</td>
</tr>
<tr>
<td>Flavanone</td>
<td>50/50MeCN/water</td>
<td>-</td>
<td>2.469*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/TEAA</td>
<td>-</td>
<td>2.495*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chlorthalidone</td>
<td>50/50MeCN/water</td>
<td>1.167</td>
<td>2.114</td>
<td>2.229</td>
<td>0.811</td>
<td>0.910</td>
<td>0.119</td>
<td>1.121</td>
</tr>
<tr>
<td></td>
<td>50/50MeCN/TEAA</td>
<td>1.710</td>
<td>2.134</td>
<td>2.235</td>
<td>0.247</td>
<td>0.307</td>
<td>0.059</td>
<td>1.238</td>
</tr>
</tbody>
</table>

*: retention time of the main peak, unresolved from the void \( t_m \); \( t_1 \) and \( t_2 \): retention of enantiomer 1 and 2 respectively, \( k_1 \) and \( k_2 \): capacity factor of enantiomer 1 and 2 respectively, \( R_s \): resolution, \( \alpha \): selectivity

Figure 3.3.5. Chromatograms of DL-norleucine to show the effect of buffer
The retention time of all analytes, except flavanone increased when a buffer was added to the mobile phase. Enantioselectivity also increased with a loss in resolution for DL-norleucine, DL-leucine and chlorthalidone when the buffer was added in the mobile phase. However, resolution was observed for DL-methionine which showed no resolution in the absence of the buffer.

Once again the presence of acetonitrile results in a decrease in resolution when 0.1% TEAA:acetonitrile was used instead of 0.1% TEAA:methanol. The use of a buffer has different effects on each compound, in some cases the resolution is increased and lost in other cases probably as a result of changing the ionisation (and hydrophobicity) of the solute.
3.3.2. DISCUSSION

Separation of racemates into enantiomers is an essential procedure in the pharmaceutical industry. The problem has been studied for a long time, but it has not yet been solved. The use of HPLC with a chiral additive to either the mobile phase or the stationary phase is one of the most important methods for chiral separations. Separation is achieved by formation of diastereomeric complexes between the chiral selector and the chiral molecule.

Chiral NMR and HPLC have been used in this study to evaluate the resolution of enantiomers by formation of diastereomeric complexes between the analyte and cyclodextrin (chiral selector). The study investigated the effects of type of β-cyclodextrin, mobile phase and the buffer on chiral discrimination.

3.3.2.1. The Effect of the Type of β-cyclodextrin

The type of cyclodextrin plays an important role as chiral selector. The β-cyclodextrins are known to form better complexes than other cyclodextrins, whereas chemically modified β-cyclodextrins form even better complexes than native β-cyclodextrins due to a change in the polarity of the cyclodextrin cavity.

Two cyclodextrins, HP-β-cyclodextrin and S-β-cyclodextrin, were evaluated as chiral discriminators with a range of chiral drugs. HP-β-cyclodextrin proved to be a better chiral selector than S-β-cyclodextrin as observed from NMR spectra of flavanone, DL-norleucine, chlorthalidone (spectra not shown) and DL-leucine (Figure 3.3.1).

DL-norleucine, DL-leucine and DL-methionine are similar in structure differing only in the alkyl chain attached to the chiral centre (Figure 3.2.1), however, chiral discrimination was only observed with DL-norleucine and DL-leucine, suggesting that the enantiomers of the two were separated due to the alkyl chain of the molecule entering the cavity with the dansyl moiety protruding from the cavity. The aromatic protons can then interact with the hydroxypropyl groups at the rim of the cavity, forming a stable inclusion complex.
The flavanone complex would probably form due to the phenol group entering the cavity. The phenolic hydroxyl (OH) protrudes through the bottom of the cavity and can thus form hydrogen bonds with the hydroxypropyl groups on the rim of the cyclodextrin, thereby increasing the stability of the complex.

The chlorthalidone complex could form with the benzene ring entering the cavity, leaving the NH/OH groups to interact with the cyclodextrin rim. Technique such as ROESY, molecular modelling or X-ray diffraction may aid in determining the structure of the complexes. DL-methionine has a sulfur group at the end of the alkyl chain which may make the molecule too large to fit into the cavity due to steric hindrance.

There was no evidence of inclusion complex formation with S-β-cyclodextrin, as shown by the spectrum of DL-leucine (Figure 3.3.1 (C)). It is unclear why this is the case, but a possible reason could be steric effects due to sulfate groups on the rim of the cyclodextrin cavity. The sulfate groups are much bigger than the hydroxyl groups, and would therefore, cause steric hindrance and thus hinder inclusion complex formation.

No chiral discrimination was observed for either cyclodextrins when used in the mobile phase in HPLC. The reason for this is not yet clear, but this could be due to insufficient interaction between the cyclodextrin and the analyte for complex formation. HPLC with a chiral stationary phase was therefore used to try and separate the enantiomers, especially as the NMR study showed resolution of enantiomers in the presence of a chiral selector. There was chiral separation using chiral stationary phase as observed from the chromatograms and from calculations obtained for the capacity factor, selectivity factor and resolution. These results have shown the usefulness of cyclodextrins in chiral separation.
3.3.2.2. The Effect of the Type and Amount of Organic Solvent

The type of organic solvent has a large effect on the separation and resolution of enantiomers. The organic solvent can complete with the solute for inclusion in the hydrophobic cavity of the cyclodextrin. Methanol proved to be a better solvent than acetonitrile as observed from NMR resonance splitting of the four compounds in methanol and further splitting for flavanone and chlorthalidone in acetonitrile (Table 3.3.1 and Figure 3.3.2).

There is a decrease in solvent polarity changing from CD$_3$OD/D$_2$O to CD$_3$CN/ D$_2$O as polarity decreases in the order D2O>CD$_3$OD>DMSO>CD$_3$CN. Separation should therefore decrease in that order as changing from a polar solvent to less polar solvent is thought to decrease or hinder the formation of the inclusion complex. This effect was observed from the spectra obtained for the compounds mentioned above (spectra not shown), with greater resolution observed with CD$_3$OD/D$_2$O compared to CD$_3$CN/D$_2$O, as shown from the spectra of chlorthalidone (Figure 3.3.2). Acetonitrile competes with the analyte to a greater extent for cyclodextrin cavity, which results in a decrease in inclusion complex formation.

A similar trend was observed in HPLC using cyclodextrin stationary phase. A decrease in resolution and capacity factor moving from methanol to acetonitrile is suggestive of an increase in competition between the analyte and the solvent for the cyclodextrin cavity (Table 3.3.5 and Figures 3.3.3 and 3.3.4). There was no enantiomeric resolution in either of the mobile phases when a cyclodextrin was used as a chiral additive in the mobile phase.

The amount of organic solvent present determines the degree of complex formation and subsequent enantiomeric resolution. Increasing the amount of organic solvent in the mobile phase weakens the strength of the inclusion complex formed between the analyte and the cyclodextrin due to an increase in competition between the solvent and the analyte molecules for the cyclodextrin cavity. This effect was observed when partial separation of flavanone was obtained when 20% acetonitrile instead of 50% (Figure 3.3.4).
3.3.2.3. The Effect of Buffer in the Mobile Phase

The main use of a buffer in the mobile phase is to increase peak resolution and peak symmetry by increasing the hydrophobicity of the analyte present in the mobile phase, which will then increase the strength of the inclusion complex formed. This effect was observed when 0.1% v/v of TEAA was used, which suggests an increase in hydrophobicity of the analyte (Table 3.3.5 and Figure 3.3.5). Figure 3.3.6 shows again the effect of the mobile phase on separation. The resolution of the enantiomers in the presence of the buffer is increased, but lost in other cases, which shows different effect of buffer on each compound as a result of changing the ionisation of the solutes.
3.4. CONCLUSION

NMR spectroscopy proved to be a powerful and fast tool for evaluation of the resolution of enantiomers since the diastereomeric complexes formed between each enantiomer and the cyclodextrin resulted in different $^1$H signals. The study has shown the importance of choice of solvent and solvent polarity on complex formation and the usefulness of cyclodextrins as chiral resolving agents. Although modified cyclodextrins are expected to form better inclusion complex than native cyclodextrins, the separation is dependent of the size of the analyte and the shape of the molecule being separated, the mobile phase and the size of the cyclodextrin cavity.

The results obtained from NMR spectroscopy were compared with the results obtained by achiral HPLC with cyclodextrin added to the mobile phase, however enantiomeric separation was not observed under similar conditions. Therefore it was not possible to use NMR to predict optimum cyclodextrin and solvent combination for chiral resolution by use of achiral HPLC. The most likely reason for this would be the cyclodextrin concentration in the mobile phase. The concentration of the cyclodextrin in the mobile phase has a dramatic effect on the retention and resolution of enantiomers and concentration of cyclodextrin used in this study was too low to form inclusion complexes (0.14 mM for HP-$\beta$-cyclodextrin and 0.08 mM for S-$\beta$-cyclodextrin).

Poor resolution was observed when acetonitrile was used in the mobile compared with methanol. Acetonitrile compete with the analyte for the cyclodextrin cavity to a greater extent, thus displaces the analyte from the cyclodextrin cavity which results in no separation. A buffer was used in order to increase the hydrophobicity of the analytes, thus increasing the interaction of the analyte with the cyclodextrin. This effect was observed from the results obtained, which showed better separation when the buffer was used.

The chiral stationary phase on the other hand has a high cyclodextrin concentration, the solutes are therefore in contact or interact with the cyclodextrin to a greater
extent, resulting in discrimination of enantiomers. The choice of solvent is also importance for enantiomer separation. The type and amount of organic solvent was also shown using chiral stationary phase to affect both the retention and resolution of enantiomers. Best results were obtained using HP-β-cyclodextrin in the stationary phase with methanol/water and methanol/TEAA.
3.5. FUTURE WORK AND RECOMMENDATIONS

The results obtained during this study suggest that the concentration of cyclodextrin used in the HPLC eluents were too low to form complexes with the enantiomers studied. Therefore, increasing the concentration of cyclodextrin in the eluent should be considered for future investigation. Additionally, the use of other cyclodextrins and selection of optimum achiral HPLC phase/column are possible areas for further work.
APPENDIX 1.1

PREPARATION OF HPLC MOBILE PHASES

PREPARATION OF METHANOL/WATER OR ACETONITRILE/WATER

In a 250 ml graduated cylinder, add 125 ml of water and 125 ml of methanol to give a solution of 50/50 water/MeOH. To this solution, add 50 mg of cyclodextrin and sonicate to dissolve. The same method was used to prepare the remaining mobile phases.

PREPARATION OF 0.1% TEAA, pH4

In a 1000 ml graduated cylinder, add 1 ml of triethylamine (TEA) per 1000 ml of deionised water to give a solution of 0.1% v/v TEA in water, degas by sonication. Adjust the pH to 4 with glacial acetic acid. To this solution add 50 mg of cyclodextrin.
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CHAPTER 4

EVALUATING THE STABILITY OF MENINGOCOCCAL A-CRM$_{197}$ GLYCOCONJUGATE VACCINE BY USE OF SPECTROSCOPIC AND CHROMATOGRAPHIC METHODS
4.1. MENINGOCOCCAL DISEASES, THERAPY AND PREVENTION

4.1.1. HISTORY OF MENINGITIS

Since its first appearance, meningococcus has caused epidemics in various places world-wide. The disease, meningococcal meningitis or "cerebrospinal fever", was first described by Gaspard Viesseux in 1805 during a small outbreak in Geneva [1,2], but it was Robert Whytt who initially described the signs and symptoms of tuberculous meningitis as "dropsy in the brain" in 1768 [3]. The organism was first described and isolated from cerebrospinal fluid (CSF) by Anton Weichselbaum, who identified it as Diplococcus intracellular meningitidis [2-4], and it was later renamed Neisseria meningitidis (N. meningitidis) after Albert Neisser [5]. The nasopharyngeal carrier state in healthy individuals was only described in 1896 by Kiefer and the serogroups were only recognised in the 1900s [6].

4.1.2. MENINGOCOCCAL DISEASE

Bacterial meningitis is a serious illness accounting for 171,000 deaths worldwide per annum [7]. The disease is caused by three species: Haemophilus influenzae type b (Hib), Streptococcus pneumoniae and Neisseria meningitidis (N. meningitidis). N. meningitidis has become one of the leading causes of meningitis worldwide since the introduction of Hib conjugate vaccines [8]. Neisseria meningitidis is unique in major bacterial agents in that it causes both endemic (sporadic) and epidemic disease in developed and developing countries worldwide [9]. The mode of infection is by direct contact with the person who is suffering from the disease or healthy individuals carrying the organism [1].
4.1.2.1. CLINICAL FEATURES

Infection with meningococcus result in one of the following conditions.

4.1.2.1.1. Asymptomatic Nasopharyngeal Carriage Meningitis

This is the most common of the clinical conditions. The nasopharynx is the only reservoir of *N. meningitidis* and asymptomatic nasopharyngeal carriers are considered the source of infection [2]. The organism is spread from host to host by contact with oral secretion or exposure to respiratory droplets. Carriers of this organism develop natural immunity as a result, typically 2 weeks after nasopharyngeal infection begins [10].

4.1.2.1.2. Meningitis

Meningitis is the most common pathological presentation (Figure 4.1.1). The disease begins with a sudden onset of fever, headache, diarrhea, vomiting, rash and stiff neck [2, 11,12]. Mortality remains higher in the developing world (~5% in children <5 years and 10-15% in adults) despite antimicrobial treatment and supportive care [2].

![Figure 4.1.1. Representation of an infant with meningococcal C disease (a) and a patient with Meningococcal A disease (b).](image)

*Figure 4.1.1. Representation of an infant with meningococcal C disease (a) and a patient with Meningococcal A disease (b).*
4.1.2.1.3. Meningococcaemia

Meningococcaemia is the most severe form of infection. It is characterised by purpuric rash, hypotension, intravascular coagulation and/or multiple-organ failure [12].

4.1.2.1.4. Metastatic Manifestation

Metastatic infections result during the bacteremic phase and are less common. This form of the disease includes arthritis, pericarditis and endophthalmitis [11,28].

4.1.2.2. DIAGNOSIS

It is important to diagnose the disease when only mild symptoms are present due to the rapid progression. Meningococcal disease can be diagnosed by culture of the blood, cerebrospinal fluid (CSF), skin lesions and infected sites [11,12,16]. Diagnosis of blood and CSF by polymerase chain reaction (PCR) has become part of the routine diagnostic methods. This approach has been used in the United Kingdom since 1996 and 35% of cases of meningococcal disease were confirmed by PCR in 1998 [4].

4.1.3. CAUSES OF MENINGOCOCCAL DISEASE

4.1.3.1. MENINGOCOCCUS

Meningococcal disease is caused by a bacteria called meningococcus, which is a gram-negative encapsulated organism found only in humans (Figure 4.1.2) [4,11,12,16]. The meningococcus has an inner and outer (cytoplasmic) cell membrane that are separated by a peptidoglycan cell wall (Figure 4.1.3 [4]).
The outer membrane consists of pili and outer membrane proteins (OMPs), lipopolysaccharide (LPS), phospholipids and capsular polysaccharide (CPS) [9]. Capsular polysaccharide is believed to be the major contributor to the virulence of *N. meningitidis* as it is used to protect the organism against environmental insults and confers resistance to phagocytosis (Table 4.1.1) [17]. The OMPs, together with the capsular polysaccharide form the principal surface antigens of the organism.
Table 4.1.1. Function of the outer-membrane components of Neisseria meningitidis

<table>
<thead>
<tr>
<th>STRUCTURE</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysaccharide capsule</td>
<td>Anti-phagocytic, reduced immunogenicity, antigen mimicry</td>
</tr>
<tr>
<td>Lipopolysaccharide (LPS)</td>
<td>Antigen mimicry, has potent endotoxic activity</td>
</tr>
<tr>
<td>Pili</td>
<td>Adhesion to epithelial and endothelial cells and erythrocytes</td>
</tr>
<tr>
<td>Outer-membrane proteins</td>
<td></td>
</tr>
<tr>
<td>Porins (PorA and PorB)</td>
<td>Create pores through which small hydrophilic solutes pass, cation-selective or anion-selective</td>
</tr>
<tr>
<td>Opacity-associated proteins</td>
<td></td>
</tr>
<tr>
<td>Opa (class 5)</td>
<td>Promotes adhesion to host cells and leukocytes</td>
</tr>
<tr>
<td>Opc (class 5c)</td>
<td>Promote adhesion to host cells</td>
</tr>
</tbody>
</table>

There are 12 different serogroups based on the chemical and immunological specificity of the capsular polysaccharide (A, B, C, H, I, K, L, W135, X, Y, Z and 29E) of which five serogroups (A, B, C, Y, and W135) account for virtually all disease-causing isolates [5,7,11,16,18,19].

4.1.3.2. TRANSMISSION OF THE MENINGOCOCCI

The organism is transmitted by contact with respiratory secretions [12]. It colonises the nasopharynx, resulting in a carrier state. This is then followed by penetration through the mucosa. It is not known why most individuals carry meningococci without experiencing illness whereas a small proportion develop serious invasive disease. The cause of progression from carriage to invasive disease is thought to be dependent on factors from both the host and the infecting organisms. The bacteria cause meningococcemia by crossing into the bloodstream or cross the blood-brain
barrier (BBB) and gain access to the cerebrospinal fluid (CSF) to cause meningitis (Figure 4.1.4) [20,21].

**Figure 4.1.4.** Colonisation of *Neisseria meningitidis* in the nasopharynx followed by entry into the bloodstream and cerebrospinal fluid. The bacteria can be transmitted to other hosts.

### 4.1.4. EPIDEMIOLOGY

Meningococcal disease is caused mainly by serogroups A, B and C. The disease can occur as endemic or epidemic cases [22]. The pattern and frequency of disease varies widely throughout the world. The annual incidence in the United States is ~1.1/100,000, with peak incidences in late winter and spring [15]. The annual incidence of invasive infection in Europe ranges from 0.3 and 7.1 per 100,000 people [22]. Serogroup A and B are responsible for the majority of cases in Europe and the Americas and true epidemics are rare [16].

Serogroups A and C predominate mainly in Asia and Africa and serogroup Y predominate in the US [4]. Serogroup A has caused major epidemics in sub-Saharan Africa (African meningitis belt) which include countries from Ethiopia to Senegal (Figure 4.1.5) [23].
The largest outbreak ever reported occurred in the meningitis belt in 1996, where 152,813 cases were reported to the WHO with 15,783 deaths [4,24].

Several factors are associated with increased risk of developing meningococcal disease. The cause of progression from carriage to invasive disease is thought to be dependent on factors from both the host and the infecting organisms [4,11,22]. These factors include household crowding, age, recent viral infection, active and passive smoking, alcohol abuse, complement deficiencies, travel to areas where epidemics are occurring, chronic illness and age [6,25-30].

Smoking increases both the carriage rate and the risk of developing the disease. Children under the age of one are at high risk of developing invasive meningococcal disease [10,31,32]. This could be related to lack of specific anti-meningococcal antibodies at this age.

Deficiencies in the complement system (C5 to C8) multiply the risk of meningococcal disease [33-37]. Absent or dysfunctional properdin, a protein that participate in the body's immune response, has also been shown to increase the risk of meningococcal disease [38,39].
4.1.5. THERAPY AND PREVENTION

The disease, in the late nineteenth century was treated by drainage of CSF by repeated lumbar punctures which was described by Heinrich Quincke in 1891 [3]. It was recognised that the abnormalities in bacterial activity of human sera are associated with increased risk for invasive meningococcal disease. This led to the introduction of animal sera, or serum therapy as the primary mode of infection [1,17,40,41]. The immune sera contained specific antibodies, which mediated therapeutic effects. The treatment was abandoned in the 1940's when antibiotics became widely available.

Introduction of sulfonamides in 1937 radically altered the outcome of meningococcal disease [2,42-45]. The sulfonamide therapy has since been abandoned by the appearance and spread of sulfa-resistant meningococcal strains, which led to the development of antibiotics and vaccines. Active immunisation has now been suggested as an alternative control measure.

4.1.5.1. ANTIBIOTIC THERAPY

The development of sulfonamide-resistance organism in the 1950's brought an end to the sulfonamide therapy era [2]. The use of antibiotics has dramatically reduced mortality due to meningococcal disease. Many antimicrobial agents, including penicillin, are active against N. meningitidis [3,12,15,46]. Aqueous penicillin G is the drug of choice given intravenously (dose of 400,000 Units/kg) for invasive meningococcal disease. Treatment with penicillin has failed in a few patients that have intermediate resistance to the drug [47], however, other patients with N. meningitidis have been treated successfully with the drug [48-50].

Several other drugs such as chloramphenicol, ceftriaxone, cefotaxime and ceftazadime have been shown to be effective in the treatment of meningococcal disease [2,4,12,15,51]. The use of these drugs is limited, however, by their extensive cost, resistance, and the lack of preventing the disease, thus vaccination is needed.
4.1.5.2. PREVENTION

4.1.5.2.1. Chemoprophylaxis

Chemoprophylaxis is used for postexposure prophylaxis against meningococcal infection. Household contacts exposed to an index case have a 500-to1000-fold increased risk of developing invasive disease [2]. Prophylaxis is given to household members, day-care centre contacts and persons exposed to oral secretions of the patient (Table 4.1.2).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampin† (oral agent)</td>
<td>Adults: 600 mg every 12 h for 2 days</td>
</tr>
<tr>
<td></td>
<td>Children (&gt;1 year): 10 mg/kg every 12 h for 2 days</td>
</tr>
<tr>
<td></td>
<td>Children (&lt;1 year): 5 mg/kg every 12 h for 2 days</td>
</tr>
<tr>
<td>Ceftriaxone (intramuscular injection)</td>
<td>Adults: 250 mg</td>
</tr>
<tr>
<td></td>
<td>Children: 125 mg</td>
</tr>
<tr>
<td>Ciprofloxacin‡ (oral agent)</td>
<td>Single dose-750 mg</td>
</tr>
<tr>
<td>Sulﬁsinoxazole (oral agent)</td>
<td>Adult: 1 g every 12 h for 2 days</td>
</tr>
<tr>
<td></td>
<td>Children (1-12 years): 500 mg every 12 h for 2 days</td>
</tr>
<tr>
<td></td>
<td>Children (&lt;1 year): 500 mg daily for 2 days</td>
</tr>
</tbody>
</table>

†Rifampin is not recommended for pregnant women, as the drug is teratogenic in laboratory animals

‡Ciprofloxacin is generally not recommended for persons under 18 years of age or pregnant or lactating women. The drug causes cartilage damage in immature laboratory animals

Rafampin was shown to be effective in eradicating meningococcal disease in 1969 [52-54] and is given as the drug of choice. Minocycline or ceftriaxone are acceptable alternative agents. Ciprofloxacin has also shown in vivo activity against N.
meningitidis [55]. During an epidemic, vaccination is recommended, as individuals are liable to become reinfected soon after prophylaxis.

4.1.5.2.2. Vaccination

There has been huge pressure from the public to find a vaccine that would eradicate the disease completely. Such a vaccine must be immunogenic in all ages and provide cross-protection against all meningococci. Vaccination is one of the most powerful approaches for protecting humankind against infectious diseases. The word 'vaccine' is derived from the Latin vacca, which means cow. Edward Jenner (1789) discovered that inoculating humans with the cowpox virus protected them against smallpox [57]. It took another century until scientists established that inoculation with inactivated microorganisms could introduce a protective immunological response.

4.1.5.2.2.1. Early Vaccines

The first vaccines against meningococcal disease used whole cell vaccines [2,5,16]. These vaccines produced severe adverse reactions, probably due to high endotoxin content, and variable efficacy. The second attempt was the use of exotoxin vaccines, which contained a mixture of bacterial constituents. There was, however, few adequately controlled trials and limited evidence of protection.

4.1.5.2.2. Capsular Polysaccharide (CP) Vaccines

The presence of capsular polysaccharide on N. meningitidis is related to its ability to cause invasive disease in humans [58]. Vaccination, therefore, attempts to induce immunity by developing bactericidal antibodies directed at the capsule of the organism [59]. Efforts have been made to develop vaccines against a variety of pathogens by using purified polysaccharide preparations.

The potential of capsular polysaccharides (CPs) was confirmed by use of multivalent pneumococcal polysaccharide vaccines [57,60,61]. These vaccines provided type-specific protection in humans against pneumococcal pneumoniae. Several CP
vaccines are licensed for use in protection of pneumococcal pneumoniae and meningitis caused by *N. meningitidis*, *Streptococcus pneumoniae* and *Hib*.

The development of vaccines against serogroups A and C were first developed in the 1960s, and this work was carried out by Gotschlich et al. [62,63]. The efficacy and effectiveness of both serogroup A and C polysaccharide vaccines has been shown in clinical trials [22,64-66]. The vaccines proved effective in prevention of group C disease in the US [67] and in controlling group A epidemics during mass vaccination in Africa [68-70]. A tetravalent polysaccharide vaccine containing serogroups A, C, Y, and W135 is available world-wide [71,72].

Vaccination with the tetravalent vaccine is recommended for prevention of disease in persons with complement deficiencies, those with asplenia and travellers to countries where the disease is epidemic [6]. Serogroups A and C have shown clinical efficacy rates of >85% in children five years of age and adults [71,72]. Serogroups Y and W135 polysaccharide are immunogenic and safe however clinical protection based on the polysaccharide is not known [4].

Vaccination with these vaccines is not recommended for childhood vaccination due to relative ineffectiveness in young children, and duration of protection is relatively short [72]. The polysaccharide antigens are T cell-independent, thus, they do not produce high-level, high affinity antibodies and do not induce T cell memory required for booster responses [5,61]. This might be overcome by conjugation to carrier protein, which provides T cell help for antibody response and induces immunological memory [25].

Several polysaccharide-protein conjugate vaccines are being developed and some have already been licensed. These vaccines are immunogenic and protective against meningococcal infection. Currently, there is no vaccine against serogroup B. The polysaccharide of serogroup B is chemically identical to polysaccharides found in human tissues. The capsule is seen as self by the immune system and is thus poorly immunogenic, even after conjugation to protein carrier [73].
Vaccines against serogroup B currently in development are based on non-capsular antigens such as outer membrane proteins (OMPs) and lipo-polysaccharide (LPS), as potential immunogens [3,5,16,17]. The structures, together with the capsular polysaccharide, form part of the principal surface of the organism (Figure 4.1.2), they can therefore be targeted in the same way as the capsular polysaccharide as vaccine candidates to induce immunogenicity against group B capsule.
4.2. MENINGOCOCCAL GLYCOCONJUGATE VACCINES

4.2.1. INTRODUCTION

Bacterial diseases are responsible for morbidity and mortality of millions of people worldwide each year. Glycoconjugate vaccines, unlike polysaccharide vaccines, are effective in protecting infants and induce immunological memory [7, 61]. Landsteiner showed that molecules such as saccharides could be converted to immunogens by coupling them to protein [74]. Avery and Goebel were the first people to extend this technology by converting *Pneumococcus* type 3 polysaccharide, which is not immunogenic in rabbits, to induce active antibodies that protected rabbits against bacteria, by binding it to horse globulin [74-76]. This approach is believed to work by recruiting T cell help for polysaccharide specific B cells, as shown in Figure 4.2.1 below [57, 77].

![Proposed mechanism of action of glycoconjugate vaccines](image)

*Figure 4.2.1. Proposed mechanism of action of glycoconjugate vaccines*

B cells with surface immunoglobulins specific for the polysaccharide binds to the conjugate, internalise and hydrolyse it and then process the protein antigen into peptides. The peptides which are T cell epitopes, are presented to T helper (TH) cells bearing specific T cell receptors (TCR) by class II major histocompatibility complex (MHC II) molecules. The activated TH cells in turn activate B cell proliferation and differentiation into antibody-secreting cells.
It has since been shown that polysaccharide-protein conjugate vaccines can produce high levels of polysaccharide-specific antibodies in infants, thus prevent the disease and colonisation of bacteria in the upper respiratory tract, which in turn reduces the chances of infection [57]. A Hib conjugate was the first conjugate vaccine to be approved for preventing meningococcal diseases in children and they have been successful by inducing high, boostable, and protective antibody levels in infants and have dramatically reduced Hib-associated diseases in countries where they are in general use [7,9]. The spectacular success of Hib conjugate and meningococcal group C (Men C) glycoconjugate vaccines have led to the development of new meningococcal conjugate vaccines such as meningococcal group A (Men A), which are currently undergoing clinical trials [9,78,79].

4.2.2. IMMUNOGENICITY AND EFFICACY OF THE CONJUGATE VACCINES

Immunogenicity of candidate vaccines can be assessed by the rise in the titre of the immune sera, which is defined as a geometric mean (GM) anti-polysaccharide antibodies [80]. There are three licensed Hib conjugate vaccines currently used in routine immunisation of infants, which have shown protective levels of antibodies [17,74]. The efficacy and safety of the Hib conjugate vaccines has been shown in Finland, UK and the US, with a reduction of > 97% [18]. The vaccines have also shown antibody levels of about 20 times higher than the protective level after 3 injections with the vaccine [74]. In the UK, a decline of 90-99% of incident of the disease has been observed in children who received the vaccine as part of the primary immunisation programme, which consists of 3 doses [81].

Clinical trials have been conducted in the UK using monovalent Men C and bivalent Men C and A glycoconjugate vaccines have shown a decline in the incident of groups C, with an estimated effectiveness of about about 97% for ages 15 to 19 years and 92% for ages 2 to 3 years [7,11,13,18,82]. Another study was carried in Gambia using Men A and C conjugate vaccine and Men C conjugate induced immunological memory as expected, whereas no evidence for this was observed with Men A [13,18]. The reason for this is still not clear, but it is suggested that it could be due to
instability of the liquid formulation of polysaccharide A under high temperatures, as is
the case in Gambia. Men C glycoconjugate vaccine is now used in routine primary
immunisation in the UK.

The development of group B polysaccharide-protein conjugate vaccine has hindered
by the dangers of induction of autoantibodies that cross-react with glycosylated host
antigens [7]. Scientists are now looking at other ways of producing non-capsular
vaccines, including the use of outer membrane proteins (OMP), lipo-polysaccharides
(LPS) and outer membrane protein vesicle-based (OMV) vaccines to produce group
B (Men B) vaccines [11,83,84].

*Jennings et al.* have modified the group B polysaccharide by substituting *N*-acetyl
with *N*-propionyl before conjugation with the carrier proteins, and these vaccines are
now under investigation [9,11,18]. *Rappuoli et al.* have come up with a new approach
called "reverse vaccinology" which promise to provide solutions for pathogens such
as group B for which vaccines are not yet available. Reverse vaccinology is involves
investigation and identification of proteins that are most likely to offer protection
against the disease using whole genomic sequence and computer analysis [85,86].

### 4.2.3. CONJUGATION STRATEGIES

Design of conjugate vaccines depend on two elements, namely, the selection of
appropriate components i.e. the saccharide and carrier protein, and the selection of
an appropriate conjugation method to link both components. Conjugation between
the polysaccharide and the carrier protein will depend on the available functional
groups on both components and the stability of labile functional groups present on
the saccharide or the protein under the reaction conditions employed for conjugation.
4.2.3.1. POLYSACCHARIDE

Polysaccharides used for conjugation are usually obtained from the bacteria due to lack of methods or strategies for oligosaccharide synthesis, and time-consuming preparation. The disadvantage of using this method is that the bacterial polysaccharides obtained exist in a wide range of molecular masses, which makes it hard to characterise and to show consistency of the vaccine in manufacturing process [87].

Several studies have been done to determine the importance of chain length of the polysaccharide on the immunogenicity of the conjugates [88]. In one case a study was performed on a conjugate prepared from small oligosaccharides from dextrans and *Salmonella typhimurium* O-antigen type 4 and these conjugates were found to be more immunogenic in animals than conjugates prepared from larger polysaccharides [60].

In a similar study on conjugates prepared from higher molecular weight in Vi antigens and *Pneumococcal* type 4, the conjugates were found to be more immunogenic than conjugates prepared from smaller oligosaccharides [89]. In group B *Streptococcus* type III conjugates, the intermediate polysaccharides (14 repeating units, 70 monosaccharides) elicited antibodies, which provided superior protection than polysaccharides with 8 and 19 repeating units [75,90]. It appears from these studies that the optimal polysaccharide size depend on the nature of the polysaccharides used, therefore no general rule could be deduced as to what optimal polysaccharide size should be used. The optimal size of the saccharide should therefore, be defined individually for each specific polysaccharide.

4.2.3.2. CARRIER PROTEIN

There are several properties that govern the use of carrier protein in conjugate vaccines and these include toxicity and immunogenicity, that is the carrier protein must be non-toxic and must be immunogenic [60,91]. Several proteins have been used for preparation of conjugate vaccines including diphtheria toxoid (DTd), tetanus toxoid (TTd), a cross-reactive mutant of diphtheria toxin (CRM197) and outer
membrane proteins (OMP) from bacteria such as *N. meningitidis* and *H. influenzae* type b [61]. The toxoids are obtained by a process called toxoiding in which the native toxin is treated with formaldehyde or other treatments, to react with some of the amino groups on lysine residues, thus destroying the toxicity [60]. Outer membrane proteins are rarely used as carrier proteins because of their hydrophobicity, they are also prone to contamination with toxic lipopolysaccharides and they have a tendency to form complexes that are hard to resolve [91].

### 4.2.3.3. SACCHARIDE-CARRIER PROTEIN CONJUGATE

The technology of conjugation of polysaccharides to carrier proteins is well established and has since been used for the development of conjugates of pneumococci, meningococci, *Staphylococcus aureus* and *Salmonella typhi* capsular polysaccharides [74]. The synthesis of conjugates involves the linkage of polysaccharides to a carrier protein by either random or selective activation of the polysaccharide [75].

The choice of conjugation method relies on the molecular size of the polysaccharide, that is large polysaccharide require random activation, which result in activation of the functional groups along the chain [60]. Smaller polysaccharides on the other hand may be selectively activated at the terminal reducing or non-reducing ends or both and thus coupled to carrier proteins at single attachment points.

The method of conjugation or carrier protein used is believed to have little effect on the immunogenicity of the conjugate, in that the conjugates exhibit the general T cell dependent response properties regardless of the method or protein used. The Hib conjugates currently on the market are made with both terminally active and random activated Hib capsular polysaccharide or oligosaccharides, different carrier proteins and different conjugation methods [92,93]. These vaccines have different immunogenic response, and have been approved for use as vaccines for infants.
4.2.3.3.1. Activation of the Capsular Polysaccharide

4.2.3.3.1.1. Random Activation (Cross-linking)

Functional groups such as O-acyl groups, phosphate groups, carboxyl groups and sialic acid residues along the polysaccharide chain, are coupled to a bifunctional spacer, e.g. adipic acid dihydrazide, to facilitate binding of capsular polysaccharide to carrier protein [76,94]. This is then followed by activation of the end groups of the spacer and coupling to carrier protein by carbodiimide-mediated condensation, which results in conjugate with multiple attachments between the polysaccharide and the carrier protein.

The disadvantage of using this method is that conjugates are cross linked and have high molecular weight, which makes it difficult to separate, reproduce consistently and difficult to define [91]. This method has been used, however, to synthesise licensed Hib conjugate vaccines (PRP-T, Act-Hib) used in primary immunisation series [18].

4.2.3.3.1.2. Selective Activation (Non-cross linking)

The advantages of using this method is that one can study the influence of structural parameters such as chain length on the immunogenicity of the conjugate vaccine, which is not possible with randomly activated polysaccharides [75]. Saccharides are activated selectively at their terminal reducing ends (or non-reducing) and coupled to carrier proteins at single attachment points.

The native capsular polysaccharide initially, undergoes size reduction to oligosaccharides by use of periodate oxidation [95], acid hydrolysis [87] or electron beam fragmentation [96] to introduce terminal aldehyde group, followed by reductive amination to form active polysaccharides. Reductive amination of the polysaccharide is by far the most commonly used conjugation chemistry for end group coupling due to its simplicity [91]. Figure 4.2.2 shows a schematic representation of some of the reactions used for selective polysaccharide activation and coupling to carrier protein [91-92,97].
Figure 4.2.2. Schematic representation of polysaccharide (PS) end group activation and coupling to carrier protein (PROT). (a) Amination of polysaccharide, (b) Thiolation of aminated polysaccharide with 2-iminothiolane (2-IT), (c) Alternative thiolation of polysaccharide with cystamine, (d) Bromoacetylation of amino groups of the carrier protein with N-hydroxysuccinimidy bromoacetate, (e) Coupling of activated polysaccharide with bromoacetylated carrier protein.

4.2.3.3.2. Activation of the Carrier Protein

The most common functional groups on the carrier proteins used during conjugation are the amino groups of lysine residues or carboxyl groups of acidic amino acid residues [60]. These groups can be activated by coupling the carrier protein to one end of the bifunctional molecule such as adipic acid dihydrazide to facilitate binding to the activated polysaccharide as described in section 4.2.3.3.1 [76,94], or by use of N-hydroxysuccinimidy bromoacetate (Figure 4.2.2).
4.2.3.3. Conjugation Methods/ Strategies

Randomly or selectively activated polysaccharides are coupled to either native or activated carrier proteins as described in Section 4.2.3.3.1 and Figure 4.2.2 above. Characterisation and purification of activated polysaccharide, carrier protein and conjugates is achieved by use of physicochemical methods to ensure consistency and quality. These methods are described in Section 4.2.4 below.

4.2.4. ANALYSIS OF CONJUGATES

Modern physicochemical techniques are being used to determine the structure of both the polysaccharide and the carrier protein moieties of the vaccine, their stability and the mechanism by which the vaccine degrades. Unlike biological assays, which provide limited information about the structural integrity of the vaccines to protect infants from infection, these techniques can give information about the identity of the materials used in the vaccine, structural information on the mechanism by which the vaccine degrades and confirm consistency of manufacture. The information can be used to determine the immunogenicity of the conjugate vaccines [98]. This allows optimisation of formulations, shelf life and storage condition of these vaccines.

Several factors are believed to affect the efficacy of the vaccine and these include the use of oligo- or poly-saccharide moieties, carrier protein, conjugation chemistry, saccharide loading, attachment sites of the saccharide chains and the extent to which the carrier protein has been denatured [99]. Mouse immunogenicity assays can be used to measure the production of specific antibodies or to confirm the capacity of the conjugate vaccines to produce specific antibodies [81]. These assays however, do not correlate well with protective immunity in infants. Physicochemical methods will thus permit the detailed characterisation of these vaccines to ensure consistency, quality and efficacy, thus providing quality control of the vaccines. Figure 4.2.3 shows some of the methods used during development for routine control purposes.
4.2.4.1. CHARACTERISATION OF THE CAPSULAR POLYSACCHARIDE

Characterisation of the polysaccharide involves composition analysis, including identity and molecular size and purity. These parameters can be obtained using several techniques, including NMR spectroscopy, size exclusion chromatography (SEC), ion exchange chromatography and mass spectroscopy [90,99-101]. NMR spectroscopy has been readily used as an alternative to immunoassays to determine the structure and identity of polysaccharides [99].

Once the structure is fully characterised, the stability of the polysaccharide is monitored using these parameters. Changes brought about by depolymerisation or degradation of the polysaccharide by heat treatment or autohydrolysis can be detected due to changes in the NMR spectra. These changes can thus be used to determine the mechanism(s) of the polysaccharide degradation. It has been shown in one study that meningococcal C polysaccharide degrades by autohydrolysis, whereas Hib polysaccharide degrades with the formation of ribose-2,3-cyclophosphate endgroups [98]. Understanding the mechanisms by which polysaccharides degrade will thus lead to optimisation of vaccine production and storage conditions of the final conjugate.
The molecular size and molecular distribution of the saccharide before conjugation can be obtained using the following methods: size exclusion chromatography with multiple angle laser light scattering (SEC-MALLS) and refractive index (RI) detection, mass spectrometry or anion exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) system and HPSEC coupled to an immuno detection (ELISA) [102]. A combination of these techniques permits the detailed physicochemical characterisation of the polysaccharide component of the vaccine, which can be used to evaluate lot to lot consistency and thereby ensure the quality of the conjugate.

4.2.4.2. CHARACTERISATION OF THE CARRIER PROTEIN

Several techniques have been used to evaluate the structural composition, purity and stability of the carrier proteins and these include:

- Optical spectroscopy
- High performance size exclusion liquid chromatography (HPLC-SEC)
- Mass spectrometry (molecular weight)
- Amino acid analysis
- N-terminal amino acid sequence
- Peptide mapping-Reverse-phase HPLC
  - MALDI-TOF mass spectroscopy

Optical spectroscopy (circular dichroism and fluorescence spectroscopy) and HPLC-SEC have been used extensively to evaluate the structure and stability of the carrier protein, especially the CRM197 carrier protein [98,103,104]. It is of utmost importance to determine the stability of the carrier protein as it was observed a century ago that toxoids such as tetanus, diphtheria and pertussis toxoids may convert to their toxic nature. The primary structure of the carrier protein may be confirmed by use of electrospray mass spectroscopy (ESMS) and a combination of proteolytic digestion and HPLC-ESMS, which is used to identify peptides, which make up the sequence of the carrier protein [98,99]. These data can also be used to determine the favoured sites of linkage to the polysaccharides.
Circular dichroism and fluorescence spectroscopy has also been used to determine the secondary and the tertiary structure of the carrier protein [104-107]. The amount of these structures has been estimated from far-UV (assess the secondary structure) or near-UV (assess the tertiary structure) spectra using programmes such as Varselec software and Yang's algorithm. These techniques have been used to determine the secondary structures of CRM197 and it was found to contain about 23% α-helix, 21% β-antiparallel, 7% β-parallel, 17% β-turn and other structures [107]. Combination of these methods provides a baseline from which the integrity, structure and stability of the carrier protein can be established.

4.2.4.3. CHARACTERISATION OF THE CONJUGATE VACCINE

Analyses of conjugates are more complex than those of the individual components, which involves the determination of the protein-polysaccharide ratio, proportions of free saccharide and unconjugated protein present and changes in both the carrier protein and the saccharide [99]. Determination of parameters such as saccharide content, protein-saccharide ratio, free saccharide, unconjugated protein, molecular size and stability of the conjugate is important due to lack of suitable animal models that could be used to predict and evaluate the quality of the conjugate vaccines [100,102].

These parameters have thus become the basis for establishing specifications of conjugate vaccines. The structure and stability of the polysaccharide and the carrier protein is based on the methodology already described in Sections 4.2.4.1 and 4.2.4.2, respectively. The data obtained provides evidence of production consistency, which can be observed from reproducibility of the results.

4.2.4.3.1. Stability of the Conjugate

The stability of the conjugate can be monitored by monitoring the free saccharide, molecular size and antigenicity of the conjugate over time [102,108]. The instability of the conjugate is thought to be due to degradation or depolymerisation of the polysaccharide. Monitoring the amount of free saccharide available over time will give
sufficient indication of how stable the conjugate is. The amount of free saccharide can be obtained as described previously, once it is separated from the conjugate mixture as done previously for meningococcal C conjugates [109]. Several methods has been used to separate the saccharide based on hydrophobic interaction, precipitation, affinity adsorption, solid-phase adsorption and size, which can be achieved by ultrafiltration [102,104,109].

The molecular size of the conjugate is another parameter considered to be important for monitoring the stability of the conjugate. This can be done by size exclusion chromatography (SEC) using either CL-2B or CL-4B gel permeation columns. However, the method has been replaced recently by HPLC using multiple angle laser scattering (MALLS) as a detector [102].

4.2.4.3.2. Unconjugated Carrier Protein

The amount of unconjugated protein is relatively low as it is separated from the conjugate during the purification process. However, the available free carrier protein can be detected mainly by HPLC, and other methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or enzyme linked immunosorbant assay (ELISA) [102].

4.2.4.3.3. Protein-Saccharide Ratio

The protein-polysaccharide ratio can be monitored by use of colorimetric assays for the saccharide moiety and HPAEC for analysis of both the saccharide and the protein [98].

4.2.4.3.4. Covalent Linkage

Several methods have been used to determine the covalent linkage of the polysaccharide to the carrier protein, mainly for Hib-CRM197 and Men C-CRM197. This has been obtained by determination of the hydroxy-ethyl lysine using an amino acid analyser, which yield distinctive amino acids, after reductive amination [102].
Size exclusion profiles of the protein and the polysaccharide is another tool that can be used to demonstrate the formation of the covalent bond.

4.2.5. MENINGOCOCCAL A-CRM\textsubscript{197} CONJUGATE VACCINES

The conjugate vaccine used in this study was developed by Chiron Vaccines, (Siena, Italy), and the conjugate consists of a meningococcal A polysaccharide and a carrier protein, CRM197. The production of the vaccine is outlined in Section 4.2.5.3 below.

4.2.5.1. MENINGOCOCCAL A CAPSULAR POLYSACCHARIDE (Men A CPS)

Meningococcal A capsular polysaccharide (Men A CPS) is isolated from serogroup A meningococcus and it consists of $N$-acetylmannosamine residues linked together by $\alpha$1-6-phosphodiester bonds (structure shown in Figure 4.2.4) [98,110,111].

![Diagram of meningococcal capsular polysaccharide](image)

**Figure 4.2.4.** Structure of meningococcal capsular polysaccharide

(-OPO$_3^-$-6-ManNAc(3OAc)$\alpha$1-OPO$_3^-$-6ManAc(3Oac)$\alpha$1-),

arrow indicates the point of cleavage by hydrolysis.

The repeating units of the polysaccharide can be obtained by a combination of chemical methods and NMR spectroscopy [112]. The structure of the group A polysaccharide, known since 1977, has been fully characterised by Lemercinier et al. by use of 1D and 2D NMR spectroscopy and it was found to contain about 70% O-acetylation in position 3 [77]. 2D $^1$H NMR spectra revealed the existence of four
distinct spin systems with 3-O-acetylated attached to H-3 \(\alpha\)-ManNAc being the major spin system [112].

### 4.2.5.2. THE CARRIER PROTEIN (CRM\(_{197}\))

The carrier protein, CRM197, is a cross-reactive mutant of diphtheria toxin and is isolated as 58.4 kDa protein [102,105,107]. The carrier protein is genetically altered at position 52 from glycine to glutamic acid (Gly\(^{52}\)\(\rightarrow\)Glu) to eliminate its enzymatic activity, which in turn eliminate its toxicity. The primary and secondary structure of the protein have been deduced by Chiron Vaccines by use of N-terminal sequence analysis (Edman degradation), analysis of the C-terminal peptide (RP-MS and MALDI-TOF MS), peptide mapping, and by use of optical spectroscopy; circular dichroism and fluorescence, respectively. The protein consists of 535 amino acid residues (amino acid sequence shown in Figure 4.2.5) and contain five tryptophan, 18 tyrosine and 18 phenylalanine residues [101,107].

![Amino acid sequence](image)

**Figure 4.2.5. Amino acid sequence of CRM\(_{197}\)**

<table>
<thead>
<tr>
<th>B!l!5!Y!!l!!!!l!!:1ll:</th>
<th>GADDVDSSK</th>
<th>SFVMENFSSY</th>
<th>HGTKPQYVD</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQKGIQKPKS</td>
<td>GTQGNYDDDW</td>
<td>KEFYSTDNKY</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>DAAGYSVNE</td>
<td>NPLSGKAGGV</td>
<td>VKVTYPGLTK</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>VLALKVDNAE</td>
<td>TIKKELGLSL</td>
<td>EPLMEQVGT</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>EEKIFRFDG</td>
<td>ASRVLVSLPF</td>
<td>AEGSSVEYI</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>NNWEAQAKALS</td>
<td>VELEINFETR</td>
<td>GKRQDAMYE</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>YMAQACAGNR</td>
<td>VRRSOGSSL</td>
<td>CINLVDWIR</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>DKTJKIESL</td>
<td>KEHGPKNKM</td>
<td>SESPNKTVSE</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>EKAKCLEEF</td>
<td>HQTALEHPEC</td>
<td>SELKTVTGAN</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>PVFAGANYAA</td>
<td>WAVNVAQVID</td>
<td>SETADNLEKT</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>TAALSILPGI</td>
<td>GSVMSADGA</td>
<td>VHHNTEEIVA</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>QSIALSSLMV</td>
<td>AQAIPVGE</td>
<td>VDIFGAAYNF</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td>VESIINLFQV</td>
<td>VHNSYNRPAY</td>
<td>PGHKTQPF</td>
<td>390</td>
<td></td>
</tr>
<tr>
<td>HDGYAVSWNT</td>
<td>VEDISRTGF</td>
<td>QGESGDHIK</td>
<td>420</td>
<td></td>
</tr>
<tr>
<td>TAENTPLPA</td>
<td>GVLLTPPGK</td>
<td>LDVNKSHTI</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>SVNGRKRIMR</td>
<td>CRAIDGDVTF</td>
<td>CRPKSPVYVG</td>
<td>480</td>
<td></td>
</tr>
<tr>
<td>NGVHANLHVA</td>
<td>FHRSSSEKIH</td>
<td>SNEISSDSIG</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td>VLGYQKTVDH</td>
<td>TKVNSKLSLF</td>
<td>FEIKS</td>
<td>535</td>
<td></td>
</tr>
</tbody>
</table>

**Residue identity:** A: Ala; C: Cys; D: Asp; E: Glu; F: Phe; G: Gly; H: His; I: Ile; K: Lys; L: Leu; M: Met; N: Asn; P: Pro; Q: Gln; R: Arg; S: Ser; T: Thr; V: Val; W: Trp; Y: Tyr
The protein contains 39 lysines, among which at least five have been shown to be highly reactive [113]. Predictions from the secondary structure indicate that the protein contains 22% α-helix, 23% β-sheet anti-parallel, 6% β-sheet parallel, 20% β-turn and 30% other [107,114]. The protein is an ideal carrier protein as it retains its native immunological properties without the danger of reversion to its enzymatic state [113].

4.2.5.3. PREPARATION OF MEN A-CRM\textsubscript{197} CONJUGATE VACCINE

Meningococcal A conjugate vaccines were produced using the same protocol used for the production of meningococcal C conjugate vaccines (Chiron Vaccines, unpublished data). The process involves fermentation, isolation and purification of the native polysaccharide, followed by hydrolysis to produce oligosaccharides with a defined size distribution. This is then followed by activation of the oligosaccharides and then coupling to the carrier protein, CRM197, using procedures described in Section 4.2.3. This process is shown in Figure 4.2.6.

The vaccine is produced by, firstly generating suitable oligosaccharide fragments by acid hydrolysis of the capsular polysaccharide [100]. The selected pool of oligosaccharides, achieved by ion-exchange chromatography, undergo reductive animation to produce amino groups which are then activated. The activated oligosaccharide is covalently attached to the amino group (lysine) on the CRM197 resulting in a conjugate vaccine. The final conjugate vaccine is characterised using the methods described in Section 4.2.4.
Figure 4.2.6. Schematic representation of the steps used to generate glycoconjugate vaccines
4.3. EXPERIMENTAL

4.3.1. MATERIALS

The vaccine, meningococcal A-CRM$_{197}$ glycoconjugate vaccine was obtained from Chiron Vaccines, Siena, Italy. The bulk vaccine contained 0.5 mg/ml of polysaccharide and 1 mg/ml of protein. Unconjugated polysaccharide, PS (500 mg) and protein, CRM (53.8 mg/ml) were also supplied.

4.3.2. STABILITY SAMPLE TREATMENT

Meningococcal A-CRM$_{197}$ bulk vaccine, 25 ml (in sterile sealed glass vials) were incubated at 4°C, RT (daily average temperature of about 25°C), 37°C and 55°C for five weeks. At the end of the incubation period, all vaccine samples were stored at 4°C. The bulk vaccine contained 0.5 mg/ml of polysaccharide and 1 mg/ml of protein. The samples were centrifuged using an Eppendorf Centrifuge 5410 before each experiment to remove any foreign materials.

4.3.3. ANALYSIS OF THE SIZE OF THE CONJUGATE VACCINE

Size exclusion chromatography (HPLC-SEC) was used to determine any changes in the hydrodynamic size of the conjugate at the different storage temperatures. Two detection systems were used, the UV detector set at 280 nm to detect the protein component and refractive index detector (RI) to detect both the protein and polysaccharide.

4.3.3.1. HPLC-SEC USING UV DETECTION

The HPLC system consisted of a ThermoSeparation SpectraSystem and SpectraSeries Gradient pump P200 and a SpectraSystem and SpectraSeries Autosampler AS100. A SpectraSeries UV100 detector was used to detect
protein absorbance at 280nm. DELTA 5.0 Chromatography Data System was used to monitor the runs. A Superdex 200 HR 10/30 gel filtration column (24 ml) with a fractionation range of $M_r$ 10000 - $6 \times 10^5$ was used.

The column was calibrated using blue dextrin, BSA (Bovine Serum Albumin) and azide. The void ($V_o$) and total ($V_t$) volumes were 11.10 ml and 31.17 ml, respectively. The samples (100 μl: 50 μg polysaccharide and 100 μg protein) were loaded onto the column and eluted isocratically in 10 mM sodium phosphate buffer, pH 7.4, 150 mM sodium chloride at a flow rate of 0.75 mL/min for 40 min. The distribution coefficient, $K_D$ of the main peak was determined and calculated as:

$$K_D = (V_e - V_o) / (V_t - V_o)$$

where $V_e$ is the volume of the elution peak of the analysed sample

$V_o$ is the void volume (blue dextrin)

$V_t$ is the total volume (azide)

The chromatographic conditions are shown in Table 4.3.1.

*Table 4.3.1. Method summary and conditions*

<table>
<thead>
<tr>
<th>Samples</th>
<th>Blue Dextrin (1), BSA (2), Azide (3) and Vaccine samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HPLC system</strong></td>
<td>Thermo Separation Products AS100</td>
</tr>
<tr>
<td><strong>HPLC column</strong></td>
<td>A Superdex 200 HR 10/30 Gel Filtration column</td>
</tr>
<tr>
<td><strong>Column temperature</strong></td>
<td>Room temperature</td>
</tr>
<tr>
<td><strong>Mobile phases#</strong></td>
<td>10 mM sodium phosphate buffer, pH 7.4; 150 mM sodium chloride</td>
</tr>
<tr>
<td><strong>Gradient profile</strong></td>
<td>Isocratic</td>
</tr>
<tr>
<td><strong>Flow rate</strong></td>
<td>0.75 ml/min</td>
</tr>
<tr>
<td><strong>Injection volume</strong></td>
<td>100μl</td>
</tr>
<tr>
<td><strong>Detection</strong></td>
<td>280nm</td>
</tr>
<tr>
<td><strong>Analysis time</strong></td>
<td>40 minutes</td>
</tr>
<tr>
<td><strong>Sample preparation</strong></td>
<td>Samples (1), (2) and (3) were dissolved in the mobile phase to make up a solution with a concentration 0.5 mg/ml for (1) and (2) and 2 mg/ml for (3).</td>
</tr>
</tbody>
</table>

*Preparation of the mobile phases is shown in Appendix 1.2*
4.3.3.2. HPLC-SEC COUPLED TO A REFRACTIVE INDEX DETECTOR

An HPLC system connected to a refractive index detector was used and the results obtained were compared with that obtained using an UV detector. A Waters Millipore Gel Permeation Chromatographic System 590 and a Waters Millipore Differential Refractometer R401 was used to detect both the protein and the oligosaccharide. The runs were monitored using a REFLOG2.PAS program. The same conditions used in HPLC-UV were applied.

4.3.4. CONFORMATIONAL ANALYSIS OF THE PROTEIN

Optical spectroscopy was used to determine any changes in the conformation of the protein with respect to change in incubation temperature.

4.3.4.1. FLUORESCENCE SPECTROSCOPY

Fluorescence spectra were obtained using an Amico SPF-500 single-photon spectrofluorometer with the 10 mm quartz cell. Excitation wavelengths of 280 and 295 nm with band pass of 5 nm and 10 nm were used for excitation and emission monochromator, respectively. Samples containing 0.2 mg/ml of the protein and 0.1 mg/ml of the polysaccharide concentration. Native carrier protein CRM197 stored at -20°C was used as a control.

4.3.4.2. CIRCULAR DICHROISM (CD)

The far-UV (180-260 nm). Circular dichroism (CD) spectra were obtained with a Jasco J-810 spectropolarimeter using quartz cell with 1 mm path length. Samples of 0.2 mg/ml protein concentration and 0.1 mg/ml polysaccharide concentration were used and native protein CRM197 was used as a control. The spectra were corrected for buffer and polysaccharide absorption and were expressed as molar ellipticity (mdeg). Preparation of the samples is reported in Appendix 1.2.
4.3.5. SACCHARIDE ANALYSIS

Analysis of the saccharide was achieved by separation of the free saccharide from the conjugate vaccine by ultrafiltration using Amicon Centricon Centrifugal Filter Devices YM-30. The filters were pre-washed three times with 0.5 ml distilled water and centrifuged at 5000xg for 30 minutes. The conjugate vaccines were applied to the pre-washed filters and centrifuged for 30 minutes, ending with a rinse to make sure all the saccharide (free) was filtered through. The retained protein was washed with 5 ml of distilled water. The samples were freeze-dried to remove the water from the samples.

4.3.5.1. TOTAL ORGANIC CARBON (TOC)

TOC was obtained using an SGE Anatoc series II total organic carbon analyser. The instrument uses light catalysed TiO₂ oxidation with detection of liberated CO₂ by a non-dispersive infrared detector. TOC is measured after removal of the inorganic carbon by acidification of the sample with HClO₄. The instrument is calibrated with 50 ppm sample of KHPthalate. The freeze-dried free saccharide obtained from 2 ml of the conjugate vaccines (~40 µg of Carbon) was dissolved in 1 ml of distilled water. The solutions, 0.5 ml (~20 µg of Carbon) were then dissolved in 9.5 ml of distilled water to make-up a 10 ml solutions. These solutions were used for TOC analysis.

4.3.5.2. NMR SPECTROSCOPY

The treated conjugated samples were freeze-dried and deuterium exchanged three times in 2 ml of D₂O, and then dissolved in D₂O for analysis. 1D NMR spectra (¹H, ¹³C and ³¹P) spectra and 2D spectra (COSY and HSQC) were recorded using a 400 MHz Varian Unity NMR spectrometer at 30°C and processed using the standard Varian Software.
4.4. RESULTS AND DISCUSSION

4.4.1. RESULTS

4.4.1.1. EFFECT OF INCUBATION TEMPERATURE ON THE CONJUGATE

4.4.1.1.1. HPLC-SEC

HPLC-SEC was used to determine or monitor the effects of incubation temperature on the molecular size of the conjugate vaccine. This is achieved by monitoring the retention volume of the conjugate, which is dependent on the hydrodynamic size of the conjugate. The chromatograms of the Men A-CRM197 conjugate stored at 4°C, RT, 37°C and 55°C are shown in Figures 4.4.1 and 4.4.2 and these were detected by UV absorption (280 nm) and refractive index (RI) detection. The UV detector was used to detect the conjugated carrier protein, while the RI detector was used to detect the free saccharide and the conjugate, which provided additional information in the changes in the conjugate.

When stored at 4°C (the recommended temperature), the conjugate elutes with a retention volume of 13.6 ml as a broad peak (Figure 4.4.1). An increase in incubation temperature from 37°C to 55°C resulted in a decrease in the main peak, which was subsequently replaced by a higher molecular weight peak. RI signals showed similar results, with additional peaks at 27 ml and between 28 and 40 ml (Figure 4.4.2).
Figure 4.4.1. Overlay chromatograms of conjugate vaccine samples stored at various temperatures using a UV detector (λ=280 nm)

Figure 4.4.2. Overlay chromatograms of conjugate vaccine samples stored at various temperatures using a refractive index detector

The distribution coefficient, $K_D$ of the main peak was calculated from the results obtained using the UV detector using the equation showed in Section 4.3 (Table 4.4.1).
The results were used in addition to monitor the size of the conjugate. The $K_D$ of the vaccine samples stored between 4°C and 37°C are similar (between 0.123 and 0.159) but decrease to $K_D$ 0.007 when the temperature was increased to 55°C. This resulted from a decrease in retention volume (Table 4.4.1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Elution Volume ($V_e$) (ml)</th>
<th>Distribution Coefficient ($K_D$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>13.60</td>
<td>0.124</td>
</tr>
<tr>
<td>RT</td>
<td>13.55</td>
<td>0.122</td>
</tr>
<tr>
<td>37°C</td>
<td>14.31</td>
<td>0.160</td>
</tr>
<tr>
<td>55°C</td>
<td>11.25</td>
<td>0.007</td>
</tr>
</tbody>
</table>

### 4.4.1.2. EFFECT OF INCUBATION TEMPERATURE ON CONFORMATION OF THE CARRIER PROTEIN

Optical spectroscopy (circular dichroism and fluorescence spectroscopy) has been used previously to determine the structure and the stability of the CRM197 carrier protein [104,108]. In this study, the techniques were used to determine the changes in conformation of the carrier protein due to unfolding or aggregation of the protein due to changes in incubation temperature.

The samples were incubated at 4°C, room temperature (RT), 37°C and 55°C for a period of five weeks and analysed. The carrier protein as mentioned in Section 4.2.5.1 consists of 535 amino acid residues and it contains five tryptophans, 18 tyrosine and 18 phenylalanine residues. The stability of the carrier protein was monitored previously, therefore the results obtained in this study could be compared with the data obtained from these previous studies performed under the same experimental conditions.
4.4.1.2.1. Fluorescence Spectroscopy

Fluorescence emission maximum \( F_{\text{max}} \) of each sample stored at different temperatures for five weeks was measured in order to determine the changes in conformation of the conjugated carrier protein. Two excitation wavelengths were used; 280 nm to monitor emission wavelengths of the tryptophan (Trp) and tyrosine (Tyr), to a lesser extent, while 295 nm was used to monitor emission wavelengths due to Trp exclusively (Table 4.4.2 and Figure 4.4.3).

**Table 4.4.2.** \( F_{\text{max}} \) of the samples at two excitation wavelengths

<table>
<thead>
<tr>
<th>Sample</th>
<th>Excitation ( \lambda ) (280 nm)</th>
<th>Sample</th>
<th>Excitation ( \lambda ) (295 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRM&lt;sub&gt;R&lt;/sub&gt;</td>
<td>340</td>
<td>CRM&lt;sub&gt;R&lt;/sub&gt;</td>
<td>322</td>
</tr>
<tr>
<td>4°C</td>
<td>339</td>
<td>4°C</td>
<td>321</td>
</tr>
<tr>
<td>RT</td>
<td>341</td>
<td>RT</td>
<td>320</td>
</tr>
<tr>
<td>37°C</td>
<td>342</td>
<td>37°C</td>
<td>320</td>
</tr>
<tr>
<td>55°C</td>
<td>345</td>
<td>55°C</td>
<td>325</td>
</tr>
</tbody>
</table>

**Figure 4.4.3.** Graphical representation to show the difference in \( F_{\text{max}} \) at 280 nm and 295 nm

The \( F_{\text{max}} \) of the native carrier protein is similar to that of the samples stored below 37°C, 340 nm (excited at 280 nm) and 322 nm (excited at 295 nm). An increase in
incubation temperature from 4°C to 37°C resulted in a shift in $F_{\text{max}}$ to longer wavelengths (from 339 to 342 nm and 321 to 320 nm) when the samples were excited at 280 and 295 nm, respectively (Table 4.4.2). A larger shift was observed in samples stored at 55°C. Similar shifts in $F_{\text{max}}$ have also been observed for tetanus toxoid [106] and diphtheria toxoid [114]. The $F_{\text{max}}$ of the CRM197 conjugated to meningococcal C and Hib polysaccharides [104,108] are different from the one obtained in this study, however, similar trends were observed.

4.4.1.2.2. Circular Dichroism (CD) Spectroscopy

The main application of circular dichroism (CD) spectroscopy is in the structural study of chiral molecules, particularly proteins. In proteins, the far-UV (180-260 nm) CD spectra is used to determine the conformation and secondary structure of the protein from the peptide backbone spectra, while the near-UV (250-320 nm) CD spectra provides information on the environment of the aromatic residues. In this study, the far-UV CD spectra of the carrier protein measured between 190 and 260 nm was recorded to determine changes in the conformation and secondary structures of the conjugated carrier protein in order to obtain information on the thermal stability of the conjugate vaccine. Figure 4.4.4 shows the far-UV CD spectra of the native carrier protein and conjugated samples stored at various temperatures.

![Figure 4.4.4](image_url)
The CD spectrum of the native CRM197 has a positive band around 195 nm and two negative bands around 207 and 218 nm, respectively. There is a shift in the CD spectra upon conjugation. For unknown reasons, the CD spectra of the conjugated samples could not be measured below 198 nm, however they all have two negative bands at the same wavelengths as that of the native CRM197.

The CD spectra of the samples stored between 4°C and 37°C are superimposable, however, a shift in minima from 208 nm to 205 nm at 55°C is observed. Furthermore, a reduction in CD intensity at about 220 nm is also observed when the storage temperature is increased from 37°C to 55°C. This results in a decrease in α-helix structures (from 23% to 15%), and an increase in β-structures (from 78% to 85%), as observed in previous studies done by Crane et al. [107,104]. However, the standard program (CD Spectra Deconvolution Programme) used did not give meaningful prediction of the secondary structures.

4.4.1.3. SACCHARIDE ANALYSIS

4.4.1.3.1. NMR Spectroscopy

NMR spectroscopy has been used in similar studies to determine or assess the stability of meningococcal type C and Hib conjugate vaccines, of which some are presently on the market. Similar principles applied to determine the polysaccharide of these vaccines were used in this study to determine the stability of the meningococcal group A capsular polysaccharide.

The structure of the Men A polysaccharide was characterised earlier from NMR analysis using 1D and 2D and the data was recently published by Lemercinier et al. [77]. The structure was found to be \( \rightarrow 6 \)-α-ManNAc-(1-OPO\(_2\)) and contain about 70% O-acetylation in position 3 as shown in Figure 4.4.5 [77].
2D $^1$H NMR spectra revealed the existence of four distinct spin systems with 3-O-acetylated attached to H-3 $\alpha$-ManNAc being the major spin system [112].

1D and 2D NMR spectra of the native (unconjugated) polysaccharide stored at -20°C were obtained for comparison. 1D $^1$H NMR showed similar signals for the ring protons, H1 (5.45 ppm) and H2 (4.58 ppm) (Figure 4.4.6). The COSY spectrum (not shown) was used to identify the major spin system and it was found to be the 3-O-acetylated spin system. The COSY spectrum was also used to assign the rest of the protons signals of the major spin system. Both the spin system and the $^1$H chemical shifts were similar to those obtained in the previous studies.

**Figure 4.4.5. Structure of the Men A capsular polysaccharide**

**Figure 4.4.6. $^1$H NMR spectra of the Men A polysaccharide stored at -20°C**
$^1$H assignments were used to assign the $^{13}$C NMR signals from an HSQC spectrum shown in Figure 4.4. The full $^1$H, $^{13}$C and $^{31}$P signals of the major spin system are shown in Table 4.4.4. The three doublets obtained due to coupling of C2, C3 and C4 with the $^{31}$P were also observed.

![HSQC spectrum](image)

**Figure 4.4.7. HSQC spectrum of the Men A polysaccharide; s is residual solvent (ethanol)**

**Table 4.4.4. Table of chemical shifts**

<table>
<thead>
<tr>
<th>$^1$H</th>
<th>Chemical shift (ppm)</th>
<th>$^{13}$C</th>
<th>Chemical shift (ppm)</th>
<th>$^{31}$P</th>
<th>Chemical shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>5.45</td>
<td>C1</td>
<td>95.09</td>
<td>monophosphate</td>
<td>3.95</td>
</tr>
<tr>
<td>H2</td>
<td>4.58</td>
<td>C2</td>
<td>50.90</td>
<td>phosphodiester</td>
<td>-2.80</td>
</tr>
<tr>
<td>H3</td>
<td>5.17</td>
<td>C3</td>
<td>72.16</td>
<td>phosphate buffer</td>
<td>1.50</td>
</tr>
<tr>
<td>H4</td>
<td>3.98</td>
<td>C4</td>
<td>63.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H5</td>
<td>4.10</td>
<td>C5</td>
<td>72.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H6</td>
<td>4.20</td>
<td>C6</td>
<td>64.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHCOCH$_3$</td>
<td>2.02</td>
<td>NHCOCH$_3$</td>
<td>22.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCOHCH$_3$</td>
<td>2.10</td>
<td>OCOHCH$_3$</td>
<td>20.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The $^{31}\text{P}$ spectrum of the native polysaccharide was also obtained and this is shown in Figure 4.4.8. The major signal at -2.60 ppm is assigned to the phosphodiester linkage between C1 and C6 of the polysaccharide chain, with the O-acetyl group at position three. The small signal at -3.4 ppm corresponds to the $^{31}\text{P}$ signal of the phosphodiester with the O-acetyl group at position four.

![Figure 4.4.8. $^{31}\text{P}$ NMR spectrum of the Men A polysaccharide](image)

The results obtained were compared with the NMR data of the conjugate samples stored at various temperatures to interpret the spectra of the conjugated polysaccharide as well as to monitor the stability of the conjugated polysaccharide. The $^1\text{H}$ NMR spectra are shown in Figures 4.4.9.

![Figure 4.4.9. $^1\text{H}$ NMR spectra of the conjugate vaccines at various temperatures](image)
$^1$H signals of the polysaccharide are situated between 2.0 and 5.5 ppm with the anomeric signal (H1, green arrow) at 5.5 ppm, H2 around 4.6 ppm and acetyl signals around 2.1 ppm. Some protein signals are situated between 0 and 2 ppm (pink arrow) and are broader. This is believed to be due to protein being less mobile than the saccharide. The signal increases as the incubation temperature increases.

Significant differences in $^1$H spectra of the samples stored between 4°C and 55°C are observed, as shown from the COSY spectra of the samples stored at 4°C, 37°C and 55°C (Figure 4.5.0 (a-c)).

![COSY spectra](image)

**Figure 4.5.0.** COSY spectra of the samples stored at various temperatures (a) 4°C, (b) 37°C, and (c) 55°C: (a) OAc at position 3, (b) OAc at position 4, (c) OAc not present, $^{\beta}$-ManNAc-6P-OH, $^{\alpha}$-ManNAc-6P-OH.
There are minor differences in the saccharide signals of the samples stored at RT (spectrum not shown) and 4°C (Figure 4.5.0 (a)). The spectrum shows the presence of the major spin system, which is evident from the crosspeaks at 3.98, 4.56 and 4.98 ppm. The appearance of the signals at 4.12 (H4,H2)b and 4.30 (H4,H3)b ppm correspond to the signals of the minor system with OAc at position 4. These results are similar to the COSY spectrum of the polysaccharide stored at -20°C and the sample stored at RT (spectra not shown).

The presence of both the major and minor spin systems is evident as shown by the presence of (H3,H2)a and (H1,H2)a, and (H4,H2)b and (H4,H3)b crosspeaks, respectively (Figure 4.5.0 (b)). However, the relative intensity of these peaks is lower compared with the intensity of the crosspeaks of the sample stored at 4°C. The development of minor signals at 3.92 (H3,H4)c and 4.40 (H1,H2)c ppm are due to the absence of the OAc group.

Further de-acetylation is evident in the sample stored at 55°C from the decrease in relative intensity of the crosspeaks at 3.92 and 4.40 ppm (Figure 4.5.0 (c)). The appearance of signals at 4.30 (H1,H2)* and 4.40 (H1,H2)** ppm is evidence of the hydrolysed Man A polysaccharide.
A similar trend was observed in the $^{31}$P NMR spectra (Figure 4.5.1).

**Figure 4.5.1.** $^{31}$P NMR spectra of the conjugate samples at various temperatures

The relative intensity of the phosphodiester signal, around -2.80 ppm, appears similar at 4°C and RT, however, it decreases in samples stored at 37°C, with a significant decrease at 55°C. The relative intensity of the monophosphate signal (at 4 ppm) is similar in samples stored below 37°C, but increases dramatically at 55°C. In fact, the relative areas of the monophosphate and phosphodiester peaks indicate that the sample is about 90% hydrolysed after five weeks at 55°C. The peak at about 1.7 ppm correspond to the signal of the phosphate buffer.

4.4.1.3.2. Total Organic Carbon (TOC)

Total organic carbon (TOC) analyser is used to record the amount of organic carbon by oxidising the organic carbon in the sample to CO$_2$, which is subsequently measured. The technique was used in this study to give an indication of how much saccharide degrade at various storage temperatures as it was observed from HPLC-IR and NMR that saccharide degradation occur at higher temperatures. This was done by measuring the concentration of carbon of the free saccharide present in the samples stored at various temperatures. The results obtained are shown in Table 4.4.5 and these were used to determine or estimate the stability of the saccharide.
Table 4.4.5. Concentration of carbon of the free saccharide present in the vaccine samples stored at various temperatures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (μl)</th>
<th>Concentration of Carbon (ppm)</th>
<th>Average (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>2000</td>
<td>14.5</td>
<td>15.25</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>2000</td>
<td>15.7</td>
<td>15.60</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>2000</td>
<td>16.4</td>
<td>16.90</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td>55°C</td>
<td>800</td>
<td>30.8</td>
<td>36.20</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>35.6</td>
<td></td>
</tr>
</tbody>
</table>

The results show an increase in the concentration of carbon form 15.25 to 36.20 ppm with an increase in incubation temperature. The concentration is fairly stable between 4°C and 37°C, with a greater increase at 55°C. This was evident from the graph plotted using the results in Table 4.4.5 (Figure 4.5.2).

**Figure 4.5.2.** Graphical representation to show an increase in the concentration of carbon of the free saccharide present in the vaccine samples
The technique, however, does not give a true quantity of the free saccharide. The true quantity of the free saccharide in the samples could not be obtained due to lack of Dionex instrumentation.

4.4.2. DISCUSSION

4.4.2.1. MOLECULAR SIZE OF THE Men A-CRM$_{197}$ CONJUGATE VACCINE

The chromatograms obtained from HPLC-SEC show that the hydrodynamic size of the conjugate vaccine increases with increase in incubation temperature, as evident by the decrease in the $K_0$ values. The vaccine samples stored at 55°C has the largest hydrodynamic size compared with samples stored below 37°C. One would expect that as more saccharide degrade at higher temperatures, the hydrodynamic size would become smaller. However, this is not observed which suggests that another effect is taking place, i.e. that the carrier protein CRM$_{197}$ unfolds as incubation temperature increases. This results in an increase in hydrodynamic size, despite the loss of saccharide.

RI detector was used to detect the protein and if possible to confirm the presence of the free saccharide. The peak eluting between 11 and 15 ml correspond to signal of the carrier protein and the chromatograms show a shift in retention volume to a higher molecular size. The results correlate with the results obtained from HPLC-UV, which suggests a change in conformation of the carrier protein with an increase in storage temperature. These conformational changes were investigated further using the optical spectroscopy.

The saccharide was identified by injecting the standard and the peak appeared at about 27 ml. The amount of free saccharide in each sample could not be quantified using this technique, however, the results confirm that the saccharide degrades at higher incubation temperature. The presence of the free saccharide was investigated further by NMR spectroscopy and TOC. The peak between 28 and 40 ml correspond to the phosphate buffer.
4.4.2.2. CARRIER PROTEIN MOIETY

The results obtained from fluorescence spectroscopy showed an increase in the mobility of the aromatic side chains of the CRM$_{197}$. The shift in $F_{\text{max}}$ to longer wavelengths (low fluorescence intensity) suggests a change in conformation of the protein, which is thought to unfold or adopt an "open" conformation, thus exposing aromatic side chains to the solvent. This result in a looser structure of the carrier protein. Similar trends have been observed for tetanus toxoid [106], diphtheria toxoid [114] and CRM$_{197}$ conjugated to Men C and Hib, with a shift in $F_{\text{max}}$ from 334 to 346 nm and from 337 to 349 nm when excited at 280 nm and 295 nm, respectively.

The CD characteristic features of the native CRM$_{197}$ suggest that the protein contain a mixture of both the $\alpha$-helix and $\beta$-type structures. The CD spectra of the conjugated samples are similar to the CD spectrum of the native protein, meaning that their structures are similar. The far-UV CD spectra of the samples stored below 37°C are superimposable on each other, suggesting that the secondary structures of the samples stored under these conditions is similar.

However, exposure to higher temperatures (37°C or 55°C) caused a shift in the CD spectra and reduction of CD intensities, which suggests that CRM$_{197}$ unfolds at 37°C or 55°C. These results correlate with the data obtained form studies done by Crane et al., which showed that an increase in temperature results in a decrease in $\alpha$-helix and an increase in $\beta$-structures as the protein unfold [105]. The results also correlate with the molecular size results (Section 4.4.2.1).

4.4.2.3. MENINGOCOCCAL GROUP A CAPSULAR POLYSACCHARIDE

The stability of the saccharide component at incubated temperatures was monitored using HPLC-IR, NMR spectroscopy and TOC. The HPLC-IR confirmed the presence of free saccharide in all the samples, however, the technique could not be used to quantify the free saccharide present.
The $^1$H and $^{31}$P spectra of the unconjugated polysaccharide are similar to the spectra of the vaccines stored at 4°C and RT with the presence of 3-OAc as the main spin system. These suggests that their structures are similar in terms of chain length of the polysaccharide. Changes in the saccharide from vaccines stored at 37°C and 55°C were observed from the appearance of minor signals between 3.9 and 5.3 ppm ($^1$H and COSY NMR spectra).

The signals are mainly due to de-acetylation and small amount of de-polymerisation at 37°C. An increase in relative intensity of the H2 signal is evidence of de-acetylation; which can be seen also from the H1 signal. Cleavage of the saccharide chain is evident at 55°C. This results in a large amount of de-polymerisation and the formation of $\alpha$ and $\beta$ ManNAc-6P-OH, with anomeric signals at 5.13 and 5.0 ppm, respectively. This is evident from the appearance of the monophosphate signal in the $^{31}$P signal at 55°C.

The amount of the free saccharide was monitored using TOC by measuring the concentration of carbon present in each sample. An increase in the number of carbons with increase in incubation temperature was observed from these measurements, which suggest an increase in the free saccharide with an increase in storage temperature. The amount is fairly similar at temperatures below 37°C but increases dramatically at 55°C. The saccharide is thus fairly stable below 37°C, however, it degrades above this temperature.
4.5. CONCLUSIONS

Physicochemical methods were used previously to determine the structure, purity, integrity and stability of various conjugate vaccines. These methods were used successfully in this study to monitor the stability of the meningococcal A-CRM\textsubscript{197} conjugate vaccine. This was done by monitoring the changes in the hydrodynamic size of the conjugate vaccine, the conformational changes of the carrier protein and the changes in the polysaccharide due to degradation.

The data obtained from HPLC-SEC showed that the size of the conjugate increases with increase in incubation temperature due to unfolding of the protein. The conformation of the protein changes and the protein adopts an open conformation, which result in exposure of the aromatic side chains to the solvent, thus increasing their mobility as shown by the optical spectroscopy.

The presence of extra peaks at 37°C and 55 °C from \textsuperscript{1}H NMR spectra is characteristic of de-acetylation, de-polymerisation and cleavage of the polysaccharide chain. This was also confirmed by the appearance of the monophosphate signal in \textsuperscript{31}P signal, and also by an increase in carbon concentration from TOC measurements. Biological implications of de-acetylation are not yet known.

We were able to show in the study that the meningococcal A-CRM\textsubscript{197} is stable at RT, however, the saccharide degrades at higher temperatures. The saccharide chain thus becomes smaller. The amount of the free saccharide was not obtained due to the unavailability of the Dionex. Quantification of the free saccharide is very important as the test is used mainly to determine the immunogenicity of the vaccine. However, part of the objective was achieved.
4.6. FUTURE STUDIES AND RECOMMENDATIONS

We have shown in the study that the vaccine is stable below physiological temperature and that incubation at 55°C enhances the degradation of the saccharide and conformational changes in the protein. Several things still need to be done;

- Quantification of the free saccharide (%), which is used by WHO as potency test.
- Extension of the conformational studies by calculation of the secondary structures using appropriate programs.
- Immunological tests to determine the effect of changes in protein conformation and changes in conjugate molecular size on the immunogenicity of the vaccine following storage under adverse temperature conditions.
APPENDICES
APPENDIX 1.2

PREPARATION OF THE MOBILE PHASES USED IN HPLC-SEC

All the weighings were done on a Mettler Toledo AB204S balance and it was calibrated according to the manufacturers specifications.

PREPARATION OF 10mM SODIUM PHOSPHATE BUFFER, pH 7.4 (SOLUTION 1)

Weight out 0.870 g of sodium phosphate dibasic (Na₂H₂PO₄) and 0.463 g of sodium phosphate monobasic (NaH₂PO₄) on an analytical balance and add 1L of Milli-Q grade water to make up a 1L solution. Adjust the pH to 7.4 with NaOH.

PREPARATION OF 150mM SODIUM CHLORIDE (SOLUTION 2)

Weigh out 8.7666 g of sodium chloride and add 1L of Milli-Q grade water.

Add 500 ml of solution 1 to 500 ml of solution 2 to make up a 1L of mobile phase.

SAMPLE PREPARATION FOR CD EXPERIMENTS

Polysaccharide (PS) (0.5 mg/ml) and protein (1 mg/ml) were prepared and used as controls. Samples containing 0.5 mg/ml and 1 mg/ml of polysaccharide and protein respectively were prepared. The solutions prepared were too concentration, and were therefore diluted to give solutions with 0.1 mg/ml and 0.2 mg/ml of polysaccharide and protein, respectively.
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CHAPTER 5

GENERAL CONCLUSIONS
5.1. CONCLUSIONS

Physicochemical methods have been used successfully and effectively for analysis of both small and complex pharmaceutical drugs. The advantages and disadvantages of each method will be outlined.

NMR SPECTROSCOPY

NMR spectroscopy proved to be a powerful, fast and better method for structural characterisation as it has the capability to provide information at the molecular level, as was evident from the results obtained from the studies conducted. The technique was used in the first study to evaluate the resolution of enantiomers of a racemic mixture using a cyclodextrin as a chiral selector. The resolution was clearly evident from the $^1$H signals of the diastereoisomers formed, which are different from the $^1$H signals of the enantiomers.

The data obtained from the second study proved further the usefulness of this technique. It was evident from the spectra obtained that both the protein and the saccharide of the conjugate undergo some changes at higher temperatures. The technique showed evidently changes in the saccharide which were not observed from HPLC and optical spectroscopy.

OPTICAL SPECTROSCOPY

Optical spectroscopy also proved to be a very useful and sensitive technique, especially in protein analysis as it can detect minor changes which are hard to observe using other techniques. Both the CD and fluorescence spectroscopy showed clearly the minor changes in conformation of the carrier protein. This was evident from the decrease in the CD intensity and a shift in $F_{\text{max}}$, respectively. These changes are evidence of change in the environment of the aromatic side chains. The study can be extended by calculation of the secondary structures, which would give further evidence of conformational changes experienced by the protein.
HPLC

HPLC has been used for separation of small molecules for decades, and this is shown from the published literature. The technique was used successfully in the first study using a chiral stationary phase where separation of enantiomers was observed. It is, nevertheless, not very sensitive for analysing complex molecules. The technique is not a very good indicator of changes experienced by the conjugate vaccine, however, it is useful in that it provides information on the relative size of the molecule.

These methods, however, complement each other and they have an advantage over animal experiments as they are less expensive and are simple to use. The methods will hopefully provide an easy way of analysing complex molecules, which were not easy to analyse before and provide parameters which can be used easily in drug discovery.