Analysis of Complex Antibiotics

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Abstract
The emergence of multidrug resistant Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* has been recognized worldwide and some clinical isolates of these bacteria are now resistant to most of the antibiotics currently available. Unfortunately, there are at present not enough new antimicrobial drugs being produced by the pharmaceutical industry to keep pace with the continuing development of antibiotic resistance.

Colistin is an old antibiotic and in the form of colistin methanesulphonate sodium (CMS) has re-emerged as a major treatment for burn patients colonised with *Acinetobacter baumannii*. However, this antibiotic can lead to profound toxicity and for this reason the blood levels of CMS should be carefully monitored. Unfortunately the methods available for measuring it in serum are not robust and are not able to differentiate between the two forms of colistin i.e. colistin sulphate (CLS) and CMS.

In this study the chemical (Thin Layer Chromatography and High Performance Liquid Chromatography) and microbiological methods for CLS and CMS analysis were investigated to develop a methodology for reproducible quantification of CLS and CMS in water or serum. Since CMS in aqueous solution has the potential to hydrolyze to produce a complex mixture of colistin sulphomethylated derivatives as well as colistin base, the optimized chemical and microbiological methods were used to determine the degradation of CLS or CMS in aqueous solution and serum.

The bacteriostatic and bactericidal activity of CLS and CMS were probed by calculations of minimum inhibitory concentrations and time survivor studies. Depending on their concentrations, both antibiotics were found to exhibit bacteriostatic and bactericidal properties against a range of Gram-negative bacteria.
Membrane damage caused by both forms of colistin was investigated using *Acinetobacter lwoflii* R46383 and determining intracellular potassium leakage and 260nm absorbing materials leakage. CLS was shown to cause substantial membrane damage, indicated by rapid, gross potassium leakage, while the effect of CMS on the membrane appeared to be more subtle, with cells exhibiting a more concentration dependent loss of potassium.

Adsorption isotherms of CLS gave results that were indicative of high affinity isotherm (H-shape), while adsorption isotherms of CMS were indicative of co-operative sorption (S-shape).

Investigations into CLS and CMS cytotoxicity were performed using normal rat kidney (NRK-52E) cell line; the investigations revealed that neither forms of colistin has a major adverse effect on the rat renal cells even at concentrations higher than the therapeutic doses.
List of Contents

Abstract .................................................................................................................3

List of Contents ........................................................................................................9

List of Figures ...........................................................................................................14

List of Tables ..........................................................................................................23

Abbreviations ...........................................................................................................27

Acknowledgements ................................................................................................30

Declaration ..............................................................................................................31

1. Introduction

1.1 History ..................................................................................................................33

1.2 Structure of the bacterial cell ................................................................................33
   1.2.1 The cell wall .....................................................................................................34
   1.2.2 The cytoplasmic membrane .............................................................................36
   1.2.3 The cytoplasm ..................................................................................................37

1.3 Factors affecting antimicrobial activity .................................................................38
   1.3.1 Concentration ..................................................................................................39
   1.3.2 pH ...................................................................................................................39
   1.3.3 Temperature ...................................................................................................40
1.3.4 Type and number of microorganisms .................................................... 40

1.4 Interactions between the bacterial cells and antibiotics ........................... 40
  1.4.1 Nature of the antimicrobial effect .................................................. 41
  1.4.2 Antibiotic interactions with Gram-negative and Gram-positive bacteria ... 41
  1.4.3 Interaction with the whole cell .................................................... 41
    1.4.3.1 S (co-operative sorption) pattern ....................................... 43
    1.4.3.2 L (Langmuir) pattern ................................................... 43
    1.4.3.3 H (High affinity) pattern .............................................. 43
    1.4.3.4 C (Constant partition) pattern ....................................... 43
  1.4.4 Lysis .................................................................................. 45

1.5 Interaction with the cytoplasmic membrane ......................................... 45
  1.5.1 Leakage of cellular constituents .................................................. 45
    1.5.1.1 Potassium leakage studies .............................................. 45
    1.5.1.2 Release of 260nm absorbing materials ............................. 46
  1.5.2 Inhibition of cytoplasmic membrane-bound enzymes ......................... 46
  1.5.3 Interactions with the cytoplasm .................................................. 46

1.6 Introduction to colistin ................................................................ 47
  1.6.1 Colistin chemistry .................................................................. 48
  1.6.2 Colistin mode of action ........................................................... 50
  1.6.3 Hydrolysis of colistin ............................................................... 51
  1.6.4 Clinical uses of colistin ............................................................. 52
  1.6.5 Dosage ................................................................................ 53
  1.6.6 Colistin pharmacokinetics ......................................................... 55
  1.6.7 Colistin pharmacodynamics ....................................................... 56
  1.6.8 Resistance to colistin ............................................................... 58
  1.6.9 Methods of quantifying colistin in the biological fluids ..................... 59
    1.6.9.1 Colistin sulphate ............................................................. 59
1.6.9.2 Colistin methanesulphonate sodium ........................................60
1.6.10 Toxicity colistin ........................................................................62

1.7 Nephrotoxicity of colistin sulphate and colistin methanesulphonate ........63
1.7.1 Kidney structure ........................................................................63
1.7.2 Renal function tests .....................................................................66
  1.7.2.1 Renal in vivo studies ...............................................................66
  1.7.2.2 Renal in vitro studies ...............................................................67
  1.7.2.3 Cultured renal cells (in vitro studies) ..........................................68
  1.7.2.4 Primary culture of proximal tubular cells ....................................69
  1.7.2.5 Renal cell lines ........................................................................69
1.7.3 Renal transportation of colistin ....................................................70

1.8 Aim and Objectives ........................................................................71
1.8.1 Aim ............................................................................................71
1.8.2 Objectives ..................................................................................71

2- Microbiological materials and general procedures
2.1 Materials .......................................................................................73
  2.1.1.1 Media culture ..........................................................................73
  2.1.1.2 Gelling agents ..........................................................................74
  2.1.1.3 Buffer solutions ........................................................................74
  2.1.1.4 Chemicals ................................................................................75
2.2 General procedures ..........................................................................76
  2.2.1 Microorganisms ..........................................................................76
  2.2.2 Growth and maintenance of microorganisms ..................................78
  2.2.3 Identification of microorganisms ..................................................78
  2.2.4 Preparation of bacterial suspension ..............................................81
  2.2.5 The plate spread technique ..........................................................82
  2.2.6 Calibration graph of cell numbers against optical density ...............82
  2.2.7 Validation of the viable count technique (plate spread technique) ....84
2.2.8 Production of growth profiles ...................................................... 85
2.2.9 Preparation of antibiotics and their discs ........................................ 86
   2.2.9.1 CLS solution ............................................................................. 86
   2.2.9.2 CMS solution ............................................................................ 86
   2.2.9.3 Antibiotics filter paper discs ..................................................... 87
2.2.10 Preparation of bioassay plates .................................................... 87
   2.2.10.1 Single layer bioassay plate ..................................................... 87
   2.2.10.2 Double layer bioassay plate ................................................... 87
2.2.11 Inoculating bioassay plates ....................................................... 88
   2.2.11.1 Streaking method ................................................................. 88
   2.2.11.2 Direct inoculation method ...................................................... 88
2.2.12 Using the multipoint applicator .................................................. 89
2.2.13 Recording and interpreting bioassay results ................. 89
2.2.14 Statistical analysis ............................................................... 89
2.2.15 Using the Bead beater (Biospec, UK) ........................................ 89

3- Optimizing microbiological assays of colistin sulphate and colistin methanesulphonate
3.1 Introduction .................................................................................. 92
3.2 Optimizing CMS bioassay
   3.2.1 Screening of bacteria by agar disc diffusion method ...................... 92
   3.2.2 Effect of the inoculum size on CMS bioassay ............................. 94
   3.2.3 Incorporating CMS in the media ................................................. 95
   3.2.4 Investigations into the diffusion of CMS into agar under different
       Experimental Conditions ................................................................. 97
       3.2.4.1 Incubation conditions and inoculum size .............................. 97
       3.2.4.2 Experimental design ............................................................ 99
   3.2.5 Investigation into the optimum combination of base and seed Layer .... 100
   3.2.6 Establishing optimum pre-diffusion time for CMS bioassay .......... 107
   3.2.7 CMS bioassay using different bacterial indicators ....................... 108
3.3 Applying previously optimized conditions to *Bordetella bronchiseptica* (NCTC 8344) .................................................................110

3.3.1 Agar disc diffusion method .....................................................110

3.3.2 Effect of the experimental design, incubation temperatures and pre-diffusion times on CMS bioassay ..............................................111

3.3.3 Effect of media ........................................................................112

3.3.4 Establishing the ideal inoculum density ......................................113

3.3.5 Standardization of CMS volume absorbed on filter paper disc ....114

3.4 CMS calibration curves using *Bordetella bronchiseptica* .............114

3.4.1 Calibration curve of CMS bioassay in aqueous solution ..............114

3.4.2 Calibration curve of CMS bioassay in human serum using standardised CMS filter Paper discs ......................................................116

3.5 CLS bioassay using previously optimized method........................117

3.6 Optimization of CLS bioassay .....................................................118

3.6.1 Investigation into a substitute gelling agent ..............................118

3.6.2 Investigation into phytogel as a seed layer ................................120

3.6.3 Effect of incubation temperatures and pre-diffusion times on CLS bioassay .................................................................121

3.6.4 Establishing the ideal inoculum density ....................................122

3.7 Calibration curve of CLS and CMS aqueous solutions using optimized agar and gel bioassay .................................................................................123

3.7.1 Bioassay of CLS and CMS using agar .....................................124

3.7.2 Bioassay of CLS and CMS using phytogel as seed layer ............124

3.8 Calibration curve of CLS bioassay in water ................................126

3.9 Calibration curve of CLS bioassay in human serum ......................127
4. Optimizing chemical assays of colistin sulphate and colistin methanesulphonate

4.1 Thin Layer Chromatography (TLC)

4.1.1 Introduction

4.1.2 Materials used in TLC studies

4.1.3 Samples preparation

4.1.4 TLC general procedure

4.1.5 Optimizing thin layer chromatographic conditions for CLS and CMS

4.1.5.1 Choosing a suitable visualizing agent

4.1.5.2 Choosing the most suitable mobile phase

4.1.5.3 Choosing the most suitable stationary phase

4.1.5.4 Standardization of CLS and CMS volume for TLC analysis

4.1.5.5 Effect of hot air drying on TLC plates

4.2 High Performance Liquid Chromatography

4.2.1 Introduction

4.2.2 Materials used in HPLC studies

4.2.3 Samples preparation

4.2.4 HPLC system

4.2.5 Optimizing HPLC chromatographic conditions for CMS

4.2.5.1 Investigation of literature methodologies

4.2.5.2 Concentrations of CMS

4.2.5.3 Testing sodium phosphate as a buffer in the mobile phase

4.2.5.4 Modifying sodium sulphate concentration in the mobile phase

4.2.5.5 Testing the effect of pH on the mobile phase

4.2.5.6 Effects of altering organic phase concentrations in the mobile phase

4.2.5.7 Effect of flow rate on CMS

4.2.5.8 CMS dissolved in mobile phase or deionised water
5. Studies into bacterial cell death and growth inhibition induced by colistin sulphate and colistin methanesulphonate

5.1 Introduction ............................................................................................................. 168

5.2 Determination of Minimum Inhibitory Concentrations (MICs) ......................... 168
  5.2.1 MICs using agar dilution technique ......................................................... 168
  5.2.2 MICs using broth dilution technique ...................................................... 173

5.3 Bacterial profiles ........................................................................................................ 175

5.4 Time-survivor curves studies for CLS and CMS ............................................. 176
  5.4.1 Neutralizing agent ....................................................................................... 177
    5.4.1.1 Assessment of the suitability of the neutralizer .................................. 177
  5.4.2 Determination of the time-survivor curves for CLS and CMS ............... 178

6. Microbiological and chemical degradation of colistin sulphate and colistin methanesulphonate

6.1 Introduction ............................................................................................................. 183

6.2 Detection of CLS and CMS degradation by microbiological Assay ............. 183

6.3 Detecting degradation of CLS and CMS by chemical methods .................. 190
  6.3.1 Detecting CLS and CMS degradation on TLC plates .......................... 190
  6.3.2 Detection of CMS degradation using HPLC ........................................... 197
7. Studies into the mechanism of action of colistin sulphate and colistin methanesulphonate

7.1 Studies into the effect of CLS and CMS on the permeability of the bacterial cytoplasmic membrane

7.1.1 Potassium leakage studies

7.1.1.1 Preparation of bacterial culture

7.1.1.2 Measuring potassium ion concentrations using Atomic Absorption Spectrophotometry

7.1.1.3 Determination of the intracellular potassium ion pool of Acinetobacter lwofii R46383

7.1.1.4 Determining potassium ions leakage from untreated bacterial cells

7.1.1.5 Effect of filtration system on potassium ion concentration

7.1.1.6 Effect of CLS and CMS on potassium ion concentrations

7.1.1.7 Leakage of intracellular potassium ions from Acinetobacter lwofii R46383 treated with CLS and CMS

7.1.2 Release of 260nm-absorbing materials

7.1.2.1 Introduction

7.1.2.2 Detecting the release of 260nm-absorbing materials from untreated bacterial Suspensions

7.1.2.3 Effect of CLS and CMS on the release of 260nm absorbing materials

7.1.2.4 Determination of total intracellular pool of 260nm-absorbing materials in Acinetobacter lwofii R46383

7.1.2.5 Release of 260nm-absorbing materials from Acinetobacter lwofii R46383 by CLS or CMS

7.2 Determination of adsorption isotherms

7.2.1 Preliminary investigations of adsorption isotherms determined by spectroscopy

7.2.2 Adsorption isotherms determined by spectroscopy

7.2.3 Preliminary investigations for the adsorption isotherms determined by microbiological assay

7.2.4 Adsorption isotherms determined by microbiological assay
8. Studies into the cytotoxicity of colistin sulphate and colistin methanesulphonate

8.1 Introduction

8.2 Cell culture

8.3 Cytotoxicity studies

8.3.1 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay

8.3.1.1 Protocol for MTT assay

8.3.1.2 Calibration of MTT assay

8.3.2 Lactate dehydrogenase (LDH) assay

8.3.3 Protocol for LDH assays

8.3.4 Calibration of LDH assay

8.4 Determining cytotoxicity of CLS and CMS

8.5 Determining cytotoxicity of degradation products of CLS and CMS

9. Discussion, conclusions and future work

9.1 Introduction

9.2 Optimization of microbiological assays for colistin sulphate and colistin methanesulphonate

9.2.1 Optimization of CMS bioassay

9.2.2 Optimization of CLS bioassay

9.3 Optimizing chemical assays of colistin sulphate and colistin Methanesulphonate

9.3.1 Optimizing the TLC methods

9.3.2 Optimizing the HPLC methods
9.4 Studies into bacterial cell death and growth inhibition induced by colistin sulphate and colistin methanesulphonate…………………………………………………………………………………………281

9.4.1 Determination of Minimum Inhibitory Concentrations (MICs)……………………………………281
9.4.1.1 Agar dilution method………………………………………………………………………………282
9.4.1.2 Broth dilution method………………………………………………………………………………284
9.4.2 Bacterial profiles………………………………………………………………………………………284
9.4.3 Time-survivor curves studies for CLS and CMS…………………………………………………285

9.5 Microbiological and chemical degradation of colistin sulphate and colistin methanesulphonate…………………………………………………………………………………………288

9.6 Studies into the mechanism of action of colistin sulphate and colistin methanesulphonate…………………………………………………………………………………………292
9.6.1 Intracellular potassium leakage……………………………………………………………………293
9.6.2 Release of 260nm absorbing materials…………………………………………………………295
9.6.3 Determination of adsorption isotherms…………………………………………………………297

9.7 Studies into the cytotoxicity of Colistin sulphate and Colistin Methanesulphonate………………299

9.8 Future work………………………………………………………………………………………………301

References……………………………………………………………………………………………………302

Appendix ………………………………………………………………………………………………………336
List of Figures

CHAPTER ONE
Figure 1.1: The structure of the bacterial cell..........................................................33

Figure 1.2: Gram-negative cell wall structure .........................................................35

Figure 1.3: Gram-positive cell wall structure .........................................................35

Figure 1.4: Types of adsorption isotherm ...............................................................42

Figure 1.5: Chemical structure of Polymyxin B.......................................................49

Figure 1.6: Chemical structure of CLS A and B .....................................................49

Figure 1.7: Chemical structure of CMS A and B ....................................................50

Figure 1.8: Longitudinal cross section of the kidney.............................................64

Figure 1.9: Basic structure of the nephron.............................................................65

CHAPTER TWO
Figure 2.1: The relationship between optical density at 420nm and viable cell number
for Bordetella bronchiseptica.................................................................83

Figure 2.2: The relationship between optical density at 420nm and viable cell number
for Acinetobacter lwaffii R46383.................................................................83

Figure 2.3: Typical growth profile of Bordetella bronchiseptica (NCTC 8344) 420nm
incubated in nutrient broth at 37°C.................................................................85
Figure 2.4: Typical growth profile of *Acinetobacter lwoffii* R46383 420nm incubated in nutrient broth at 37°C………………………………………………………………………86

CHAPTER THREE

Figure 3.1: Inhibition zones for CMS using different indicator bacteria…………………………109

Figure 3.2: Effect of various incubation conditions and pre-diffusion times on CMS bioassay. (Single layer of agar was used)…………………………………………………………..110

Figure 3.3: Effect of various incubation conditions and pre-diffusion times on CMS bioassay (double layer of agar was used)…………………………………………………………111

Figure 3.4: Effect of the inoculum size of *Bordetella bronchiseptica* on CMS bioassay..113

Figure 3.5: CMS in water calibration plot using *Bordetella bronchiseptica*…………………..115

Figure 3.6: CMS bioassays in human serum……………………………………………………………116

Figure 3.7: CMS and CLS bioassay using previously optimized bioassay…………………118

Figure 3.8: CLS inhibition zones under different incubation Conditions………………….121

Figure 3.9: CLS inhibition zones under different pre-diffusion time conditions………..122

Figure 3.10: Effect of the inoculum size of *Bordetella bronchiseptica* on CLS bioassay…………………………………………………………………………………………123

Figure 3:11: CLS bioassay in agar and gel……………………………………………………………125

Figure 3.12: CMS bioassay in agar and gel……………………………………………………………125
Figure 3.13: Bioassay of CLS in water........................................................................................................126

Figure 3.14: Bioassay of CLS in human serum.................................................................127

CAPTEER FOUR

Figure 4.1: RP TLC of CLS using 60% 2M NaCl: 40% Acetone + drop of phosphoric acid
..................................................................................................................................................133

Figure 4.2: NP TLC of CMS using 90% of Methanol and 10% of Acetone + drop of
phosphoric acid .................................................................135

Figure 4.3: Effect of drying methods on CLS TLC plate................................................139

Figure 4.4: Effect of drying methods on CMS TLC plate...........................................139

Figure 4.5: CMS 0.5667mmol/L at 200nm.................................................................144

Figure 4.6: CMS 0.1133mmol/L at 200nm.................................................................145

Figure 4.7: CMS 0.0227mmol/L at 200nm.................................................................146

Figure 4.8: CMS 0.5667mmol/L at 210nm.................................................................147

Figure 4.9: CMS 0.5667mmol/L at 210nm for 120 minutes.................................148

Figure 4.10: CLS 0.5667mmol/L at 200nm for 60 minutes........................................150

Figure 4.11: CMS using 10mM of sodium sulphate in mobile phase......................151

Figure 4.12: CMS in mobile phase pH3........................................................................153
Figure 4.13: CMS in mobile phase pH4………………………………………………………154
Figure 4.14: CMS in mobile phase pH5………………………………………………………155
Figure 4.15: CMS in mobile phase pH 5.5……………………………………………………..156
Figure 4.16: CMS in mobile phase pH6………………………………………………………157
Figure 4.17: CMS in mobile phase pH6.6……………………………………………………158
Figure 4.18: Effects of altering organic phase concentrations in the mobile phase………..160
Figure 4.19: CMS in mobile phase of 75% and 77% acetonitrile…………………………..161
Figure 4.20: CMS in mobile phase of 70%, 75% and 80% acetonitrile …………………..162
Figure 4.21: Effect of the Flow Rate on the CMS retention times. ………………………..163
Figure 4.22: CMS in 75% acetonitrile, flow rate of 1.5ml/min…………………………….164
Figure 4.23: CMS dissolved in the mobile phase……………………………………………165
Figure 4.24: CMS in deionised water for HPLC………………………………………………166

CHAPTER FIVE
Figure 5.1: Acinetobacter lwofii profile treated with CLS or CMS…………………………176
Figure 5.2: Survivor plot for bacteria treated with CLS………………………………………179
Figure 5.3: Bacterial survival treated with CMS………………………………………………180
CHAPTER SIX

Figure 6.1: Degradation of CLS and CMS in water and serum at -20°C over nine days
...........................................................................................................................................185

Figure 6.2: Degradation of CLS and CMS in water and serum at 4°C over nine days…..186

Figure 6.3: Degradation of CLS and CMS in water for and serum at 25°C over nine

days........................................................................................................................................188

Figure 6.4: Degradation of CLS and CMS in water and serum at 37°C over nine days....189

Figure 6.5: TLC of CLS stored at 37°C for four days.................................................192

Figure 6.6: TLC of CMS stored at -20°C.................................................................193

Figure 6.7: TLC of CMS stored at 4°C.................................................................194

Figure 6.8: TLC of CMS stored at 25°C. .................................................................195

Figure 6.9: TLC of CMS stored at 37°C.................................................................196

Figure 6.10. Fresh and stored CMS at -20°C over five days.........................198

Figure 6.11: CMS stored at 4°C over two days .................................................200

Figure 6.12: CMS stored at 4°C over five days .................................................201

Figure 6.13: AUC of CMS stored at 4°C..........................................................202

Figure 6.14: CMS stored at 25°C over 24 hours.................................................203
Figure 6.15: CMS stored at 25°C over five days ..............................204

Figure 6.16: CMS at 25°C .............................................................205

Figure 6.17: CMS stored at 37°C over five hours .............................206

Figure 6.18: CMS stored at 37°C over 12 hours .............................207

Figure 6.19: CMS stored at 37°C over four days .............................208

Figure 6.20: CMS at 37°C over three days .................................209

CHAPTER SEVEN

Figure 7.1: The relationship between potassium ion concentrations and emission ..........216

Figure 7.2: Determination of total intracellular pool of Acinetobacter lwoffii R46383 ....218

Figure 7.3: Potassium ion leakage from untreated bacterial suspension .................220

Figure 7.4: Potassium ion leakage from Acinetobacter lwoffii R46383 in the presence of increasing concentrations of CLS ........................................224

Figure 7.5: Potassium ion leakage from Acinetobacter lwoffii R46383 in the presence of increasing concentrations of CMS ............................................225

Figure 7.6: 260nm absorbing material leakage from untreated bacterial suspension ....229

Figure 7.7: Determination of total intracellular pool of 260nm-absorbing materials of Acinetobacter lwoffii R46383 ..................................................231
Figure 7.8: Determination of release of 260nm-absorbing materials from CLS treated *Acinetobacter lwoffii R46383*………………………………………………………………………………233

Figure 7.9: Determination of release of 260nm-absorbing materials from CMS treated *Acinetobacter lwoffii R46383*………………………………………………………………………………234

Figure 7.10: Percentage of residual CLS and CMS over time measured by absorption at 210nm………………………………………………………………………………238

Figure 7.11: Calibration graphs for uptake isotherm studies. (CLS aqueous solutions)….241

Figure 7.12: Determination of uptake studies (CLS aqueous solution)…………………242

Figure 7.13: Calibration graphs for uptake isotherm studies (CMS aqueous solutions)…243

Figure 7.14: Determination of uptake studies (CMS aqueous solution)…………………244

**CHAPTER EIGHT**

Figure 8.1: MTT calibration plot………………………………………………………………………………249

Figure 8.2: LDH calibration plot………………………………………………………………………………251

Figure 8.3A: LDH released from cells after exposure to a single dose of antibiotics and were incubated at STCE for two days…………………………………………………………253

Figure 8.3B: MTT assay after cells were exposed to a single dose of antibiotics and were incubated at STCE for two days…………………………………………………………254

Figure 8.4A: LDH released from cells topped up with antibiotic every 12 hours without changing media for two days at STCE…………………………………………………………255
Figure 8.4B: MTT released from cells topped up with antibiotic every 12 hours without changing media for two days at STCE ..........................256

Figure 8.5A: LDH released from cells where media containing antibiotics were changed every 24 hours at STCE for two days..........................257

Figure 8.5B: MTT released from cells where media containing antibiotics were changed every 24 hours at STCE for two days..........................258

Figure 8.6A: LDH released from cells where media containing antibiotics were changed every 12 hours at STCE or two days.........................259

Figure 8.6B: MTT released from cells where media containing antibiotics were changed every 12 hours at STCE for two days.........................260

Figure 8.7A: LDH released from cells topped up with antibiotics every 12 hours and medium was changed every 24 hours for four days at STCE.................261

Figure 8.7B: MTT released from cells topped with antibiotics every 12 hours and medium was changed every 24 hours for four days at STCE.................262

Figure 8.8A: LDH assay on cells exposed to freshly prepared antibiotics and those previously degraded at STCE for two days.................................263

Figure 8.9A: LDH assay on cells exposed to a freshly or stored antibiotics at STCE for two days. .................................................................264

Figure 8.9B: MTT assay on cells exposed to a freshly or stored antibiotics at STCE for two days.................................................................265
Figure 8.10A: LDH assay on cells exposed to a freshly or stored antibiotics at STCE for two days. .................................................................266

Figure 8.10B: MTT assay on cells exposed to a freshly or stored antibiotics at STCE for two days. .................................................................267

CHAPTER NINE

Figure 9.1: Degradation of CMS in water or serum.................................................288
List of Tables

CHAPTER ONE
Table 1.1: Factors influencing antibacterial activity .......................................................38
Table 1.2: Types and examples of adsorption isotherms ..............................................44
Table 1.3: MICs of CMS against Gram-negative bacteria ..........................................57
Table 1.4: MIC90 (mg/L) against P. aeruginosa. .........................................................80

CHAPTER TWO
Table 2.1: Isolation history of Sussex strains. .............................................................77
Table 2.2: Biochemical profile of Bordetella bronchiseptica NCTC 8344 using API 20NE ..................................................................................................................79
Table 2.3: Biochemical profile of Acinetobacter lwofii R46383 using API 20NE ..........80
Table 2.4: Bacterial counts for Bordetella bronchiseptica NCTC 8344 .......................84

CHAPTER THREE
Table 3.1: Results of CMS diffusion agar assay .........................................................94
Table 3.2: Effect of CMS on Gram-negative bacteria .................................................96
Table 3.3: Effect of different incubation conditions on CMS bioassay ......................98
Table 3.4: Effect of different incubation conditions and double layer media on CMS bioassay .................................................................100
Table 3.5.A: Base layer: ISA pH 7.4. Seed layer as shown in table
CMS made up in distilled water......................................................102

Table 3.5.B: Base layer: ISA pH 7.4. Seed layer as shown in table
CMS made up in buffer pH6......................................................102

Table 3.6.A: Base layer: DSTA agar. Seed layer as shown in table.
CMS made up in distilled water ...............................................103

Table 3.6.B: Base layer: DSTA. Seed layer as shown in table
CMS made up in buffer pH6......................................................103

Table 3.7.A: Base layer: TSA pH 7.3. Seed layer as shown in table.
CMS made up in distilled water...............................................104

Table 3.7.B: Base layer: TSA pH 7.3. Seed layer as shown in table.
CMS made up in buffer pH6......................................................104

Table 3.8.A: Base layer: Antibiotic media No.3 pH 6.5. Seed layer as shown in table
CMS made up in distilled water...............................................105

Table 3.8.B: Base layer: Antibiotic media No.3 pH 6.5. Seed layer as shown in table.
CMS made up in buffer pH6......................................................105

Table 3.9.A: Base layer: NA pH 7.4. Seed layer as shown in table
CMS made up in distilled water...............................................106

Table 3.9.B: Base layer: NA pH 7.4. Seed layer as shown in the table
CMS made up in buffer pH6......................................................106
Table 3.10: Base layer: R²A Seed layer as shown in table, CMS made up in distilled water

Table 3.11: Effect of the different pre-diffusion times on CMS bioassay.

Table 3.12: Effect of media on CMS bioassay using Bordetella bronchiseptica indicator

Table 3.13: Investigation into the substitute gelling agents

CHAPTER FOUR
Table 4.1: Mobile phases used in RP CLS TLC system

Table 4.2: Mobile phases used for CMS NP TLC system

Table 4.3: CLS spot volume in TLC

Table 4.4: CMS spot volume in TLC

CHAPTER FIVE
Table 5.1: MIC of CMS measured by an agar dilution technique, (bacterial concentrations of \(10^7\)Cfu/ml)

Table 5.2: MIC of CMS measured by an agar dilution technique (bacterial concentrations of \(10^4\)Cfu/ml)

Table 5.3: MIC of CLS measured by an agar dilution technique

Table 5.4: MIC of CLS measured by an agar and broth dilution

Table 5.5: MIC of CMS measured by an agar and broth dilution techniques
CHAPTER SIX
Table 6.1: Detection of CMS degradation at different temperatures and by different methods .................................................................210

Table 6.2: Detection of CLS degradation at different temperatures and by different methods .................................................................211

CHAPTER SEVEN
Table 7.1: Effect of CLS and CMS on potassium ion concentrations ..................222

Table 7.2: Effect of filtration on the absorbance at 260nm ...........................228

Table 7.3: Effect of CLS and CMS on 260nm absorbing materials ..................230

Table 7.4: Absorbance of different concentrations of CLS at 210 ..................236

Table 7.5: Absorbance of different concentrations of CMS at 210 ..................236

Table 7.6: Absorption of various inocula at 210nm .....................................237

Table 7.7: Comparison between filtered an unfiltered samples .....................240
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. baumannii</strong></td>
<td>Acinetobacter baumannii</td>
</tr>
<tr>
<td><strong>A. lwoffii</strong></td>
<td>Acinetobacter lwoffii</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine TriPhosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under Curve</td>
</tr>
<tr>
<td><strong>B. bronchiseptica</strong></td>
<td>Bordetella bronchiseptica</td>
</tr>
<tr>
<td>BP</td>
<td>British Pharmacopeia</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood Urea Nitrogen</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>Cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CLS</td>
<td>Colistin Sulphate</td>
</tr>
<tr>
<td>CMS</td>
<td>Colistin Methanesulphonate Sodium</td>
</tr>
<tr>
<td>Conc</td>
<td>Concentration</td>
</tr>
<tr>
<td>d</td>
<td>Day(s)</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>Dab</td>
<td>2, 4-Diaminobutyric acid</td>
</tr>
<tr>
<td>DAP</td>
<td>Diaminopimelic acid</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSTA</td>
<td>Diagnostic Sensitivity Test Agar</td>
</tr>
</tbody>
</table>
$E.\ coli$  $Escherichia\ coli$

GFR  Glomerular Filtration Rate

h  Hour(s)

HCl  Hydrochloric acid

HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC  High Performance Liquid Chromatography

ISA  Isosensitest Agar

ISB  Isosensitest Broth

IU  International Unit

KCl  Potassium Chloride

$\text{KH}_2\text{PO}_4$  Potassium dihydrogen Phosphate

LDH  Lactate Dehydrogenase

LPS  Lipopolysaccharides

MIC  Minimum Inhibitory Concentration

MTT  3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

NA  Nutrient Agar

NaCl  Sodium Chloride

NAD  Nicotinamide Adenine Dinucleotiside

Na$_2$HPO$_4$  Disodium hydrogen phosphate

NaOH  Sodium hydroxide

NB  Nutrient Broth

NCTC  National Collection of Type Culture

NP  Normal Phase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRK</td>
<td>Normal Rate Kidney</td>
</tr>
<tr>
<td>OPA</td>
<td>Orthophthalaldehyde</td>
</tr>
<tr>
<td>p</td>
<td>Probability value</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>Psi</td>
<td>Pound-force per square inch</td>
</tr>
<tr>
<td>PT</td>
<td>Proximal Tubule</td>
</tr>
<tr>
<td>PW</td>
<td>Peptone Water</td>
</tr>
<tr>
<td>RF</td>
<td>Retardation Factor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse Phase</td>
</tr>
<tr>
<td>Rpm</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>Stdev</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptone Soya Agar</td>
</tr>
<tr>
<td>TS broth</td>
<td>Tryptone Soya broth</td>
</tr>
<tr>
<td>U.V</td>
<td>Ultra Violet</td>
</tr>
</tbody>
</table>
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I would also like to express my biggest thanks to my husband Riyadh. Finishing this project is of immense relief to both of us. Thank you Riyadh for your unending patience and unfailing moral support over the last years, without which, this project would not have been possible.

Last but by no means least; I would like to express my thanks to the Ministry of Health of Oman for offering me this valuable opportunity to undertake this project. I will remain appreciative and thankful to all my colleges at the Ministry of Health for their sincere and genuine support.
Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed Nabila AL-Lawati

Dated 15/5/2012
CHAPTER ONE

Introduction
1.1 History

In the history of modern medicine, few developments have had such a profound effect on human life as the progress of the power to control the growth of microorganisms. Over 2000 years ago, treatments for infection in ancient Chinese medicine were using plants with antimicrobial properties (Lindblad, 2008). Many other ancient cultures such as the ancient Egyptians and the ancient Greeks used moulds and plants to treat infections (Forrest, 1982). Louis Pasteur observed that, "if we could intervene in the antagonism observed between some bacteria, it would offer perhaps the greatest hope for therapeutics" (Kingston, 2008).

The understanding of bacterial structure and antibiotic interaction with different cells provides important information regarding the design of antimicrobials. In addition, it can predict the capacity of combinations of antibiotics and provide clues of unwanted toxicity as well as knowledge about resistance mechanisms.

1.2 Structure of the bacterial cell

The main components (Figure 1.1) of the bacterial cell can be divided into three groups: the cell wall, the cytoplasmic membrane and the cytoplasm (Madigan et al, 2002). These components of bacterial cells are discussed below.

Figure 1.1: The structure of the bacterial cell

1.2.1 The cell wall

The cell wall resides outside the cytoplasmic membrane. Its primary function is to maintain cell integrity by withstanding the internal osmotic pressure. It is also responsible for the maintenance of cell shape and is involved in the cell division process (Beveridge and Graham, 1991). If the cell wall is injured due to the effect of antibiotics such as the beta lactams or the polymyxin group, the permeability of the cell wall will alter causing cell death (Hancock and Chapple, 1999; Zhang et al, 2000).

The cell wall contains peptidoglycan which is made up of N-acetylglucosamine, N-acetylmuramic acid (these are sugar derivatives) and amino acids including L-alanine, D-alanine, D-glutamic acid and either lysine or diaminopimelic acid (DAP). The structure of peptidoglycan is a thin sheet in which the glycan chain formed by the sugars is connected by cross-links of amino acids. The glucosidic bonds which connect the sugars in the glucan chain are strong, but need to be in groups to provide rigidity. The full strength of peptidoglycan structure is realized only when these chains are cross linked with amino acids (Rogers et al, 1980).

Bacteria can be divided into two major classes, Gram-negative (Figure 1.2) and Gram-positive (Figure 1.3) bacteria, based on the physical properties of their cell walls and their ability to retain the dye crystal violet when washed by ethanol (decolourization). Bacteria that retain the crystal violet and stain purple are identified as Gram-positive bacteria, while these that lose it and stain pink (or red) with safranin are identified as Gram-negative bacteria (Beveridge, 2001).

Gram-negative bacterial walls are composed of a thin peptidoglycan layer and an overlying lipid-protein bilayer (outer membrane) containing lipopolysaccharide, while the Gram-positive cell wall is composed of a thicker layer of peptidoglycan interspersed with molecules of teichoic and lipoteichoic acids (Beveridge, 2001).
Figure 1.2: Gram-negative cell wall structure

(Taken from http://invsee.asu.edu).

Figure 1.3: Gram-positive cell wall structure

(Taken from http://invsee.asu.edu/invsee/invsee.htm).
Antibiotics such as peptide antibiotics including colistin produce their antibacterial action by interfering with the bacterial cell wall by interacting with the protein and phospholipid layers available in the outer membrane of the bacterial cell (Hancock and Chapple, 1999; Zhang et al, 2000).

The outer membrane of Gram-negative bacteria is relatively permeable to small molecules such as hydrophobic molecules but not to larger molecule such as enzymes. It also prevents certain enzymes, which are present outside the cytoplasmic membrane from diffusing away from the cells. These enzymes are present in an area called the periplasm, which is an important physiological feature of Gram-negative bacteria. It is gel-like in consistency, due to the abundance of periplasmic enzymes found there and because this area is filled with a loose network of peptidoglycan. The periplasm of Gram-negative bacteria contains different types of enzymes (Paustian and Roberts, 2010).

The outer cell wall of Gram-negative bacteria is composed of more complex layers compared to Gram-positive bacteria and offers an intricate barrier system to antibiotics, regulating and sometimes preventing their entry to the cell and hence to their target sites (Madigan and Martinko, 2005).

**1.2.2 The cytoplasmic membrane**

Both Gram-positive and Gram-negative bacteria have a cytoplasmic membrane which resides inside the cell wall and consists of phospholipids (20-30%) and (50-70%) proteins. Phospholipids are made up of a charged polar head and two uncharged non-polar tails, which in aqueous solution adopt a bilayer formation. In this configuration the polar portion of the molecule faces the outside aqueous environment and the non-polar portion faces inwards. Proteins are able to move around in the phospholipids and this is known as the fluid mosaic model (Singer and Nicolson, 1972). Major proteins in the cytoplasmic membrane have hydrophobic external surfaces in the region that spans the membrane and have hydrophilic surfaces exposed on both the inside and outside of the cell. The cytoplasmic membrane is stabilized by hydrogen bonds and hydrophobic interactions. Ions such as magnesium and calcium also help to stabilise the membrane by combining ionically with the negative charge of the phospholipid.
Materials such as sugars, amino acids, vitamins, proteins, nucleic acids and other soluble materials involved in catabolic and anabolic reactions, all occur inside the cell. The hydrophobic nature of the cytoplasmic membrane endows it with selective permeability characteristics, allowing passage of small hydrophobic molecules and the specific transport of hydrophilic and charged molecules. Water is able to penetrate between the phospholipid molecules and the presence of aquaporins can raise the speed of water transport (Alberts et al, 2002; Paustian and Roberts, 2010).

Transport systems can be used for nutrient uptake, waste excretion, and protein export. Membranes are the site of much enzymatic activity and are essential for the life of the bacterium. Membranes contain components of the respiratory chain including cytochromes, flavins and ubiquinones and several enzymes feeding reducing equivalents into it in addition to the associated ATPase on the inner surface of the membrane (Thony - Meyer, 1997).

### 1.2.3 The cytoplasm

The cytoplasm is an aqueous fluid containing the genetic material of the cell. The bacterial cytoplasm lacks the equivalent of the endoplasmic reticulum in eukaryotic cells, but has high density due to the presence of ribosomes which are the site of protein synthesis.

The bacterial chromosome is a singular circular molecule of DNA. It resides in the cytoplasm in a highly condensed state without any nuclear membrane envelope. The bacterial cytoplasm also contains other materials such as extra chromosomal elements which may be present as episomes or plasmids. Episomes are fragments of the main chromosome that have become detached, whilst plasmids are small pieces of DNA which may carry a limited amount of genetic information. Plasmids are often associated with conferring resistance against antimicrobial agents or enhancing overall virulence (Madigan et al, 2002).
1.3 Factors affecting antimicrobial activity

The rate and extent of antimicrobial action are influenced by many factors (intrinsic and extrinsic to the organism). Intrinsic factors relate to the nature, structure and condition of the microorganisms and their ability to resist the action of the antibiotic or biocide of interest. Extrinsic factors influencing antibiotic action relate to the external environment in which the antibiotic is present (e.g. concentration of antibiotic, pH and temperature). These can all significantly influence the action of an antibiotic. The factors which influence antimicrobial activity are summarised in Table 1.1.

Table 1.1: Factors influencing antibacterial activity

<table>
<thead>
<tr>
<th>Factor</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>The activity of antibiotics increases with concentration; however the relationship is not necessarily linear and varies between antibiotics.</td>
</tr>
</tbody>
</table>
| pH                  | The activity of many antibacterial agents are influenced by pH for the following reasons:  
                         1- An effect on the molecule: e.g. changes in pH may alter the dissociation of the molecule, the undissociated species making the greatest contribution to killing.  
                         2- An alteration in bacterial cell surface charge: a change in pH may alter the surface charge on the bacterium and hence influence the binding of the antibiotic. |
| Temperature         | The antimicrobial activity of most chemicals increases with temperature coefficient. |
| Time of exposure    | The death of a microorganism exposed to an antimicrobial takes place over period of time depending on the nature of the antibiotic and its concentration. |
| Type of organism    | The variation in response of various bacteria to antibiotics is a sign of the nature of the organism of interest. |
1.3.1 Concentration
The rate of kill of a bacterial population may depend on the concentration of the antimicrobial agent. Slight changes in the concentration of certain agents can significantly increase or decrease their bactericidal effects.

The relationship between concentration and activity is an exponential one and is called the concentration exponent or dilution coefficient denoted by the Greek letter $\eta$. The relevance of $\eta$ is that it gives direct information as to the effect of dilution upon the speed with which an antibiotic can kill (Watson, 1908). Antibacterial agents with high $\eta$ values lose their activity rapidly on dilution, while, those with low $\eta$ values retain much of their original activity.

1.3.2 pH
Raising or lowering the pH of the environment may affect the activity and stability of antimicrobial agents as well as the rate of growth of bacterial cells and the physiochemical state of their surfaces (Sjostrom and Larsson, 1996). A pH of 6-8 is optimal for the growth of the most bacteria and their growth will be affected negatively on either side of this range.

The activity of many antibiotics is determined by their ability to cross the lipid membranes of the cell and if they are weak acids or weak bases the pH of the external environment will profoundly influence their lipid solubility. These molecules will be more active in their un-ionised form and so weak acids will have increased activity as low pH values and weak bases will be more active at higher pH values. As the external pH increases the cell surface will become more negatively charged. Cationic agents which react with negatively charged groups, will be more effective at increased pH, whilst anionic agents will be repelled (Madigan et al, 2002).
1.3.3 Temperature
The effect of temperature is more marked with some antimicrobial agents than others, and is complicated by the temperature dependency of the organism in question. This can be expressed in mathematical terms by determining the times ($t_1$ and $t_2$) to kill a suspension of microorganisms at temperature $T_1$ and $T_2$ respectively, from which the temperature coefficient $\theta$ can be calculated using the following equation (Denyer and Wallhaeusser, 1990):

$$\theta^{T_2-T_1} = \frac{t_1}{t_2}$$

$\theta$ refers to the effect of temperature per 1 °C rise, and usually has a value between 1 and 1.5 as a consequence of the apparent geometrical relationship between temperature and activity (Denyer and Wallhaeusser, 1990). It is more usual to use to $\theta^{10}$ value which refers to the change in activity over a 10°C rise in temperature.

1.3.4 Type and number of microorganisms
The activity of an antimicrobial agent depends on the type of microorganism (i.e. Gram-positive or Gram-negative bacteria) and also on the number of organisms present, and their intrinsic resistance. If a high number of microorganisms are present, higher concentrations of the antimicrobial or more prolonged exposure time will be required to kill (Johnston et al, 2000).

1.4 Interactions between the bacterial cells and antibiotics
In order to display an antibacterial action the target agent must reach an effective concentration at its biochemical sites of action. Factors affecting antibacterial chemistry and microbial physiology will affect the antibacterial passage and its interactions with its target sites. Antibacterial agents must be able to penetrate bacterial cells and overcome these barriers in order to display their effects (Russell, 1991).
1.4.1 Nature of the antimicrobial effect

Antibiotics produce both bacteriostatic and bactericidal effects, although the mechanism of action responsible for each may be different. It is considered that bacteriostatic effects arise from metabolic injury, and can be reversed after removal or neutralisation of the antibiotic, while bactericidal injury is irreversible even after removal or neutralization of the antibiotic, and is thought to arise from irreparable damage to a vital cellular structure or function (Denyer and Wallhaeusser, 1990).

The interaction of an antibacterial agent and a bacterium takes place in several stages:
- Uptake of the agent by the bacterial cells
- Partition/passage of the agent to its target
- Concentration of the agent at the target site
- Damage to the target

1.4.2 Antibiotic interactions with Gram-negative and Gram-positive bacteria

There are different types of permeability/target constraints between both Gram-negative and Gram-positive bacteria, due to their different structures.

The walls of Gram-positive bacteria are composed of peptidoglycan and teichoic acid while the outer layer of Gram-negative bacteria is more complex and offers an intricate barrier to the antibiotics (Madigan and Martinko, 2005). Thus there is increasing concern over the emergence of antibiotic-resistant Gram-negative bacteria, in particular \textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa}) and \textit{Acinetobacter baumannii} (\textit{A. baumannii}) (Li and Nation, 2006).

1.4.3 Interaction with the whole cell

The uptake of antimicrobial agents can involve one or more of processes, such as: adsorption, absorption, chemisorption, partition or diffusion (Hugo and Russell, 1992). The absolute amount adsorbed will depend upon factors such as the concentration of the drug, time of exposure, the pH of the solution and the number of organisms present.
Uptake isotherms are important parameters to understand the mode of action of antibiotics, and provide information on the availability of target sites on the cells. At specific exposure time points, uptake isotherms record the amount of the antibiotic bound to the cells as a function of the residual level of antibiotic. Results from uptake studies are expressed as adsorption isotherms. The values of equilibrium concentrations are plotted on the horizontal axis against the amount of antibiotic absorbed to the cells.

A classification system for the uptake isotherms was described and developed by Giles and co-workers (Giles et al., 1960 and 1974) who studied the mechanism of solute adsorption onto solid surfaces. Isotherms were divided into four main classes (Figure 1.4) according the initial slope as described below.

![Figure 1.4: Types of adsorption isotherm](image)

**Figure 1.4: Types of adsorption isotherm** (as proposed by Giles *et al.*, 1960 and 1974)

A = S-shaped; B = L-shaped; C = H-shaped and D = C-shaped.
1.4.3.1 S (co-operative sorption) pattern
The "S" pattern is obtained when the solute molecule is mono-functional and has a large hydrophobic residue (C > 5). The molecule has moderate intermolecular attraction, which will make it to orientate vertically in the adsorbed layer and meets strong competition for substrate sites from molecules of the solvent or by another adsorbed species.

1.4.3.2 L (langmuir) pattern
In the ‘‘L’’ pattern, as more sites are filled it becomes increasingly hard for bombarding solute molecules to find an empty position. The adsorbed solute molecule is either not vertically orientated or there is strong competition from the solvent. If the molecules from the solvent are adsorbed vertically, then there is a strong intermolecular attraction between the adsorbed molecules.

1.4.3.3 H (high affinity) pattern
The H-curve is a special case of the L-curve, in which the solute has such high affinity for the adsorbent that in dilute solutions it is taken up completely, so that the initial part of the curve is vertical.

1.4.3.4 C (constant partition) pattern
The "C" pattern is found when there is a constant partition of the solute between solution and substrate, right up to the point of maximum adsorption, where an abrupt change to a horizontal plateau is observed. One of the main conditions favouring the C-curve is that the solute has more penetrating power than the solvent and can therefore penetrate porous substrates.

An additional pattern not shown above is the Z type pattern which is described as a complex isotherm. It has very seldom been observed and was first recorded by Giles & Tolia (1974) when studying the uptake of p-nitrophenol from organic solvents by dry cellulose fibres.
In the "Z" pattern a sharp break in the isotherm was observed associated thereafter by an increased uptake. Leakage studies and electron microscopy revealed correlations between the concentration of drug initiating the inflection and causing marked damage to the cell envelope thus increasing the surface area for adsorption.

The adsorption isotherms, their characteristics and agents found to produce them are listed in Table 1.2.

**Table 1.2: Types and examples of adsorption isotherms.**

<table>
<thead>
<tr>
<th>Adsorption isotherm pattern</th>
<th>Description of adsorption process</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-shaped (co-operative sorption)</td>
<td>Slope increases at first with concentration, as in co-operative adsorption, sites capable of retaining an antibiotic molecule increase. Eventually the slope decreases.</td>
</tr>
<tr>
<td>L-shaped (langmuirian)</td>
<td>Slope falls with rise in concentration of antibiotic, as vacant sites become harder to find with progressive coverage of the surface.</td>
</tr>
<tr>
<td>H-shaped (high affinity)</td>
<td>Absorbate-substrate affinity is especially high; antibiotic is most completely adsorbed.</td>
</tr>
<tr>
<td>C-shaped (constant partition)</td>
<td>Antibiotics penetrate more rapidly into adsorbate than does the solvent; surface available for adsorption expands proportionally with amount of solute adsorbed.</td>
</tr>
<tr>
<td>Z-shaped (enhanced uptake)</td>
<td>Sharp inflection in isotherm is followed by an increase in uptake.</td>
</tr>
</tbody>
</table>
1.4.4. Lysis

True lysis in bacteria is seen when the cell wall is dissolved (wholly or partially). The cytoplasmic membrane will then rupture in a hypotonic environment due to internal solute pressure. Whole cell lysis is considered to be caused by gross, irreparable, damage to the cell wall and cytoplasmic membrane (Fastrez, 1996).

1.5 Interaction with the cytoplasmic membrane

The adsorption of antibacterial agents to the bacterial cell is to serve the purpose of directing the drug to its active site rather than being the fatal event itself. The cytoplasmic membrane is a vital structural and functional component of the bacterial cell. It has a large surface area and is near to the external aqueous environment, increasing the chance of successful antibacterial agent interaction. Cationic antimicrobial peptides such as polymyxins kill Gram-negative bacteria by activating the initial interaction with the Lipopolysaccharides (LPS) negatively charged surface molecule. This results in self-promoted uptake through the outer membrane, stimulating the interaction with the negatively charged cytoplasmic membrane of bacteria. This interaction leads to permeability changes and leakage of intracellular material (Wu et al, 1999).

1.5.1 Leakage of cellular constituents

1.5.1.1 Potassium leakage studies

The leakage of intracellular materials such as potassium ions into the surrounding environment has long been recognised as an indicator of minor membrane damage. The rate and levels of leakage provide an insight into the level of damage inflicted at the membrane which may permit the recovery or lead to cell death (Lambert and Hammond, 1973). Damage to the bacterial cytoplasmic membrane can be detected by leakage of a variety of intracellular species such as potassium ions, pentose sugar and 260nm-absorbing materials. Due to the small size of potassium ions (K⁺), it is the most sensitive indicator of cytoplasmic membrane damage, and therefore it is an effective detection tool for alterations to the cell membrane permeability.
Potassium leakage through the membrane and cell growth inhibition has led to the belief that for some membrane active compounds, potassium leakage is an effective marker for inhibitory activity (Miynarcík et al, 1992).

1.5.1.2 Release of 260nm absorbing materials

Potassium leakage is an indication of subtle cell membrane damage and is the first marker to exit the cell (Kroll and Anagnostopoulos, 1981). Release of other cell constituents such as pentose, purines, pyrimidines and even nucleotides is suggestive of more substantial bacterial membrane damage. Pentose is a substrate used for metabolism and macromolecular synthesis. It may exist within the cell in free from or as pentose-containing material. It is possible to correlate pentose and 260nm-absorbing material leakage due to their similar leakage patterns: ribose sugars can be detected in nucleosides and nucleotides, which absorb at 260nm. Purines and pyrimidines are essential for RNA/DNA synthesis and absorb at 260nm. The 260nm-absorbing materials detected upon leakage may be materials such as purines and pyrimidines or larger materials such as nucleosides and nucleotides. Longer antibiotic exposure times may cause further breakdown of intracellular materials, which are marker of autolysis which is an intracellular event where the bacterial cell enters a self-destructive state and becomes committed to death (Hugo and Bloomfield, 1971).

1.5.2 Inhibition of cytoplasmic membrane–bound enzymes

The effect of an antimicrobial agent on the cytoplasmic membrane may be inferred from its effect on the activity of membrane–bound enzymes (Gilbert et al, 1977). The cytoplasmic membrane contains a range of proteins many of which are enzymes, for example: those associated with the electron transport chain.

1.5.3 Interactions with the cytoplasm

General coagulation (irreversible) of cytoplasmic constituents is a severe lesion observed at drug concentrations much higher than those producing general cell lysis or leakage (Hugo, 1992).
1.6 Introduction to colistin

Colistin is one of the polymyxin antibiotics, isolated from *Bacillus polymyxa* subsp. *Colistinus*. Polymyxins were discovered in 1947 (Ainsworth *et al.*, 1947; Benedict and Langlykke, 1947) and colistin was first reported in 1950 by Koyama and co-workers (Koyama *et al.*, 1950).

Colistin in the form of colistin sulphate (CLS) was introduced as a therapeutic agent for the treatment of Gram negative infections in 1950s; unfortunately this form of colistin exhibited serious side effects such as nephrotoxicity and neurotoxicity which limited its use (Kunin and Boggy, 1971; Beringer, 2001). In 1961, colistin methanesulphonate sodium (CMS) was formulated which was found to have reduced toxicity (Barnett *et al.*, 1964; Hancock and Chapple, 1999). After being used for a few years however, the need for the drug diminished as less toxic antibiotics were introduced (Evans *et al.*, 1999).

Since CLS was brought into clinical use in the 1950s before contemporary drug development procedures had been devised, it was never subjected to those regulatory processes required for medicines today (Li *et al.*, 2006a).

In recent years however a number of important Gram-negative pathogens have developed resistance to most of the current commercially available antibiotics (Travis, 1994; Levy, 2002). For example, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are multidrug resistant, Gram-negative bacteria which cause nosocomial infections (Andreas and Raoult, 2002) particularly in burn patients (Trottier *et al.*, 2007). Since there have been no new antibiotics developed for Gram-negative bacterial infections in recent years (Livermore, 2003 and 2004; Payne *et al.*, 2007; Falagas *et al.*, 2008), clinicians and researchers have been reappraising the clinical worth of colistin (Li *et al.*, 2006a).

However, CMS still remains a toxic drug although some early clinical reports of severe nephrotoxicity and neurotoxicity associated with its use are likely to have occurred due to higher than recommended doses and inappropriate monitoring (Li *et al.*, 2005b).
1.6.1 Colistin chemistry

Colistin is white powder whose salts are soluble in water (Schwartz et al, 1959) and it is amphipathic, exhibiting both hydrophobic (due to the fatty acid moiety) and basic properties (due to the 5 unmasked amino groups). It is a multi-component cationic polypeptide antibiotic consisting of at least 30 of very closely related decapeptides (Orwa et al, 2000 and 2001; Govaerts et al, 2003), comprising at least 85% colistin A (polymyxin E₁) and colistin B (Polymyxin E₂) (Gobin et al, 2010).

Colistin minor components include Polymyxin E₃ and E₄ (Thomas et al, 1980), norvaline-polymyxin E₁, valine polymyxin E₂, valine polymyxin E₁, isoleucin-polymyxin E₁, isoleucin-polymyxin E₂ (Ikai et al, 1998b), polymyxin E₇ and isoleucine polymyxin E₈ (Orwa et al, 2001). Colistin has a molecular weight about 1200 Dalton (Da) (Decolin et al, 1997). It is a polycationic peptide ring containing ten amino acids with a high content of the 2, 4-diaminobutyric acid (Dab) residue. It also has a fatty acid side chain attached to the peptide ring (Evans et al, 1999). Colistin A (Polymyxin E₁) and colistin B (Polymyxin E₂) contain the same amino acids but different fatty acids (6-methyloctanoic acid and 6-methylheptanoic acid respectively) (Orwa et al, 2000). The only difference between the structure of polymyxin B (Figure 1.5) and colistin (Figure 1.6) are in the amino acid components; D-phenylalanine replaces leucine in polymyxin B (Kwa et al, 2007).

CMS (Figure 1.7) is formulated by the use of a sulphotmethylation technique where free amino groups are treated with formaldehyde followed by sodium bisulphite to give the sulphotmethyl derivative. Sulphotmethylation of CLS has been shown to minimize side affects without destroying antibacterial activity (Beveridge and Martin, 1966).
Figure 1.5: Chemical structure of Polymyxin B (Kwa et al, 2007)

Figure 1.6: Chemical structure of CLS A and B (Kwa et al, 2007).
Thr: Threonine; Leu: Leucine. Dab: α, γ- Diaminobutyric acid. α, and γ indicate the respective- NH involved in the peptide linkage.
Colistin A, fatty acid is 6-methyloctanoic acid
Colistin B, fatty acid is 6-methylheptanoic acid
1.6.2 Colistin mode of action

Generally polymyxins are bacteriostatic at low concentrations and bactericidal at high concentrations with a narrow spectrum of antibacterial activity against Gram-negative bacteria (Li et al., 2005b). Most studies into mechanisms of action have been carried out on polymyxin B, which is considered as a model for polymyxins in general and as colistin base has a similar structure to polymyxin B it is believed that it has an identical mechanism of action (Storm et al., 1977).

Polymyxins interfere with the structure and function of the bacterial cell wall and both colistin and polymyxin B have potent antiendotoxin activity (Gudmendsson and Craig, 1986; Rogers and Cohen, 1986; Porro, 1994; Jerala and Porro, 2004; Kwa et al., 2007) by binding to lipid A and neutralising many of the biological effects of the endotoxin (Corrigan and Bell, 1971; Corrigan et al., 1974). They interact with the protein and phospholipid layers of the outer membrane, causing it to increase permeability of the cell envelope which consists of cell wall and cytoplasmic membrane (Newton, 1956; Zhang et al., 2000), so affecting the osmotic integrity of the cell wall. As a result, intracellular
constituents, such as nucleic acids and protein, leak out causing cell death (Koike et al., 1969; Lorian, 1971; David and Rastogi, 1985; Fekety, 1990; Hancock and Chapple, 1999).

Colistin acts on the bacterial cell wall and bacterial cytoplasmic membrane (Koike et al., 1969; Hancock and Chapple, 1999; Zhang et al., 2000). Initial binding to the outer membrane takes place when the cationic side of colistin displaces divalent cations (like calcium and magnesium that normally stabilize lipopolysaccharides molecules in the bacterial outer membrane) (Leive, 1974; Schindler and Osborn, 1979) from the negatively charged phosphate groups on the membrane lipids, and by binding to the outer membrane polysaccharides (Newton, 1953). Additional complexing with lipopolysaccharides is facilitated by hydrophobic interactions between the lipid A portion of lipopolysaccharides and the fatty acids of colistin (Morrison and Jacobs, 1976).

### 1.6.3 Hydrolysis of colistin

CLS in the dry state is stable at room temperatures for up to 12 months (Ninger and Schwartz, 1962), while it is less stable in water at pHs above 6 (Orwa et al., 2002). CMS is not stable in vitro (Li et al., 2003b) or in vivo (Li et al., 2003a) and has the potential to hydrolyze in aqueous solution to produce a complex mixture of colistin sulphomethylated derivatives as well as colistin base. These products have considerably increased antimicrobial activity (Beveridge and Martin, 1966; Li et al., 2001a).

Recent studies have revealed that CMS is an inactive prodrug of colistin base (Bergen et al., 2006). Using HPLC methods it has been demonstrated that the conversion of CMS to partially sulphomethylated derivatives of colistin occurs in biological fluids, while the formation of colistin base from CMS in-vivo has been demonstrated in patients (Li et al., 2002 and 2003b) and rats (Li et al., 2004). This suggests that separate standards would be necessary for these different entities (Lightbown et al., 1973).

The international unit for CMS was defined as “the activity contained in 0.00007874mg of the International Reference Preparation of colistin Methane Sulfonate”. These authors went on to state: “Colistin methane sulfonate is perhaps the most complex antibiotic mixture for which a biological standard has had to be established” (Lightbown et al., 1973).
1.6.4 Clinical uses of colistin

Polymyxin B is only formulated as polymyxin B sulphate which is administered intravenously, intramuscularly, intrathecally, by inhalation or by topical routes (Kwa et al, 2007; Dhar et al, 2010). However, colistin is available in two forms for clinical use: CLS for oral use as tablets or syrup for bowel decontamination and topical for treating skin infections (Giamarellou, 2006) while CMS is used for parenteral routes. This results in significant absorption (Bergan and Fuglesang, 1982; Jensen et al, 1987; Fekety, 1990) and CMS was formulated to give reduced toxicity (Bergan and Fuglesang, 1982). Both forms of colistin can be delivered by inhalation (Li et al, 2006a).

Current data support the use of CMS for the treatment of *A. baumannii* and *P. aeruginosa* infections (Geles et al, 2001; Pitt et al, 2003) which is a major cause for concern in patients with burns, cystic fibrosis (CF) and in those who are immunocompromised (Beringer, 2001). *P. aeruginosa* is reported from national nosocomial infection surveillance studies to be ranked as the second most commonly isolated Gram-negative pathogen (Tummler and Kiewitz, 1999). Colistin is sometimes the only available active antibiotic against most Gram-negative bacteria (Livermore, 2002, 2003 and 2004; Li et al, 2004 and 2005a; Falagas and Kasiakou, 2005; Falagas et al, 2005a; Mukhopadhyay et al, 2008; Healy et al, 2011) such as *A. baumannii* (Villalon et al, 2010). Colistin has the advantage of rapid bactericidal activity against Gram-negative bacilli and only slowly leads to the development of resistance (Conway et al, 2000; Medina et al, 2011).

There have been a substantial number of clinical reports on the successful use of colistin in neonates (Jajoo et al, 2011), children (Celebi et al, 2009; Rosanova et al, 2009) and adults (Jimenez et al, 2000; Beringer, 2001; Reed et al, 2001; Garnacho et al, 2003; Linden et al, 2003; Markou et al, 2003; Al-aloul et al, 2005; Falagas et al, 2005a; Michalopoulos et al, 2005a and 2005b; Reina et al, 2005) against infections caused by multidrug-resistant *P. aeruginosa* (Maeda et al, 2008), *A. baumannii* (Tan et al, 2007; Giamarellou, 2010) and *Klebsiella pneumoniae* (Lim et al, 2010; Sader et al, 2010).
Numerous reports have been published on the efficacy of intravenous CMS in patients with or without cystic fibrosis (CF) (Falagas et al., 2005b; Kasiakou et al., 2005a and b). CMS has been successfully used intrathecally and/or intraventricularly as direct administration to the infection site has been suggested to be less problematic (Berlana et al., 2005; Bukhary et al., 2005; Gump and Walsh, 2005; Quinn et al., 2005; Baiocchi et al., 2010).

Several studies have reported the use of CLS or CMS by inhalation in patients with CF (Jensen et al., 1987; Frederiksen et al., 1997; Marquette et al., 2004; Westerman et al., 2004; Dhar et al., 2010). Inhalation of CMS reduced the number of lower respiratory samples containing *P. aeruginosa* in patients with CF (Littlewood et al., 1985; Conway, 2005), while side effects only comprised chest tightness and bronchospasm (Dodd et al., 1997; Alothman et al., 2005) however, with high doses of aerosol colistin therapy, hypersensitivity pneumonitis may occur (Leong et al., 2010).

A recent study revealed that combining the aerosolized colistin with intravenous colistin did not provide any therapeutic advantage over intravenous colistin alone in patients with multidrug-resistant ventilator-associated pneumonia due to Gram-negative bacteria (Kofteridis et al., 2010; Rattanaumpawan et al., 2010).

### 1.6.5 Dosage

Pharmacokinetic information is important to set accurate clinical dose regimens (Li et al., 2005b) and there are no standard dosing regimens for clinically ill patients (Plachouras et al., 2009) needing haemodialysis or continuous renal replacement therapy (Imberti et al., 2010).

There are many commercial brands of CMS on the market. The two most commonly available CMS formulations are Colomycin manufactured by Dumex-Alpharma A/S, Denmark and distributed by Forest laboratories, UK.

The other product is Coly-Mycin M manufactured by Parkedale Pharmaceuticals, USA and distributed by Monarch Pharmaceuticals, USA. Both are parenteral formulations containing CMS as dry powder for reconstitution before administration (Li et al., 2006a).
Colomycin (UK) is labelled as international units (IU); it is labelled as 500000 IU, 1 million and 2 million IU per vial and since there are about 12500 units per milligram of CMS (Sweetman et al, 2005); there are about 40, 80 and 160mg respectively of CMS in each vial. The advised dose for Colomycin for a patient over 60kg and with normal renal function is 1-2 million IU three times daily (240-480mg per day) (Li et al, 2006a; British National Formulary, 2010).

The other commercially available product, Coly-Mycin M (USA) is labelled as "150mg colistin base activity per vial", but Li et al (2006a) have stated that the mass of CMS dry powder per vial is actually 400mg which is equivalent to 5 million IU. The recommended dose is 2.5-5 mg/kg colistin base activity per day in divided doses. Li et al (2006a) have calculated this to be equivalent to 6.67-13.3mg/kg of CMS per day and on this basis have claimed that for a 60kg patient with normal renal function the dose is almost double (400-800mg/day) that for Colomycin. However, when the calculation is performed on the basis of the molecular weights of colistin and colistin sulphomethate the recommended dose of 2.5-5mg/kg colistin base is found to be equivalent to 3.65 -7.29mg/kg CMS. This brings the values (220-440 mg/day for 60 Kg patient) to the same as that for Colomycin.

The discrepancy between the doses reported by Li et al (2006a) seems to have been neglected in most published work and often the brand of colistin used in the studies is not specified. The optimal way to avoid confusion of CMS dosing is to use the IU of the drug (Falagas and Kasiakou, 2006b).
1.6.6 Colistin pharmacokinetics

Generally there is a lack of human studies on colistin pharmacokinetics and pharmacodynamics (Nation and Li, 2007; Michalopoulos et al., 2010), most of the colistin kinetics data have been generated from the microbiological assays of biological fluids (Mackay and Kaye, 1964; Froman et al., 1970) but these assays lack sensitivity, accuracy and are unable to quantify CMS and colistin base separately. Two pharmacokinetics studies have been conducted to determine concentrations in the plasma following doses of CMS using specialised methods such as HPLC (Brun et al., 2000; Li et al., 2003a) but still most data are collected from microbiological assays.

Colistin (CLS and CMS) is poorly absorbed from gastrointestinal tract, mucosal surfaces, inflamed surfaces or burns. Information on the volume of distribution of CMS is inadequate. Recently a value of 0.09 ± 0.02 L/kg in CF patients was reported using an HPLC technique (Reed et al., 2001). Another study also using HPLC (Li et al., 2002) found the volume of distribution of CMS in CF patients to be 0.34 ± 0.10 L/kg after an intravenous dose (1.63–3.11 mg/kg every 8 h) at steady state (Li et al., 2003a).

Following the intravenous administration of CMS (1.63–3.11 mg/kg every 8 h) to CF patients at steady state, the plasma concentration at one hour was between 2.6 and 9.8 mg/L, while those at 6 h were between 0.36 and 2.5 mg/L; substantial colistin (base) was also measurable in all of the collected samples, with concentrations ranging from 1.0 to 3.1 mg/L at 1 h and from 0.23 to 1.7 mg/L at 6 h. The $t_{1/2}$ of CLS and CMS were 251 ± 79 min and 124 ± 52 min respectively (Li et al., 2003a).

Profiles for the plasma concentrations of CMS and colistin (base) versus time after an intravenous bolus in rats show that there was substantial colistin within 5 minutes after administration of CMS. Compared with the in vitro stability of CMS (Li et al., 2003b), these results suggest that there are different mechanisms in blood/plasma-mediated hydrolysis leading to the rapid in vivo formation of colistin (base) (Li et al., 2003a and 2004).
The differences in chemistry between the two forms of colistin also translate into differences in pharmacokinetics and pharmacodynamics. CMS is eliminated by the kidney and the urinary excretion involves renal tubular secretion, while CLS is eliminated by the non-renal route because the compound (at least a portion) undergoes very extensive renal tubular re-absorption (Li et al, 2003c and 2004). CMS is excreted by the renal route but a portion is hydrolysed to partially sulphomethylated derivatives of colistin or colistin base (Li et al, 2004). The colistin is thus cleared by non renal mechanisms in processes that are not yet fully characterised. In those patients suffering from renal impairment CMS elimination by the kidneys will be decreased and a greater amount of CMS will be converted to colistin. Therefore, the dose of CMS in renal impaired patients must be reduced. CMS is excreted in the urine, high levels being reached within 30 minutes of intramuscular injection (Boger and Gavin, 1961; Barnett et al, 1964).

1.6.7 Colistin pharmacodynamics

Most information on CLS or CMS pharmacodynamics is on in-vitro studies (Barnett et al, 1964; Eickhoff and Finland, 1965; Tan and Schilling, 2005). In-vitro kill time studies with CLS or polymyxin B showed concentration dependent killing against P. aeruginosa (Tan and Schilling, 2005) and A. baumannii (Li et al, 2005b). Colistin is active in-vitro against many Gram-negative bacteria, while it is not active against Gram-positive bacteria and most fungi (Schwartz et al, 1959).

The in-vitro activity (Minimum Inhibitory Concentrations - MICs) of CMS against Gram-negative bacteria measured by the agar dilution technique is shown below (Catchpole et al, 1997). Against Acinetobacter (23 strains), E. coli (50 strains) and P. aeruginosa (94 strains), CMS displayed acceptable activity (breakpoint of 4mg/L), while Serratia strains were resistant to CMS (Table 1.3).
Table 1.3: MICs of CMS against Gram-negative bacteria (Catchpole et al, 1997).

<table>
<thead>
<tr>
<th>Organism (no of strains)</th>
<th>MIC$_{50}$ mg/L</th>
<th>MIC$_{90}$ mg/L</th>
<th>Range</th>
<th>Percentage susceptibility at breakpoint of 4mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter spp (23)</td>
<td>1</td>
<td>2</td>
<td>1-128</td>
<td>96%</td>
</tr>
<tr>
<td>E. coli (50)</td>
<td>0.5</td>
<td>1</td>
<td>0.5-1</td>
<td>100%</td>
</tr>
<tr>
<td>P. aeruginosa (94)</td>
<td>2</td>
<td>4</td>
<td>0.5-32</td>
<td>97%</td>
</tr>
<tr>
<td>Serratia spp. (24)</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>16-128</td>
<td>-</td>
</tr>
</tbody>
</table>

Li and co-workers (2001b) demonstrated that CMS had a lower (about threefold) overall bactericidal activity than CLS against 23 strains of *P. aeruginosa* isolated from patients with CF with a mean MIC of 7.1mg/L versus 3.1mg/L respectively for all susceptible strains tested (P<0.05). In another study performed by Li et al (2001a), it was found that the MIC for CLS was 1 to 4mg/L for the sensitive organisms, while it was higher for CMS (4 to 16mg/L). A difference in MIC is to be expected as the molecular weight of CMS is higher than CLS and hence a given weight will contain more molecules of CLS than CMS. However, even taking this into account the differences are larger than expected. The MICs may vary between the studies (Li et al, 2001a) since CLS and CMS are unequal mixtures of the salt of two components, colistin A and colistin B in which various formulations may differ in activity of the *in vitro* studies. Furthermore, CMS derivatives are mixtures of mono-, di-, tri-, tetra-, and penta-substituted compounds, thus different pharmaceutical formulation may differ in the degree of hydrolysis and thus in antibacterial activity (Evans et al, 1999).

The MICs in broth reported in these studies were considerably different from those measured by Barnett and co-workers (1964) who tested the activity of colistin (CLS and CMS) against *P. aeruginosa*. The results are shown in the table below and the difference could be due to different techniques used for measuring the MIC, or because of an increase in resistance (*Table 1.4*).
Table 1.4: MIC90 (mg/L) against \textit{P. aeruginosa} (Barnett \textit{et al}, 1964).

<table>
<thead>
<tr>
<th></th>
<th>Nutrient broth (NB)</th>
<th>Nutrient agar (NA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLS</td>
<td>0.46</td>
<td>0.52</td>
</tr>
<tr>
<td>CMS</td>
<td>1.97</td>
<td>2.34</td>
</tr>
</tbody>
</table>

Barnett and co-workers (1964) pointed out that the activity of the CMS may be overestimated because of the conversion to colistin base during the 18-hour incubation period. This possibility was proved by increasing the number of organisms so that growth in the NB in the absence of antibiotics was visible after three hours incubation.

Additional evidence that the CMS form is less active than the CLS was gained by further experiments. Activities were measured against \textit{Klebsiella pneumoniae} which showed that for CMS, the concentration needed to kill 50% of the organisms in 30 minutes incubation was 19.7 mg/L, after one hour was 5.6mg/ L and 2.2 mg/L after three hours. It was thus observed that the concentration needed to kill 50% of the organisms reduced over time as CMS converted to colistin base (Barnett \textit{et al}, 1964).

Since there are known differences in the activities between CLS and CMS, it is critical to take this into account as CLS may often be used for susceptibility testing \textit{in-vitro}, while the sodium salt of CMS is clinically used.

\textbf{1.6.8 Resistance to colistin}

There have been reports on colistin resistance with *A. baumannii*. In a recent study performed by Gilad and co-workers (2005) a strong link between the use of colistin and the development of resistance in *A baumannii* clinical isolates collected in Israel between the year 2001 and 2004 was observed. Other studies reported heteroresistance to colistin in clinical isolates of colistin-susceptible multidrug-resistant *A. baumannii* (Li *et al*, 2005a; Poudyal *et al*, 2008).

Since colistin is considered as a last resort against the serious clinical threat posed by multi-resistant *Acinetobacter* species, the increasing threat of resistance to colistin has emphasised the urgent need to define appropriate CMS dose regimens (Li and Nation, 2006; Li *et al*, 2006b).

### 1.6.9 Methods of quantifying colistin in the biological fluids

#### 1.6.9.1 Colistin sulphate

Many analytical techniques have been developed for colistin such as paper chromatography (Suzuki *et al*, 1963), thin layer chromatography (TLC) (Thomas and Holloway, 1978; Thomas *et al*, 1980); high speed counter-current chromatography (Ikai *et al*, 1998a), capillary zone electrophoresis (Kristensen and Hansen, 1993; Kang *et al*, 2000) and HPLC (Elverdam *et al*, 1981; Wall, 1981; Decolin *et al*, 1997; Brun *et al*, 2000; Orwa *et al*, 2000; Li *et al*, 2001b; Gmur *et al*, 2003). Nevertheless, microbiological assays are currently the only official method for colistin analysis in many pharmacopoeias including the British Pharmacopoeia (Orwa *et al*, 2000; British Pharmacopoeia 2010).

Microbiological assays may be performed by diffusion or turbidimetric methods, details of which are presented in the British Pharmacopoeia 2010 (see appendix). The diffusion assay is performed by using *Bordetella bronchiseptica* NCTC 8344 or *Escherichia coli* NCIB 8879.

TLC, capillary electrophoresis and HPLC methods have been applied for the separation of the components of CLS but only limited HPLC (Decolin *et al*, 1997; Brun *et al*, 2000; Li *et al*, 2001b; Gmur *et al*, 2003) assays have been extended to measuring concentrations in biological fluids.
An HPLC method developed by Orwa and co-workers (2000), showed good separation but the analysis time was about 45 minutes. Despite the difficulty of assaying colistin in biological samples due its complex chemistry (Li et al., 2005b) and the hydrolytic conversion of CMS to colistin (Li et al., 2003b) HPLC methods have been developed for colistin levels in human serum and plasma but most of them have been applied to the sulphate form of colistin (Leive, 1974). These methods involved a derivatization step because colistin has a weak ultraviolet absorption and doesn’t have native fluorescence (Li et al., 2001a).

Li and co-workers (2001b) have developed a selective and sensitive HPLC method for determination of CLS in plasma. However, this method is not straightforward and has proved difficult to replicate. Reverse-phase HPLC was obtained by derivatization with fluorescent 9-fluorenylmethyl chloroformate in the same solid-phase extraction cartridge used to separate colistin from plasma; CLS can be measured without interference with methanesulphonate derivatives. This method has been employed for investigating the pharmacokinetics of colistin following the administration of CLS (Li et al., 2003c).

1.6.9.2 Colistin methanesulphonate sodium (CMS)

“Colistin methane sulfonate is perhaps the most complex antibiotic mixture for which a biological standard has had to be established” (Lightbown et al., 1973).

As with CLS, microbiological assays are the only official method for CMS analysis in many pharmacopoeias (British Pharmacopeia, 2010). The microbiological method has been widely used in monitoring CMS concentrations; however it lacks sensitivity, selectivity and requires at least an 18 hour incubation time. The colistin base formed from CMS in-vivo (in plasma or urine) (Schwartz et al., 1959; Barnett et al., 1964; Beveridge and Martin, 1966; Li et al., 2001a) and during microbiological assays (37ºC incubation overnight) in-vitro, will affect the accuracy and reproducibility of the microbiological assays, which rely on the total antibacterial activity within the sample.
It is apparent that plasma or serum samples contain many intermediates of CMS hydrolysis products with mono, di, tri, tetra and penta methanesulphonate groups located at different amine groups of colistin (Li et al, 2003b). CMS contains five methanesulphonate groups and it is used for preparing calibration curves for microbiological assays. The conversion of CMS to colistin base means that CMS must lose all five methanesulphonate groups, therefore samples containing such intermediates will produce colistin more rapidly during incubation in the microbiological assays, compared with samples contains CMS for calibration curves.

Colistin (base) is positively charged and may therefore be expected to diffuse more slowly than CMS (negatively charged) in agar which is also negatively charged. This molecular charge may thus contribute to the inability of the antibiotic to diffuse through the agar. However, another potentially cationic peptide antibiotic (bacitracin) diffuses well in agar and so the picture is clearly more complex. The charge on bacitracin, however, will vary according to the pH of the medium. At pH 7 the net charge on the molecule is likely to be neutral whereas colistin will carry a net positive charge.

Microbiological assays are unable to measure CMS and colistin separately, and the reliability of the reported results on the determination of the concentrations of CMS is most likely compromised by the occurrence of the more microbiologically active compound (colistin) and its partially sulphomethylated derivatives (Sande and Kaye, 1970).

HPLC methods that have been developed to assay colistin in biological fluids by Decolin et al (1997) and Brun et al, (2000), involved the complex derivatization of CLS using orthophthalaldehyde (OPA), unfortunately this method is not suitable for CMS as OPA only reacts with primary amine (-NH₂) groups which are unavailable in CMS (Li et al, 2001b).
The HPLC method for CMS developed by Li et al (2002) depended on the acceleration of hydrolysis of CMS to colistin in biological fluids, extraction of colistin from the sample, then derivatization prior to HPLC analysis. Another successful method was developed by Li et al (2003b) using Strong Anion Exchanger (SAX).

Due to the complexity of the hydrolytic products of CMS, it remains a challenge to separate and measure the concentrations of CLS and CMS with sufficient sensitivity, even with advanced techniques such as HPLC or capillary electrophoresis coupled to mass spectrometry (Li et al, 2002).

1.6.10 Toxicity of colistin

In early clinical studies, a high incidence of toxicity with intravenous CMS was reported (Wolinsky and Hines, 1962; Perkins, 1964; Elwood et al, 1966; Ryan et al, 1969; Vinnicombe and Stsmey, 1969; Bosso et al, 1991). However, Wolinsky (Wolinsky and Hines, 1962), Ledson and colleagues (Ledson et al, 1998) and Conway’s group (Conway et al, 2000) reported satisfactory safety profiles with doses of 160mg every 8 hours of Colymycin in CF patients with normal renal function.

Recent investigations have demonstrated that CMS is not as nephrotoxic as previously reported in patients with CF, (Etherington et al, 2004; Al-Aloul et al, 2005; Hakim et al, 2008; Cheng et al, 2010; Ganapathy et al, 2010). The toxicities observed in the early studies contributed to the lack of understanding of colistin pharmacokinetics, pharmacodynamics, toxicodynamics and correct and accurate dosing (Lim et al, 2010).

The avoidance of parallel administration of nephrotoxic and/or neurotoxic drugs, together with properly monitored dosage, may be some of the reasons for the discrepancy between reports in the old and recent literature (Li et al, 2005b; Falagas and Kasiakou, 2006a).
1.7 Nephrotoxicity of colistin sulphate and colistin methanesulphonate

Antibacterial agents which cause profound damage to bacterial membranes might also be expected to cause adverse effects to the mammalian host cell membranes thus leading to side effects. Nephrotoxicity and neurotoxicity were the main side effects of CLS (Beringer, 2001); therefore CMS was formulated to reduce these toxicities. In order to understand the effects of CLS and CMS on the kidney it is necessary to have knowledge of its structure and function. Some basic information on kidney structure is provided here and is taken from Brenner & Rector's text book (The Kidney, Brenner and Rector's, 2003) which should be referred to for more detailed information.

1.7.1 Kidney structure

The kidneys are paired organs with many functions. They are an essential part of the urinary system and also involved in the regulation of electrolytes, maintenance of acid-base balance, regulation of blood pressure and production of particular hormones. They act mainly as the body’s natural filter for the blood. The kidneys also excrete a variety of waste products produced by metabolism, including the nitrogenous waste urea, from protein catabolism, and uric acid, from nucleic acid metabolism.

The kidney has a bean-shaped structure; each kidney has a concave and convex surfaces. The concave surface or the renal hilum is the point at which the renal artery enters the organ, the renal vein and ureter leave. The kidney is enveloped by fibrous tissue or the renal capsule, which surrounded by perinephric fat, renal fascia and paranephric fat. The kidney (Figure 1.8) is divided into two major structures: superficial renal cortex surrounding a portion of renal medulla. These structures take the shape of 8 to 18 cone-shaped renal lobes, each lobe containing renal cortex and the renal medulla called a renal pyramid, between the renal pyramids are projections of cortex which are known as renal columns.
The vital functional unit of the kidney is the nephron. Each kidney consists of approximately 1-1.5 million nephrons. Each nephron consists of two major structures: the glomerular portion (Bowman's capsule) and the Tubule, which is divided into three major areas: the Proximal Tubule (PT), the loop of Henle and the distal nephron (Figure 1.9). The nephron can also be sub-classified into two distinct groups, the cortical nephrons and the juxtamedullary nephrons.

The cortical nephrons have glomeruli in the outer two-thirds of the cortex with short loops of Henle. They may extend a short distance into the medulla or may not reach the medulla. These account for 85% and 30% of the nephrons in man and rat respectively. The juxtamedullary nephrons have glomeruli in the inner third of the cortex with long loops of Henle, extending deep into the medulla. They account respectively for 15 and 70% of the nephrons in man and rat (Valtin, 1977).
The kidney is considered to filter large quantities of plasma which occurs in the glomerular membrane (Bowman's capsule). The filtration barrier made up of three layers: the capillary endothelium, the basement membrane and the epithelium. The capillary endothelium is like the other capillaries elsewhere in the body. The glomerular endothelial cells have several holes or in their cytoplasm. The basement membrane consists of collagen and proteoglycans with no cells present. It envelops the glomerular capillaries. Overlying the basement membrane is epithelial cell layer of the Bowman's capsule.

Figure 1.9: Basic structure of the nephron.
(Taken from www.anselm.edu/.../jpitocch/genbio/nephron.JPG.)
The tubular portion of the nephron is responsible for re-absorption of the essential substances from and secretion of waste substances into the tubular fluid. The tubular nephron consists of an epithelial cell layer that is continuous with the epithelial cell layer forming the Bowman's capsule. The epithelial cells in the various regions of the tubule differ in terms of size, shape, the number of mitochondria and number of microvillus on the luminal surface. However, certain structural features are common to all tubular epithelial cells. In the description of tubular epithelium, the luminal and basal surfaces are often known as mucosal or apical and the serosal or peritubular surfaces respectively.

1.7.2 Renal function tests

1.7.2.1 Renal in vivo studies

The production of urine and excretion of water-soluble waste products of metabolism is the unique function of the kidney. Our knowledge of renal function has been obtained from in vivo animal and human studies. In vivo nephrotoxicity assessment usually uses three different approaches. These include the following:

- Urine analysis using the assessment of proteinuria, enzymuria, glucosuria, pH, water and electrolyte excretion as toxicity endpoints.
- The use of endpoints such as drug uptake, enzyme levels, adenosine triphosphate (ATP), glutathione, metabolic activities, molecular expression of specific markers and pathology.
- Serum collection for Blood Urea Nitrogen (BUN) or creatinine assessment including measurement of Glomerular Filtration Rate (GFR).

Creatinine assessment is a more accurate index of renal function than BUN. Unfortunately, serum concentrations of creatinine vary highly with GFR which is affected with renal diseases, while BUN level is highly affected by variations in urine flow rate and by the diet, increasing with high protein intake. Furthermore, BUN fluctuates more widely during the day than serum creatinine. BUN is also increased by surgery, infection, trauma, and steroid and tetracycline therapy. It is decreased by old age and chronic liver disease.
Another disadvantage of the \textit{in-vivo} studies is that the kidney consists of 15 to 20 cell types that differ from each other in function, metabolism and ultra structure (Kriz and Bankir, 1988). Some chemicals may be concentrated selectively in discrete areas of the kidney and it is difficult in \textit{in-vivo} studies to determine the exact site that the particular substance produces its toxic effect. In addition, it is possible to obtain false positive results due to the high susceptibility of the kidney to internal and external influences such as variations in blood pressure and the variations in the \textit{in-vivo} renal function between species probably due to the different physiology (Pincus and Abraham, 2006).

\subsection*{1.7.2.2 Renal \textit{in vitro} studies}
\textit{In vitro} studies are commonly used as they overcome many of the problems encountered with \textit{in vivo} studies. The main advantage of an \textit{in vitro} over the \textit{in vivo} system is that it is very unlikely to produce false positive results. Therefore, if potentially nephrotoxic compounds actually exert toxic effects or inhibit the cell survival mechanisms this will be detected. Moreover, experimental designs can easily be repeated \textit{in vitro} using isolated structures or cell cultures. However, the main disadvantage of an \textit{in vitro} system is that it may produce false negative results. Therefore, if a drug is potentially cytotoxic, the test may not confirm it due to factors such as, the lack of chemical solubility, short exposure time or low cellular permeability and the absence of extra-renal metabolism of some chemicals into nephrotoxic metabolites. For instance, the halogenated alkenes require glutathione conjugation in the liver, as a prerequisite for nephrotoxicity (Vamvakas \textit{et al}, 1989).

Unfortunately the \textit{in vitro} test cannot replicate the complex interactions of animals, as it lacks the experimental design to model proteinuria, urinary pH, and water and electrolyte excretion. In addition, serum collection for creatinine and BUN are compounds excreted by the kidney; there is no equivalent \textit{in vitro} experimental design to model these assessments.
In vitro systems have been classified into two classes: short term systems which include the use of whole perfused kidneys, renal slices, perfused isolated tubular and cell suspensions for a period of hours and long term systems which utilize cell lines and primary cultures for days or weeks (Boogaard et al, 1990).

1.7.2.3 Cultured renal cells (in vitro studies)

Cell cultures are most commonly used as primary cultures or cell lines. Cultures are directly prepared from the tissues of an organism and are called a primary culture. This culture continues to be primary until it is successfully subcultured or passaged (secondary cultures), then it is classified as a cell line. Cultured cells have several advantages, such as:

- The extended life span of cultured cells (several days to weeks) enables them to be studied for a long period of time;
- They usually grow best when attached to a solid surface and they produce a continuous or confluent layer over the surface of the culture dish;
- They can be studied in a controlled and well-defined environment and the effect of the substance in question can be observed;
- They can be grown in large numbers with high cell purity allowing a large number of experiments to be performed on cells originating from one organ;
- They allow the utilization of a minimal amount of the investigated agent;
- The use of cultured cells reduces the number of animals required in renal research;

They allow the drug to directly access the monolayer and if grown upon membrane filters, separate access to the basolateral and apical surfaces of the epithelia (Adler, 2006). The basolateral surface is usually the surface attached to the culture dish and the surface facing the culture medium is the apical surface.
The main disadvantage of using cells in culture is that they may lose expression of their differentiated properties; therefore care must be taken in extrapolating the functional characteristics of cells in culture environment. This may be of different cell type or origin to that required. Thus, it is vital to be aware of exact identity of cells and the way in which cell markers may modify with time in culture.

In spite of these disadvantages, renal primary cultures are considered as a more reliable though difficult system to study basic renal cellular function and their modulation by nephrotoxins. Thus, many in vitro nephrotoxicity studies have been performed on permanent or continuous renal epithelial cell lines (Pfaller and Gstraunthaler, 1998).

1.7.2.4 Primary culture of proximal tubular cells
A xenobiotic is a chemical which is found in an organism but which is not normally produced or expected to be present in it. It can also cover substances which are present in much higher concentrations than are usual. Particularly, antibiotics are xenobiotic in humans since the human body does not produce them itself.

The Proximal Tubular (PT) is the main site of xenobiotic induced injury. Thus in vitro models of this segment of the nephron are important for the evaluation of the safety of xenobiotics with regards to kidney, thus the majority of in vitro investigations are carried on permanent or continuous epithelial cells.

1.7.2.5 Renal cell lines
Although cell lines may have maintained dedifferentiated functions from their in vivo ancestor cells, they have dedifferentiated in culture (Gstraunthaler, 1988) therefore; cells of PT origin may display a combination of features, which are characteristic of different parts of nephron. The most commonly used PT cell line in nephrotoxicity studies includes the NRK-52E cells derived from the normal rat kidney.
Although the biochemical function of Normal rate kidney NRK-52E cells have not been extensively characterized, they are still considered as first choice for physiological and toxicological studies because of their origin and characterization close to PT cells of the rat kidney.

1.7.3 Renal transportation of colistin

CMS is excreted from the body by the kidney and the urinary excretion involves renal tubular secretion. CLS is excreted by the non-renal route because part of the compound undergoes very extensive renal tubular re-absorption, CLS secretion is not fully understood (Li et al, 2003c and 2004; Ma et al, 2009).

The toxicity of Polymyxins may be due to the contents of D-amino acid and fatty acid. It is suggested that polymyxins produce renal toxicity by increasing the transepithelial conductance of the urinary bladder epithelium. Renal toxicity is dependent on concentration and length of exposure. Nephrotoxicity associated with the administration of colistin is dose dependent (Lewis and Lewis, 2004).

Human fibroblast and keratinocytes cells were exposed to CMS for 48 hours. Cytotoxic effects of CMS on the skin (CMS use in infections related to burn patients) were examined using MTT; no cytotoxicity was observed when the particular cells were exposed to CMS for 48 hours at concentrations even higher than the therapeutic plasma concentrations (Damour et al, 1992).
1.8 Aim and objectives

Burn patients can become colonised with *P. aeruginosa* and *A. baumannii* which are very difficult to control or treat. CMS is often the only effective drug which can be used but because of its toxicity profile it must be carefully monitored. The methods available for measuring CMS in serum are not robust and are not able to differentiate between CLS or CMS and the breakdown products of CMS.

1.8.1 Aim:

The aim of this thesis is to develop methodologies for the reproducible quantification of CLS and CMS in water or serum and explore the relative toxicities of the different forms of colistin.

1.8.2 Objectives:

i. To investigate the microbiological assay of CLS and CMS and develop optimized procedures for its quantification in water and serum.

ii. Improve the currently used HPLC and TLC methods for CLS and CMS and develop procedures for their quantification in water.

iii. Utilise the developed assay procedures to investigate the degradation of CMS on storage at different temperatures and in water and serum.

iv. Investigate the mode of action of CLS and CMS on *Acinetobacter lwofii* (*A. lwofii*) R4638 in terms of their killing effect and leakage of intracellular components.

v. Investigate the relative toxicities of CLS and CMS on mammalian cell cultures.
CHAPTER TWO

Microbiological materials and general procedures
2.1 Materials

2.1.1 Culture media, gelling agents, buffer solutions and chemicals used in the microbiological studies including their preparations

CLS was purchased from Sigma (Hertfordshire, UK). CMS as Colymycin was obtained as a gift from Forest Laboratories (Kent, UK). All glassware and plastics were purchased from Fisher Scientific (Loughborough, UK). Chemicals were supplied by Sigma, BDH (Dorset, UK), Neveon (USA) and Biomedicals (Ohio, USA). Culture media were supplied by Oxoid Ltd (Hampshire, UK), Fisher Scientific and Difco Ltd (Beckton Dickinson and company, USA). Human serum was purchased from Immune Systems Limited (Devon, UK).

2.1.1.1 Culture media

Media and broths (as mentioned below) were reconstituted using distilled water according to the manufacturer’s instructions and then autoclaved at 121°C (15 psi) for 15 minutes (Autoclave model, 300/75LR, Boxer laboratory equipment, Hertfordshire, UK).

The base layer agar:
Nutrient Agar (NA) (Fisher Scientific, CM0003B)
Tryptone Soya Agar (TSA) (Fisher Scientific, CM131B)
M1 Antibiotic media (Oxoid Ltd, CM0327)
Diagnostic Sensitivity Test Agar (DSTA) (Fisher Scientific, CM261B)
Isosensitest Agar (ISA) (Fisher Scientific, CM471B)
Dehydrated culture media (R2A) (Oxoid Ltd, CM0906)

The seed layer agar
Nutrient Broth (NB) (Fisher Scientific, CM001B) + Technical Agar 0.4% (Oxoid Ltd, LPO138)
Isosensitest Broth (ISB) (Oxoid Ltd, CM0473) + Technical Agar 0.4%
Peptone water (PW) (Fisher Scientific, CM009B) + Technical Agar 0.4%
Antibiotic Medium No.3 (Oxoid Ltd, CM0287) + Technical Agar 0.4%
Tryptone Soya Broth (TS broth) (Fisher Scientific, CM129B) + Technical Agar 0.4%

**Culture broth**

Tryptone Soya broth (TS broth) (Fisher Scientific, CM129B)
Nutrient Broth (NB) (Fisher Scientific, CM001B)
Synthetic Broth AOAC (Becton Dickinson, MD, USA)

**2.1.1.2 Gelling agents**

Sodium alginate (BDH, Dorset UK)
Carrageenan type I and type II (Sigma, UK)
Gum xanthan (Sigma, UK)
Gum guar (Sigma, UK)
Carbopol ultrez 21 (Neveon, USA)
Phytogel (Sigma, UK)

Specific amounts (indicated in the text) of the above materials were added to boiling water, TS broth or NB on the hot stirrer plate (Fisher, UK) until the powder was dissolved. The solutions were distributed in aliquots, autoclaved at 121 ºC for 15 minutes and then allowed to cool to room temperature.

**2.1.1.3 Buffer solutions**

**Buffer pH6** was chosen as it is the buffer recommended in the BP for CMS assay (British Pharmacopeia, 2010). It was prepared as 100ml of 0.1M of $\text{KH}_2\text{PO}_4$ (BDH, UK), to which 11.2ml of 0.1M NaOH (BDH, UK) was added to give pH of 6. The solution was sterilised at 121 ºC (15psi) for 15 minutes. The pH of the solution was checked after autoclaving to ensure that autoclaving had no effect on pH (buffer solutions were prepared freshly).

**HEPES buffer** (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Biomedicals, Ohio, USA) solution was chosen for investigation as it has an effective buffering capacity (dipolar compound). HEPES buffer was prepared at a concentration of 0.05M. The free acid (119.15g) was added to 350ml of distilled water, then was titrated with sodium
hydroxide pellets (about 2.5g) to produce a final solution pH of 7.4; the volume was made up with distilled water to a litter and then sterilised at 121 °C (15psi) for 15 minutes. The pH of the solution was checked after autoclaving to ensure that autoclaving had no effect on pH (buffer solutions were prepared freshly).

**Phosphate Buffer Saline (PBS)**

8.00g of NaCl (BDH, UK), 0.20g of KCl (BDH, UK), 1.44g of Na$_2$HPO$_4$ (BDH, UK) and 0.24g of KH$_2$PO$_4$ (BDH, UK) were dissolved in 800ml of distilled water, the solution was adjusted the pH to 7.4 with HCl or NaOH, and then the volume was made up to one litter with distilled water and then was sterilised at 121 °C (15psi) for 15 minutes. pH of the solution was checked after autoclaving to ensure that autoclaving had no effect on pH.

**2.1.1.4 Chemicals**

0.5% and 1% w/v Calcium chloride, CaCl$_2$ (BDH, UK)
1mM Cetrimide (Thornton and Ross, UK)
10% Glycerol (BDH, UK)
0.5% and 1% w/v Calcium chloride (BDH, UK)
1mM Cetrimide (Thornton and Ross, UK)
10% Glycerol (BDH, UK)
0.1% and 0.2% Soya bean lecithin (BDH, UK)
0.1M Magnesium chloride (BDH, UK)
0.1% and 2% w/v Potassium chloride (BDH, UK)
0.9% Sodium chloride, Saline solution (BDH, UK)
1% and 4% w/v Sodium hydroxide (BDH, UK)
1% and 5% Trichloroacetic acid (BDH, UK)
1% and 2% Tween 80 (BDH, UK)
1:10 Tritonx 100 (Sigma, UK)
1% and 5% Trichloroacetic acid (BDH, UK)

The above solutions were prepared in distilled water and autoclaved at 121°C (15 psi) for 15 minutes.
2.2 General procedures

2.2.1 Microorganisms

Different species of Gram-negative bacteria were grown overnight at 37°C in TS broth and used as bio-indicators in the establishment of a microbiological agar diffusion assay for CLS and CMS. These species were tested to select the bacterium that showed the best performance in terms of size and clarity of the inhibition zones.

During preliminary investigations, the starting concentrations of all cultures employed in this study (except *B. bronchiseptica* NCTC 8344 and *A. lwoffii* R46383) were prepared as follows: one loopful from each bacterium was taken from the original TS broth culture into 10ml single strength TS broth (media prepared as detailed in section 2.1.1.1) and incubated at 37°C overnight. After incubation this culture was standardised to approximately 10^8 Colony forming unit/ml (Cfu/ml) by assuming that an absorbance of 0.1 was equivalent to a bacterial count of approximately 10^8 Cfu/ml, this was prepared in TS broth and optical density was measured at wavelength of 420nm using a UV-visible spectrophotometer (Model 6300, Jenway, UK) with a 1cm cell length, against TS broth as a blank. 1ml of these standardised cultures were diluted into 9ml of TS broth to produce cultures at concentrations of 10^7 Cfu/ml, further dilutions were made as required. These optical densities were estimated without any calibration plots as these were used in the early investigations and were found not to be efficient. The routinely used bacteria such as *B. bronchiseptica* NCTC 8344 and *A. lwoffii* R46383 were calibrated as in section 2.2.6.

*B. bronchiseptica* (NCTC 8344) was obtained from the National Collection of Type Cultures (Health Protection Agency, Colindale, London UK), as freeze dried cultures. Nine viable clinical *Acinetobacter* spp. isolates (from burn patients, not an endemic strain) (*Table 2.1*) were donated as frozen cultures by Dr. J. A. Child, Consultant Microbiologist at the Surrey and Sussex NHS Trust in December 2000, together with relevant antibiotic disc sensitivity data and some isolation history.*A. lwoffii* R46383 used in this study was one of the clinical isolates donated by Dr. Child and was subjected to an API identification test (*Table 2.3*).
### Table 2.1: Isolation history of Sussex strains. (Information as provided with the culture)

<table>
<thead>
<tr>
<th>STRAIN SHORT CODE</th>
<th>Strain isolation history</th>
<th>Outbreak strain</th>
<th>Source</th>
<th>MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1973</td>
<td>From patient with severe burns showed Amikacin resistant.</td>
<td>Yes</td>
<td>QVH</td>
<td>Yes</td>
</tr>
<tr>
<td>R2751</td>
<td>From wound of patient on surgical ward. Treated with IV colistin.</td>
<td>Yes</td>
<td>QVH</td>
<td>Yes</td>
</tr>
<tr>
<td>R3417</td>
<td>Representative of an outbreak strain at a Sussex Hospital, other than QVH.</td>
<td>No</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>R4474</td>
<td>Blood-culture isolate from patient with severe burns, who later died, <em>Acinetobacter</em> infection was contributory factor</td>
<td>Yes</td>
<td>QVH</td>
<td>-</td>
</tr>
<tr>
<td>R45502</td>
<td>From a patient with severe burns showed Amikacin resistant</td>
<td>Yes</td>
<td>QVH</td>
<td>Yes</td>
</tr>
<tr>
<td>R46383</td>
<td>From burns patient, not an epidemic strain.</td>
<td>No</td>
<td>QVH</td>
<td></td>
</tr>
<tr>
<td>W5211</td>
<td>From blood culture of patient with 80% burns who later died.</td>
<td>Yes</td>
<td>QVH</td>
<td>Yes</td>
</tr>
<tr>
<td>W6108</td>
<td>Blood culture isolate from a patient on a general medical ward</td>
<td>-</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>W6492</td>
<td>Blood-culture isolate from a medical patient</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Legend Table 2.1:**

* Outbreak strain ‘Yes’ identifies a strain described as an ‘outbreak strain’ in the letter obtained with the samples
* Source ‘QVH’ identifies strains clearly identified as isolated at the Queen Victoria Hospital, East Grinstead.
* - denotes where insufficient information were provided
2.2.2 Growth and maintenance of microorganisms

One loopful from each culture was reconstituted in TS broth, incubated at 37°C overnight, then sub-cultured into TS broth and incubated overnight at the same temperature. For the bacterial suspension of *B. bronchiseptica* (NCTC 8344) and *A. lwoffii* R46383, sterile glycerol (BDH) was added as a cryo-protectant to a final concentration of 10%, and 2ml aliquots of the suspensions were aseptically transferred in cryo-tubes and stored in a –70°C freezer (Canyo electric company, model No. MDF-U71V). Sufficient stock of the bacterial cultures was stored to last the duration of the research project.

At the beginning of each month, a frozen bacterial sample was reconstituted; the thawed culture was aseptically added to 100ml of NB and incubated at 37°C overnight. From the suspension, six TSA isolation plates were incubated at 37°C for 24 hours and stored at 4°C as stock plates for the month. The purity of the cultures was verified by API tests (section 2.2.3).

Each week, starter cultures of 10ml of TS broth were produced by inoculating media with single colony from the stock plates (stored at 4°C) and incubation at 37°C overnight.

2.2.3 Identification of microorganisms

There are various brands of identification kits on the market. API 20NE (bioMérieux, Lyon, France) were used due to the specific design for use with non-fermentative Gram-negative bacteria.

API 20NE test kit (bioMérieux, Lyon, France) was used according to manufacturer’s instructions for the identification of isolates. Freshly subcultured colonies on TSA were used to inoculate the test strips. According to the manufacturer’s literature the Analytical Profile Index I.D. API20NE was not valid before 48 hour incubation for all strains, which warranted the maximum incubation time at 30°C.

Typical biochemical profiles for *B. bronchiseptica* and *A. lwoffii* R46383 strains used in this project are indicated below (**Table 2.2 and Table 2.3**).
Table 2.2: Biochemical profile of *Bordetella bronchiseptica*
NCTC 8344 using API 20NE (n=10)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction/Enzyme</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium nitrate</td>
<td>Reduction of nitrates to nitrites</td>
<td>+</td>
</tr>
<tr>
<td>L-tryptophane</td>
<td>Indole production</td>
<td>-</td>
</tr>
<tr>
<td>D-glucose</td>
<td>Fermentation (GLucose)</td>
<td>-</td>
</tr>
<tr>
<td>L-arginine</td>
<td>Arginine DiHydrolase</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>UREase</td>
<td>+</td>
</tr>
<tr>
<td>Esculin ferric citrate</td>
<td>Hydrolysis (B-glucosidase)</td>
<td>-</td>
</tr>
<tr>
<td>Gelatine (bovine origin)</td>
<td>Hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>4-nitophenyl-BD-gelactopyranoside</td>
<td>B-galactosidase</td>
<td>-</td>
</tr>
<tr>
<td>D-glucose</td>
<td>Assimilation (GLucose)</td>
<td>-</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>Assimilation (ARabinose)</td>
<td>-</td>
</tr>
<tr>
<td>D-mannose</td>
<td>Assimilation (ManNosE)</td>
<td>-</td>
</tr>
<tr>
<td>D-mannitol</td>
<td>Assimilation (MANnitol)</td>
<td>-</td>
</tr>
<tr>
<td>N-acetyl-glucosamine</td>
<td>Assimilation (N-Acetyl-Glucosamine)</td>
<td>-</td>
</tr>
<tr>
<td>D-maltose</td>
<td>Assimilation (MALtose)</td>
<td>-</td>
</tr>
<tr>
<td>Potassium gluconate</td>
<td>Assimilation (potassium GlucoNate)</td>
<td>-</td>
</tr>
<tr>
<td>Capric acid</td>
<td>Assimilation (capRIC Acid)</td>
<td>-</td>
</tr>
<tr>
<td>Adipic acid</td>
<td>Assimilation (ADIPIC ACID)</td>
<td>+</td>
</tr>
<tr>
<td>Malic acid</td>
<td>Assimilation (MalaTe)</td>
<td>+</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>Assimilation (trisodium CITrate)</td>
<td>+</td>
</tr>
<tr>
<td>Phenylacetic acid</td>
<td>Assimilation (PhenylAcetic acid)</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>Cytochrome oxidase</td>
<td>+</td>
</tr>
</tbody>
</table>

Strain short code: (NCTC 8344)
API20NE reading (48 hours): 0200067
Species: *B. bronchiseptica*, percentage confidence 95%
Table 2.3: Biochemical profile of *Acinetobacter lwoffii* R46383 using API 20NE (n=10)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction/Enzyme</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium nitrate</td>
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<tr>
<td>L-tryptophane</td>
<td>Indole production</td>
<td>-</td>
</tr>
<tr>
<td>D-glucose</td>
<td>Fermentation (GLUcose)</td>
<td>-</td>
</tr>
<tr>
<td>L-arginine</td>
<td>Arginine DiHydrolase</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>UREase</td>
<td>+</td>
</tr>
<tr>
<td>Esculin ferric citrate</td>
<td>Hydrolysis (B-glucosidase)</td>
<td>-</td>
</tr>
<tr>
<td>Gelatine (bovine origin)</td>
<td>Hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>4-nitophenyl-BD-gelactopyranoside</td>
<td>B-galactosidase</td>
<td>-</td>
</tr>
<tr>
<td>D-glucose</td>
<td>Assimilation (GLUcose)</td>
<td>-</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>Assimilation (ARAbinose)</td>
<td>-</td>
</tr>
<tr>
<td>D-mannose</td>
<td>Assimilation (ManNosE)</td>
<td>-</td>
</tr>
<tr>
<td>D-mannitol</td>
<td>Assimilation (MANnitol)</td>
<td>-</td>
</tr>
<tr>
<td>N-acetyl-glucosamine</td>
<td>Assimilation (N-Acetyl-Glucosamine)</td>
<td>-</td>
</tr>
<tr>
<td>D-maltose</td>
<td>Assimilation (MALtose)</td>
<td>-</td>
</tr>
<tr>
<td>Potassium gluconate</td>
<td>Assimilation (potassium GlucoNate)</td>
<td>-</td>
</tr>
<tr>
<td>Capric acid</td>
<td>Assimilation (capRIC Acid)</td>
<td>+</td>
</tr>
<tr>
<td>Adipic acid</td>
<td>Assimilation (ADIPIC ACID)</td>
<td>-</td>
</tr>
<tr>
<td>Malic acid</td>
<td>Assimilation (MalaTe)</td>
<td>+</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>Assimilation (trisodium CITrate)</td>
<td>-</td>
</tr>
<tr>
<td>Phenylacetic acid</td>
<td>Assimilation (PhenylAcetic acid)</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>Cytochrome oxidase</td>
<td>-</td>
</tr>
</tbody>
</table>

Strain short code: R46383
API20NE reading (48 hours): 0000063
Species: *A. lwoffii*, percentage confidence 95%
2.2.4 Preparation of bacterial suspension

10ml of TS Broth was inoculated with one to three colonies from a stock plate (stored at 4°C) of bacteria and incubated at 37°C overnight. This culture was then used to inoculate a large assay plate containing 200ml of TSA and incubated overnight to produce a bacterial lawn. Growth of the inocula as a lawn enabled high cell densities to be reached with minimal trauma to the cells and with minimal carryover of nutrients (Gilbert and Brown, 1991).

The surface of the agar was then washed with 20ml of HEPES buffer (0.05M) and the lawn was removed with a sterile plastic spreader. This bacterial suspension was centrifuged (Sorvall RT6000B, Hertfordshire, UK) at 3000g for 30 minutes and washed twice with 20ml of HEPES buffer. The optical density of cell suspensions in HEPES buffer were determined at a wavelength of 420nm using a UV-visible spectrophotometer (Model 6300, Jenway, UK) with a 1cm cell length, against HEPES buffer as a blank.

Dilutions of the bacterial suspension were conducted in HEPES buffer measured at 420nm and adjusted to obtain a reading of 0.1.

An absorbance of 0.1 was measured for *B. bronchiseptica* and *A. lwofii* and found to correspond to 0.99 x 10^8 and 1.14 x 10^8 Cfu/ml respectively (as detailed in section 2.2.6).

1ml of *B. bronchiseptica* suspension or *A. lwofii* was diluted in 9ml HEPES buffer to produce 0.99 x 10^7 Cfu/ml or 1.14 x 10^7 Cfu/ml respectively, further dilutions were made in the same manner if required. The Cfu/ml for the stock suspension was confirmed by spread plate counts (section 2.2.5).

*B. bronchiseptica* was applied in this study using 20µl in 5ml or 200µl in 50ml depending on the size of plate used; assuming a starting concentration of approximately 1 x 10^8 Cfu/ml this would be equivalent to 0.4 x 10^6 Cfu/ml (*Figure 2.1 for B. bronchiseptica*, *Figure 2.2 for A. lwofii R46383*).
2.2.5 The plate spread technique

The number of bacteria in suspension may be determined by using the spread plate technique. In this technique, the bacterial sample was diluted to reach bacterial count of 30-300 per plate. A small aliquot (0.2ml) was transferred to an agar plate; the bacteria were then distributed evenly over the surface by a sterile plastic spreader. After overnight incubation, colonies were counted and the number of bacteria in the original sample calculated as Cfu/ml. In this technique it is assumed that each viable bacterium in the suspension would grow to form an individual colony Cfu/ml.

2.2.6 Calibration graph of cell numbers against optical density

Accurate determinations of cell number were made on cultures of B. bronchiseptica NCTC 8344 and A. lwoffii R46383.

Bordetella bronchiseptica NCTC 8344 and Acinetobacter lwoffii R46383 were grown as detailed in section 2.2.4. The suspensions were diluted in HEPES buffer to give a range of concentrations from 100% to 20% and optical density readings were recorded between 0 and 0.5 at a wavelength of 420nm using a UV-visible spectrophotometer against a HEPES blank. Suspensions of different bacterial concentrations were serially diluted in HEPES buffer and 0.2ml samples from each dilution were plated in triplicate, on TSA plate using the plate spread technique as in section 2.2.5, this experiment was performed three times. Counts were recorded from plates containing colonies ranging from 30 and 300. The Cfu/ml values and optical densities were plotted, (Figure 2.1 for Bordetella bronchiseptica, Figure 2.2 for Acinetobacter lwoffii R46383).
Figure 2.1: The relationship between optical density at 420nm and viable cell number for *Bordetella bronchiseptica*. n=3± Stdev

Figure 2.2: The relationship between optical density at 420nm and viable cell number for *Acinetobacter lwofii* R46383. n=3± Stdev
Figures 2.1 and 2.2 indicate that an absorbance of 0.1 for *B. bronchiseptica* and *A. lwofii* corresponds to viable counts of $0.99 \times 10^8$ and $1.14 \times 10^8$ Cfu/ml respectively.

### 2.2.7 Validation of the viable count technique (plate spread technique)

This experiment was performed to validate the viable plate count technique which was used in the enumeration of the bacterial suspensions (section 2.2.4). 10ml of NB was inoculated with one to three colonies from the stock plate of bacteria (stored at 4°C) and incubated at 37°C overnight; this process was performed with three tubes of NB (each time from different plate). The optical densities of these cultures were adjusted with HEPES buffer to give readings of 0.1 at 420nm against HEPES as a blank using a spectrophotometer.

*B. bronchiseptica* stock suspensions were subjected to serial dilutions in sterile HEPES buffer and 0.2ml of each bacterial suspension was spread on the surface of three plates of NA. The plates were then incubated at 37°C overnight and the number of bacterial colonies was counted (Table 2.4). The results were represented by the mean values of replicate plates. Since each colony could be formed by single or a clump of cells, the concentration of bacteria was expressed in terms of Cfu/ml. This experiment was performed three times.

### Results

**Table 2.4: Bacterial counts for *Bordetella bronchiseptica* NCTC 8344.**

*n=3± Stdev*

<table>
<thead>
<tr>
<th>Tube</th>
<th>Colony count (plate 1)</th>
<th>Colony count (plate 2)</th>
<th>Colony count (plate 3)</th>
<th>Average of each tube</th>
<th>Stdev of each tube</th>
<th>ANOVA between plates of each tube</th>
<th>ANOVA between different tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td>183.3</td>
<td>200</td>
<td>191.1</td>
<td>191.6</td>
<td>8.3</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Tube 2</td>
<td>310</td>
<td>236.6</td>
<td>241.7</td>
<td>262.7</td>
<td>40.9</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Tube 3</td>
<td>213.3</td>
<td>168.4</td>
<td>228.3</td>
<td>203.3</td>
<td>31.16</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>
Table 2.4 shows the number of Cfu/ml. The analysis of variance (ANOVA) shows no significant differences between the variation within the plates in each tube and the variation between the three tubes (P>0.05). Coefficient of variance of tubes 1, 2 and 3 were equal to 4.3%, 15.5% and 15.2% respectively.

2.2.8 Production of growth profiles

Growth profiles for *B. bronchiseptica* (NCTC 8344) and *A. lwaffii* R46383 were required in order to characterise the normal growth behaviour of the organisms. 1ml of an overnight culture of each bacterium was placed in each of 5 flasks containing 100ml of NB, and incubated at 37°C with shaking at 80 rotations per minute (rpm) in a Grant orbital shaking water bath (Fisher Scientific, UK). 2ml samples were removed from each flask at 30 minute intervals for analysis over a period of 8 hours.

The optical densities of these samples were determined at 420nm against NB as a blank using the spectrophotometer (model: 6300, Jenway, UK).

These data enabled growth profiles for the organism to be produced over an eight hour period (Figure 2.3 and Figure 2.4). This experiment was performed in triplicate.

Figure 2.3: Typical growth profile of *Bordetella bronchiseptica* (NCTC 8344) 420nm incubated in nutrient broth at 37°C. n=3± Stdev
Figure 2.4: Typical growth profile of *Acinetobacter lwoffii* R46383 420nm incubated in nutrient broth at 37°C. n=3± Stdev

These data show the slow growth of *B. bronchiseptica* and *A. lwoffii*, as the cell number doubles about every two hours (Optical densities increased from 0.04 to 0.08 from within 120 minutes).

### 2.2.9 Preparation of antibiotics and their discs

#### 2.2.9.1 CLS solution

Both CLS and CMS are soluble in water. An accurate amount of CLS was aseptically weighed and dissolved in sterile distilled water, HEPES buffer or sterile human serum to give the required concentrations. CLS solutions were freshly prepared on the day of the experiment, unless otherwise stated.

#### 2.2.9.2 CMS solution

An accurate amount of CMS was aseptically weighed and dissolved in sterile distilled water, HEPES buffer, horse blood or human serum to give the required concentrations. CMS solutions were freshly prepared on the day of the experiment, unless otherwise stated.
2.2.9.3 Antibiotics filter paper discs

After the antibiotic solutions were freshly prepared, 6mm diameter filter paper discs (Whatman AA disks, FDH-700-060F) were touched into the surface of the prepared CLS or CMS concentrations and placed onto previously prepared and inoculated agar plates. Four antibiotic filter paper discs were placed on each sterile 90mm Petri dishes, with the aid of sterile forceps. 36 or 49 antibiotics filter paper discs were placed on each large antibiotic assay plate 245mm x 245mm x 25mm with the aid of sterile forceps. The filter paper discs were arranged on the agar in a random Latin square design to eliminate bias from irregularities.

2.2.10 Preparation of bioassay plates

2.2.10.1 Single layer bioassay plate

Specified agars were prepared and autoclaved as detailed in section 2.1.1.1, melted by boiling in water bath and then cooled to 50°C. 20ml of the molten agar was dispensed aseptically into sterile 90mm petri dishes and allowed to set for 30 minutes in a laminar air flow cabinet. The plates were prepared and used within 2 days. These plates were inoculated using the streaking method detailed in section 2.2.11.1.

2.2.10.2 Double layer bioassay plate

A double layer technique was used in the CLS and CMS bioassay. This technique facilitates the penetration of both antibiotics into the agar or gel, as both forms of colistin (CLS and CMS) have poor diffusion properties through the usual strength agar giving small inhibition zones.

The double layer technique consists of two layers either of agar or gel: the usual strength agar (hard agar) at the bottom of the plate, and the top layer which is the soft (seed) agar that contains a low content of agar (0.4% agar) or gel mixed with indicator organism. Specified usual strength agar (base layer agar) was prepared and autoclaved as detailed in section 2.1.1.1. 20ml of the molten base agar was dispensed aseptically into sterile 90mm Petri dishes and allowed to set for 30 minutes in a laminar air flow cabinet.
Specified seed layers, either gel (prepared and autoclaved as detailed in section 2.1.1.2) or agar layer which contained 0.4% agar (prepared and autoclaved as detailed in section 2.1.1.1). 5ml of the seeded gel or agar was dispensed carefully onto the solidified base layer and allowed to set for 30 minutes. The seed layer was inoculated by the direct inoculating method as detailed in section 2.2.11.2.

For the large antibiotic assay plates 245mm x 245mm x 25mm (Fisher Scientific, DIS-988-010M), the above procedure was followed using 200ml of base layer and 50ml of seed layer.

2.2.11 Inoculating bioassay plates
The plates of single agar layer were inoculated by the streaking method, while plates of double layers were inoculated by the direct inoculating method.

2.2.11.1 Streaking method
Within 20 minutes after adjusting the concentration of the bacterial suspension, a sterile cotton swab was dipped into the suspension by pressing firmly against the inside wall of the tube just above the fluid level, the swab was rotated to remove excess liquid. The swab was streaked over the surface of the dried medium three times and the plate was rotated after each application to ensure an even distribution of the inoculum.

2.2.11.2 Direct inoculation method
Since the seed layers used in the double layer bioassay were either gel or soft agar layer (0.4% agar), inoculation by streaking would damage the soft layer; therefore the direct inoculation method was used.

For the 90mm Petri dishes, 20µl of specified inoculum concentration was added to 5ml of the gel or molten agar at about 40°C and mixed without introducing air bubbles, the seed layer was dispensed carefully onto the base layer and allowed to set for 30 minutes in a laminar air flow cabinet.

For large antibiotic assay plates (245mm x 245mm x 25mm), the above procedure was carried out using 200µl of inoculum added to 50ml of the top layer.
2.2.12 Using the multipoint applicator

A multipoint applicator A400 (Denley, Bootle, UK) is a device where many different bacteria can be inoculated simultaneously onto each agar plate. It consists of a Perspex tray where the agar plate is placed and 16 channels each loaded with a different organism (about 0.3ml of each bacterium is added into each channel). The user places the agar plate on the Perspex tray and the applicator automatically will deliver 2µl of each of the 16 different bacterial suspensions onto each plate.

2.2.13 Recording and interpreting bioassay results

After the filter paper discs were placed on the plates, they were incubated under the specified conditions for the period of time detailed. After incubation, the diameters of the inhibition zones were measured (excluding the diameter of the disc, 6mm) and recorded in millimetres to an accuracy of 0.1mm using callipers. Zones with hazy edges were measured by neglecting the haze zone and measuring to the point at which there was no effect on the growth of the indicator organism.

2.2.14 Statistical analysis

The calculations of significant differences between the means were determined by one-and two way ANOVA using Tukey’s method. Probability values (p-value) were considered significant when less than 0.05 (95% confidence). These calculations were performed using the SPSS v17.0 statistical package.

2.2.15 Using the Bead beater (Biospec, UK)

The Bead Beater is a commercially available device that will disrupt over 90% of the cells in a suspension in less than 5 minutes of operation by high speed blending with slurry of small beads.
The homogenisation procedure involves a cell 'cracking' action where cell membranes will still appear to be intact when observed under the microscope to minimize over-heating the Bead Beater was operated with an ice water jacket for one minute and then the homogenisation was stopped for one minute, cycling until homogenisation was complete. The bead size used in this project was 0.1mm diameter as recommended for bacteria.
CHAPTER THREE

Optimizing microbiological assays of Colistin sulphate and colistin methanesulphonate
3.1 Introduction
Generally antibiotics are assayed by both chemical and microbiological assays. Chemical methods such as HPLC are more accurate and more specific than the microbiological assays but confidence in a chemical assay depends on its harmonisation with the microbiological assay (Higuchi and Conners, 1961). In some cases the chemical assay might measure a breakdown product which has no microbiological activity.

Serum concentrations of CLS and CMS can be measured by using standard microbiological agar techniques with *B. bronchiseptica* as indicator organism. However, these methods lack sensitivity and selectivity (Leroy et al, 1989) and require long incubation period where colistin base will formed (Schwartz et al, 1959; Barnett et al, 1964; Beveridge and Martin, 1966; Li et al, 2001a; Bergen et al, 2006).

The objective of this study was to carry out experiments in an attempt to optimize this analytical technique for future application in the project.

The current standard microbiological method for the analysis of colistin is given in the British Pharmacopoeia 2010. In attempting to develop a more accurate and discriminating assay system two approaches were therefore possible:

- To take the current standard method and modify to try to optimise performance.
- To start with a clean sheet and analyse each component of the assay to engineer the most favourable conditions for each part of the system.

It was decided to opt for the second strategy and then compare the optimised system with the current standard.

3.2 Optimizing CMS bioassay

3.2.1 Screening of bacteria by agar disc diffusion method
The agar disc diffusion method is normally performed to determine the sensitivity of a microorganism to a specified antibiotic. The method which was employed here used a wide range of Gram-negative bacteria on a single layer of agar to screen the most appropriate bacterial indicator for CMS bioassay.
One loopful from each bacterium (Table 3.1) was taken from the original TS broth culture into 10ml single strength TS broth (media prepared as detailed in section 2.1.1.1) and incubated at 37°C overnight. After incubation, this was standardised to produce $10^7$ Cfu/ml in TS broth (as detailed in section 2.2.1). Cultures of $10^7$ Cfu/ml were streaked (as detailed in section 2.2.11.1) onto plates of ISA (prepared as detailed in section 2.2.10.1).

Aqueous solutions of various CMS concentrations of 0.0023, 0.0045, 0.0068 and 0.0091mmol/L (4, 8, 12 and 16mg/L) were prepared as detailed in section 2.2.9.2. Sterile 6mm diameter filter paper discs were touched onto the surface of the prepared CMS solutions and placed onto previously prepared and inoculated agar plates. Triplicate plates (each plate contained four discs of various CMS concentrations) were incubated at 37°C for 16-18 hours.

**Results**

Although all the bacteria grew well on each plate, none of the plates showed any inhibition zones with any of the CMS concentrations used in the experiment (Table 3.1).

The lack of inhibition zones may be due to a number of reasons such as:

a. The bacterial inoculum may have been too heavy  
b. The CMS may have been inactive  
c. The bacteria employed in the experiment may not have been sensitive to the antibiotic.  
d. The agar may have caused interference with CMS.  
e. The CMS may not have been able to diffuse through the agar.

These reasons will be tested individually to detect which one of them is the reason behind the lack of inhibition zones.
Table 3.1: Results of CMS diffusion agar assay.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter</em> CANADA 140 (Brighton University)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td>A. <em>lwoffii</em> R46383 (Brighton University)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em> (ATCC 8090)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em> (NCTC 10006)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>Enterobacter cloaca</em> (Brighton University)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>E. coli</em> (NCIB 8879)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>E. coli</em> (NCTC 8196)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em> (ATCC 11228)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (NCTC 11228)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (ATCC 9027)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (NCTC 6749)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (NCIMB 10548)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>Salmonella abony</em> (Brighton University)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>Salmonella arizona</em> (ACTC 8297)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>Serratia liquifaciens</em> (ATCC 27595)</td>
<td>No inhibition zone</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> (Brighton University)</td>
<td>No inhibition zone</td>
</tr>
</tbody>
</table>

3.2.2 Effect of the inoculum size on CMS bioassay

This experiment was carried out to investigate whether bacterial inoculum size used in the agar diffusion assay was responsible for the lack of inhibition zones. If the bacterial culture concentrations used in the previous experiment were too high, increased concentrations of antibiotic or lower concentrations of bacterial culture would be required.

*P. aeruginosa* was used as it is known to be sensitive against colistin (Geles *et al*, 2001; Pitt *et al*, 2003), the procedure 3.2.1 was carried out in triplicate with three strains of *P. aeruginosa* (NCIMB 10548), (ATCC 9027) and (NCTC 6749) using an inoculum size of $10^6$, $10^7$ or $10^8$ Cfu/ml (prepared and standardized as detailed in section 2.2.1).
Sterile discs were touched onto the surface of the prepared CMS solution of concentration of 0.0091mmol/L (16mg/L) and placed onto previously prepared and inoculated agar plates.

**Results**

For bacterial concentration of $10^6$ Cfu/ml, light bacterial growth was observed, while for other inoculum sizes such as $10^7$ Cfu/ml and $10^8$ Cfu/ml, good bacterial growth was evident but the antibiotic solution did not produce any inhibition zones. These results show that a high bacterial culture concentration was not the reason for the lack of zones of inhibition.

### 3.2.3 Incorporating CMS in the media

Since the agar diffusion experiments did not produced any inhibition zones against the employed Gram-negative bacteria, this experiment was conducted to determine whether CMS had an antibacterial effect at the concentrations of 0.00007mmol/L to 0.0045mmol/L (0.125 to 8mg/L). This experiment was also designed to test the sensitivity of the indicator bacteria to CMS. This experimental design eliminated the need for the CMS molecules to diffuse through the agar as the antibiotic was pre-mixed with the agar.

CMS aqueous solutions were prepared as detailed in section 2.2.9.2. These solutions were incorporated in the molten ISA single layer agar plates prepared as detailed in section 2.2.10.1 so that the final concentrations of CMS in 20ml of agar were: 0.00007, 0.0001, 0.0003, 0.0006, 0.0011, 0.0023 and 0.0045mmol/L (0.125, 0.25, 0.5, 1, 2, 4 and 8mg/L).

Bacterial cultures of various Gram-negative bacteria were standardised to give $10^8$ Cfu/ml in TS broth as detailed in section 2.2.1 and pipetted into the cavities of a Perspex tray and a multipoint applicator (as detailed in section 2.2.12) was used to transfer the suspensions to the agar plates. After inoculation the plates were incubated overnight at 37°C and growth on the surface of the plates was detected. This experiment was conducted in triplicates with a control plate free from antibiotic.
Results

Table 3.2: Effect of CMS on Gram-negative bacteria.

<table>
<thead>
<tr>
<th>Organism 10^8 Cfu/ml.</th>
<th>CMS concentration mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00007</td>
</tr>
<tr>
<td>Acinetobacter CANADA 140</td>
<td>2</td>
</tr>
<tr>
<td>(Brighton University)</td>
<td></td>
</tr>
<tr>
<td>E. coli (NCIB 8879)</td>
<td>2</td>
</tr>
<tr>
<td>P. aeruginosa (NCIMB 10548)</td>
<td>2</td>
</tr>
<tr>
<td>Salmonella abony (Brighton University)</td>
<td>2</td>
</tr>
<tr>
<td>Salmonella arizona (ACTC 8297)</td>
<td>2</td>
</tr>
</tbody>
</table>

Growth of each microorganism was initially recorded in numerical terms (0, 1 and 2) in direct relationship with its density. Thus ‘0’ signified no growth, ‘1’ indicated a visible degree of growth but less then that shown by the control and ‘2’ was a growth equal to that on the control plate.

The control (bacteria without antibiotic) showed positive growth for all the organisms. This experiment revealed that CMS had antibiotic activity at the concentrations used 0.0045mmol/L (8 mg/L) in the agar diffusion assay (Table 3.2) and thus eliminated the possibility of the bacteria used being insensitive to the CMS. This experiment clearly demonstrated that the agar itself did not interfere with the activity of the CMS but the problem appeared to be diffusion through the agar.
E. coli (NCIB 8879) showed an increased sensitivity (MIC of 0.0023mmol or 4mg/L) to CMS compared to the other Gram-negative bacteria tested (MIC of 0.0045mmol or 8mg/L) (Table 3.2). Further experiments were conducted in order to facilitate CMS diffusion through the agar.

3.2.4 Investigations into the diffusion of CMS into agar under different experimental conditions

The previous experiment (3.2.3) showed that CMS had poor diffusion properties through the agar. To investigate these diffusion problems, various conditions of incubation temperatures, pre-diffusion times and bacterial inoculum size were tested in order to optimize the CMS bioassay. The experimental design was also modified by using a double layer agar to facilitate CMS diffusion through the gel network. It consisted of normal strength agar as a base layer and a top seed layer which contained a lower content of agar (0.4% agar). This seed agar contained the indicator organism and the lower strength of the gelling agent thus facilitating the penetration of CMS through the agar.

3.2.4.1 Incubation conditions and inoculum size

Different incubation temperatures affect bacterial growth and therefore have an impact on the size and clarity of the inhibition zones; CMS bioassay plates were incubated at 30°C or 37°C with or without a pre-diffusion period at 4°C. Pre-diffusion time allows CMS to diffuse into the agar while the bacterial growth is inhibited by the reduced temperature.

E. coli NCIB 8879 (prepared as detailed in section 2.2.1), which showed increased sensitivity to CMS among the other Gram-negative bacteria, was selected as assay indicator organism to indicate further the most appropriate methodology.

The experiment described in section 3.2.1 was performed using E. coli (NCIB 8879) suspensions at concentrations of $10^4$, $10^5$, $10^6$, $10^7$ or $10^8$ Cfu/ml (prepared and standardized as detailed in section 2.2.1). Sterile discs of CMS solution 0.0181mmol/L (32mg/L) were placed on previously prepared and inoculated agar plates. This experiment was conducted in triplicate.
These plates were incubated under the following conditions:
A- Immediately incubated at 37°C overnight.
B- Pre-diffused for four hours at 4°C, and then incubated at 37°C overnight.
C- Immediately incubated at 30°C overnight.
D- Pre-diffused for four hours at 4°C, and then incubated at 30°C overnight.

Results

Table 3.3: Effect of different incubation conditions on CMS bioassay.

<table>
<thead>
<tr>
<th>Incubation conditions:</th>
<th>$10^4$ Cf u/ml</th>
<th>$10^5$ Cf u/ml</th>
<th>$10^6$ Cf u/ml</th>
<th>$10^7$ Cf u/ml</th>
<th>$10^8$ Cf u/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediately incubated at 37°C.</td>
<td>No clear zone of inhibition.</td>
<td>No clear zone of inhibition.</td>
<td>1.5mm Not clear inhibition Zone.</td>
<td>No inhibition Zone.</td>
<td>No inhibition Zone.</td>
</tr>
<tr>
<td>Pre-diffused 4 hours at 4°C, and then incubated at 37°C.</td>
<td>No clear zone of inhibition.</td>
<td>No clear zone of inhibition.</td>
<td>3.2mm Fairly clear zone</td>
<td>3mm Fairly clear zone</td>
<td>No inhibition Zone</td>
</tr>
<tr>
<td>Immediately incubated at 30°C.</td>
<td>No clear zone of inhibition.</td>
<td>No clear zone of inhibition.</td>
<td>Hazy zone</td>
<td>Hazy zone</td>
<td>No inhibition Zone</td>
</tr>
<tr>
<td>Pre-diffused 4 hours at 4°C, and then incubated at 30°C.</td>
<td>No clear zone of inhibition.</td>
<td>No clear zone of inhibition.</td>
<td>Hazy zone</td>
<td>Hazy zone</td>
<td>No inhibition Zone</td>
</tr>
</tbody>
</table>
Bacterial growth was not even at bacterial concentrations of $10^4$ or $10^5$ Cfu/ml, while concentrations of $10^8$ Cfu/ml produced a dense lawn.

These results (Table 3.3) show that *E.coli* (NCIB 8879) at a concentration of $10^6$ or $10^7$ Cfu/ml, in a system pre-diffused for four hours at 4°C, and then incubated at 37°C overnight produced slightly improved results but still not sufficiently good to be used as an assay method.

### 3.2.4.2 Experimental design

As colistin (both forms) diffuse poorly in the agar, a double layer agar technique combined with pre-diffusion time at 4°C for four hours was used to facilitate CMS diffusion through the agar. A double agar technique was tested using CMS to investigate whether the lack of inhibition zones in the agar diffusion assay was a result of the poor diffusion properties of CMS, perhaps due to its large molecular weight.

The agar disc diffusion experiment was performed in 90mm Petri dishes by dispensing 20ml of the molten ISA as a base layer followed by 5ml of molten ISB mixed with 0.4% agar (prepared as detailed in section 2.2.10.2) which was inoculated with 20µl of $10^4$, $10^5$, $10^6$, $10^7$ or $10^8$ Cfu/ml *E.coli* (NCIB 8879), prepared and standardized as detailed in section 2.2.1.

Sterile discs were touched onto the surface of the prepared 0.0181mmol/L (32mg/L) CMS solution (prepared as detailed in section 2.2.9.2) and placed onto previously prepared and inoculated agar plates (in triplicates).

These plates were incubated under conditions same as experiments 3.2.4.1.

### Results

Bacterial growth was not uniform at bacterial concentrations of $10^4$ or $10^5$ Cfu/ml.

These results (Table 3.4) reveal that *E.coli* at concentrations of $10^7$ or $10^8$ Cfu/ml, in a system pre-diffused for four hours at 4°C, and then incubated at 37°C overnight in double layer media produced fairly clear but still small zones of inhibition.
Table 3.4: Effect of different incubation conditions and double layer media on CMS bioassay.

<table>
<thead>
<tr>
<th>Incubations condition:</th>
<th>$10^4$ Cfu/ml</th>
<th>$10^5$ Cfu/ml</th>
<th>$10^6$ Cfu/ml</th>
<th>$10^7$ Cfu/ml</th>
<th>$10^8$ Cfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediately incubated at 37°C.</td>
<td>No clear zone of inhibition.</td>
<td>No clear zone of inhibition.</td>
<td>Unreadable zone.</td>
<td>4.2mm Not clear.</td>
<td>3mm Not clear</td>
</tr>
<tr>
<td>Pre-diffused 4 hours at 4°C, and then incubated at 37°C.</td>
<td>No clear zone of inhibition.</td>
<td>No clear zone of inhibition.</td>
<td>5.3 mm Fairly clear</td>
<td>5.1mm Very clear</td>
<td>5.1mm Very clear</td>
</tr>
<tr>
<td>Immediately incubated at 30°C.</td>
<td>No clear zone of inhibition.</td>
<td>No clear zone of inhibition.</td>
<td>Unreadable zone.</td>
<td>3.1mm Not clear</td>
<td>3.3mm Not clear</td>
</tr>
<tr>
<td>Pre-diffused 4 hours at 4°C, and then incubated at 30°C.</td>
<td>No clear zone of inhibition.</td>
<td>No clear zone of inhibition.</td>
<td>Unreadable zone.</td>
<td>3.2mm Not clear</td>
<td>2.2mm Not clear</td>
</tr>
</tbody>
</table>

3.2.5 Investigation into the optimum combination of base and seed layer

Since CMS has poor diffusion properties in agar, various combination of base and seed layer were tested in an attempt to improve the bioassay system.
The experiment 3.2.4.2 was carried out with a various combinations (pH ranged from 6.5-7.4) of base and seed agar (Tables from 3.5 to 3.10), using 20µl of *E.coli* (NCIB 8879) at a concentration of $10^8$ Cfu/ml (prepared and standardized as detailed in section 2.2.1). Sterile filter paper discs were touched onto the surface of the CMS solutions prepared in distilled water and buffer pH6 as described in section 2.1.1.3 at concentrations of 0.0181, 0.0363 and 0.0725mmol/L (32, 64 and 128mg/L). Duplicate plates were pre-diffused for 4 hours at 4°C and then incubated at 37°C overnight.

Those combinations which gave the largest and clearest zones were replicated up to three times.

**Results**

*Tables 3.5 to 3.10* show CMS inhibition zone diameters (mm) using combinations of base and seed agar, CMS was diluted in distilled water and buffer pH6 (Tables from 3.5 to 3.10).

Some of these combinations (*Tables 3.6A, 3.8A, and 3.9A*) showed reasonable results in term of size, clarity and sharpness of the inhibition zones.

The most suitable media (*Table 3.6A*) in terms of size of the inhibition zones was 20ml of DSTA as a base layer and 5ml of PW (to which technical agar 0.4% was added) as seed layer.
Table 3.5.A
Base layer: ISA pH 7.4. Seed layer as shown in table
CMS made up in distilled water (n=2).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No. 3 broth + Agar 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181 mmol/L</td>
<td>4.9mm</td>
<td>5.0mm</td>
<td>5.1mm</td>
<td>3.8mm</td>
<td>3.5mm</td>
</tr>
<tr>
<td>0.0363 mmol/L</td>
<td>6.4mm</td>
<td>6.6mm</td>
<td>6.8mm</td>
<td>5.5mm</td>
<td>5.3mm</td>
</tr>
<tr>
<td>0.0725 mmol/L</td>
<td>7.1mm</td>
<td>7.0mm</td>
<td>7.4mm</td>
<td>6.2mm</td>
<td>6.4mm</td>
</tr>
</tbody>
</table>

Table 3.5.B
Base layer: ISA pH 7.4. Seed layer as shown in table
CMS made up in buffer pH6 (n=2).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS Broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No. 3 broth + Agar 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181 mmol/L</td>
<td>5.1mm</td>
<td>4.2mm</td>
<td>3.5mm</td>
<td>3.9mm</td>
<td>2.9mm</td>
</tr>
<tr>
<td>0.0363 mmol/L</td>
<td>6.5mm</td>
<td>6.4mm</td>
<td>5.6mm</td>
<td>6.2mm</td>
<td>5.4mm</td>
</tr>
<tr>
<td>0.0725 mmol/L</td>
<td>7.0mm</td>
<td>7.5mm</td>
<td>6.1mm</td>
<td>7.0mm</td>
<td>6.1mm</td>
</tr>
</tbody>
</table>
Table 3.6.A

Base layer: DSTA, pH 7.4. Seed layer as shown in table.

CMS made up in distilled water (n=3).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS Broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No3 broth + Agar 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181mmol/L</td>
<td>6.0mm</td>
<td>6.3mm</td>
<td>6.7mm</td>
<td>6.2mm</td>
<td>2.3mm</td>
</tr>
<tr>
<td>0.0363mmol/L</td>
<td>6.5mm</td>
<td>7.1mm</td>
<td>7.1mm</td>
<td>6.4mm</td>
<td>5.2mm</td>
</tr>
<tr>
<td>0.0725mmol/L</td>
<td>7.0mm</td>
<td>8.3mm</td>
<td>8.8mm</td>
<td>6.9mm</td>
<td>6.9mm</td>
</tr>
</tbody>
</table>

Table 3.6.B

Base layer: DSTA. Seed layer as shown in table.

CMS made up in buffer pH6 (n=2).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No. 3 broth + Agar 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181mmol/L</td>
<td>2.9mm</td>
<td>4.3mm</td>
<td>5.5mm</td>
<td>3.8mm</td>
<td>5.5mm</td>
</tr>
<tr>
<td>0.0363mmol/L</td>
<td>5.5mm</td>
<td>6.2mm</td>
<td>5.9mm</td>
<td>6.4mm</td>
<td>6.4mm</td>
</tr>
<tr>
<td>0.0725mmol/L</td>
<td>6.5mm</td>
<td>7.3mm</td>
<td>7.0mm</td>
<td>6.4mm</td>
<td>7.2mm</td>
</tr>
</tbody>
</table>
Table 3.7.A

Base layer: TSA pH 7.3. Seed layer as shown in table.

CMS made up in distilled water (n=2).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No. 3 broth + Agar 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181mmol/L</td>
<td>2.4mm</td>
<td>4.2mm</td>
<td>5.0mm</td>
<td>4.5mm</td>
<td>3.1mm</td>
</tr>
<tr>
<td>0.0363mmol/L</td>
<td>5.5mm</td>
<td>5.9mm</td>
<td>6.9mm</td>
<td>6.7mm</td>
<td>5.3mm</td>
</tr>
<tr>
<td>0.0725mmol/L</td>
<td>7.6mm</td>
<td>6.4mm</td>
<td>7.8mm</td>
<td>7.7mm</td>
<td>6.5mm</td>
</tr>
</tbody>
</table>

Table 3.7.B

Base layer: TSA pH 7.3. Seed layer as shown in table.

CMS made up in buffer pH6 (n=2).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No. 3 broth + Agar 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181mmol/L</td>
<td>2.5mm</td>
<td>3.2mm</td>
<td>3.5mm</td>
<td>4.4mm</td>
<td>2.8mm</td>
</tr>
<tr>
<td>0.0363mmol/L</td>
<td>5.1mm</td>
<td>5.2mm</td>
<td>4.5mm</td>
<td>6.1mm</td>
<td>5.0mm</td>
</tr>
<tr>
<td>0.0725mmol/L</td>
<td>6.9mm</td>
<td>6.7mm</td>
<td>5.2mm</td>
<td>7.8mm</td>
<td>7.4mm</td>
</tr>
</tbody>
</table>
Table 3.8.A
Base layer: Antibiotic media No.3 pH 6.5. Seed layer as shown in table.
CMS made up in distilled water (n=3).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS broth+ Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No. 3 broth + Agar0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181mmol/L</td>
<td>5.0mm</td>
<td>2.6mm</td>
<td>5.3mm</td>
<td>3.7mm</td>
<td>3.2mm</td>
</tr>
<tr>
<td>0.0363mmol/L</td>
<td>6.8mm</td>
<td>5.2mm</td>
<td>7.0mm</td>
<td>5.4mm</td>
<td>4.5mm</td>
</tr>
<tr>
<td>0.0725mmol/L</td>
<td>7.2mm</td>
<td>7.1mm</td>
<td>7.8mm</td>
<td>7.0mm</td>
<td>7.1mm</td>
</tr>
</tbody>
</table>

Table 3.8.B
Base layer: Antibiotic media No.3 pH 6.5. Seed layer as shown in table.
CMS made up in buffer pH6 (n=2).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No. 3 broth + 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181mmol/L</td>
<td>6.4mm</td>
<td>2.3mm</td>
<td>3.8mm</td>
<td>3.2mm</td>
<td>2.9mm</td>
</tr>
<tr>
<td>0.0363mmol/L</td>
<td>6.8mm</td>
<td>4.4mm</td>
<td>6.0mm</td>
<td>5.0mm</td>
<td>4.8mm</td>
</tr>
<tr>
<td>0.0725mmol/L</td>
<td>7.2mm</td>
<td>6.9mm</td>
<td>6.8mm</td>
<td>6.5mm</td>
<td>5.7mm</td>
</tr>
</tbody>
</table>
### Table 3.9.A
Base layer: NA pH 7.4. Seed layer as shown in table
CMS made up in distilled water (n=3).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No. 3 broth + Agar 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181mmol/L</td>
<td>2.8mm</td>
<td>2.1mm</td>
<td>5.5mm</td>
<td>4.6mm</td>
<td>3.5mm</td>
</tr>
<tr>
<td>0.0363mmol/L</td>
<td>4.3mm</td>
<td>5.7mm</td>
<td>7.2mm</td>
<td>6.4mm</td>
<td>5.2mm</td>
</tr>
<tr>
<td>0.0725mmol/L</td>
<td>5.3mm</td>
<td>5.9mm</td>
<td>7.9mm</td>
<td>7.1mm</td>
<td>6.9mm</td>
</tr>
</tbody>
</table>

### Table 3.9.B
Base layer: NA pH 7.4. Seed layer as shown in the table
CMS made up in buffer pH6 (n=2).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>NB + Agar 0.4%</th>
<th>TS broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No. 3 broth + Agar 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181mmol/L</td>
<td>2.2mm</td>
<td>2.4mm</td>
<td>3.3mm</td>
<td>3.7mm</td>
<td>2.3mm</td>
</tr>
<tr>
<td>0.0363mmol/L</td>
<td>4.0mm</td>
<td>5.6mm</td>
<td>5.7mm</td>
<td>5.9mm</td>
<td>5.4mm</td>
</tr>
<tr>
<td>0.0725mmol/L</td>
<td>4.9mm</td>
<td>6.2mm</td>
<td>6.2mm</td>
<td>6.1mm</td>
<td>6.0mm</td>
</tr>
</tbody>
</table>
Table 3.10

Base layer: R2A Seed layer as shown in table

CMS made up in distilled water (n=2).

<table>
<thead>
<tr>
<th>Concentration of CMS</th>
<th>PW + Agar 0.4%</th>
<th>TS broth+ Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0181mmol/L</td>
<td>5.2mm</td>
<td>3.9mm</td>
</tr>
<tr>
<td>0.0363mmol/L</td>
<td>6.7mm</td>
<td>5.5mm</td>
</tr>
<tr>
<td>0.0725mmol/L</td>
<td>7.4mm</td>
<td>7.1mm</td>
</tr>
</tbody>
</table>

3.2.6 Establishing optimum pre-diffusion time for CMS bioassay

A pre-diffusion time of four hours at 4°C for CMS bioassay was used previously. This experiment was carried out to select an optimum pre-diffusion time that gave the best results for CMS bioassay. The most suitable media were chosen on the basis of the size and sharpness of the inhibition zones as in experiment 3.2.5.

The agar disc diffusion experiment was performed in 90mm Petri dishes by dispensing 20ml of DSTA as a base layer followed by 5ml of molten seeded agar (PW + technical agar 0.4%) (Media prepared as detailed in section 2.2.10.2). The experiment was performed using 20µL of *E.coli* (NCIB 8879) suspension at a concentration of $10^8$ Cfu/ml prepared as described in section 2.2.1.

Sterile discs were touched onto the surface of the prepared CMS solution of concentration of 0.0181mmol/L or 32mg/L (prepared as detailed in section 2.2.9.2) and placed onto previously prepared and inoculated agar plates.

These triplicate plates were allowed to pre-diffuse at 4°C for two, three, four, five and 24 hours and then incubated at 37°C overnight.
Results

Table 3.11: Effect of the different pre-diffusion times on CMS bioassay.

<table>
<thead>
<tr>
<th>Pre-diffusion time (Hours)</th>
<th>Inhibition zones (mm)</th>
<th>Stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.6</td>
<td>0.28</td>
</tr>
<tr>
<td>3</td>
<td>3.9</td>
<td>0.26</td>
</tr>
<tr>
<td>4</td>
<td>6.8</td>
<td>0.36</td>
</tr>
<tr>
<td>5</td>
<td>6.7</td>
<td>0.43</td>
</tr>
<tr>
<td>24</td>
<td>7</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Table 3.11 showed that the pre-diffusion of CMS at 4°C for four hours was necessary to optimize the bioassay. There was no significant difference between pre-diffusion at 2 or 3 hours (P>0.5), while significant difference was observed between pre-diffusion time of 3 and 4 hour (P<0.5).

These results revealed that pre-diffusion for 24 hours (inhibition zone was 7mm) had little enhanced effect compared to four hours on CMS bioassay (P>0.5), calculated as detailed in section 2.2.14.

Since not much difference was observed in pre-diffusion time of 4 and 24 hours, the optimal pre-diffusion time was chosen to be four hours.

3.2.7 CMS bioassay using different bacterial indicators

A CMS bioassay was performed using different indicator bacteria including the bacterium recommended by the British Pharmacopoeia 2010 (see appendix).

This experiment was conducted in order to select the most sensitive organism to the antibiotics under investigation.

The agar disc diffusion experiment was performed by dispensing 200ml of the DSTA as a base layer followed by 50ml of the molten seeded agar (PW + technical agar 0.4%) prepared as detailed in section 2.2.10.2 in a large bioassay plate 245mm x 245mm x 25mm.
The molten seed layer was inoculated with 200µL of $10^8$ Cfu/ml *E.coli* (NCIB 8879) or *B. bronchiseptica* (NCTC 8344) prepared as in section 2.2.1.

Sterile discs were touched onto the surface of the CMS concentrations of 0.017, 0.034, 0.0737, 0.085, 0.1133, 0.1417, 0.17, 0.1983, 0.2267, 0.225, 0.2833 and 0.34mmol/L (30, 60, 130, 150, 200, 250, 300, 350, 400, 450, 500 and 600mg/L) (prepared as detailed in section 2.2.9.2) and placed onto previously prepared and inoculated plates (each experiment involved a set of 6 CMS concentrations on each large bioassay plate; duplicate plates were used for each organism).

**Results**

**Figure 3.1** demonstrated that *B. bronchiseptica* was the most suitable organism for CMS bioassay in terms of size of inhibition zones and linearity of the dose response.

![Inhibition zones for CMS using different indicator bacteria.](image)

**Figure 3.1:** Inhibition zones for CMS using different indicator bacteria.

(The two lines of each experiment indicates the duplicate experiments using the same organism)
3.3 Applying previously optimized conditions to *Bordetella bronchiseptica* (NCTC 8344)

3.3.1 Agar disc diffusion method

The previous experiment (3.2.7) revealed that *B. bronchiseptica* was the most sensitive organism among the tested organisms for CMS. Therefore it was used for the CMS bioassay as it produced the best results in terms of clarity and sharpness of inhibition zones.

The experiment 3.2.1 (single agar layer) was repeated using $10^8$ Cfu/ml of *B. bronchiseptica* (NCTC 8344) prepared as detailed in section 2.2.4. Sterile discs were touched onto the surface of the prepared 0.017mmol/L (30mg/L) CMS solution and placed onto previously prepared and inoculated agar plates. Triplicate plates were incubated as detailed in figure 3.2.

**Results**

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Inhibition zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Incubation at 37°C overnight.</td>
<td>![Bar graph A]</td>
</tr>
<tr>
<td>B. Pre-diffusion 4 hours at 4°C, and then incubated at 37°C overnight.</td>
<td>![Bar graph B]</td>
</tr>
<tr>
<td>C. Incubation at 30°C overnight.</td>
<td>![Bar graph C]</td>
</tr>
<tr>
<td>D. Pre-diffusion 4 hours at 4°C, and then incubated at 30°C overnight.</td>
<td>![Bar graph D]</td>
</tr>
</tbody>
</table>

**Figure 3.2:** Effect of various incubation conditions and pre-diffusion times on CMS bioassay. (Single layer of agar was used). n=3± Stdev
This experiment indicated that agar diffusion assay using single layer of the agar produced small inhibition zones even with \textit{B. bronchiseptica} (Figure 3.2).

A statistically significant difference was observed for the mean of the different incubation conditions used in the experiment. $P$ value < 0.05 calculated as detailed in section 2.2.14.

3.3.2 Effect of the experimental design, incubation temperatures and pre-diffusion times on CMS bioassay

Experimental design, incubation temperatures and pre-diffusion times are important parameters in microbiological assays and have an impact on the CMS bioassay; therefore this experiment was performed to check if the previously optimized experimental design, incubation temperatures and pre-diffusion times on \textit{E.coli} were applicable to \textit{B. bronchiseptica} (NCTC 8344).

Experiment 3.2.4.2 (double layer agar) was performed using 20$\mu$l of $10^8$ CfU/ml \textit{B. bronchiseptica} prepared as in section 2.2.4. Triplicate plates were incubated under different conditions as detailed in figure 3.3.

Results

Figure 3.3: Effect of various incubation conditions and pre-diffusion times on CMS bioassay (double layer of agar was used). $n=3\pm$ Stdev
These results (Figure 3.3) showed that 37°C was more suitable incubation temperature for CMS bioassay than 30°C in terms of size and clarity of the inhibition zones. In addition, pre-diffusion for four hours at 4°C prior to overnight incubation at 37°C gave enhanced zone diameter and clarity of zone edge. It was shown that conditions optimized for *E. coli* applied also to *B. bronchiseptica*.

A statistically significant difference was observed for the mean of the different incubation conditions used in the experiment. *P* value < 0.05 calculated as detailed in section 2.2.14.

### 3.3.3 Effect of media

Many different media were tested to choose the most suitable media when using *B. bronchiseptica* (NCTC 8344) prepared as detailed in 2.2.4 as indicator organism. The experiment 3.3.2 was carried out with number of combinations of base and seed agar (Table 3.12). 20μL of 10⁸ Cfu/ml *B. bronchiseptica* was employed.

Duplicate plates were pre-diffused for four hours at 4°C and then incubated at 37°C overnight.

#### Results

**Table 3.12: Effect of media on CMS bioassay using *Bordetella bronchiseptica* indicator.**

<table>
<thead>
<tr>
<th>Seed layer</th>
<th>Base Layer</th>
<th>NB + Agar 0.4%</th>
<th>TS broth + Agar 0.4%</th>
<th>PW + Agar 0.4%</th>
<th>Antibiotic No.3 broth + Agar 0.4%</th>
<th>ISB + Agar 0.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ISA</strong></td>
<td></td>
<td>14.4mm</td>
<td>11.5mm</td>
<td>13.2mm</td>
<td>10.5mm</td>
<td>6.4mm</td>
</tr>
<tr>
<td><strong>TS broth</strong></td>
<td></td>
<td>13.0mm</td>
<td>12.1mm</td>
<td>14.1mm</td>
<td>9.9mm</td>
<td>7.0mm</td>
</tr>
<tr>
<td><strong>DSTA</strong></td>
<td></td>
<td>10.6 mm</td>
<td>13.8mm</td>
<td>15.6mm</td>
<td>11.5mm</td>
<td>8.5mm</td>
</tr>
</tbody>
</table>
The results (Table 3.12) revealed that the conditions optimized for *E. coli* apply to *B. bronchiseptica* as well (DSTA as base layer and PW as seed layer).

### 3.3.4 Establishing the ideal inoculum density

The procedure detailed in experiment 3.3.2 was carried out using 20µl volume of $10^6$, $10^7$, $10^8$ and $10^9$ Cfu/ml of *B. bronchiseptica* (prepared as in section 2.24). The triplicate plates were pre-diffused for four hours at 4°C and then incubated at 37°C overnight.

#### Results

![Inhibition zone diameters](image)

**Figure 3.4: Effect of the inoculum size of *Bordetella bronchiseptica* on CMS bioassay. n=3± Stdev**

The inoculated plates with 20µl of a $10^6$ Cfu/ml inoculum gave zones that were hazy and difficult to read with a poorly defined edge (these results not included in figure 3.4). However, an inoculum of 20µl of $10^7$ Cfu/ml gave zones which were of better quality but still did not provide sufficient clarity for use in a reproducible assay.
This experiment indicated that *B. bronchiseptica* with an inoculum size of 20µl of \(10^8\) Cfus/ml produced reasonable inhibition zone diameters (Figure 3.4) with good degree of clarity and sharpness of the inhibition zones. Inoculum size of 20µl of \(10^9\) Cfus/ml produced small inhibition zones. Inoculum size of 20µl of \(10^8\) Cfus/ml will be used for all future experiments.

3.3.5 Standardization of CMS volume absorbed on filter paper disc

An experiment was conducted to compare the amount of absorption of CMS in distilled water and in serum onto filter paper discs. 10 Empty filter paper discs were weighed and then were touched onto the surface of CMS aqueous solution or CMS in horse blood. The amount of the solutions absorbed was determined by weight difference.

**Results**

An average of 0.020g was absorbed onto filter paper discs from the CMS aqueous solution (Stdev = 0.0001), while the amount of CMS solution in blood was absorbed into the disc was 0.026 g (Stdev = 0.0037). This experiment revealed that standardization of the volume absorbed into the filter paper was required; 25µl was chosen to fill the filter paper.

3.4 CMS calibration curves using *Bordetella bronchiseptica*

3.4.1 Calibration curve of CMS bioassay in aqueous solution

\((0.0003 – 0.017\text{mmol/L})\)

The agar disc diffusion experiment was performed by dispensing 200ml of the DSTA as a base layer followed by 50ml of the molten seeded agar (PW + technical agar 0.4%) (Media prepared as detailed in section 2.1.1.1) in the large bioassay plate. The experiment was performed using 200µL of the \(10^8\) Cfus/ml of *B. bronchiseptica* (NCTC 8344) prepared as described in section 2.2.4. Volumes of 25µl of freshly prepared CMS solution of 0.0003, 0.0009, 0.0011, 0.0014, 0.0017, 0.0023, 0.0028, 0.0057, 0.0085, 0.0113, 0.0142 and 0.017mmol/L (0.5, 1.5, 2, 2.5, 3, 4, 5, 10, 15, 20, 25 and 30mg/L) (prepared as detailed in section 2.2.9.2) were pipetted on a sterile filter paper discs and placed onto
previously prepared and inoculated agar plates (2.2.11.2). Various concentrations were included in triplicate large bioassay plates and were pre-diffused for four hours at 4°C and then incubated at 37°C overnight.

Results

![Graph showing the relationship between log CMS concentration and inhibition zone diameter.](image)

**Figure 3.5:** CMS in water calibration plot using *Bordetella bronchiseptica*. 

CMS at concentrations of below 0.001 mmol/L (2 mg/L) produced very small inhibition zones. Therefore inhibition zones for these concentrations were considered unreliable and not recordable.

These results show that the size of the CMS inhibition zones increased with increasing CMS concentration. The graph of log CMS concentration against zone diameters was linear over the range 0.0011 mmol/L to 0.017 mmol/L (2 mg/L to 30 mg/L) (Figure 3.5).
3.4.2 Calibration curve of CMS bioassay in human serum using standardised CMS filter paper discs

In the clinical situation CMS would be assayed in the presence of blood or serum and so the bioassay was repeated with the CMS diluted in human serum. For this experiment an attempt was made to standardise the volume of solution absorbed onto each disc.

The procedure in experiment 3.4.1 was repeated using large antibiotic bioassay plates. 25µl sterile solutions of CMS in human serum at concentrations of 0.0003, 0.0006, 0.0009, 0.0011, 0.0014, 0.0017, 0.0023, 0.0028, 0.0057, 0.0085, 0.0113, and 0.017mmol (0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 10, 15, 20 and 30mg/L) were pipetted accurately on the discs. This experiment was performed four times (each set of 6 concentrations were included in one bioassay plate). The plates were pre-diffused for four hours at 4°C and then incubated at 37°C overnight.

**Results**

Figure 3.6: CMS bioassays in human serum. n=4± Stdev
For concentrations below 0.0011mmol/L (2mg/L), the inhibition zones were very small and were concealed under the viscous serum which diffused into the agar from the disc. Therefore inhibition zones for these concentrations were considered unreliable and not recordable.

The results shown in figure 3.6 indicate that the relationship between log CMS concentration and zone diameter was linear over the range of 0.0011 to 0.017mmol/L (2-30mg/L).

The blood levels of CMS in patients are likely to range from 10 to 15mg/L or 125-200units/ml (British National Formulary, 2010). Thus figure 3.6 suggests that the bioassay is sufficiently sensitive to be able to detect CMS in patients.

3.5 CLS bioassay using previously optimized method

The previously optimized CMS bioassay was applied to CLS at the same molar concentrations in distilled water and the results were compared.

The procedure 3.4.1 was repeated using 25µl of 0.0028, 0.0057, 0.0113, 0.0227, 0.0453, 0.0907mmol/L of CLS and CMS in distilled water (equivalent to 4, 8, 16, 32, 64, 128mg/L of CLS and 5, 10, 20, 40, 80, 160mg/L of CMS respectively), this experiment was performed in duplicate plates.

Results

This experiment (Figure 3.7) showed that CLS has poor diffusion properties through the soft agar (PW + 0.4% agar). The limit of detection of CLS was reduced to 0.0113mmol/L (16mg/L), compared to 0.0028mmol/L (5mg/L) with CMS. Further experiments were therefore carried out to find a suitable agar substitute gelling agent that might facilitate CLS diffusion.
Figure 3.7: CMS and CLS bioassay using previously optimized bioassay
(The two lines of each experiment indicates the duplicate experiments using the same organism)

3.6 Optimization of CLS bioassay

3.6.1 Investigation into a substitute gelling agent

Since CLS has poor diffusion properties through the soft agar, substitute gelling agents were investigated for inclusion in the seed layer (Table 3.13). Agar has a negative charge and since CLS molecules have a positive charge this may contribute to the inability of the antibiotic to diffuse through the agar.

The agar disc diffusion experiment was performed by dispensing 20ml of the molten DSTA prepared as detailed in section 2.1.1.1 as a base layer in a Petri dish (the base layer contained cross-linking agent) followed by 5ml of various substitute gelling agents (Table 3.13) prepared as detailed in section 2.1.1.2 which contained 20µl of $10^8$ Cfu/ml of *B. bronchiseptica* prepared as detailed in section 2.2.4. Filter paper discs were pipetted with 25µl of CLS solution of 0.0227mmol/L (32mg/L) prepared as detailed in section 2.2.9.1
and placed onto the prepared plates. Triplicate plates were pre-diffused four hours at 4 °C and then incubated at 37°C overnight.

**Results**

**Table 3.13: Investigation into the substitute gelling agents.**

<table>
<thead>
<tr>
<th>Seed layer</th>
<th>Cross-link agents</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Sodium alginate in distilled water</td>
<td>1% CaCl₂</td>
<td>- The base layer was turbid (due to the cross-linking agent)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Gel formed at room temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Hazy inhibition zones were observed</td>
</tr>
<tr>
<td>0.5% Carrageenan type I and II in distilled water</td>
<td>2% KCl</td>
<td>- Base layer was clear</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Gel formed at room temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Light bacterial growth was observed</td>
</tr>
<tr>
<td>1% Gum guar in distilled water</td>
<td>1% CaCl₂</td>
<td>- Very hard to dissolve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Gel formed at room temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Light bacterial growth was observed</td>
</tr>
<tr>
<td>1% Gum xanthan in distilled water</td>
<td>1% CaCl₂</td>
<td>- Gum xanthan, very hard to dissolve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Light bacterial growth was observed</td>
</tr>
<tr>
<td>0.5%, 1% and 2% Carbopol ultrez 21 in distilled water (pH=3)</td>
<td>4% NaOH</td>
<td>- 0.5% did not gel on the plate within 30min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 1% and 2%, formed gels after 5min but light bacterial growth was observed</td>
</tr>
<tr>
<td>0.25% Phytogel in distilled water</td>
<td>1% CaCl₂</td>
<td>- Turbidity of the base layer occurred (due to the cross-linking agent)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- The phytogel 0.25% in distilled water was easy to spread, Solidified within 5min, allowed bacterial growth and produced a measurable inhibition zone size with CLS (bigger than produced by agar, about 7.4 mm).</td>
</tr>
</tbody>
</table>
As Phytogel 0.25% produced larger inhibition zones than agar, it was chosen as the most appropriate gelling agent; it was crossed linked with calcium chloride in order to form a firmer gel at lower concentrations.

### 3.6.2 Investigation into phytogel as a seed layer

Phytogel is an agar substitute produced from a bacterial fermentation composed of glucuronic acid, rhamnose and glucose. It produces a clear, colourless, high strength gel which aids in detection of microbial contamination (Sigma Aldrich, 2010). Phytogel forms a gel when it is added to distilled water at a high concentration. It may also form gels at lower concentrations when crossed linked with divalent cations such as calcium (Sigma Aldrich, 2010).

The experiment 3.6.1 was carried out with Phytogel 0.25% as a gelling agent; it was crossed linked with calcium chloride in order to form a firmer gel at lower concentrations, 1% or 0.5% of CaCl$_2$ were added and mixed with the base layer (DSTA) to allow diffusion into the seed layer. Other techniques were used to cross link the phytogel by adding different aliquots (0.1, 0.2, 0.4 and 0.5ml) of 1% CaCl$_2$ to the solidified the gel on the plate. To improve the bacterial growth (*B. bronchiseptica*) in the new gelling agent, phytogel was made up with TS broth and NB as diluents. These experiments were performed in triplicate.

### Results

When using phytogel as a gelling agent, it was crossed linked with calcium chloride. Initially, 1% CaCl$_2$ was mixed with the base layer (DSTA): however, turbidity occurred due to CaCl$_2$ precipitation in DSTA. In order to rectify this, two approaches were tested; the concentration of CaCl$_2$ was reduced from 1% to 0.5% but still turbidity occurs. The other approach was to prepare DSTA base agar free from CaCl$_2$ and to cross link the phytogel 0.25% by adding 0.1ml of 1% CaCl$_2$ to the solidified DSTA on the plate (gel was added at the end), this approach was effective and eliminated the turbidity problem in the base layer. In order to improve the bacterial growth in the gelling agent, phytogel was made up with TS broth and NB as diluents; both diluents resulted in solutions which did not gel.
3.6.3 Effect of incubation temperatures and pre-diffusion times on CLS bioassay

Incubation temperature and pre-diffusion times are important parameters in the bioassays. This experiment was carried out to select the most appropriate incubation conditions and optimum pre-diffusion times that optimum results in the CLS bioassay.

The agar disc diffusion experiment was performed by dispensing 20ml of the molten DSTA prepared as detailed in section 2.1.1.1 as a base layer followed by 5ml of 0.25% of phytogel prepared as detailed in section 2.1.1.2, which contained 20µl of 10^8 Cfu/ml of *B. bronchiseptica* prepared as detailed in section 2.2.4. The phytogel was cross linked with 0.1ml of 1% CaCl₂ on the DSTA layer before adding the gelling agent. 25µl of volumes of CLS solution at 0.0227mmol/L (32mg/L) prepared as detailed in section 2.2.9.1 were pipetted accurately on the discs and placed onto the prepared plates. Triplicate plates were incubated under different conditions indicated in figure 3.8.

The plates were allowed to pre-diffuse at 4°C for 0, 2, 3, 4, 5, and 24 hours and then incubated at 30°C or 37°C overnight.

**Results**

![Graph showing inhibition zones under different incubation conditions](image)

**A** Incubation at 37°C overnight.

**B** Pre-diffusion 4 hours at 4°C, and then incubated at 37°C overnight.

**C** Incubation at 30°C overnight.

**D** Pre-diffusion 4 hours at 4°C, and then incubated at 30°C overnight.

**Figure 3.8: CLS inhibition zones under different incubation conditions.**

*n=3± Stdev*
These results (Figure 3.8) showed that 37°C is a more suitable incubation temperature for CLS bioassay than 30°C in terms of size and clarity of the inhibition zones. In addition, the experiment (Figure 3.9) showed the pre-diffusion of CLS bioassay at 4°C for four hours is necessary to optimize the bioassay.

### 3.6.4 Establishing the ideal inoculum density

The procedure detailed in experiment 3.6.3 was carried out using a culture of *B. bronchiseptica*. 20µl volumes of $10^7$, $10^8$ and $10^9$ Cfu/ml prepared as detailed in section 2.2.4 were used as inocula. Triplicate plates were pre-diffused for four hours at 4°C and then incubated at 37°C overnight.
Results

![Graph showing inhibition zone diameters for different inoculum sizes.](image)

**Figure 3.10**: Effect of the inoculum size of *Bordetella bronchiseptica* on CLS bioassay. \( n=3\pm\) Stdev

An inoculum of 20µl of \(10^7\) CFU/ml gave good size inhibition zones but did not provide sufficient clarity for use in a reproducible assay. The experiment showed that using *B. bronchiseptica* with an inoculum of 20µl of \(10^8\) CFU/ml produced reasonable inhibition zones size with good degree of clarity and sharpness of the inhibition zones. (Figure 3.10). Consequently an inoculum of 20µl of \(10^8\) CFU/ml will be used for further investigations.

### 3.7 Calibration curve of CLS and CMS aqueous solutions using optimized agar and gel bioassay

This experiment was performed in order to prepare a standard calibration curve for CLS and compare it with CMS in distilled water using the bioassays optimized for both antibiotics. This calibration plot will enable the determination of unknown concentrations of CLS in aqueous or serum solutions.
3.7.1 Bioassay of CLS and CMS using agar

The agar disc diffusion assay was carried out using 200ml of DSTA as a base layer followed by a seed layer of 50ml of PW + 0.4% agar contained 200µL of the $10^8$ Cfu/ml of *B. bronchiseptica* (NCTC 8344) prepared as in 2.2.4. Sterile filter paper discs were pipetted accurately with 25µl solutions of antibiotic in distilled water at concentrations of 0.0014, 0.0028, 0.0057, 0.0113, 0.0227 and 0.0453mmol/L (equivalent to 2, 4, 8, 16, 32 and 64mg/L of CLS and 2.5, 5, 10, 20, 40 and 80mg/L of CMS) prepared as detailed in section 2.2.9. A set of 6 concentrations were placed on each plate. Triplicate large antibiotic assay plates were pre-diffused for four hours at 4°C and then incubated at 37°C overnight.

3.7.2 Bioassay of CLS and CMS using phytogel as seed layer

This experiment was performed in large antibiotic assay plates. 200ml of DSTA was used as a base agar layer followed by seeded layer of 50ml of 0.25% of phytogel containing 200µl of $10^8$ Cfu/ml *B. bronchiseptica* (NCTC 8344) prepared as detailed in section 2.2.4. The phytogel was cross linked with 1ml of 1% CaCl$_2$ on the DSTA base layer before adding the gelling agent. Sterile filter paper discs were pipetted accurately with 25µl of antibiotic in distilled water at concentrations of 0.0014, 0.0028, 0.0057, 0.0113, 0.0227 and 0.0453mmol/L (equivalent to 2, 4, 8, 16, 32 and 64mg/L of CLS and 2.5, 5, 10, 20, 40 and 80mg/L of CMS). A set of 6 concentrations were placed on each plate. Triplicate large antibiotic assay plates were pre-diffused for four hours at 4°C and then incubated at 37°C overnight.
Results

Figure 3:11: CLS bioassay in agar and gel n=3±Stdev

Figure 3.12: CMS bioassay in agar and gel. n=3±Stdev
CMS gave reasonable results in agar but even better results in gel in terms of the size of the inhibition zones. A concentration of 0.0453mmol/L (80mg/L) CMS produced a 14.8mm inhibition zone in agar while it produced a 15.6mm in gel.

CMS has limit of detection of 0.0014mmol/L (2.5mg/L) in both systems, while CLS is more sensitive to the gel than agar, sensitivity limit was 0.0113mmol/L (16mg/L) in agar and 0.0028mmol/L (4mg/L) in gel (Figure 3.11 and 3.12).

### 3.8 Calibration curve of CLS bioassay in water

The procedure 3.7.2 was carried out to bioassay CLS in distilled water using sterile filter paper discs pipetted accurately with 25µl solutions of CLS at concentrations of 0.0014, 0.0028, 0.0057, 0.0113, 0.0227 and 0.0453mmol/L (equivalent to 2, 4, 8, 16, 32 and 64mg/L of CLS).

**Results**

$$y = -7967.4x^2 + 622.25x - 0.3086$$

$$R^2 = 0.9946$$

![Calibration curve of CLS bioassay in water](image)

Figure 3.13: Bioassay of CLS in water. n=3± Stdev
For concentrations below 0.0028mmol/L (4mg/L), no inhibition zone was detected. The results shown in figure 3.13 indicate that CLS in distilled water can be measured at concentrations down to 0.0028mmol/L (4mg/L).

### 3.9 Calibration curve of CLS bioassay in human serum

The procedure 3.7.2 was carried out to bioassay CLS in human serum using sterile filter paper discs pipetted accurately with 25µl solutions of CLS at concentrations of 0.0011, 0.0014, 0.0017, 0.0023, 0.0028, 0.0057, 0.0113, 0.017, 0.034 and 0.0453mmol/L (equivalent to 1.6, 2, 2.4, 3.2, 4, 8, 16, 24, 48 and 64mg/L of CLS).

**Results**

![Graph showing the calibration curve of CLS bioassay in human serum](image)

**Figure 3.14:** Bioassay of CLS in human serum. n=3± Stdev
For concentrations below 0.0017mmol/L (2.4mg/L), the inhibition zones were very small and were concealed under the viscous serum which diffused into the agar from the disc. The results shown in figure 3.14 indicate that CLS in serum can be measured at concentrations down to 0.0017mmol/L (2.4mg/L).
CHAPTER FOUR

Optimizing chemical assays of colistin sulphate and colistin methanesulphonate

Thin Layer Chromatography
High Performance Liquid Chromatography
4.1 Thin Layer Chromatography (TLC)

4.1.1 Introduction

TLC provides a good method for separating and identifying components in a mixture by utilizing the relative affinities which compounds in a mixture have for a stationary phase. As the mobile phase moves past the sample spot applied to the TLC plate, equilibrium takes place for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are in solution. The components will differ in their solubility and therefore some components will be eluted farther up the plate than others. When the solvent reaches the top of the plate, the plate is removed, dried, and the separated components of the mixture are visualized by different methods such as fluorescence quenching or specific spray reagents. It is a widely used technique due to its simplicity, sensitivity, and low cost (Beckett, 1988).

TLC uses a stationary phase, usually alumina or silica, which is highly polar (normal phase NP) or non polar (reverse phase RP).

The British Pharmacopoeia (2010) (see appendix) method for TLC is recommended for the identification of both forms of colistin: CLS and CMS. It uses a silica gel plate as a stationary phase and 25:75 parts of water: phenol as a mobile phase. This method involves heating of the CLS or the CMS at 135 ºC with mixture of equal volumes of HCl and water for five hours and compares it with standard solutions of the amino acids such as leucine and threonine. Both forms of colistin contain these amino acids. Thus this method does not differentiate between both forms of colistin and therefore, was not applied in this study.

4.1.2 Materials used in TLC studies

Reagents:
Methanol, acetone, NaCl, ninhydrin sprays, iodine vapour, acetic acid and phosphoric acid (BDH, UK).
TLC developing plates:
- TLC plates Macherey-Nagel silica gel 60A 20cm x 20cm, on flexible aluminium sheets with F254 fluorescent indicator (NP).
- TLC plates Macherey-Nagel C18 reversed phase silica gel 5cm x 2cm, on glass with F254 fluorescent indicator (RP).
All the above materials were purchased from Fisher Scientific (Loughborough, Fisher UK).

4.1.3 Sample preparation
An accurate amount of CLS or CMS was aseptically weighed and dissolved in deionised water or human serum to give the required concentrations. CLS and CMS solutions were freshly prepared on the day of the experiment, unless otherwise stated.

4.1.4 TLC general procedure
The TLC tank was allowed to equilibrate with solvent vapour for at least 20 minutes. When the tank was equilibrated, the prepared NP or RP TLC plates were developed by placing in a vertical position. After about 20 minutes, the plates were removed, dried at room temperature and sample spots were visualized. The methods for this are the subject of the section below.

4.1.5 Optimizing thin layer chromatographic conditions for CLS and CMS
TLC conditions for CLS and CMS were tested and the optimized conditions were used to detect the degradation processes over time as discussed in chapter six.

The following parameters were investigated:

A- The most suitable visualizing agent
B- The most suitable mobile phase
C- The most suitable stationary phase
D- The antibiotic volume to be used in TLC
E- The effect of applying hot air drying on TLC plates of CLS and CMS
4.1.5.1 Choosing a suitable visualizing agent

A suitable method for visualizing CLS and CMS on TLC plates was required.
A 20µl spot of 0.5667mmol/L (1000mg/L of CMS and 800mg/L of CLS) of CLS or CMS aqueous solution was spotted on NP or RP TLC plates, dried by applying hot air and eluted as in section 4.1.4. These plates were visualized by different methods such as using a UV (254nm) lamp; exposure to iodine vapour or by spraying with ninhydrin and then drying in a pre-heated oven at 100°C.

Results

The most suitable technique to visualize CLS and CMS was spraying the plate with ninhydrin spray and then heating for 4-5 minutes in a pre-heated oven at 100°C. This procedure was used in all further experiments.

4.1.5.2 Choosing the most suitable mobile phase

A variety of mobile phases were used in order to find the most suitable mobile phase for the CLS and CMS TLC system. The mobile phase for CLS used with the RP plate was adapted from Ikai et al (1998a) and then concentrations of organic phase to aqueous phase were modified (Ikai et al, 1998a used RP-18 TLC plate, solvent system 50% 2M NaCl: 50% Acetone)

TLC plates were spotted with 20µl of 0.5667mmol/L of CLS and CMS (prepared as detailed in section 4.1.3), developed in the different mobile phases (as in section 4.1.4), sprayed with ninhydrin spray and then heated for 4-5 minutes in a pre-heated oven at 100°C (mobile phases shown in Table 4.1 and 4.2).
Results for CLS

Table 4.1: Mobile phases used for RP CLS TLC system.

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% 2M NaCl : 50% Acetone</td>
<td>Only one spot was observed</td>
</tr>
<tr>
<td>60% 2M NaCl : 40% Acetone</td>
<td>2 well separated spots</td>
</tr>
<tr>
<td>70% 2M NaCl : 30% Acetone</td>
<td>2 Fairly separated spots</td>
</tr>
<tr>
<td>80% 2M NaCl : 20% Acetone</td>
<td>2 Well separated spots</td>
</tr>
<tr>
<td>60% 2M NaCl: 40% Acetone+ 1 drop of phosphoric acid.</td>
<td>2 Very well separated spots with one hazy spot</td>
</tr>
</tbody>
</table>

Results from table 4.1 revealed that 60% 2M NaCl: 40% Acetone + drop of phosphoric acid (Figure 4.1) was the most suitable mobile phase for the RP CLS TLC system, (two separated TLC spots which most likely were colistin A and B and one unknown spot (Ikai et al, 1998a). (CLS spots obtained by this method disappeared shortly after development).

Figure 4.1: RP TLC of CLS using 60% 2M NaCl: 40% Acetone + drop of phosphoric acid (labeling of spots is adopted from Ikai et al, 1998a)
Results for CMS

Table 4.2: Mobile phases used for CMS NP TLC system.

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Methanol + 1 drop of acetic acid.</td>
<td>The spot did not elute completely</td>
</tr>
<tr>
<td>100% Methanol + 1 drop of phosphoric acid.</td>
<td>The spot did not elute completely but better than the previous one.</td>
</tr>
<tr>
<td>50% Methanol: 50% Acetone + 1 drop of phosphoric acid.</td>
<td>The spot did not elute</td>
</tr>
<tr>
<td>70% Methanol: 30% Acetone + 1 drop of phosphoric acid.</td>
<td>The spot did not elute completely</td>
</tr>
<tr>
<td>80% Methanol: 20% Acetone + 1 drop of phosphoric acid.</td>
<td>Fair separation with tailing</td>
</tr>
<tr>
<td>90% Methanol: 10% Acetone + 1 drop of phosphoric acid.</td>
<td>Good separation</td>
</tr>
<tr>
<td>90% Methanol: 10% Acetone</td>
<td>Fair separation with tailing</td>
</tr>
</tbody>
</table>

Results from table 4.2 reveal that 90% of Methanol: and 10% of Acetone + 1 drop of phosphoric acid was the most suitable mobile phase for the CMS NP TLC system (Figure 4.2).

A suitable stationary phase for the CMS (CMS is less polar than CLS) was found to be the highly polar NP and the mobile phase was non polar, thus the compound eluted with the mobile phase and produced two spots near the top of the plate. These are suggested to be CMS-5 or CMS -4, while the light streak appearing from the original spot towards the top of the plate is suggested to be CMS-3 or CMS-2 CMS-5 and CMS- 4 contain less polar groups and tend to be eluted with the non-polar mobile phase more than the CMS-3 and CMS-2 as these contain more polar groups, thus interacting more with the highly polar stationary phase and form a streak (Figure 4.2).
TLC plates of CMS (Figure 4.2) shows two separated spots and a streak appears from the original spot towards the two separated spots, this streak represents the closely related components of colistin. Since TLC plate development takes about 40 minutes, degradation of CMS to colistin base may occur which will result in methylsulphonated derivatives such as CMS-1, CMS2, CMS-3, CMS-4, CMS-5 and colistin base, these methylsulphonated groups are shown in Figure 4.2.

The spot appearing at the bottom of the plate (Figure 4.2) is suggested to be CLS itself, as the CLS is more polar (as it contains five amine groups) and tends to stick to the highly polar stationary phase, this was confirm as the CLS spot did not elute on the NP plate as shown in section 4.1.5.3.

Figure 4.2: NP TLC of CMS using 90% of Methanol and 10% of Acetone + drop of phosphoric acid
4.1.5.3 Choosing the most suitable stationary phase

This experiment was performed to select the stationary phase (NP and RP plates) for both antibiotics.

20μl of 0.5667mmol/L of CLS or CMS in deionised water or serum (prepared as detailed in section 4.1.3) were developed as in section 4.1.4 using 60% 2M NaCl: 40% Acetone + 1 drop of phosphoric acid for RP plates (for CLS) and 90%: 10% Methanol: Acetone + 1 drop of phosphoric acid as a mobile phase for NP plates (for CMS). Plates were sprayed with ninhydrin spray and then heated for 4-5 minutes in a pre-heated oven at 100°C.

Results

CLS in deionised water on a RP plate, mobile phase was 60% 2M NaCl: 40% Acetone + 1 drop of phosphoric acid: The two CLS spots with one unknown spot (colistin A and B as suggested by Ikai et al, 1998a) were separated (Figure 4.1). These spots faded and subsequently disappeared within a few minutes after developing.

CLS in deionised water on NP plate, mobile phase was 90%: 10% Methanol: Acetone + 1 drop of phosphoric acid: The CLS original spot did not move and no additional spots were detected. CLS and breakdown products are polar (due to the five amine groups) and do not elute on a NP (polar) stationary phase, also the non-polar mobile phase does not help the compound to elute.

CLS in serum on a RP plate, mobile phase was 60% 2M NaCl: 40% Acetone + 1 drop of phosphoric acid: The CLS original spot did not move and remained at the bottom of the plate. This suggests that serum was bound to the silica gel and CLS was protein bound.

CLS in serum on NP plate, mobile phase was 90%: 10% Methanol: Acetone + 1 drop of phosphoric acid: The CLS original spot did not move and remained at the bottom of the plate.
CMS in deionised water on NP plate, mobile phase was 90%: 10% Methanol: Acetone + 1 drop of phosphoric acid: The two CMS spots (suggested to be colistin A and B) were separated (figure 4.2) with an increasing intensity of the spot at the origin with aging of the solution. This suggests that CMS was converted to partially sulphonmethylated derivatives of colistin as well as colistin base over time; this suggestion is deduced from the fact that CLS on NP remains un-eluted.

CMS in deionised water on RP plate, mobile phase was 60% 2M NaCl: 40% Acetone + 1 drop of phosphoric acid: A small amount of the original spot was eluted and produced a faint spot; most of the spot did not elute. A spot with similar Rf value was seen when CLS was analyzed under the same conditions.

CMS in serum on NP, mobile phase was 90%: 10% Methanol: Acetone + 1 drop of phosphoric acid: The CMS original spot did not move and remained at the bottom of the plate. This suggests that serum was bound to the silica gel and CMS was protein bound.

CMS in serum on RP, mobile phase was 60% 2M NaCl: 40% Acetone + 1 drop of phosphoric acid: The CMS original spot did not move and remained at the bottom of the plate.

### 4.1.5.4 Standardization of CLS and CMS volumes for TLC analysis

This experiment was performed to optimize the TLC system by adjusting the volume of the antibiotic spot to give the most clearly observable and separated spots.

Concentrations of 0.5667mmol/L of CLS or CMS in deionised water on NP (for CMS) or RP TLC plates (for CLS) were used. Spots of 2, 5, 10, 20, 40 and 60µl were used. The triplicate TLC plates were developed and visualized as detailed in experiment 4.1.5.3.
Results

Table 4.3: CLS spot volume in TLC

<table>
<thead>
<tr>
<th>Volume of CLS spot</th>
<th>2µl</th>
<th>5µl</th>
<th>10µl</th>
<th>20µl</th>
<th>40µl</th>
<th>60µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results</td>
<td>Spots were not observable</td>
<td>Light streak</td>
<td>Faint spots</td>
<td>Faint spots</td>
<td>Two separated spots</td>
<td>Heavy streak</td>
</tr>
</tbody>
</table>

Table 4.4: CMS spot volume in TLC

<table>
<thead>
<tr>
<th>Volume of CMS spot</th>
<th>2µl</th>
<th>5µl</th>
<th>10µl</th>
<th>20µl</th>
<th>40µl</th>
<th>60µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results</td>
<td>Spots were not observable</td>
<td>Light streak</td>
<td>Faint spots</td>
<td>Two separated spots</td>
<td>Two overlapping spots</td>
<td>One heavy spot</td>
</tr>
</tbody>
</table>

The results show that a CLS 40µl spot of 0.5667mmol/L in deionised water produced the clearest results (Table 4.3). For CMS, 20µl spot of 0.5667mmol/L in deionised water produced the clearest results (Table 4.4).

4.1.5.5 Effect of applying hot air on TLC plates of CLS and CMS

Li et al (2003b) showed that CMS was sensitive to higher temperatures. The effect of hot air drying when applying the spot drop by drop on the TLC plates was tested and compared with the cold air drying and room temperature drying. The effect of heat drying was tested on CLS and CMS.

Experiment 4.1.5.4 was carried out using 40µl of 0.5667mmol/L of CLS and 20µl of CMS in deionised water.
Results

When this experiment was performed on CLS using RP plates the results showed that CLS was not affected by the applying of the hot air method of drying as it is much more stable than CMS (Figure 4.3). The chromatograms obtained from all drying methods were identical.

Figure 4.3: Effect of different methods of drying on CLS TLC plate

Figure 4.4: Effect of drying methods on CMS TLC plate
The results obtained (Figure 4.4) indicated that CMS was sensitive to the application of heat during preparation of the TLC plate as the two spots arising from the hot air drying were not well separated, while drying at room temperature or using cold air produced similar results of two well separated spots, therefore the TLC plates were dried at room temperature. (NB: The TLC plates produced results which were clear when observed by eye but did not reproduce well in photographs and so are consequently not shown). They are thus of some qualitative value and simply indicate the ease and rapidity with which degradation takes place.

The updated conditions for TLC chromatography were found to be: 40µl of 0.5667mmol/L of CLS or 20µl of 0.5667mmol/L of CMS in deionised water (prepared as detailed in section 4.1.3) and were developed as in section 4.1.4 by using 60% 2M NaCl: 40% Acetone + 1 drop of phosphoric acid for RP (for CLS) and 90%: methanol: and 10% of acetone + 1 drop of phosphoric as a mobile phase for NP (for CMS) plates. Plates were sprayed with ninhydrin spray and then heated for 4-5 minutes in a pre-heated oven at 100°C.

This updated method will be used later (in chapter six) to determine the degradation of aqueous CLS and CMS over various temperatures, although this method can not provide quantitative data, it can still support degradation studies along with bioassays and HPLC analysis.
4.2 High Performance Liquid Chromatography

4.2.1 Introduction
High Performance Liquid Chromatography (HPLC) is an analytical technique used for the separation of components within a mixture. The mobile phase is pumped through a column at a known constant flow rate (FR), and a sample of solution is injected onto the top of the column. The eluent passes through the column to a post-column detector, which monitors the concentration of analytes as they leave the column (James and Marcel, 1984; Snyder et al, 2009).

The stationary phase
The stationary phase refers to the material (typically hydrophobic saturated carbon chains bound to silica gel) contained within the column over which the mobile phase continuously flows. The sample is injected through the injector port and flows with the mobile phase. As the sample solution runs with the mobile phase through the stationary phase, the components of that sample will elute depending on the binding properties of the compounds with the stationary and mobile phases. The relative physico-chemical interactions of the sample components with the stationary phase and the mobile phase, determine the degree of elution and separation of the constituents contained in the sample. (James and Marcel, 1984; Snyder, 2009)

4.2.2 Materials used in HPLC studies
Reagents
Sodium sulphate, sodium phosphate and acetonitrile were all purchased from Fisher Scientific (Loughborough, Fisher UK).

4.2.3 Sample preparation
An accurate amount of CMS was weighed and dissolved in deionised water for HPLC, to give the required concentrations. CMS solutions were freshly prepared unless otherwise stated.
4.2.4 HPLC system

The system of Li et al. (2003b) was used to perform CMS analysis. It comprised a pump (Waters 2695) and photodiode array detector (Waters 2996) connected to a multi-instrument data acquisition and processing system (Empower software). The column was a stainless steel 150 x 4.6mm Strong Anion Exchanger (SAX) with 5µm particle size. The mobile phase consisting of acetonitrile: 1.2mM sodium sulphate (70:30) was pumped at 1ml/min. The UV absorbance of the eluent was measured at 210nm. Due to the hydrolysis of CMS, it still remains a challenge to separate and determine the concentrations of CMS with sufficient sensitivity, even with advanced techniques such as HPLC (Li et al., 2002, 2003b).

4.2.5 Optimizing HPLC chromatographic conditions for CMS

4.2.5.1 Investigation of literature methodologies.

A number of parameters were varied, based on the Li method, to achieve optimum separation of CMS components. The following parameters were investigated in order to achieve clear separated peaks:

- The CMS concentration was varied.
- The mobile phase was changed from sodium sulphate to disodium hydrogen phosphate.
- Sodium sulphate concentration in mobile phase was increased from 1.2mM to 10mM.
- The pH of the mobile phase was tested to include a range from 3 to 6.6.
- Various proportions of organic phase and aqueous phase were tested.
- Flow rate was changed from 1ml/min to 1.5ml/min.

All trials were based on duplicate injection into the HPLC system.

4.2.5.2 Concentrations of CMS

Concentrations of CMS were varied to determine the most suitable concentration which gives clear peaks and free from noise.
0.5667, 0.1133, 0.0227mmol/L (1000, 200 or 40mg/L) of CMS were freshly made in the mobile phase (30% 1.2mM sodium sulphate: 70% acetonitrile) and a sample of 20µl of CMS solution was injected into the HPLC system. The flow rate was 1ml/min. Detection was tested at 200 or 210nm. These conditions were obtained from the method of Li et al, (2003a).

**Results**

Detection at 200nm showed CMS peak absorbance values which correlated with the CMS concentrations. CMS concentrations of 0.5667mmol/L (calculated as 100%), 0.1133mmol/L (20%) and, 0.0227mmol/L (4%) resulted in peak absorbance values of 0.045 (100%), 0.008 (18%), and 0.002 (4.4%) respectively (**Figure 4.5, 4.6 and 4.7**).

These results (**Figure 4.5, 4.6 and 4.7**) showed that CMS peaks appeared to start at about 7 minutes at 200nm, as the peaks before that time did not change with CMS concentration; which suggested that those peaks were solvent front peaks.

The results showed (**Figure 4.5**) that a CMS concentration of (0.5667mmol/L) was the most suitable concentration of those tested to be used in HPLC system, lower concentrations such as 0.0227mmol/L showed too much background noise.
Figure 4.5: CMS 0.5667mmol/L at 200nm
Figure 4.6: CMS 0.1133 mmol/L at 200nm
Figure 4.7: CMS 0.0227mmol/L at 200nm
Detection at 210nm was tested to see if there was any better resolution, as interference tends to be worse at shorter wavelengths. Results showed that the CMS peak appeared few minutes later, at about 10 minutes and the peaks were better separated (figure 4.8). This is probably because a newly prepared mobile phase was used each time and the retention time appears to be strongly affected by very minor differences in mobile phase composition. 0.5667mmol/L (1000mg/L) of CMS detected at 210nm produced reasonable separated peaks.

Figure 4.8: CMS 0.5667mmol/L at 210nm
CMS dissolved in the mobile phase was run using the HPLC system at the same previous conditions for two hours to ensure that no delayed peaks were present. The results showed the absence of peaks after 30 minutes (Figure 4.9).

Figure 4.9: CMS 0.5667mmol/L at 210nm for 120 minutes
4.2.5.3 Testing sodium phosphate as a buffer in the mobile phase

Since sodium sulphate which used in the mobile phase did not offer buffering action, it was replaced by disodium hydrogen phosphate in the mobile phase. This experiment was performed to find a better mobile system which offered superior buffering action, thus obtaining better CMS separation. 0.5667mmol/L of CMS was freshly made in the mobile phase (30% 1.2mM disodium hydrogen phosphate: 70% acetonitrile) and 20µl of the sample was injected into the HPLC system. The flow rate was 1ml/min and detection was at 210nm.

Results

The results showed that no better separation was achieved (same peaks appeared) by switching the sodium sulphate with disodium hydrogen phosphate (Na$_2$HPO$_4$) in the mobile phase (Figure 4.10).
4.2.5.4 Modifying sodium sulphate concentration in the mobile phase

This experiment was performed to optimize the mobile system in order to separate the CMS components. Sodium sulphate concentration in mobile phase was increased from 1.2mM to 10mM. It was postulated that this might have the effect of increasing competition with the stationary phase.

The procedure 4.2.5.3 was carried out using a mobile phase of 30% 10mM of sodium sulphate: 70% acetonitrile.

Figure 4.10: CMS in mobile phase (30% 1.2mM sodium phosphate 70% acetonitrile)
Results

No better separation was achieved by increasing the concentration of sodium sulphate in the mobile system (Figure 4.11).

Figure: 4.11: CMS using 10mM of sodium sulphate in mobile phase
4.2.5.5 Testing the effect of pH on the mobile phase

The performance of ion exchange columns can be considerably affected by the pH of the mobile phase. With a strong anion exchanger as used here, the active site is likely to be a quaternary amine group. In order to optimize the HPLC system the pH of the mobile phase was adjusted to try to achieve better separation of the components of CMS.

The procedure described in section 4.2.5.3 was carried out using 1.2mM 30% sodium sulphate: 70% acetonitrile as a mobile phase. Different pH values were tried ranging from 3 to 6.6.

Results

Similar peaks were observed when using a range of pH3 to pH5.5 with retention time from 10 minutes to 13 minutes. This experiment shows that a range of pH3 to pH5.5 is suitable for CMS detection (Figure 4.12, 4.13, 4.14 and 4.15).

pH6 and pH6.6 produced un-separated peaks of retention times less than eight minutes, these experiments showed that a pH above 6 is not suitable for CMS detection (Figure 4.16 and 4.17).

These results revealed that the system produced better separation of CMS when the mobile phase was at a pH below 5. A mobile phase of pH 5 was therefore chosen to be used in future analysis.
Figure 4.12: CMS in mobile phase pH3
Figure 4.13: CMS in mobile phase pH4
Figure 4.14: CMS in mobile phase pH5
Figure 4.15: CMS in mobile phase pH 5.5
Figure 4.16: CMS in mobile phase pH6
Figure 4.17: CMS in mobile phase pH6.6

4.2.5.6 Effects of altering organic phase concentrations in the mobile phase

HPLC employs various types of stationary phase; analyte retention time varies according to the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase.

Mobile phase proportions of aqueous phase to the organic phase are critical in HPLC analysis, as adjusting these proportions alters retention times and therefore may result in better separation. With all types of column, the mobile phase needs to have the appropriate hydrophilic / lipophilic balance for the optimum resolution of components of a mixture.
The procedure detailed in section 4.2.5.3 was carried out using different proportions of sodium sulphate to acetonitrile within the mobile phase (pH5). Beginning with 100% of buffer without any acetonitrile, the concentration of acetonitrile in the mobile phase was increased in the range 0%, 50%, 60%, 65%, 70%, 75%, 77% and 80%. The mobile phase was made up to 100% with 1.2mM sodium sulphate.

**Results**

When 100% of 1.2mM sodium sulphate (acetonitrile 0%) was used as mobile phase, early peaks were produced very close to the solvent peaks. These results confirm the necessary use of acetonitrile in the mobile system.

Acetonitrile concentrations below 60% produced early peaks that interfered with the solvent front and retention time was less than three minutes.

These results (Figure 4.18) showed an increase in the CMS retention times as the acetonitrile concentrations increased from 60% to 80% in the mobile phase. Increasing the acetonitrile by just 2% from 75% to 77% resulted in a delay in CMS retention time from 16 to 20 minutes (Figure 4.19). Further increasing the acetonitrile from 75% to 80% resulted in a further delay in CMS retention time from 16 to 30 minutes (Figure 4.20).

There is clearly a very sharp increase in retention times above 75% acetonitrile (Figure 4.18). Although this leads to increased separation of peaks, the longer residence times on the column lead to peak broadening, smaller peak heights and no better resolution. These results suggested that optimum results for CMS analysis in HPLC were obtained by using 25% 1.2mM sodium sulphate: 75% acetonitrile as mobile phase, pH5.
Figure 4.18: Effects of altering organic phase concentrations in the mobile phase.
Figure 4.19: CMS in mobile phase of 75% and 77% acetonitrile
4.2.5.7 Effect of flow rate on CMS

A possible means of improving resolution could be to use an increased acetonitrile concentration to give better separation, but increase the flow rate to keep peak widths narrow. A flow rate of 1.5ml/min was used in an attempt to improve separation in the HPLC system. Experiment 4.2.5.3 was carried out with a flow rate of 1.5ml/min using, 75% of acetonitrile as a mobile phase. The mobile phases were made up to 100% with sodium sulphate, pH5.

Figure 4.20: CMS in mobile phase of 70%, 75% and 80% acetonitrile
Results

Results showed an early appearance of CMS peaks with a flow rate of 1.5ml/min compared with flow rate of 1ml/min (Figure 4.21). No better resolution was achieved with a flow rate of 1.5 ml/min (Figure 4.22).

A flow rate of 1ml/min was used in the further work.

Figure 4.21: Effect of Flow Rate on CMS retention times.
The previous experiments were conducted using solutions of CMS dissolved in the mobile phase. However, future degradation studies will be performed on CMS in deionised water for HPLC; therefore it was necessary to determine the influence on the analytical process of CMS dissolved in deionised water for HPLC. 0.5667mmol/L of CMS was freshly made in the mobile phase or deionised water for HPLC. 20µl of the sample was injected into the HPLC system using 25% 1.2mM sodium sulphate: 75% acetonitrile as mobile phase, pH5. The flow rate was 1ml/min. and detection was at 210nm.

Figure 4.22: CMS in 75% acetonitrile, flow rate of 1.5ml/min

4.2.5.8 CMS dissolved in mobile phase or water
Results

The CMS chromatogram in deionised water for HPLC was identical to that in mobile phase (Figure 4.23 and 4.24).

![CMS chromatogram](image)

**Figure 4.23**: CMS dissolved in the mobile phase
This updated method will be used later (in chapter six) to determine the degradation of aqueous CMS over various temperatures.
CHAPTER FIVE

Studies into bacterial cell death and growth inhibition induced by colistin sulphate and colistin methanesulphonate
5.1 Introduction

Before attempting to assess the mechanism of action of CLS and CMS, quantitative measurements of Minimum Inhibitory Concentrations (MICs) should be made while consideration should also be given to establishing the rate of action (Denyer and Hugo, 1991). In this chapter, MICs, bacterial profiles and time-survivor curve studies for CLS and CMS will be studied.

5.2 Determination of Minimum Inhibitory Concentrations (MICs)

The MIC has been defined by Andrew (2001) as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation.

MIC is a fundamental parameter in antibacterial pharmacodynamics and is used as a guide in clinical management of the patient (Li et al, 2006a).

Agar and broth dilution methods were used to determine MIC concentrations of CLS and CMS.

5.2.1 MICs using agar dilution technique

Initial investigations employed the agar dilution method to determine the MICs of CLS and CMS against many Gram-negative bacteria (Table 5.1, Table 5.2, and Table 5.3). Two different concentrations of cells (1 x 10^7 and 1 x 10^4 Cfu/ml) were used to determine MICs of CMS in order to investigate the effect of a greater number of cells on the activity of the antibiotic.

Fresh CMS and CLS aqueous solutions were prepared as detailed in section 2.2.9 and were incorporated in the molten ISA single layer agar plates as detailed in section 2.2.10.1 (method for using ISA was adopted from Andrew, 2001) so the final concentrations of CMS or CLS in 20ml of agar were:
Bacterial cultures of $10^7$ or $10^4$ Cfu/ml (prepared as detailed in section 2.2.4) were pipetted into the cavities of a Perspex tray and a multipoint applicator (as detailed in section 2.2.12) was used to transfer the suspensions to the agar plates (three plates were inoculated for each concentration of CLS or CMS). After inoculation the plates were incubated at 37°C. At 24 and 48 hours, all plates were recorded for visual growth. In addition, positive controls containing bacteria on the agar plate and negative controls containing only CLS or CMS on the agar plate were included in the experiment.

Determining MICs for CLS employed only selected Gram-negative bacteria. The selection was based on the sensitivity to CMS.

*B. bronchiseptica* (NCTC 8344) and *E. coli* (NCIB 8879) were selected as both organisms have low MICs and they are recommended for the bioassays of CLS and CMS in the British Pharmacopoeia (British Pharmacopoeia, 2010). *A. lwofii* was selected since CMS is the most effective treatment available. *Serratia marcescens* was selected as it has the highest MIC value among the bacteria employed in the study (these conclusions were drawn from the experiment conducted in section 3.2.3).

**Results**

Growth of each microorganism was initially recorded in numerical terms (0, 1 and 2) in direct relationship with its density. Thus ‘0’ signified no growth, ‘1’ indicates a visible degree of growth but less then that shown by the control and ‘2’ is a growth equal to that on the control plate (*Table 5.1, 5.2 and 5.3*).
Table 5.1: MICs of CMS measured by agar dilution technique (Bacterial concentrations of $10^7$ Cfu/ml)

<table>
<thead>
<tr>
<th>Organism $10^7$ Cfu/ml</th>
<th>CMS Concentration (mg/L)</th>
<th>MIC (mg/L)</th>
<th>MIC (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25 0.5 1 2 4 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter</em> CANADA 140 (Brighton University)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>A. Iwoffii</em> R46383 (Brighton University)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em> (NCTC 8344)</td>
<td>1 0 0 0 0 0.5</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td><em>Citrobacter freundii</em> (ATCC 8090)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em> (NCTC 10006)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em> (Brighton university)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>E. coli</em> (NCIB 8879)</td>
<td>2 2 2 2 0 4</td>
<td>0.0023</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> (NCTC 8196)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em> (ATCC 11228)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (NCTC 11228)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (NCIMB 10548)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (ATCC 9027)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (NCTC 6749)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>Salmonella abony</em> (Brighton University)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>Salmonella arizona</em> (ACTC 8297)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>Serratia liquifaciens</em> (ATCC 27595)</td>
<td>2 2 2 2 2 8</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> (Brighton University)</td>
<td>2 2 2 2 2 &gt;8</td>
<td>&gt;0.0045</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2: MICs of CMS measured by agar dilution technique. (Bacterial concentrations of $10^4$ CFu/ml)

<table>
<thead>
<tr>
<th>Organism $10^4$ CFu/ml</th>
<th>CMS Concentration (mg/L)</th>
<th>MIC (mg/L)</th>
<th>MIC (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter CANAD140 (Brighton University)</td>
<td>2 2 2 1 0 0</td>
<td>4</td>
<td>0.0023</td>
</tr>
<tr>
<td>A. lwofii R46383 (Brighton University)</td>
<td>2 2 2 1 0 0</td>
<td>4</td>
<td>0.0023</td>
</tr>
<tr>
<td>B. bronchiseptica (NCTC 8344)</td>
<td>0 0 0 0 0 0</td>
<td>&lt;0.25</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Citrobacter freundii (ATCC 8090)</td>
<td>2 2 2 2 0 0</td>
<td>4</td>
<td>0.0023</td>
</tr>
<tr>
<td>Enterobacter aerogenes (NCTC 10006)</td>
<td>2 2 2 2 1 0</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td>Enterobacter cloacae (Brighton university)</td>
<td>2 2 2 2 1 0</td>
<td>4</td>
<td>0.0022</td>
</tr>
<tr>
<td>E. coli (NCIB 8879)</td>
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<td>2</td>
<td>0.0011</td>
</tr>
<tr>
<td>E. coli (NCTC 8196)</td>
<td>2 2 2 2 0 0</td>
<td>4</td>
<td>0.0023</td>
</tr>
<tr>
<td>Klebsiella pneumoniae (NCTC 11228)</td>
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<td>0.0023</td>
</tr>
<tr>
<td>P. aeruginosa (NCIMB 10548)</td>
<td>2 2 2 2 0 0</td>
<td>4</td>
<td>0.0023</td>
</tr>
<tr>
<td>P. aeruginosa (ATCC 9027)</td>
<td>2 2 2 2 0 0</td>
<td>4</td>
<td>0.0023</td>
</tr>
<tr>
<td>P. aeruginosa (NCTC 6749)</td>
<td>2 2 2 2 1 0</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td>Salmonella abony (Brighton University)</td>
<td>2 2 2 2 1 0</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td>Salmonella arizona (ACTC 8297)</td>
<td>2 2 2 2 0 0</td>
<td>4</td>
<td>0.0023</td>
</tr>
<tr>
<td>Serratia liquifaciens (ATCC 27595)</td>
<td>2 2 2 2 1 0</td>
<td>8</td>
<td>0.0045</td>
</tr>
<tr>
<td>Serratia marcescens (Brighton University)</td>
<td>2 2 2 2 2 2</td>
<td>&gt;8</td>
<td>&gt;0.0045</td>
</tr>
</tbody>
</table>
Table 5.3: MICs of CLS measured by agar dilution technique

<table>
<thead>
<tr>
<th>Organism 10⁷Cfu/ml</th>
<th>CLS Concentration (mg/L)</th>
<th>0.01</th>
<th>0.1</th>
<th>0.2</th>
<th>0.4</th>
<th>0.8</th>
<th>1.6</th>
<th>3.2</th>
<th>6.4</th>
<th>12</th>
<th>MIC mg/L</th>
<th>MIC mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. lwoffii R46383 (Brighton university)</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.2</td>
<td></td>
<td>0.0023</td>
</tr>
<tr>
<td>B. bronchiseptica (NCTC 8344)</td>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td></td>
<td>0.00007</td>
</tr>
<tr>
<td>E. coli (NCIB 8879)</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td></td>
<td>0.0006</td>
</tr>
<tr>
<td>Serratia marcescens (Brighton university)</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>&gt;13</td>
<td></td>
<td>&gt;0.0091</td>
</tr>
</tbody>
</table>

Visual growth was determined after 24 hours incubation and no further drift of MIC was observed after 48 hours incubation. Therefore, 24 hour data were used to determine MIC values.

The positive control (bacteria without antibiotics) showed positive growth for all the organisms, while the negative control (antibiotic only) showed no growth for both CLS and CMS. For bacterial cultures at a concentration of 10⁷ Cfu/ml, the MICs values were higher (Table 5.1) than those obtained with bacterial concentrations of 10⁴ Cfu/ml (Table 5.2).

CLS was more effective, i.e. produced lower MIC values compared to CMS against all the bacteria employed in this study. CMS which was used against many Gram-negative bacteria used in this study at a concentration of 10⁷ Cfu/ml showed MICs value of 0.0045mmol/L or 8mg/L against most organisms, while CLS had MIC of 0.0023mmol/L or 3.2mg/L or even less. Both CLS and CMS were considerably more effective, against B. bronchiseptica (MIC= 0.00007mmol/L or 0.1mg/L and 0.0003mmol/L or 0.5mg/L respectively) and E. coli (NCIB 8879) (MIC= 0.0006mmol/L or 0.8mg/L and 0.0023mmol/L or 4mg/L respectively). (Tables 5.1, 5.2 and 5.3).
Serratia marcescens is highly resistant to CLS and CMS even at high concentration such as 0.0091mmol/L (13mg/L) or 0.0045mmol/L (8mg/L) respectively.

5.2.2 MICs using broth dilution technique

Preliminary MIC investigations showed that both CLS and CMS have poor diffusion properties in agar (as detailed in chapter four), and this investigation was conducted to identify MICs values for CLS and CMS against many Gram-negative bacteria in an AOAC broth medium.

CLS or CMS solutions (prepared as detailed in section 2.2.9) were freshly prepared and diluted in 9ml of AOAC broth medium (prepared as detailed in section 2.1.1.1), so that the final concentrations of CLS or CMS were: 0.00006, 0.0001, 0.0003, 0.0006, 0.0011, 0.0023, and 0.0045mmol/L (equivalent to 0.1, 0.2, 0.5, 1, 2, 4 and 8mg/L of CMS or 0.08, 1.6, 0.4, 0.8, 1.6, 3.2, 6.4mg/L of CLS).

Bacterial cultures of different Gram-negative bacteria (Table 5.4) were prepared as detailed in section 2.2.1 to give concentrations of $10^8$ Cfu/ml; 1ml of these suspensions were diluted with 9ml of AOAC broth so the final bacterial concentrations were $10^7$ Cfu/ml.

In addition, positive controls containing (bacteria in AOAC broth) and negative controls (CLS or CMS in AOAC broth) were included in the experiment; triplicate tubes were used for each concentration of CLS or CMS.

All tubes were maintained in a 37°C incubator under static conditions. At 24 hours, all tubes were recorded for visual growth.
Results

Table 5.4: MICs of CLS measured by agar and broth dilution

<table>
<thead>
<tr>
<th>Organism 10^7 Cfu/ml</th>
<th>NB</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. lwofii R46383 (Brighton University)</td>
<td>0.08mg/L 0.00006mmol/L</td>
<td>3.2mg/L 0.0023mmol/L</td>
</tr>
<tr>
<td>B. bronchiseptica (NCTC 8344)</td>
<td>&lt;0.0008mg/L &lt;0.000006mmol/L</td>
<td>0.1mg/L 0.00007mmol/L</td>
</tr>
</tbody>
</table>

Table 5.5: MICs of CMS measured by agar and broth dilution techniques

<table>
<thead>
<tr>
<th>Organism 10^7 Cfu/ml</th>
<th>NB</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. lwofii R46383 (Brighton University)</td>
<td>0.2mg/L 0.0001mmol/L</td>
<td>8mg/L 0.0045mmol/L</td>
</tr>
<tr>
<td>B. bronchiseptica (NCTC 8344)</td>
<td>0.1mg/L 0.0006mmol/L</td>
<td>0.5mg/L 0.0003mmol/L</td>
</tr>
</tbody>
</table>

The positive control (bacteria without antibiotic) showed growth for all the organisms, while the negative control (antibiotic only) showed no growth for both CLS and CMS. In this study MICs values in NA were higher than in NB (Table 5.4 and 5.5). These results were consistent with those of Barnett et al (1964). These authors demonstrated the activity of CMS against *P. aeruginosa* by using broth and agar dilution technique and found that MICs values were higher in agar than in broth.

Although MICs are a useful tool in characterising the antimicrobial profile of an antibiotic, they provide no information on the rate of killing or dilution effects, therefore their importance in defining bactericidal efficacy should not be overestimated.
Since determining MICs in broth during overnight incubation at 37°C, hydrolysis of CMS to partially sulphotaurinated derivatives of colistin or colistin base occurs via a complex series of methanesulphonated intermediates, the killing properties of this complex mixture, which changes over incubation, are likely to be even more complicated (Li et al, 2006a).

5.3 **Bacterial profiles**

True death in bacteria is observed when the cell wall is dissolved. After the cell wall is wholly or partially dissolved, the cytoplasmic membrane is ruptured in a hypotonic environment due to the internal solute pressure (Mcquillen, 1958). Bacterial experiments were performed in an attempt to understand the antibacterial action of CLS and CMS.

CLS and CMS at concentrations of 0.0057 and 0.0453mmol/L (8 or 64mg/L of CLS and 10 or 80mg/L of CMS) were prepared in distilled water as detailed in section 2.2.9. 5ml of *Acinetobacter lwofii* R46383 at a concentration of 1x10⁸Cfu/ml was prepared as detailed in section 2.2.4 and added to a flask containing 45ml of the CLS or CMS aqueous solutions, so the final bacterial concentration in the antibiotic solutions was 1x10⁷Cfu/ml. The suspensions were then incubated at 37°C with shaking at 80 rpm in a Grant orbital shaking water bath (Fisher Scientific, UK). 2ml samples were removed from both antibiotic flasks at 0, 1, 2 and 4 hours. Optical densities of these samples were determined at 420nm against water as a blank using the spectrophotometer (model: 6300, Jenway UK). This experiment was performed in triplicate.
**Results**

**Figure 5.1**: *Acinetobacter lwoffii* profile treated with CLS or CMS. 

\( n=3 \pm \text{Stdev} \)

**Figure 5.1** shows that CLS at high concentrations such as 0.0453mmol/L (64mg/L) cause bacterial cells to lyse (absorbance is 30% less than the initial value), while at lower concentrations of CLS and all concentrations of CMS employed in the study the values appeared unchanged from the initial point.

The loss of absorbance observed in the presence of high concentrations of CLS can be attributed to lysis of the bacterial cells caused by osmotic damage to the cytoplasmic membrane (Denyer and Hugo, 1991).

**5.4 Time-survivor curves studies for CLS and CMS**

Time-survivor studies give information on the rates at which organisms are killed and therefore, unlike MICs, they provide information on the time course of bactericidal action.
Kronig and Paul (1897) were the first to plot the logarithm of the surviving organisms against time, which they found gave an approximately linear response. Time survivor curves are produced by counting the number of viable cells after exposure to various concentrations of an agent at different time intervals, and can provide vital information regarding suitable formulations as well as giving an insight into the effects of dilution on activity (Hurwitz and McCarthy, 1985).

5.4.1 Neutralizing agent

5.4.1.1 Assessment of the suitability of the neutralizer

For accurate assessment of surviving organisms after exposure to antibiotics, activity must be stopped at the time of sampling. It is also important that antibiotic concentrations, which may inhibit growth, are not transferred to the recovery media. This is usually achieved by employing a neutralizing agent able to overcome antibiotic activity. The choice of the neutralizer must meet two important criteria: efficacy and safety to the inoculum (MacKinnon, 1974; USP, 2011).

In this investigation, tween 80 with lecithin at various concentrations was tested as a neutralizing agent. Concentration of 2% w/v of Tween 80 and 0.2% lecithin was found to be an effective naturalizing agent for CLS and CMS.

To evaluate the efficacy of tween 80 with lecithin in neutralizing the antimicrobial action of both forms of colistin, CLS and CMS aqueous solutions were added to the neutralizing agent and left for five minutes to be neutralized. 1ml of *A. lwofii* at a concentration of 1 x 10⁷ Cfu/ml (prepared as detailed in section 2.2.4) was added to 9ml of antibiotic/neutralizer mixture, so the final concentration was 1 x 10⁶ Cfu/ml. After about one minute, samples were removed, serially diluted in HEPES buffer and 0.2ml was plated on NA plates in triplicates. Plates were incubated overnight at 37°C and the numbers of colonies were counted.
A toxicity control was performed by adding bacterial cells to the neutralizing agent and left for five minutes before sampling to detect whether it had any antimicrobial effect. These results were compared with cell counts in HEPES buffer (HEPES control). Neutralizer free control was employed in this study by adding bacterial cells to the both forms of antibiotics without neutralizing agent.

**Results**

No growth was observed on plates prepared from the neutralizer free control (cells plus antibiotics); while there was a considerable growth (average counts were $0.7 \times 10^6$ Cfu/ml compared to HEPES control counts were $0.6 \times 10^6$ Cfu/ml) on plates with the neutralizer (cells plus antibiotic with neutralizer, efficacy test). This experiment should demonstrate that the average of counts on agar plates for efficacy test is not less than 70% of counts for HEPES control (USP, 2011). These results demonstrated that 2% tween 80 with 0.2% lethicin was an effective neutralizing agent for CLS and CMS. Five minutes was chosen as a satisfactory time interval for complete neutralization.

There was no increase or decrease in the viable cell counts (average counts were $0.5x10^6$) of the plates with neutralizing agent (toxicity control) compared to HEPES control (average counts were $0.6x10^6$). These results demonstrate that the neutralizing agent employed in the study is not toxic to the bacteria.

**5.4.2 Determination of the time-survivor curves for CLS and CMS**

CLS and CMS at different concentrations were prepared in distilled water as detailed in section 2.2.9. Overnight cultures of *A. lwaffii* R46383 were prepared, harvested and washed in HEPES buffer. The bacterial suspension was then standardised to $1 \times 10^7$ Cfu/ml (this suspension was prepared by harvesting the organism as detailed in section 2.2.4).
18ml of different CLS and CMS concentrations (shown in figure 5.2 and 5.3) were left to equilibrate at 37°C. 2ml of A. lwaffii 1 x 10^7 Cfu/ml were added to the suspensions (final bacterial concentration was 1 x 10^6 Cfu/ml). At appropriate time intervals (as indicated in figure 5.2 and 5.3), 1ml samples were removed and added to 9ml of neutralizing agent to neutralise the effect of CLS and CMS, and left for 5 minutes. Serial dilutions were performed on the sample using 0.05M HEPES buffer and 0.2ml of each dilution plated out in triplicate on NA plates and incubated at 37°C overnight. Plates with 30 to 300 colonies were counted and then number of surviving cells at a particular time was calculated (the number of bacteria were counted and multiplied by dilution factor). A graph of log_{10} number of surviving cells was plotted against time.

**Results**

![Figure 5.2](image)

**Figure 5.2: Survivor plot for bacteria treated with CLS. n=3± Stdev**
Figure 5.3: Bacterial survival treated with CMS. n= 3± Stdev.

The time survivor curves for *A. lwofii* exposed to CLS and CMS are shown in figure 5.2 and figure 5.3.

A concentration of 0.0227 mmol/L (32 mg/L) of CLS produced a 3.8 log reduction in viable counts after 30 minutes contact with the antibiotic. The zero time point was in reality approximately one minute as this was the time taken for the bacteria to be added, mixed and the diluted in the neutralizing agent.

CLS at a concentration of 0.0113 mmol/L (16 mg/L) also demonstrated rapid and extensive killing with just less than 2.5 log reductions by 30 minutes. Cells were reduced by just less than 2.5 log reductions within one hour at a concentration of 0.0057 mmol/L (8 mg/L), while the concentration of 0.0028 mmol/L (4 mg/L) produced 1.5 log reductions within one hour of exposure to the antibiotic. Killing rate is thus concentration dependent, while the control (cells plus antibiotic with the neutralizing agent) did not cause any killing action (Figure 5.2).
Concentrations of 0.0028, 0.0057 and 0.0113mmol/L (5, 10 and 20mg/L) of CMS did not cause more than 0.5 log reduction, higher concentrations such as 0.0227 and 0.0453mmol/L (40 and 80mg/L) produced about one log reduction within one hour contact with the antibiotic (Figure 5.3).
CHAPTER SIX

Microbiological and chemical degradation of colistin sulphate and colistin methanesulphonate
6.1 Introduction

Colistin contains five primary amine groups and when synthesizing CMS, all of the five groups have the potential to be sulphomethylated. During degradation it can be assumed that hydrolysis of the five sulphomethylated groups is random, and therefore there could be up to 64 possible products arising from the hydrolysis of CMS in aqueous solution including colistin itself (Li et al, 2003b and 2004; Bergen et al, 2006). CMS in aqueous solution is converted to a mixture of colistin sulphomethylated derivatives and colistin itself over a period of time and the degradation is more rapid at higher temperatures such as 37°C (Li et al, 2003b). These hydrolysed products have been shown to possess increased antibacterial activities (Barnett et al, 1964; Wallace et al, 2008).

In this study CLS and CMS were stored in aqueous solution and in human serum at different temperatures (-20°C, 4°C, 25°C and 37°C) in order to investigate the characteristics of decomposition. Previously updated microbiological (chapter three) and chemical assays including TLC and HPLC methods (chapter four) were employed in this study.

This information will be helpful in understanding the pharmacokinetics and pharmacodynamics of CLS and CMS.

6.2 Detection of CLS and CMS degradation by microbiological assay

CMS has the potential to hydrolyze in aqueous solution to liberate the microbiologically active and more toxic parent compound, colistin base. "Liberation of colistin during storage of pharmaceutical formulations may potentiate the toxicity of CMS". (Wallace and Rayner, 2008).
CLS and CMS solutions were freshly prepared in distilled water or human serum and stored at -20°C, 4°C, 25°C or 37°C over nine days. Samples of these solutions were analysed using previously optimized microbiological methods (CMS in water or serum as detailed in section 3.4, CLS in water or serum as detailed in section 3.7.2 and 3.8).

**CLS assay**
The agar disc diffusion experiment was performed using a large antibiotic assay plate by dispensing 200ml of DSTA as a base layer followed by 50ml of phytogel solution which contained 200µL of $10^8$ CFU/ml of *B. bronchiseptica* (NCTC 8344) prepared as in section 2.2.4. The phytogel was cross linked with 1ml of 1% CaCl$_2$ on the DSTA layer before adding the gelling agent.

**CMS assay**
The agar disc diffusion experiment was performed using a large antibiotic assay plate by dispensing 200ml of DSTA as a base layer followed by 50ml of molten seeded agar (PW + technical agar 0.4%) which contained 200µL of $10^8$ CFU/ml of *B. bronchiseptica* (NCTC 8344) prepared as in section 2.2.4.

Sterile filter paper discs were inoculated with 25µl of CLS or CMS in distilled water or human serum at a concentration of 0.017mmol/L (equivalent to 24 of CLS or 30mg/L of CMS) prepared as detailed in section 2.2.9 and placed on each previously prepared plate. A fresh control solution of 0.017mmol/L was prepared freshly on the day of assay, assayed and was calculated as 100% concentration. Hence, although some degradation would be expected to occur during the assay this is accounted for in the control and the degradation studies are compared to this value.

The duplicate plates were allowed to pre-diffuse for four hours at 4°C and then incubated at 37°C overnight. After incubation the zones of bacterial growth inhibition were recorded and interpreted as detailed in section 2.2.13.
Results

Storage of CLS and CMS at -20°C

Figure 6.1: Degradation of CLS and CMS in water or serum at -20°C over nine days.

The activities of both CLS and CMS either in distilled water or serum showed stability over nine days when stored at -20°C, (all the values between about 97% and 104%) (Figure 6.1).
Storage of CLS and CMS at 4°C

Figure 6.2: Degradation of CLS and CMS in water or serum at 4°C over nine days.
CLS activity either in water or serum remained stable for the period of nine days when stored at 4°C (all values between 97% and 103%) (Figure 6.2). A literature review demonstrated that CLS in aqueous solution was stable for up to 60 days when stored at 4°C, this review was based on an HPLC method (Li et al, 2003b).

The activity of CMS remained stable for up to one day in aqueous solution or serum, and then it started to lose microbiological activity within three days of storage to reach about 95%. CMS activity in water or serum continued to decrease within nine days to reach about 75% or 72% of its original activity respectively (Figure 6.2). Although the conversion of CMS to the more active colistin base occurs over time, microbiological activity of CMS was reduced after three days and further reduced within nine days. This observation may be due to the fact that microbiological activity was determined every 48 hours, while the conversion may have been much faster than that, perhaps within hours. The microbiological activity might be expected to increase due to the hydrolysis of CMS to colistin base and then subsequently decrease however, the optimized system used for determination of CMS does not allow the proper diffusion of colistin base (as shown in section 3.5).

These results were in line with results reported by Li et al, (2003b), who stated that CMS was stable for two days when stored at 4°C.

The hydrolysis which occurred in human plasma was more rapid than that in water at 4°C, suggesting that this hydrolysis probably also takes place in the body to a greater extent.
Storage of CLS and CMS at 25°C

Figure 6.3: Degradation of CLS and CMS in water or serum at 25°C over nine days.
The activity of CLS in water or in serum remained stable for nine days (all values between 98% and 103%) (Figure 6.3), while the activity of CMS in water or serum remained fairly stable for one day at 25°C (activity was above 92%), and then reduced until it reached about 65% or 63% after nine days of storage respectively (Figure 6.3).

**Storage of CLS and CMS at 37°C**

![Graph showing degradation of CLS and CMS at 37°C over nine days.](image)

*Figure 6.4: Degradation of CLS and CMS in water or serum at 37°C over nine days.*
CLS activity in water and in serum remained stable for one day (activity was above 95%), and then it declined to reach about 70% after the third day and then remained stable during the storage at 37°C (Figure 6.4).

These results are different from the results reported by Li et al., (2003b), who found that CLS aqueous solution was stable for 120 hours (five days) at 37°C; however, these results were obtained using the more sensitive HPLC method as the analytical method.

CMS in water and in serum showed degradation within the first day of storage at 37°C to reach activity of about 74% or 71% respectively (Figure 6.4). These results agree with the results of Li et al (Li et al, 2003b) who reported that CMS in aqueous solution was completely degraded after 12 hours at 37°C (by HPLC method), this may be due to the fact that CMS completely converts to colistin base which has increased microbiological activity (as concluded by Li et al, 2003b) within 12 hours of incubation at 37°C, where the antibacterial activity should increased, however, these results are for CMS after 24 hours where colistin base is already formed and may undergone degradation. In addition, the assay method for CMS dose not shows activity of colistin due to lack of diffusion.

6.3 Detecting degradation of CLS and CMS by chemical methods

6.3.1 Detecting CLS and CMS degradation on TLC plates

Since the microbiology bioassays involved overnight incubation at 37°C, the degradation rate information is rather complicated. To an extent this has been accounted for by the inclusion of a positive control in the form of a freshly prepared solution of antibiotic. The stability samples are all referenced to this solution and so the degradation rates are comparable. Chemical methods such as TLC however, may give a better understanding of the degradation rate of CLS or CMS.

Freshly prepared CLS and CMS aqueous solutions were stored at -20°C, 4°C, 25°C or 37°C. Samples of these solutions were spotted onto TLC plates (in duplicate) at different time intervals, dried at room temperature and developed according to the updated method in chapter four as following.
**CLS** 40µl of 0.5667mmol/L (800mg/L) of CLS in deionised water or serum were developed by using 60% 2M NaCl: 40% Acetone + 1 drop of phosphoric acid for RP plates.

**CMS** 20µl of 0.5667mmol/L (1000mg/L) of CMS in deionised water or serum (prepared as detailed in section 5.1.3) were developed using 90%:Methanol: and 10% Acetone + 1 drop of phosphoric acid as a mobile phase for NP plates.

After running the analysis the duplicate plates were sprayed with ninhydrin spray and then dried in a pre-heated oven at 100°C for a few minutes. This experiment was repeated three times.

The degradation was performed in aqueous solutions of CLS and CMS only, as preliminary investigations showed that both forms of colistin did not elute in serum (refer to section 4.1.5.3).

**Results**

**CLS degradation**

CLS was stable when stored at -20, 4 and 25°C over eight days of storage. CLS on RP plates gave three separated spots. When the solutions were stored over eight days the position of the spots remained unchanged but faded fairly quickly after being developed. This made the capture of a photographic image very difficult and so diagrams are included for clarity.

Fresh CLS solution resulted in three separated spots, these spots merged to become one spot after four days of storing at 37°C (Figure 6.5).
This experiment indicated that CLS was stable when stored at -20, 4 and 25°C over eight days as the chromatographs were identical to the fresh one (as in Figure 4.1), while it was stable for only four days when stored at 37°C as the spots were merged to one spot only after four days of storing. The results given here were almost consistent with those of Li et al (2003b) who showed that aqueous CLS was stable for 60 days and five days when stored at 4°C or 37°C respectively.

![Figure 6.5: TLC of fresh and stored CLS at 37°C after four days.](image_url)
CMS degradation

Storage of CMS at -20°C

Figure 6.6: TLC of CMS stored at -20°C.

TLC analysis of the fresh CMS solution shows two separated spots. Storage of CMS solution at -20°C showed that it was stable for eight days as the chromatogram showed the same two separated spots (Figure 6.6).
Storage of CMS at 4°C

Figure 6.7: TLC of CMS stored at 4°C.

CMS chromatograms showed two separated spots with a light streak from the original spot towards the top of the plate within two days of storage at 4°C (Rf values were 0.63 and 0.56) which were similar to the fresh CMS. After three days both spots disappeared and the light streak became heavier at the bottom of the plate. The fresh solution’s original spot intensity increased gradually as time passed; this intensity was considerably increased after three days of storage. Eight days later the CMS solution had been almost completely hydrolyzed to colistin, this was concluded as the colistin spot remained without elution at the bottom of the plate (figure 6.7).

TLC of fresh CMS solution showed two well-defined spots with some slight streaking. Over time the intensities of these spots decreased and spots of lower Rf value could be seen. Eventually the main spots were seen at and just above the point of application. The streaking is believed to be due to the very large number of possible breakdown products of CMS. There are two forms of CMS, and for each of these there are 64 possible degradation products involving loss of methylsulphonate groups. With a total of 64 possible compounds
of quite similar structure, it would be almost impossible to completely separate all of them, hence the apparent streaking observed. As the number of methylsulphonate groups decreases, the Rf values would be expected to decrease and this was observed.

When degradation by loss of methylsulphonate groups is complete and colistin is formed the RF value equals zero. After eight days, it appeared that there were probably a small number of methylsulphonate groups remaining on some molecules. The solution was relatively stable for two days (though there was clearly some material at low Rf values). After eight days degradation appeared to be essentially complete.

**Storage of CMS at 25°C**

![Diagram showing TLC of CMS stored at 25°C](image)

Figure 6.8: TLC of CMS stored at 25°C.

The fresh CMS chromatogram showed two separated spots with Rf values of 0.63 and 0.56 with a light streak from the bottom to the top of the plate. After one day of storage at 25°C both spots merged to become one spot with an RF value of 0.51. Two days later, the single spot disappeared and the streak became heavier (Figure 6.8).
Degradation appeared to be faster at 25°C than at 4°C. The solution was relatively stable for one day but after eight days degradation appeared to be essentially complete (Figure 6.8).

**Storage of CMS at 37°C**

![TLC diagram showing CMS degradation at 37°C]

**Figure 6.9: TLC of CMS stored at 37°C**

The TLC plates showed clearly that degradation was rapid at 37°C and it was essentially complete after one day.

Fresh CMS chromatograms showed two separated spots of Rf values of 0.63 and 0.56 with a light streak. However, after two hours of storage at 37°C the two spots merged, 12 hours later, both spots disappeared and the streak become heavier (Figure 6.9).

The fresh solution’s original spot intensity was increased considerably after 12 hours of storage. After one day the CMS solution almost hydrolyzed to colistin (figure 6.9). The results for CMS degradation shown here using TLC plates were consistent with the results of Li *et al* (2003b). They reported that aqueous CMS solution was stable for two days at 4°C and was degraded within 12 hours when stored at 37°C.
The TLC results in this thesis offer supportive evidence to the bioassays and HPLC methods but cannot be relied upon when used in isolation.

6.3.2 Detection of CMS degradation using HPLC

Freshly prepared CMS in deionised water for HPLC at a concentration of 0.5667mmol/L (1000mg/L) was stored at -20°C, 4°C, 25°C, or 37°C. Samples of these solutions were taken at different time intervals (over five days) and injected into the HPLC system using 25% 1.2mM of sodium sulphate: 75% Acetonitrile, pH5 as mobile phase. The flow rate was 1ml/min. Detection was at 210nm (method was developed as in section 4.2.5). These experiments were performed twice (duplicate injection for each sample).

Degradation of CMS in serum was not included in this study as preliminary investigations showed that it was too difficult to extract CMS from the serum. This additional procedure would have been necessary as it is not possible to inject the serum directly onto the HPLC column.
Results

Detection of CMS degradation at -20°C

Figure 6.10. Fresh and stored CMS at -20°C over five days. (The chromatograms have been shifted vertically for clarity).

Peak A: Short retention peak suggested as being colistin base or CMS-1
Peak B: Short retention peak suggested as being colistin base or CMS-1
Peak C: Peak suggested as being CMS-2
Peak D: Long retention peak suggested as being CMS-3
Peak E: Long retention peak suggested as being CMS-4 and CMS-5
These peaks were suggested to be as described in figure 6.10 by the author. These conclusions were assumed as the long retention peaks resulted from the binding between active sites on the SAX column (stationary phase) and highly sulphonated compounds such as CMS-4 or CMS-5, as these compounds have high affinity to the stationary phase. The less sulphonated compounds (short peaks) such as colistin base or CMS-1 which elute faster as they have greater affinity to the less polar mobile phase.

The chromatograms showed a large number of peaks even when the solution was freshly prepared. This suggests that the original material was not a mixture of completely methylsulphonated colistin A and B. Presumably the sample contained colistin with a variable number of methylsulphonated groups. However from the overlaid chromatograms it is apparent that no degradation had occurred. The relative intensities of all the peaks were very similar for the fresh sample and that which was stored for five days at -20°C (Figure 6.10).

**Detection of CMS degradation at 4°C**

After 48 hours storage at 4°C the chromatogram showed almost the same peaks with the same areas under the curve (AUC) as the fresh CMS solution (Figure 6.11). This chromatogram indicated that CMS was stable for two days when stored at 4°C.
Figure 6.11: CMS stored at 4°C over two days (The chromatograms have been shifted vertically for clarity).
Figure 6.12: CMS stored at 4°C over five days (The chromatograms have been shifted vertically for clarity).

For the first two days, samples of CMS were taken at 0, 24, and 48 hour intervals; Areas under the curves of the peaks were measured by manual integration but changes less than 3% were ignored due to the measuring errors in the sample chromatogram. After two days storage of CMS solution at 4°C, the chromatogram peaks appeared earlier and their areas were changed (Figure 6.12). Fresh CMS solution peaks appeared after 15 minutes, while they appeared after about 7 minutes when stored at 4°C over five days.
The chromatograms showed a decrease in the area under curve (AUC) of the long retention time peaks and a corresponding increase in the AUC of the shorter retention time peaks due to less methylsulphonated compounds.

**Figure 6.13** shows the change in peak areas from the HPLC of CMS when stored at 4°C over four days. All peaks maintained constant areas for the first two days showing that CMS in aqueous solution was stable for two days when stored at 4°C. After two days of storage the AUC of peak A and B (suggested as being colistin base or CMS-1) were increased which suggests that this peak represents the conversion of 5,4,3,2 or 1-sulphomethylated group of CMS to CMS of 1-sulphomethylated group or colistin itself (multi-sulphonated CMS to lesser substituted compounds). The peak assigned as colistin base or CMS-1 appeared even in a fresh solution suggesting that CMS contained an amount of unsulphonated material. This was corroborated by the TLC results where fresh solutions always showed a spot of the colistin.
The AUC of peak C decreased slightly, while peaks D and E dropped after the third day then slightly increased. This decrease followed by increase may be due to the conversion of the more sulphonated groups to the less sulphonated ones. This overall decrease in peak C, D and E which were suggested as being CMS-2, CMS-3, CMS-4 and CMS-5 corresponds to conversion of these highly sulphonated groups to CMS-1 or colistin base.

Detection of CMS degradation at 25°C

![Graph showing CMS degradation](image_url)

**Figure 6.14:** CMS stored at 25°C over 24 hours (The chromatograms have been shifted vertically for clarity).
**Figure 6.14** shows that the chromatogram peaks have eluted earlier and their areas have changed ([Figure 6.14](#)) after storage for 12 hours at 25°C. The chromatograms show a decrease in the AUC of the long retention time peaks (due to more highly methylsulphonated material) and an increase in the shorter retention time peaks due to less methylsulphonated compounds. The chromatogram shows that aqueous CMS was degraded within 12 hours when storing at 25°C.

**Figure 6.15:** CMS stored at 25°C over five days (The chromatograms have been shifted vertically for clarity).

**Figure 6.15** shows that the chromatogram peaks eluted earlier and their areas changed gradually over a period of five days.
Figure 6.16: CMS at 25°C. (For peaks A, B, C, D, E refer to Figure 6.10)

Figure 6.16 shows the change in area under the curve (AUC) of CMS when stored at 25°C over three days. The AUC of peak A (suggested as being colistin or CMS-1) increased gradually during storage over 3 days; thus suggesting that this peak represents the conversion of highly sulphonated compounds to colistin or CMS-1 (multi sulphonated group to the less sulphonated).

The AUC of peaks B, D and E decreased after three days where CMS-3, CMS-4 and CMS-5 convert to CMS-2, CMS-3 and CMS-4 respectively, until most of the highly sulphonated groups have converted to CMS-1 or colistin base. Peak C suggested as being CMS-2 follows a random pattern; this may be due to the constant conversion of highly sulphonated to less sulphonated compounds.
Detection of CMS degradation at 37°C

Figure 6.17: CMS stored at 37°C over four hours. (The chromatograms have been shifted vertically for clarity).
Figure 6.18: CMS stored at 37°C over 12 hours. (The chromatograms have been shifted vertically for clarity).
When analysing the CMS samples incubated in water bath at 37°C using the HPLC method, the chromatograms showed a considerable increase in all the shorter retention time peaks after one hour of storage (Figure 6.17). In addition, the peaks appeared earlier in the stored samples than the fresh solution. The chromatograms show that peaks appear at 10 minutes on the second day of storage, while they appeared at 5 minutes after the third and fourth day of incubation at 37°C. This was probably due to the conversion of highly methylsulphonated compounds to the less methylsulphonated compounds, thus it affects CMS binding affinity to the active sites on the HPLC column leading to elution of the CMS much earlier.

It is clear from the substantial change in the chromatogram that a large portion of CMS was converted to CMS-1 or colistin itself after storage at 37°C within a few hours (Figure 6.18 and 6.19).
Figure 6.20: CMS at 37°C over three days. (For peaks A, B, C, D, E refer to Figure 6.10)

Figure 6.20 shows the change in peak area under the curve of CMS solution when stored at 37°C over three days. Peaks A and B reduced over a period of 12 hours and 6 hours respectively, and this may represent the conversion of CMS-1 to colistin itself or conversion of colistin to other degradation products since the storage temperatures were high. Subsequently, the AUC of Peaks A and B followed a random pattern.

Peaks C and D (suggested as being CMS-2 and CMS-3) decreased more rapidly during storage for the first 6 hours, after which peak C remained constant while peak D continued to decrease. This suggests that CMS was converting to colistin within 6 hours as the CMS-2 and CMS-3 were converting to the less sulphonated groups. After five hours, the AUC of peak E (suggested to be CMS-4 and 5) decreased as the highly sulphonated group was converted to the less sulphonated groups; this conversion was accelerated at the elevated temperatures. Subsequently, these peaks increased dramatically due to the degradation of the formed colistin base.
Generally, the results from these experiments (microbiological assay, TLC and HPLC assay) indicate that stability of both CLS and CMS is temperature dependent. The hydrolysis of both antibiotics is clearly evident at temperatures such as 37°C.

Table 6.1 and table 6.2 summarise the results of all the work carried out in this chapter.

Table 6.1: Detection of CMS degradation at different temperatures and by different methods

<table>
<thead>
<tr>
<th></th>
<th>-20°C</th>
<th>4°C</th>
<th>25°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioassay (9 days experiment)</td>
<td>Stable for 9 days</td>
<td>Stable for one day, starts to degrade within three days</td>
<td>Fairly stable for one day.</td>
<td>Degraded within one day</td>
</tr>
<tr>
<td>TLC (8 days experiment)</td>
<td>Stable for 8 days</td>
<td>Stable for two days</td>
<td>Degraded within one day</td>
<td>Starts to degrade within two hours, completely degraded within one day</td>
</tr>
<tr>
<td>HPLC (5 days experiment)</td>
<td>Stable for 5 days</td>
<td>Stable for two days</td>
<td>Degraded within 12 hours</td>
<td>Degraded within 2 hours</td>
</tr>
<tr>
<td>HPLC by Li et al (2003b)</td>
<td>-</td>
<td>Stable for two days</td>
<td>-</td>
<td>Degraded completely within 12 hours</td>
</tr>
</tbody>
</table>
Experiments in this chapter indicate that CMS in aqueous solution or in serum are stable at \(-20^\circ\text{C}\) for at least 5 days (by HPLC).

Aqueous solutions of CMS at \(4^\circ\text{C}\) have been shown to be stable for only two days (Table 6.1) which was confirmed by testing CMS solution microbiologically and chemically. When tested microbiologically, results showed that CMS was stable for one day and was degraded within three days, this was due to the test frequency at 0,1,3,5,7 and 9 days.

CMS stored at \(25^\circ\text{C}\) was shown to degrade within one day. The more sensitive HPLC method suggests that the CMS degrades within 12 hours.

CMS stored at \(37^\circ\text{C}\) was shown to degrade within one day when tested microbiologically. The more sensitive TLC and HPLC methods showed that CMS starts to degrade within two hours. According to Li et al, (2003b), CMS is completely degraded within 12 hours when stored at \(37^\circ\text{C}\).

Table 6.2: Detection of CLS degradation at different temperatures and by different methods

<table>
<thead>
<tr>
<th></th>
<th>(-20^\circ\text{C})</th>
<th>(4^\circ\text{C})</th>
<th>(25^\circ\text{C})</th>
<th>(37^\circ\text{C})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioassay</td>
<td>Stable for 9 days</td>
<td>Stable for 9 days</td>
<td>Stable for 9 days</td>
<td>Starts to degrade within three days</td>
</tr>
<tr>
<td>(9 days experiment)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLC</td>
<td>Stable for 8 days</td>
<td>Stable for 8 days,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8 days experiment)</td>
<td></td>
<td>Stable for 8 days</td>
<td>Stable for 4 days</td>
<td></td>
</tr>
<tr>
<td>HPLC by Li et al (2003b)</td>
<td>-</td>
<td>Stable for 60 days</td>
<td>-</td>
<td>Stable for five days</td>
</tr>
</tbody>
</table>

Experiments in this chapter indicate that CLS in aqueous solution or in serum are both stable at \(-20^\circ\text{C}\) for at least 8 days.
The results of storing CLS at 4°C showed stability for the complete period of the experiment which complies with the findings of Li et al (2003b), while they differ from the results of Li et al (2003b) when stored at 37°C. These differences may be due to the method used as the HPLC which was used by Li et al was more sensitive than TLC and does not need incubation as in the bioassay.
CHAPTER SEVEN

Studies into the mechanism of action of
Colistin sulphate and colistin methanesulphonate
7.1 Studies into the effect of CLS and CMS on the permeability of the bacterial cytoplasmic membrane

The bacterial cytoplasmic membrane is a delicate organelle and is metabolically highly active. It acts primarily as a selective permeability barrier between the cytosol within the cell and the external cell environment of the bacterium (Al-Adham et al, 1998). It is the principal site of action for a number of antimicrobial agents where membrane damage leads to leakage of cell contents and ultimately death.

The mechanisms of action of both forms of colistin, CLS and CMS were investigated against A. *lwoffii* R46383. Potassium leakage studies together with the release of 260nm absorbing materials were used to assess different levels of membrane damage as a result of interaction between both CLS and CMS and the bacterial cell.

**7.1.1 Potassium leakage studies**

The sensitivity of the cytoplasmic membrane of the bacterial cell to an antimicrobial agent may be determined by studying the leakage of intracellular constituents from the cell (Lambert and Hammond, 1973). Depletion of the internal potassium pool by around 30% is believed to trigger the activation of latent ribonucleases pre-synthesised within the cell (Lambert and Smith, 1976). The study of potassium ion leakage from bacteria therefore provides an immediate insight into minor alterations in membrane permeability caused by CLS and CMS.

**7.1.1.1 Preparation of bacterial culture**

A culture of *A. lwoffii* R46383 was grown overnight on a large Petri dish containing 200ml of synthetic broth AOAC solidified with 1.5% technical agar no.5 (prepared as detailed in section 2.1.1.1). Potassium chloride solution (prepared as detailed in section 2.1.1.4) was added to the agar to promote enhanced intracellular potassium ion concentration. The bacteria were harvested by washing the surface of the agar with 20ml aliquots of 0.1M magnesium chloride solution (prepared as detailed in section 2.1.1.4).
The magnesium enables the bacteria to resist cellular stress, and hence reduces the possibility of leakage of potassium ions during the harvesting process (Tempest and Strange, 1966). The cell suspension was centrifuged at 300g for 25 minutes, and the pellets washed twice with 20ml of magnesium chloride solution. The washed cell pellet was then re-suspended in deionised water and standardized to give a cell concentration of $8 \times 10^8$ or $1 \times 10^{10}$ CFU/ml.

7.1.1.2 Measuring potassium ion concentrations using Atomic Absorption Spectrophotometry

Potassium ion concentration was determined using a Perkin Elmer Atomic Absorption Spectrophotometer (Perkin Elmer Beaconsfield, UK) in the Flame Emission mode.

Prior to analysing samples the spectrophotometer was calibrated using a series of standard solutions of potassium chloride (BDH analytical grade) in deionised water and HEPES buffer at concentrations of 0.1, 0.2, 0.4, 0.6 and 1mg/L (figure 7.1). For all concentrations of HEPES buffer, the potassium emission was too high due to the interference of potassium with the sodium ions available in HEPES buffer. With deionised water, a linear relationship was found between potassium chloride concentration and emission (figure 7.1). Standard solutions of potassium chloride in deionised water were measured at intervals during the analysis. This ensured that any systemic errors with the aspiration of the sample during analysis would be detected.

For each point, three potassium readings were taken. This experiment was performed three times.
Results

Figure 7.1: The relationship between potassium ion concentrations and emission. n=3 ± Stdev

7.1.1.3 Determination of the intracellular potassium ion pool of *Acinetobacter lwoffii* R46383

The intracellular potassium ion pool of *A. lwoffii* R46383 was determined in order to calculate the maximum amount of potassium that could be released during the experiment. This was important so that the potassium leakage could be calculated as percentage leakage.

2.5ml of bacterial suspension at a concentration of $8 \times 10^8$ Cfu/ml (prepared as detailed in section 7.1.1.1) was diluted with 10ml of deionised water containing 1mM Cetrimide (equilibrated at 37°C) and incubated at 37°C for 10 minutes (final bacterial concentration was $1.6 \times 10^8$ Cfu/ml). Cetrimide (prepared as detailed in section 2.1.1.4) is known to cause the release of the intracellular potassium pool from the cells, it is a cationic surfactant and disrupts the cell membrane.
A 2.5ml sample was removed after 10 minutes, filtered through a 0.45µm cellulose nitrate membrane filter (Nalgene) to remove the cells from the samples (due to filtration difficulties in filtering such a dense suspension, three filters were used for each sample), diluted with 2.5ml of deionised water (samples were diluted so the potassium leakage would be in the range of 0.1 to 1mg/L as the standard solutions) and potassium ion concentration was measured using Perkin Elmer Atomic Absorption Spectrophotometer in the Flame Emission mode as detailed in section 7.1.1.2.

In order to confirm that this represented a true total pool value, 2.5ml aliquots of the bacterial suspension at a concentration of 8 x 10⁸ CFU/ml were also treated with 10ml of 1:10 Triton X100 (1%) and 1% trichloroacetic acid, (final bacterial concentration was 1.6 x 10⁸ CFU/ml) while others were subjected to boiling for one hour. All these samples (2.5ml) were collected after treatment, filtered through 0.45µm cellulose nitrate membrane filter (three filters) to remove the cells from the samples, diluted with 2.5ml of deionised water (samples were diluted so the potassium leakage would be in the range of 0.1 to 1mg/L as the standard solutions) and potassium ion concentrations were measured using Perkin Elmer Atomic Absorption Spectrophotometer in the Flame Emission mode as detailed in section 7.1.1.2.

Mechanical disruption of the bacterial cells was carried out using a Bead Beater (as described in section 2.2.15). A 2.5ml sample of A. lwofii suspension at a concentration of 8 x 10⁸ CFU/ml (prepared as detailed in section 7.1.1.1) was diluted with 10ml of deionised water (final bacterial concentration was 1.6 x 10⁸ CFU/ml). This diluted culture in deionised water was introduced in the beater surrounded by an ice jacket (to prevent degradation of RNA and proteins due to the high temperatures) with an either quarter or half chamber of beads for 3, 4 and 5 cycles. Each cycle lasted 30 seconds with 30 seconds between each cycle. 2.5ml samples were removed after each cycle, transferred to sterile centrifuge tube, centrifuged to separate the supernatant liquid and were diluted in 2.5ml of deionised water. In order to confirm complete bacterial death, further experiments were setup to compare the viable cell count after different periods of beading with either quarter or half chamber of beads.
From the counts, it was concluded that beating with a half chamber of beads for three cycles was the optimum system required to disrupt the cells.

**Results**

![Graph showing potassium ions release mg/L for different treatments](image)

**Figure 7.2: Determination of total intracellular pool of *Acinetobacter lwofii* R46383. n=3 ± Stdev** (Results were multiplied by a dilution factor of 2)

**Figure 7.2** shows that the use of cetrimide yielded the largest potassium pool (1.6mg/L), while mechanically disrupting the cells with the bead beater produced a rather smaller pool of 1.4mg/L.

Potassium ions leaked by 1% trichloroacetic acid varied with each experiment, which may be due to the original potassium content of the acid. Chemicals such as 1:10 Triton X100 (1%) leaked only 0.9mg/L potassium ions, while bacteria subjected to boiling for one hour leaked 1.2mg/L potassium.

Since 1mM cetrimide released the greatest amount of potassium from the cells, it was considered as a 100% potassium intracellular pool.
7.1.1.4 Determining potassium ions leakage from untreated bacterial cells

The mechanism of action of CLS and CMS was detected by potassium ion leakage using *A. lwofii* as indicator organism. This strain is sensitive to colistin which is widely used clinically to treat *A. lwofii* infections.

Prior to the measurement of intracellular potassium leakage after exposure to both forms of colistin it was important to determine the extent of intracellular potassium ions leakage from bacterial cells in the absence of CLS and CMS. This experiment measured potassium leaked from the bacterial suspension in deionised water at 37°C over one hour.

30ml aliquots of deionised water were pipetted into a glass tube, placed in a water bath at 37°C and left to equilibrate at this temperature for 10 minutes. 7.5ml of *A. lwofii* R46383 at a concentration of $8 \times 10^8$ Cfu/ml (prepared as detailed in section 7.1.1.1) was added in each tube so the final bacterial concentration was $1.6 \times 10^8$ Cfu/ml. 2.5ml of sample was immediately removed with a syringe and further samples were removed at various time intervals for a period of one hour. These samples were passed through of 0.45µm cellulose nitrate membrane filter (due to filtration difficulties in filtering such a dense suspension, three filters were used for each sample) to remove the cells from the samples and the potassium ion concentrations in the filtrates were measured using a Perkin Elmer Atomic Absorption Spectrophotometer in the Flame Emission mode as detailed in section 7.1.1.2. Potassium ion concentration in the diluent (deionised water) controls was determined as well.
Results

Figure 7.3: Potassium ion leakage from untreated bacterial suspension. n=3± Stdev

These results (Figure 7.3) show that A. lwofii R46383 produced an amount of potassium ion in deionised water at 37°C. The graph demonstrates that intracellular potassium leaked from the bacterial cells increased slightly within one hour, from 0.53mg/L at 2.5 minutes to 0.71mg/L at one hour time (potassium ions concentration of the diluent (deionised water) was undetectable).

It was observed that the cytoplasmic membrane was naturally ‘leaky’ to potassium ions when the cells in the stock suspension were maintained in deionised water, and in addition the bacterial harvesting procedure as detailed in section 7.1.1.1 may cause some leakage.
HEPES buffer was usually used as a diluent to maintain osmolarity but using it in this study was not possible as it contains sodium ions which interfered with potassium ions during analysis.

The potassium ions leaked from the bacterial cells in the presence of deionised water will affect the concentrations of intracellular potassium leaked from A. lwoffii R46383 treated with CLS and CMS. Therefore the amounts of potassium released from untreated bacteria were subtracted from the intracellular potassium released from the bacterial cells treated with CLS and CMS.

### 7.1.1.5 Effect of filtration system on potassium ion concentration

To determine potassium ion concentrations leaked from bacteria treated with both forms of colistin, a filtration system was used to remove the cells from the tested samples. The effect of the filtration system on potassium ion concentrations in the analysed sample was detected.

Three solutions of potassium chloride in deionised water were filtered (0.4, 0.6 and 1mg/L); passed through 0.45µm cellulose nitrate membrane filter (due to filtration difficulties in filtering such a dense suspension, three filters were used for each sample) and then samples of 2.5ml were analysed for potassium ion concentration. Samples of the same solutions without filtering were analysed (as detailed in section 7.1.1.2) for potassium ions to determine if the potassium ions might adsorbed to the filter system. This experiment was repeated three times.

**Results**

No differences were found between the filtered samples and the unfiltered samples. This experiment reveals that filtration has no effect on potassium ion concentration in the sample (One way ANOVA P>0.05).
7.1.1.6 Effect of CLS and CMS on potassium ion concentrations

The effect of both forms of colistin, CLS and CMS on potassium ion concentrations was determined. These preliminary investigations were carried out to ensure that potassium released from the samples is totally due to the effect of the antibiotics on the cells.

A potassium chloride solution at a concentration of 1mg/L was compared with potassium chloride 1mg/L to which either 0.0453mmol/L of CLS or CMS (64mg/L of CLS or 80mg/L of CMS) was added so the final concentration of potassium in the antibiotic solution was 1mg/L. 2.5ml of the samples of these solutions were analysed for potassium ion concentrations as detailed in section 7.1.1.2.

Results

Table 7.1: Effect of CLS and CMS on potassium ion concentration.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Av. Potassium ion concentration.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium chloride solution 1mg/L</td>
<td>0.99mg/L</td>
</tr>
<tr>
<td>Potassium chloride solution 1mg/L + CLS</td>
<td>0.96mg/L</td>
</tr>
<tr>
<td>Potassium chloride solution 1mg/L + CMS</td>
<td>0.95mg/L</td>
</tr>
</tbody>
</table>

Table 7.1 shows that neither CLS nor CMS affected the potassium ion determination of samples.

7.1.1.7 Leakage of intracellular potassium ions from *Acinetobacter lwaffii* R46383 treated with CLS and CMS

To determine the intracellular potassium ion leakage caused by CLS and CMS from a bacterial suspension, 5ml of 8 x 10^8 Cfu/ml of *A. lwaffii* R46383 suspension (prepared as in section 7.1.1.1) was added to 20ml of aqueous solution of CLS or CMS (final bacterial concentration was 1.6 x 10^8 Cfu/ml) in a range of concentrations of 0.0003, 0.0028, 0.0057, 0.0113 and 0.0227mmol/L at 37°C (equivalent to 0.5, 5, 10, 20 and 40 of CMS and 0.4, 4, 8, 16 and 32 of CLS).
2.5ml samples were removed at 2.5, 5, 10, 20, 30, 45, and 60 minutes (zero time was not practical, as the time taken to remove the sample and filter it was approximately two minutes), filtered through a 0.45µm cellulose nitrate filter to remove the bacterial cells (due to filtration difficulties in filtering such a dense suspension, three filters were used for each sample) and then were diluted with 2.5ml of deionised water (samples were diluted so the potassium concentrations would be in the range of 0.1 to 1mg/L as the standard solutions). The filtrates were then analysed for potassium ion concentration as detailed in section 7.1.1.2.

The antibiotic free bacterial suspension was analysed for potassium ion release over one hour and these potassium amounts were subtracted from the tests results (as detailed in section 7.1.1.3). Results were plotted as intracellular potassium leaked of the total pool (cetrimide).

**Results**

Results shown in **Figure 7.4 and 7.5** are calculated by excluding the values for potassium ions released form the untreated bacteria then the obtained results were calculated according to the cetrimide pool value.
Figure 7.4: Potassium ion leakage from *Acinetobacter lwofii* R46383 in the presence of increasing concentrations of CLS. n=3 ± Stdev
Figure 7.5: Potassium ion leakage from *Acinetobacter lwofii R46383* in the presence of increasing concentrations of CMS. n=3 ± Stdev.
The results shown in Figure 7.4 and 7.5 demonstrate that both CLS and CMS induced leakage of intracellular potassium, indicating that both forms of colistin cause damage to the cytoplasmic membrane of *A. lwaffii* R46383.

Exposure of the cells to CLS at high concentrations such as 0.0227mmol/L (32mg/L) caused immediate intracellular potassium leakage reaching the maximum pool value within ten minutes of contact. A concentration of 0.0113mmol/L (16mg/L) CLS released maximum pool potassium levels within 45 minutes. The introduction of a lower CLS concentration of 0.0057mmol/L (8mg/L) caused a slower leakage of intracellular potassium but still gave rise to nearly total potassium release by the end of one hour (Figure 7.4). Very low concentration of CLS such as 0.0028mmol/L (4mg/L) produced low amounts of potassium leakage to about only 30% of the maximum pool potassium, while concentrations of 0.0003mmol/L (0.4mg/L) seems not to cause damage to the cytoplasmic membrane even after one hour of exposure to the antibiotics.

A different leakage profile was observed for CMS. Exposure of the cells to CMS at high concentrations such as 0.0227mmol/L (40mg/L) caused 30% intracellular potassium leakage within ten minutes of contact followed by release of total pool levels over 45 minutes. Slow intracellular potassium leakage was observed with CMS concentrations of 0.0113, 0.0057 and 0.0003mmol/L (20, 10, and 0.5mg/L) where the potassium leakage reached a plateau and did not exceed 40% of pool even at the end of one hour (Figure 7.5). Exposure to very low concentrations of CMS such as 0.0003mmol/L (0.5mg/L) seems not to cause damage to the cytoplasmic membrane even after one hour of exposure to the antibiotics (Figure 7.5).

The pool extraction procedure using cetrimide did not seem to be efficient at releasing all of the intracellular pool potassium ions since the use of high concentrations of CLS gave readings in excess of 100%.
In this thesis, potassium leakage of 100% was suggestive of maximum pool release for a given leakage marker. Although large amounts of potassium were released from the treated cells, this does not rule out the possibility of potential survivors. Cells exposed to high concentrations of CLS may have:
(a) Received a lethal dose and were killed immediately
(b) Received a sub-lethal dose and were able to recover by making good the loss of intracellular materials
(c) Avoided injury due to the reduction of the active dose by killed cells, overall population effect was observed, so some cells may well survive.
(d) Only selected cell population is being tested

7.1.2 Release of 260nm-absorbing materials

7.1.2.1 Introduction
Leakage of intracellular constituents from bacterial cells follows a defined pattern. The first marker of membrane damage is the release of potassium ions (Lambert and Hammond, 1973) which is indicative of subtle perturbations in structure; this is followed by the release of 260nm absorbing materials indicating more extensive damage and the release of larger molecules such as purines, pyrimidines, DNA and RNA (Hugo and Bloomfield, 1971).

7.1.2.2 Detecting the release of 260nm-absorbing materials from untreated bacterial suspensions
Prior to the measurement of the release of 260nm-absorbing materials from cells treated with both forms of colistin it was necessary to determine the release of 260-absorbing materials from A. lwaffii R46383 in the presence of diluent alone. This experiment measured the release of 260nm-absorbing materials from a bacterial suspension over one hour at 37°C.
18ml of deionised water was pipetted into a glass tube, and placed in a water bath at 37°C and left to equilibrate at this temperature for 10 minutes. 2ml of a $1 \times 10^{10}$ Cfu/ml of *A. lwaffii* R46383 stock suspension (prepared as detailed in section 7.1.1.1 without the addition of potassium chloride) was added to the tube so the final bacterial concentration was $1 \times 10^9$ Cfu/ml. The contents were mixed and 3ml samples were removed at different time intervals over one hour and passed through 0.45µm cellulose nitrate membrane filter (Nalgene) to remove the cells from the samples (due to filtration difficulties in filtering such a dense suspension, three filters were used for each sample) and 260nm absorbing materials were measured using a Perkin Elmer spectrophotometer at a wavelength 260nm. A matched pair of UV cuvettes (Hellma precision synthetic far-UV quartz 200-2500, light path 10mm) was used to measure each filtrate sample, blanked with deionised water.

It was not practical to measure absorbance at zero time due to the time taken in sampling and filtration (using three filters of 0.45µm cellulose nitrate). Samples of 3ml of deionised water were filtered through three separate membrane filters (1ml in each filter) to ensure that the membrane filters do not leach the surfactant which is used as wetting agent. The absorbance of these filtrates was measured using a Perkin Elmer spectrophotometer at a wavelength of 260nm against deionised water as a blank. This value could be considered as a zero point time.

**Results**

**Table 7.2: Effect of filtration on the absorbance at 260nm**

<table>
<thead>
<tr>
<th></th>
<th>Measured absorbance at 260nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>0.010</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0.008</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>0.005</td>
</tr>
<tr>
<td>mean</td>
<td>0.008</td>
</tr>
<tr>
<td>Stdev</td>
<td>0.003</td>
</tr>
</tbody>
</table>
Figure 7.6: 260nm absorbing material leakage from untreated bacterial suspension. n=3± Stdev

Table 7.2 shows that membrane filters do not leach the surfactant which is used as wetting agent in the filter membranes as the absorbance values were considered negligible (Stdev 0.003), thus zero time was considered as zero absorbance.

These results showed (Figure 7.6) that untreated *A. lwaffii* R46383 released 260-absorbing materials when suspended in deionised water at 37°C over one hour (0.73 absorbance after one hour).

The leakage detected from the bacterial cells under these conditions will affect the concentrations of the released 260nm absorbing materials from *A. lwaffii* R46383 treated with CLS and CMS. Therefore the amounts of 260nm-absorbing material released from untreated bacteria were subtracted from the values released from the bacterial cells treated with CLS and CMS.
7.1.2.3 Effect of CLS and CMS on the 260nm absorbance values

The effect of both forms of colistin, CLS and CMS on the 260nm absorbance values was determined.

CLS and CMS aqueous solutions at various concentrations as shown in table 7.3 were prepared, samples of 3ml were filtered through 0.45µm cellulose nitrate membrane filter (Nalgene) and 260nm absorbing materials were measured using Perkin Elmer spectrophotometer at wavelength 260nm. A matched pair of UV cuvettes (Hellma precision synthetic far-UV quartz 200-2500, light path 10mm) was used to measure each filtrate sample, blanked with deionised water.

Results

Table 7.3: Effect of CLS and CMS on absorbance at 260nm

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Absorbance at 260nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLS 0.0453mmol/L</td>
<td>0.018</td>
</tr>
<tr>
<td>CLS 0.0227mmol/L</td>
<td>0.010</td>
</tr>
<tr>
<td>CLS 0.0057mmol/L</td>
<td>0.0083</td>
</tr>
<tr>
<td>CMS 0.0453mmol/L</td>
<td>0.016</td>
</tr>
<tr>
<td>CMS 0.0227mmol/L</td>
<td>0.012</td>
</tr>
<tr>
<td>CMS 0.0057mmol/L</td>
<td>0.0097</td>
</tr>
</tbody>
</table>

Table 7.3 shows that absorbance of CLS and CMS at 260nm can be neglected as it is considered to be minor compared to the absorbance values observed in the release from untreated bacteria as in section 7.1.2.2.
7.1.2.4 Determination of total intracellular pool of 260nm-absorbing materials in *Acinetobacter lwoffii* R46383

To determine the total intracellular pool of 260-absorbing materials for *A. lwoffii* R46383, 1ml of bacterial suspension at a concentration of 1 x 10^{10} Cfu/ml (prepared as detailed in section 7.1.1.1 without the addition of potassium chloride) was diluted with 9ml of deionised water containing either 1mM Cetrimide;1:10 Triton X100 or trichloroacetic acid (1% and 5%) (Prepared as detailed in section 2.1.1.4), while other cells were boiled for one hour at 100°C or autoclaved at 121°C (15 psi) for 15 minutes. Some cells were exposed to the bead beating technique (as detailed in section 2.2.15).

3ml of these solutions (except the samples produced from the bead beading) were filtered through 0.45µm cellulose nitrate membrane filter (three filters). Bacterial cells which were exposed to the bead beading process were treated as detailed in section 7.1.1.3.

Results

![Graph showing absorbance at 260nm for different methods of cell treatment.](image)

**Figure 7.7: Determination of total intracellular pool of 260nm-absorbing materials of *Acinetobacter lwoffii* R46383. n=3 ± Stdev**
There was no apparent release of 260nm-absorbing materials from cells treated with Triton X100 but this may be due to the interference of the surfactant with the analytical process. The Bead Beater produced the greatest release of 260nm-absorbing materials and therefore this value was taken to be the total intracellular pool (Figure 7.7).

7.1.2.5 Release of 260nm-absorbing materials from Acinetobacter lwoffii R46383 treated with CLS or CMS.

*A. lwoffii* R46383 suspension was prepared as detailed in section 7.1.1.1 (without the addition of potassium chloride) and 2ml at the concentration of $1 \times 10^{10}$ Cfu/ml was added to 18ml of varying concentrations (as shown in figure 7.8) of aqueous CLS or CMS, so the final cell concentration was $1 \times 10^9$ Cfu/ml. These cells were shaken at 37°C in an orbital shaking water bath. 3ml samples were removed at different time intervals over one hour and passed through 0.45µm cellulose nitrate membrane filter (Nalgene) to remove the cells from the samples (due to filtration difficulties in filtering such a dense suspension, three filters were used for each sample) and the concentrations of 260nm absorbing materials were measured using a Perkin Elmer spectrophotometer at a wavelength of 260nm.
Results

Figure 7.8: Determination of release of 260nm-absorbing materials from CLS treated *Acinetobacter lwofii R46383*. n=3 ± Stdev
Figure 7.9: Determination of release of 260nm-absorbing materials from CMS treated *Acinetobacter lwoffii* R46383. n=3 ± Stdev

260nm-absorbing materials leaked from cells treated with CLS leakage was rapid at all concentrations used (40 to 60% at zero time), while leakage of 260nm-absorbing materials was much less with cells treated with CMS (below 20% with a concentration of 0.0453mmol/L or 80mg/L) (Figure 7.8 and 7.9).

It is clear from the above figures that release of 260nm absorbing materials was concentration dependent for both forms of colistin.
7.2 Determination of adsorption isotherms

A systematic approach may be used to understand the mode of action of both forms of colistin by determining their adsorption isotherm properties. The affinity of an antibiotic for a given cell may be determined by adsorption isotherms, which may provide valuable information on the availability of target sites. At a specific exposure time point, uptake isotherms record the amount of the antibiotic bound to cells versus the residual level of antibiotic.

The adsorption of both forms of colistin, CLS and CMS, to cells was studied by adding *A. lwaffii* R46383 suspension to different concentrations of both antibiotics. At specific time intervals over two hours, samples were removed, cells were separated by passing through 0.45µm cellulose nitrate membrane filters and the residual amount of the antibiotics in solution were determined by UV spectroscopy at a wavelength of 210nm. A matched pair of UV cuvettes (Hellma precision synthetic far-UV quartz light path 10mm) was used to measure each filtrate sample, blanked with deionised water. Isotherms may be plotted from these data, and information on the rate and total uptake determined.

7.2.1 Preliminary investigations of adsorption isotherms determined by spectroscopy.

In order to determine the best wavelength to detect both forms of colistin, aqueous CLS and CMS at various concentrations (*Table 7.4 and 7.5*) were scanned from the wavelengths of 200nm to 300nm. Both CLS and CMS absorbed at wavelengths of 210 and 220nm, while below 210nm absorption was unclear as all ions absorbed there. Since absorption at 210 was greater, this wavelength was chosen to conduct adsorption isotherms experiment.

Preliminary investigations were also undertaken to detect the lowest concentration of each antibiotic that could be detected using this technique at the specified wavelength.
Table 7.4: Absorbance of different concentrations of CLS at 210 and 220nm.

<table>
<thead>
<tr>
<th>mmol/L</th>
<th>210nm</th>
<th>220nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.085</td>
<td>0.753</td>
<td>0.376</td>
</tr>
<tr>
<td>0.0453</td>
<td>0.599</td>
<td>0.266</td>
</tr>
<tr>
<td>0.0227</td>
<td>0.291</td>
<td>0.09</td>
</tr>
<tr>
<td>0.0113</td>
<td>0.133</td>
<td>0.036</td>
</tr>
<tr>
<td>0.0057</td>
<td>0.107</td>
<td>0.0308</td>
</tr>
</tbody>
</table>

Table 7.5: Absorbance of different concentrations of CMS at 210 and 220nm.

<table>
<thead>
<tr>
<th>mmol/L</th>
<th>210nm</th>
<th>220nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.085</td>
<td>1.03</td>
<td>0.353</td>
</tr>
<tr>
<td>0.0453</td>
<td>0.593</td>
<td>0.268</td>
</tr>
<tr>
<td>0.0227</td>
<td>0.261</td>
<td>0.108</td>
</tr>
<tr>
<td>0.0113</td>
<td>0.083</td>
<td>0.0185</td>
</tr>
<tr>
<td>0.0057</td>
<td>0.01</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Table 7.4 and 7.5 indicated that CLS and CMS absorbed more at 210 than 220nm; therefore the wavelength of 210nm was used in all subsequent work.

0.0227mmol/L (32mg/L for CLS and 40mg/L for CMS) was the lowest concentration of both antibiotics that could be measured at 210nm with limited accuracy, as absorbance readings below 0.2 were considered inaccurate (Table 7.4 and 7.5).
Other investigations were performed on the filtrate of the bacterial cells to ensure that the filtrate of bacterial cells do not interfere with the antibiotic absorption at 210nm. Bacterial filtrate samples of 3ml were removed and filtered (due to the concentrated bacterial culture three filters were used for each sample) through 0.45µm cellulose nitrate membrane filters (Nalgene) to remove cells. A UV scans of the filtrate of various concentrations of A. hwoffii R46383 such as 1 x 10^8 and 1 x 10^9 Cfu/ml (prepared as detailed in section 7.1.1 without the addition of potassium chloride) at 210nm were performed. This experiment was repeated three times.

**Table 7.6: Absorption of various inocula at 210nm. n=3 ± Stdev**

<table>
<thead>
<tr>
<th>Concentrations of Bacteria</th>
<th>Absorbance</th>
<th>Stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^8 Cfu/ml at 210nm</td>
<td>0.065</td>
<td>0.0015</td>
</tr>
<tr>
<td>1 x 10^9 Cfu/ml at 210nm</td>
<td>0.083</td>
<td>0.0018</td>
</tr>
</tbody>
</table>

Table 7.6 indicated that leaked cellular constituents from cells after filtration at concentrations of 1 x 10^8 or 1 x 10^9 Cfu/ml do not interfere with the absorption of the antibiotics at 210nm, as the absorbance readings were below 0.1.

**7.2.2 Adsorption isotherms determined by spectroscopy.**

A. hwoffii R46383 suspension was prepared and harvested (as detailed in section 7.1.1.1 without the addition of potassium chloride). 2ml of cell suspension at a concentration of 1 x 10^{10} Cfu/ml were added to 18ml of aqueous CLS and CMS at concentrations of 0.0227, 0.0453 and 0.085mmol/L (32, 64 and 120mg/L of CLS or 40, 80 and 150mg/L of CMS), so the final bacterial concentration was 1 x 10^9 Cfu/ml, the mixture was shaken at 37°C in an orbital shaking water bath. Samples of 3ml were removed at time intervals over two hours, filtered (due to the concentrated bacterial culture three filters were used for each sample) through 0.45µm cellulose nitrate membrane filters (Nalgene) to remove cells and the antibiotic concentrations were determined by UV spectroscopy at a wavelength of 210nm. A matched pair of UV cuvettes (Hellma precision synthetic far-UV quartz light path 10mm) was used to measure each filtrate sample, blanked with deionised water.
Results

Figure 7.10: Percentage of residual CLS and CMS over time measured by absorption at 210nm (Starting concentration was 0.085mmol/L). n=4 ± Stdev

For concentrations below 0.085mmol/L (120mg/L of CLS and 150mg/L of CMS), experiments were repeated for four times, but the results were highly variable possibly due to the low sensitivity of the used method.

Figure 7.10 shows the residual amounts of CLS and CMS in the bacterial suspension at intervals during the experiment.

The residual amount of CMS in the solution was reduced to about 75% within two minutes and it continued to reduce at a lower rate until it reached about 60% after two hours. The residual amount of CLS dropped to about 25% within two minutes and was undetectable at end of the two hours. A control experiment to demonstrate that the antibiotics were not adsorbed onto the membrane during the course of filtration was conducted as described in section 7.2.3. This showed that adsorbance of the antibiotics was minimal (Table 7.7).
The results from the experiments described above were obtained using UV absorbance as a means of analysis, however this method was found not to be sensitive enough to detect some of the lower concentrations employed in this investigation. Therefore, it was decided to attempt to measure the residual antibiotic using a microbiological bioassay.

### 7.2.3 Preliminary investigations for the adsorption isotherms determined by microbiological assay

In order to confirm that filtration doesn’t affect the activity of CLS or CMS, aqueous solutions of CLS and CMS were prepared at a concentration of 0.0453mmol/L (80mg/L of CMS and 64mg/L of CLS), samples of 3ml of both antibiotics were filtered through 0.45μm cellulose nitrate membrane filters (due to the concentrated bacterial culture three filters were used for each sample) and were compared with unfiltered samples, all samples obtained from the duplicated experiments were assayed microbiologically.

Preliminary investigations were undertaken to ensure that there was no change in antibiotic uptake after 60 minutes. This experiment was conducted by preparing aqueous solutions of CLS and CMS at a concentration of 0.0453mmol/L (80mg/L of CMS and 64mg/L of CLS), these solutions were exposed to *A. lwoffii* R46383 suspension (prepared as in section 7.2.4) and samples of 3ml of both antibiotics were filtered through 0.45μm cellulose nitrate membrane filters (due to the concentrated bacterial culture three filters were used for each sample) and the antibiotic concentrations were determined by the updated microbiological assay at time intervals of 2, 20, 30, 60 and 120 minutes.

### Results

No difference was observed for the mean of the filtered and unfiltered samples (Table 7.7).

For CMS, no difference was found after 60 minutes and 120 minutes exposure to *A. lwoffii* R46383 suspension (prepared as in section 7.2.4), while no difference was found after two minutes and 20, 30 or 60 minutes for CLS.
Table 7.7: Comparison between filtered and unfiltered samples (microbiologically assayed).

<table>
<thead>
<tr>
<th></th>
<th>Filtered samples (Inhibition zones)</th>
<th>Unfiltered samples (Inhibition zones)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLS 0.09mmol/L</td>
<td>27.7mm</td>
<td>27.5mm</td>
</tr>
<tr>
<td>CMS 0.09mmol/L</td>
<td>33mm</td>
<td>33.2mm</td>
</tr>
</tbody>
</table>

7.2.4 Adsorption isotherms determined by microbiological assay

A. lactalis R46383 suspension was prepared and harvested (as detailed in section 7.1.1.1 without the addition of potassium chloride) 2ml of cell suspension at a concentration of \(1 \times 10^{10}\) CFU/ml was added to 18ml of different concentrations (0, 0.0113, 0.0227, 0.0453 and 0.0907mmol/L of CLS or 0, 0.0057, 0.0113, 0.056, 0.113 and 0.226mmol/L of CMS) of both forms of colistin in distilled water, so the final bacterial concentration was \(1 \times 10^{9}\) CFU/ml, the mixture was shaken at 37°C in an orbital shaking water bath. Samples of 3ml were removed at specific time (60 minutes for CMS and two minutes for CLS), filtered (due to concentrated bacterial suspension, three filters were used for each sample) through 0.45µm cellulose nitrate membrane filters (Nalgene) to remove cells and the antibiotic concentrations were determined by the updated microbiological assay (as detailed in chapter three) using *B. bronchiseptica* NCTC 8333. The antibiotic concentrations were calculated with reference to a calibration curve conducted at the same time on bioassay plates with a random Latin square design.

Determination of zero time values were not practical due to the long sampling procedure (about two minutes) therefore values for zero time were calculated from the control samples. Control samples were prepared free from bacteria; 9ml of antibiotic solution at different concentrations were added to 1ml of distilled water (the same conditions were used in the actual experiment but without cells).

The standard curves were made using various concentrations of aqueous CLS and CMS (same concentrations were used in the actual experiment). These solutions were assayed microbiologically as detailed chapter three.
The calibration points for CLS or CMS (figure 7.11 and 7.13) were assayed in triplicates (6 discs for each point in each plate), while each concentration at 60 minutes was assayed with the control or the zero point (in triplicates plates, 6 discs for each point). Samples were collected and stored at 4°C until the last time interval and were assayed at the same time (samples were not stored longer than three hours).

The residual concentrations of antibiotic were calculated from the equations developed from the calibration curves (figures 7.11 and 7.13). These values were then subtracted from the original antibiotic challenge concentrations to determine the amounts of antibiotic bound to the bacterial cells.

**Results**

![Figure 7.11: Calibration graphs for uptake isotherm studies. (CLS aqueous solutions). n=3 ± Stdev](image)

\[ y = 77.496x + 6.9872 \]
\[ R^2 = 0.9025 \]
Figure 7.12: Uptake isotherm studies. (CLS aqueous solutions).

$\text{n}=3 \pm \text{Stdev}$
Figure 7.13: Calibration graphs for uptake isotherm studies. (CMS aqueous solutions). n=3 ± Stdev
Figure 7.14: Uptake isotherm studies. (CMS aqueous solutions).

Figures 7.12 and 7.14 illustrate adsorption isotherms for CLS and CMS respectively. The graph for CLS indicates an H (high affinity) pattern which is a special case of the L (Langmuir) curve. Here the affinity of the solute (CLS) for the adsorbent (the bacterial cell) is so high that in dilute solutions it is adsorbed completely and the initial part of the curve is vertical. The affinity of the solute for the absorbent is so high that in many concentrations such as 0, 0.0113, 0.0227, 0.0453 and even at high concentration such as 0.0907mmol/L the solute is completely absorbed and the equilibrium is equal to zero in all used concentrations within two minutes.

The graph for CMS shows an S-shaped pattern which is indicative of co-operative adsorption. As molecules of antibiotic are adsorbed to the cell they encourage the adsorption of further molecules until all binding sites are eventually filled.
CHAPTER EIGHT

Studies into the cytotoxicity of colistin sulphate and colistin methanesulphonate
8.1 Introduction
CLS was used clinically quite widely until the late 1950s, but serious side effects such as nephrotoxicity and neurotoxicity led to a marked reduction in its use (Beringer, 2001). Although CMS was formulated to have reduced toxicity, less toxic antibiotics became available which subsequently limited the use of CMS. This study was conducted to determine the toxicity of CLS on rat kidney cell culture and compare it with the less toxic compound CMS.

The *in vitro* nephrotoxicity of both forms of colistin was evaluated using cultures of the normal rat kidney NRK-52E cell line. Cultured cells retain most of the properties of their original cells *in vivo* and NRK-52E cells were derived from the proximal tubule (PT) of normal rat kidney. They are able to express receptors for epidermal growth factor (EGF) and they have also been shown to undergo both apoptotic and necrotic cell death (Leussink *et al.*, 2002). Since most nephrotoxic effects are selective to the PT cells of the kidney, NRK-52E cells of normal rat PT origin were considered as an appropriate choice for nephrotoxicity studies.

8.2 Cell culture
NRK-52E cells were obtained from the European Collection of Cell Cultures (ECACC) and were maintained through serial passages in 80cm² Nunc culture flasks using Dulbecco’s Modified Eagles Medium (DMEM) containing 4.9g/L glucose and 0.584g/L L-glutamate (this is the medium of choice and is suitable for most types of cells, including rat cells). The cells were incubated at 37°C in a humidified incubator (Haraeus, Germany) containing 5% carbon dioxide and 95% air (Standard Tissue Culture Environment STCE). The medium was supplemented with 10% (v/v) foetal calf serum (FCS) (the serum is important for optimal growth and function of the cultured cells), and 1% non-essential amino acid solution. The medium (20ml in each flask) was changed the day after passaging and therefore on alternative days. When the cells were about 85-90% confluent (usually between 3 – 4 days, depending on the original numbers of cells), they were passaged for subsequent seeding into 24-well plates at a density of 3.2 x 10⁵ cells/well.
Passaging of cells involved the following process: (i) detaching of the cell monolayer from the base of the culture vessel flask by removal of the old media, washing cells with phosphate buffered saline (PBS), adding trypsin solution (0.1% w/v), followed by 5-10 minutes incubation at 37°C; (ii) inactivation of the trypsin solution by the addition of double volumes of the medium of choice containing 10% FCS; (iii) re-collection of the cells by centrifugation of the cell suspension at 500g for 5 minutes; and (iv) removal of the supernatant liquid with subsequent re-suspension (in duplicate flasks) of the cell pellet in the medium of choice.

To count the cell number in the flask, the pellet was mixed well with 1ml of the medium of choice, 100µl aliquots of the passaged cells were diluted with 100µl of trypan blue solution and cells were counted using a haemocytometer under a light microscope. The count gives an idea of the amount of diluted cells to be added in each well. If the cell counts were higher than the required density, the cells were diluted in the medium to give the required density.

Aliquots containing the passaged NRK-52E cells were seeded at a density of 3.2 x 10^5 cells/cm^2 into each well of a 24-well plate and maintained with the medium of choice at STCE. The medium was supplemented with 10% FCS, 1% non-essential amino acid solution.

8.3 Cytotoxicity studies
The cytotoxicity effect of both forms of colistin (CLS and CMS) on 85 to 90% confluent NRK-52E cells was investigated with the use of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays. MTT assays measure mitochondrial/respiratory function (viability of the cells) while LDH assays evaluate membrane integrity and therefore cell death (Abe and Matsuki, 2000).
8.3.1 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

The MTT assay is based on the ability of the viable cells to take up and reduce the tetrazolium salt MTT to a water-insoluble purple or blue coloured formazan product with the aid of the mitochondrial dehydrogenase enzyme system. The formazan formed is impermeable to the cell membrane so it accumulates within healthy cells only. Therefore, solubilisation of the cells using a solvent such as dimethyl sulphoxide (DMSO) or methanol, results in the liberation of the purple or blue formazan crystals. The intensity of the colour can be measured colorimetrically or spetrophotometrically and it is directly proportional to the number of viable cells (Mosmann, 1983).

8.3.1.1 Protocol for MTT assay

Depending upon the cell counts obtained from the passaging process (section 8.2), the cells were diluted in the medium of choice so that each 0.5ml contained passaged NRK-52E cells at a density of $3.2 \times 10^5$ cells/cm$^2$ and the 0.5ml of the seeded media were placed into each well of a 24-well plate. These cells were incubated at STCE for 24 hours to reach 85 to 90% confluence and also to recover from any trypsin effect (from the passaging process).

The MTT assay was performed on 85 to 90% confluent NRK-52E cells grown in 24 well plates. The culture medium on these cells was removed with the use of a Pasteur pipette and a vacuum pump within a laminar air flow hood (Haraeus, Germany) and replaced with 0.5ml of a medium containing increasing concentrations of either CLS or CMS. Media of some wells were left in the plate with the addition of antibiotic diluted with media (antibiotic topping up).

At the end of the cells exposure period to the antibiotics, the medium containing antibiotics was removed and replaced with fresh 1ml of 0.2% MTT solution dissolved in the medium of choice and incubated at STCE for an additional one hour. Thereafter, the MTT solution was removed followed by the addition of 200µl of dimethyl sulphoxide (DMSO) to each well. Aliquots of 50µl of the purple or blue formazan solution formed were transferred to 96 well plates in triplicate. A microplate reader set at 540nm wavelength was used to measure the colour intensity of the dissolved formazan. Absorbance readings were
corrected for background (i.e. DMSO only) and expressed as a percentage of the control reading (i.e. the reading obtained from the untreated cells).

### 8.3.1.2 Calibration of MTT assay

The MTT assay was calibrated to demonstrate that: (i) MTT is metabolised by the NRK-52E cells; (ii) absorbance is directly related to the density of live cells and (iii) absorbance obtained from the MTT assays of the treated cells occurred within the linear portion of the calibration curve. NRK-52E cells were seeded at varying densities (500,000 to 1,000,000 cells/well) into 24 well plates. These cells were maintained with the medium and were incubated at STCE for 24 hours. At the end of the incubation period, the MTT assay as described in section 8.3.1.1 was performed.

![MTT calibration plot](image)

**Figure 8.1: MTT calibration plot. n=6± Stdev**

Figure 8.1 shows the linearity of MTT assay indicating that the absorbance is directly related to the number of viable cells.
8.3.2 Lactate dehydrogenase (LDH) assay

LDH is an intracellular enzyme which is impermeable to the plasma membrane. When the membrane is damaged, the intracellular content of the cells, including LDH, leaks out of the cell. Thus, the LDH assay measures the release of LDH from damaged cells and it is therefore used in cytotoxicity studies as an index of membrane integrity and cell death. A colorimetric method is used to determine the released LDH (Abe and Matsuki, 2000).

This method is based on the same principle as that reported by Korzeniewski and Callewaert (1983), where the β-NAD (Nicotinamide Adenine Dinucleotide) reduced to NADH through the conversion of lactate to pyruvate by LDH. The NADH produced then reduces soluble tetrazolium dyes to insoluble formazan dyes in the presence of electron coupling agents. However, unlike the above authors, Abe and Matsuki (2000) utilized the tetrazolium salt MTT instead of 2-p-iodophenyl-3-p-nitrophenyl tetrazolium chloride in the LDH substrate mixture. The conversion of MTT to the insoluble blue MTT formazan by NADH is directly proportional to LDH activity. The MTT formazan is however solubilised by the addition of Triton X100 (t-octylphenoxypolyethoxyethanol) to the LDH substrate mixture (Abe and Matsuki, 2000).

8.3.3 Protocol for LDH assays

Depending upon the cell counts obtained from the passaging process (section 8.2), the cells were processed as detailed in section 8.3.1.1

The LDH assay was performed on 85-90% confluent NRK-52E grown in 24 well plates. When the cells reached 85-90% confluence, the medium was removed and replaced with 0.5ml of fresh medium containing increasing concentrations of antibiotic; some wells were left with the same media and were topped up with media containing increasing concentration of antibiotics. Cells containing antibiotics were incubated at STCH for the required period of time. One hour prior to each incubation period, 250µl of 1% (v/v) Triton X100 was added into three wells of the untreated (cells without antibiotics) cells per 24 well plates. At the end of the incubation period an aliquot of 50µl of the culture medium was transferred into 96 well plates in triplicate. Thereafter, 50µl of the LDH substrate
mixture was added to each well of a 96-well plates and the reaction was allowed to proceed for 10-15 minutes at 37°C. At the end of the cells exposure time an aliquot of 100µl dimethylformamide (DMF) solution was added to each well to stop the reaction. The absorbance was subsequently measured at 530nm using a microplate reader. The absorbance reading was corrected for background and expressed as a percentage of that obtained with cells lysed by Triton X100.

**8.3.4 Calibration of LDH assay**

The optimization of the LDH assay used in this study was performed to justify its use in cytotoxicity studies involving the NRK-52E cells. This was undertaken in order to verify that the absorbance obtained from the LDH assays occurred within the linear portion of the calibration curve. Aliquots of 10, 20, 50, 100 and 200µl containing various cell densities (1,000 to 1,000,000 cells/well) were placed into the wells of a 24 well plate. These cells were maintained with the culture medium and they were incubated at STCE for 24 hours. One hour before the end of the incubation period, 250µl of 1% Triton X100 at a final concentration of 0.2% was added into each well of the 24 well plates. At the end of the incubation period the LDH assay (as detailed in section 8.3.3) was performed.

![Graph](image)

**Figure 8.2: LDH calibration plot. n=6± Stdev**
Figure 8.2 shows the linearity of LDH assay indicating that the absorbance was directly related to the amount of LDH released.

8.4 Determining cytotoxicity of CLS and CMS

The cytotoxicity of CLS and CMS was detected using the MTT and LDH assays as detailed in section 8.3. These tests were performed on 85 to 90% confluent NRK-52E cells grown in 24 well plates, 500µl of the medium of choice was used as detailed in section 8.2. Various concentrations of fresh antibiotics were employed in the study.

The effects of degradation products of both CLS and CMS on the target cells were investigated by comparing the effects of freshly prepared CLS and CMS with the one stored at 37°C for five and two days respectively. All experiments were performed in triplicate wells.

Results

The NRK-52E cells were grown into 24 well plates (85-90% confluent) and were exposed to either CLS or CMS at concentrations of 0.0011, 0.0023 or 0.023mmol/L (equivalent to 2, 4, 40mg/L of CMS or 1.6, 3.2, 32mg/L of CLS). LDH and MTT assays were performed after two days of incubation at STCE (without changing the media). Each plate contained three replicate wells of the three concentrations for each antibiotic, three wells of untreated cells and the cells in the other three wells were treated with Triton X100.

Neither antibiotics (CLS or CMS) had an adverse effect on cell viability as only about 45% of LDH was released from the cells, and a toxic effect is generally considered to have occurred when cell counts reduced by 50% . Cell viability also appeared to be unaffected as the MTT assays were considered as being toxic when cell viability was reduced to below 80% (Haslam et al, 2000) (Figure 8.3A and 8.3B). Some results for the MTT assay were higher than 100% possibly as a result of the target cells trying to compensate and the mitochondria becoming more active as a defence mechanism.
Figure 8.3A: LDH released from cells after exposure to a single dose of antibiotics and were incubated at STCE for two days. n=5± Stdev
In the previous experiment the target cells were exposed to a single dose of antibiotic and cells were incubated at STCE for two days without changing the media, where the antibiotic dose may be insufficient to produce significant changes in cell injury indicators such as LDH. Therefore the target cells were exposed to repeated doses of both antibiotics at concentrations of 0.0011, 0.0023 or 0.023mmol/L every 12 hours (The t1/2 of CLS and CMS are 251 ± 79 min and 124 ± 52 min respectively according to Li et al, 2003a) by topping up the antibiotic without changing the media (adding a concentrated solution of antibiotic so the total antibiotic concentration remained the same). Thus, the availability of the drug at the target cells increased and the number of doses increased as well, LDH and MTT assays were performed after two days of incubation at STCE.

Figure 8.3B: MTT assay after cells were exposed to a single dose of antibiotics and were incubated at STCE for two days. n=5± Stdev
Increasing the availability of the drug to the target cells by topping up the antibiotics every 12 hours had no increased toxic effect as demonstrated by either the LDH or MTT assays. Only about 20-30% LDH (except CLS at a concentration of 0.0023 mmol/l which produced about 40%) was released from the cells compared to about 40% released from cells when exposed to a single dose only. This may be due to the fact that the LDH enzyme is unstable, thus this reduced LDH values especially when the media remained unchanged for the two days with topping up the doses (Figure 8.4A and Figure 8.4B).

![Figure 8.4A: LDH released from cells topped up with antibiotic every 12 hours without changing media for two days at STCE. n=3± Stdev](image-url)
Figure 8.4B: MTT released from cells topped up with antibiotic every 12 hours without changing media for two days at STCE. n=3± Stdev

During the above experiments, the medium was not changed over the incubation period of two days at STCE. The LDH which is an enzymatic reaction produced upon cell injury may have degraded during incubation at 37°C for two days. Thus, the medium containing antibiotic (final antibiotics concentrations in each well were 0.0023 and 0.023mmol/L) was changed every 24 hours for a two days.

LDH and MTT assays were performed before the media change every 24 hours, LDH is a non cell destructive assay, while two plates for MTT assays were grown and treated with antibiotics, one plate was used each time for the MTT assay.

Changing the medium had no effect on the cell death or LDH levels (the LDH assay was calculated by adding the results from day one to the results from day two of the antibiotic-treated and Triton X100-treated cells). Assays of MTT showed no toxic effect on cell viability when treated with CMS at concentrations of either 0.0023 or 0.023mmol/L, while it produced a slightly toxic effect (reduction in cell viability by 70%) on target cells when treated with CLS at concentrations of 0.023mmol/L. Toxicity was noted at the end of the incubation period of two days.
(Figures 8.5A and 8.5B). However, a reduction in cell viability down to 70% is a relatively weak marker of toxicity as generally in these studies lower values would be expected.

Figure 8.5A: LDH released from cells where media containing antibiotics were changed every 24 hours at STCE for two days. n=3± Stdev
Figure 8.5B: MTT released from cells where media containing antibiotics were changed every 24 hours at STCE for two days. n=3± Stdev.

Another experiment involved the exposure of the target cells to the antibiotics for a period of two days at STCE during which the medium with the antibiotics were changed more frequently every 12 hours (similar to the previous experiment, but media containing antibiotic were changed every 12 hours instated of 24 hours). This will affect the drug availability to the target cells, which may produce a significant effect of LDH levels as the dose will double up compared to the last experiment, and more nutrition will be available to the cells as medium is changed more frequently.

LDH and MTT assays were performed before the media change every 12 hours.
The LDH levels show a negligible effect of the antibiotics as on the target cells. The MTT assay for CMS showed a negligible toxic effect, while the MTT assays for CLS at a concentration of 0.023mmol/L gave reduced cell viability levels (about 70%) , however, a reduction in cell viability down to 70% is a relatively weak marker of toxicity (Figure 8.6A and 8.6B).

The cellular injury and death of target cells produced by CLS or CMS may increase with increasing incubation period. In order to explore this effect the experiment was repeated using longer incubation periods.

Figure 8.6A: LDH released from cells where media containing antibiotics were changed every 12 hours at STCE or two days. n=3± Stdev
Figure 8.6B: MTT released from cells where media containing antibiotics were changed every 12 hours at STCE for two days. n=3± Stdev.

A further experiment was therefore performed, where the target cells were exposed to the topping up of the antibiotics in the medium every 12 hours and the medium was changed every 24 hours (media containing the antibiotic) for a period of four days. This experiment reduced the risk of the cell constituents dissolved in the media as the media were changed once a day rather than twice a day, while the drug availability was increased as the drug was topped up every 12 hours. In addition, this experiment imposed the drug availability at the target cells for longer periods which may be sufficient to produce effects either on cell death indicators or cell validity.

LDH was performed before the media change, on the first, second, third and fourth day of incubation at STCE and the LDH values for four days were added together, while MTT assays were performed after four days of incubation at STCE.
Negligible effects were found on MTT or LDH assays (Figure 8.7A and 8.7B) with incubation of four days, cells started to show death after four days of incubation (Figure 8.8A), cell death was confirmed as the control cells (untreated cells) showed LDH levels of 90%.

Figure 8.7A: LDH released from cells topped up with antibiotics every 12 hours and medium was changed every 24 hours for four days at STCE. n=3± Stdev
Figure 8.7B: MTT released from cells topped with antibiotics every 12 hours and medium was changed every 24 hours for four days at STCE. n=3± Stdev
Figure 8.8A: LDH released from cells topped with antibiotics every 12 hours and medium was changed every 24 hours after four days at STCE. n=3± Stdev

8.5 Determining cytotoxicity of degradation products of CLS and CMS

The degradation products of both CLS and CMS on the target cells were investigated by comparing the effects of freshly prepared CLS or CMS with samples stored at 37°C for five or two days respectively, these incubation periods either of five or two days were chosen as the CLS is degraded within five days at 37°C, while CMS is degraded within two days (Li et al, 2003b). In all the experiments, MTT assays were performed on untreated cells (control, antibiotic free well) and LDH assays were performed on cells treated with TritonX100.

The NRK-52E cells were grown in 24 well plates and were exposed to freshly prepared or stored CLS and CMS (CLS or CMS stored at 37°C for five and two days respectively) at concentrations of 0.0023 and 0.023mmol/L (3.2 and 32mg/L of CLS or 4 and 40mg/L of CMS). LDH and MTT assays were performed after two days of incubation at STCE.
The results showed that the degraded antibiotics had no greater deleterious effect on the cells than the freshly prepared samples (Figure 8.9A and 8.9B).

Figure 8.9A: LDH assay on cells exposed to freshly prepared antibiotics and those previously degraded at STCE for two days. n=3± Stdev. (F= Fresh antibiotic, S= Stored antibiotic at 37°C).

NB. CLS was stored for 5 days, while CMS was stored for 2 days at 37°C before adding the antibiotics to the target cells.
Figure 8.9B: MTT assay on cells exposed to freshly prepared antibiotics and those previously degraded at STCE for two days. n=3± Stdev (F= Fresh antibiotic, S= Stored antibiotic at 37°C).

In the previous experiment the target cells were exposed to a single dose of antibiotic for two days at STCE which may be insufficient to produce a significant change in the markers of cell injury, thus the cells were exposed to the fresh or stored antibiotic dose every 12 hours (CMS half life= 5h) by topping up the antibiotic and media was changed every 24 hours.

LDH was performed before the media change, on the first and second days, while MTT assays were performed after two days of incubation at STCE.

The results are shown in figures 8.10A and 8.10B and indicate that no differences were observed by exposing the target cells to more frequent antibiotic doses and daily media changing.

These experiments either on the fresh or degraded antibiotics (CLS and CMS) shows negligible effects on levels of cell death (LDH assays) or cell viability (MTT assays).
Figure 8.10A: LDH assay on cells exposed to a freshly or stored antibiotics at STCE for two days. Cells were topped up with antibiotic every 12 hours and media was changed every 24 hour. n=3± Stdev. (F= fresh antibiotic, S= Stored antibiotic at 37°C).
Figure 8.10B: MTT assay on cells exposed to a freshly or stored antibiotics at STCE for two days. Cells were topped up with antibiotic every 12 hours and media was changed every 24 hours. n=3± Stdev. (F= Fresh antibiotic, S= Stored antibiotic at 37°C).
CHAPTER NINE

Discussions, conclusions and future work
9.1 Introduction

Section 1.6.1 gave details of the complex chemistry of the polymyxin group of antibiotics. In common with many other natural products they comprise not a single chemical entity but a complex mixture of closely related compounds. The situation is further complicated by the industrial synthesis of CMS where the free amino groups of colistin are sulphomethylated in order to produce an antibiotic with reduced toxicity. However, following the synthesis of CMS it is not possible to determine whether all of the amino groups have been sulphomethylated and if not which groups are left unchanged. Consequently, the final product may be a mixture of compounds each with a different number and/or range of sulphomethylated groups.

Some workers have expressed the view that CMS is a prodrug of colistin (Bergen et al, 2006). The CMS molecule is however, highly unstable and degrades rapidly in aqueous solution to colistin base which has marked antibacterial activity. The degradation pathway of CMS is uncertain and the relative antimicrobial and toxicological properties of the degradation products are also unknown. The complexity of the large peptide antibiotic itself, together with the array of subtle variations in structure shown by different members of the group, and their inherent instability make analysis of this antibiotic fraught with difficulties.

The aim of this thesis was to attempt to shed some light on this process by exploring the methods of analysis available and investigating the antimicrobial and toxicological properties of CLS and CMS.
9.2 Optimization of microbiological assays for colistin sulphate and colistin methanesulphonate

Most of the pharmacokinetic data on colistin are obtained using microbiological assays. These assays have many limitations such as limited sensitivity and they require overnight incubation at 37°C (Schwartz et al, 1959; Evans et al, 1999; Li et al, 2005a), thus accelerating the conversion of CMS to colistin base which will influence the accuracy and reproducibility of the microbiological assays, which rely on the total antibacterial activity within the sample.

Also the intermediates from the hydrolysis of CMS to colistin base will produce colistin more rapidly during incubation in the microbiological assays, compared with the samples containing CMS for calibration curves. The reliability of the reported results on the determination of the concentrations of CMS in the biological fluids measured by microbiological methods is most likely overstated by the availability of the more microbiologically active compound (colistin, the base) and its partially sulphomethylated derivatives (Sande and Kaye, 1970; Li et al, 2005a).

Since microbiological assays (agar diffusion or turbidimetric assay) are currently the only official methods for CMS analysis in many pharmacopoeias such as the British Pharmacopoeia (British Pharmacopoeia, 2010), the microbiological method (agar diffusion method) was investigated in order to determine the levels of CLS and CMS in water and serum. Primary optimization was carried out using antibiotic solutions prepared in water and this was then followed by investigation of the assay using antibiotics prepared in serum.

Turbidimetric assays are also recommended in the British Pharmacopoeia but this type of assay is less commonly used than agar diffusion assays because their precision is poorer. Difficulties can also be encountered with solutions that are not clear such as may be found in the presence of blood or serum. In addition, bacterial suspensions that are pigmented or have a tendency to clump can give rise to inaccuracies. For these reasons the use of the turbidimetric assay would only have been explored if the agar diffusion method proved to be unsatisfactory.
9.2.1 Optimization of CMS bioassay

The agar disc diffusion method for CMS as recommended in the British Pharmacopoeia (2010) was performed with a range of CMS concentrations from 0.0023 to 0.0091 mmol/L (4 – 16 mg/L). The recommended bacteria in the British Pharmacopoeia are *E. coli* (NCIB 8879) or *B. bronchiseptica* (NCTC 8344). At the outset, efforts were made to find more sensitive indicator organisms but were ultimately unsuccessful. Preliminary experiments were conducted using several Gram-negative bacteria as bio-indicators at inoculum size of $10^7$ but no bacterial growth inhibition zones were observed (Table 3.1).

Subsequently, many investigations were carried out in order to determine the optimal experimental parameters for the CMS bioassay such as inoculum size, incubation temperature, pre-diffusion time, experimental design and type of medium (all these investigations were conducted using the BP (British Pharmacopeia, 2010) recommended strain *E. coli* (NCIB 8879) as indicator organism).

**Inoculum size:** Diffusion assays are a dynamic interplay between the diffusion of the antimicrobial molecule through the agar and growth of the indicator organism. As the antibiotic diffuses through the agar it exhibits a concentration gradient from the source across the plate. At the outer edges of the diffusion area the antibiotic concentrations will be too low to have any effect. At some region moving inwards however, there will be a critical concentration that is inhibitory. As the indicator organism, which is inoculated throughout the plate, grows it will be unhindered in those outer regions of the plate where no antibiotic has yet reached. As we get nearer to the source of the antibiotic the concentration increases until it is too great to permit growth. When the critical cell density encounters that critical concentration the zone edge is formed and further incubation will not change its position.
Since this process is interplay between antibiotic diffusion and cell growth, the size of the inoculum will influence the size of the inhibition zones. Higher inoculum concentrations will take a shorter time to reach the critical cell density and during that growth period the critical concentration will not have diffused so far into the agar. Hence a heavy inoculum will produce small zones of inhibition and vice versa. In these development experiments inocula such as $10^6$ Cfu/ml produced light bacterial lawns on the surface of the plate and while the zones were larger the edges were not distinct. Good results in terms of inhibition zone clarity were found when using inoculum sizes of $10^7$ or $10^8$ cfu/ml.

**Incubation temperature**: The choice of temperature is clearly a compromise as it will influence the growth of the test organism but also have an impact on the diffusion of the molecules through the agar and degradation of the antibiotic. Investigations revealed that 37°C was the incubation temperature which gave the largest and clearest zones of inhibition. This is the optimal growth temperature for the majority of the human pathogens.

**Pre-diffusion time**: Given the dynamic interplay outlined above it is clear that larger zones of inhibition will be obtained if the antibiotic can be allowed to diffuse through the agar in advance of the growth of the indicator bacteria. Pre-diffusion is a process where the completed agar plate is placed at a non-growth temperature (usually 4°C) for a period of time to allow the antibiotic molecules to diffuse outwards from their source. The plates are then placed at the appropriate incubation temperature to allow the indicator organism to grow and for the zone to develop.

Experiments conducted in section 3.2.6 demonstrated that a four hour pre-diffusion at 4°C had a significant (P<0.5) impact on the CMS assay and produced larger inhibition zones compared to plates which were not pre-diffused. Extending the pre-diffusion time to 24 hours did not produced any significant increase (P>0.5) in the inhibition zone size over the four hour pre-diffusion time suggesting that most of the CMS had diffused during the four hour incubation time (**Table 3.11**).
Experimental design and choice of medium: Initial experiments showed that CMS produced small, indistinct zones of inhibition. Moreover, CMS appeared to give more satisfactory results than CLS even though it is reported to be less microbiologically active. This may have been due to the fact that either the antibiotic was not effective against the indicator organism at the concentrations used or there was an inability to diffuse through the agar. During later experiments on MIC determination (3.2.3) inhibition of growth was observed when CMS at low concentrations of 0.00007 to 0.0045mmol/L (0.125 – 8mg/L) was incorporated into the agar itself.

These results clearly suggest that an inability of the colistin molecule to readily diffuse through the agar might be responsible for the lack of inhibition zones seen in the bioassay. There are a variety of possible reasons for this, including the size of the molecule and its charge.

Molecular size: All other things being equal, as the size of the molecule increases so the rate of diffusion through agar will tend to decrease and this will result in smaller zones of inhibition. Ultimately, very large molecules will not diffuse at all and, even if they are potent antimicrobial agents, will not form zones of inhibition. Colistin base has a molecular weight of approximately 1200 Da and similarly sized molecules are able to produce zones of inhibition. Indeed, CMS has a higher molecular weight (1750 Da) than CLS (1400 Da) and has been shown to produce larger zones. Thus it would appear that the lack of zones of inhibition is not due to molecular size.

Charge: Agar carries a net negative charge and so molecules which are highly positively charged will tend to bind to the polymer and diffuse poorly. CLS has five amino groups which will therefore impart on the molecule a net positive charge. CMS however, is prepared by the sulphomethylation of these amino groups resulting in a molecule which is essentially uncharged. It might be surmised therefore that CMS will be able to diffuse through the negatively charged agar whereas CLS will not and this is indeed what we see. However, other cationic peptide antibiotics such as bacitracin are able to produce zones of inhibition in agar gels. This can be explained by examining the bacitracin molecule which
contains the amino acids aspartic acid and glutamic acid (these carry a net negative charge) as well as the positively charged amino acids histidine, isoleucine and ornithine. The net charge which this molecule carries will depend on the pH and the pKas of the different ionisable groups. The following table details the values of the pKas for bacitracin and demonstrates that at neutral pH the bacitracin molecule would have essentially no net charge. There would be positive regions of the molecule that could bind to agar, but binding would likely be much less than with colistin sulphate containing 5 NH$_3^+$ groups. The difference in zone sizes observed between CLS and CMS can thus be attributed to molecular charge at neutral pH.

<table>
<thead>
<tr>
<th>pKa figure</th>
<th>Form at pH7</th>
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<tbody>
<tr>
<td>Aspartate group</td>
<td>3.6</td>
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<tr>
<td>Glutamate group</td>
<td>4.4</td>
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<tr>
<td>Histidine group</td>
<td>6.4</td>
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<tr>
<td>Iso-leucine</td>
<td>7.6</td>
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<tr>
<td>Ornithine</td>
<td>9.7</td>
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pKa figures taken from Castagnola et al. (2003).

From the above discussion it was apparent that the experimental conditions of the bioassay would have to be changed in order to produce larger, clearer inhibition zones and thus improve assay sensitivity. The use of standard strength agar would appear to be a limiting factor in the diffusion of the antibiotic molecule. Thus the experimental designs were changed to a new approach using a double layer of agar which was an adaptation from the method described in the British Pharmacopoeia. It consisted of normal strength agar as a base layer and a top seed layer which contained a lower content of agar (0.4% w/v). This seed agar was inoculated with the indicator organism and facilitated the penetration of CMS through the more open gel network of the lower strength agar rather than the 1 to 1.5% agar content found in the normal agar media. This modification had a positive impact on the CMS assay and when coupled with a 4-hour pre-diffusion at 4°C produced clear zones of measurable size (Table 3.4).
Having established a protocol which gave improved sensitivity for the CMS bioassay the test organism was changed from the first BP (British Pharmacopeia, 2010) recommended species *E. coli* NCIB 8879 to the other recommended species *Bordetella bronchiseptica* NCTC 8344. The use of this organism gave greater sensitivity with larger clearer zones of inhibition (Figure 3.1).

Using this model as a basis, various media (pH ranged from 6.5-7.4) were employed during further optimization experiments for use as both base layer and seed layer. The constituents of the agar may have a major impact on the bioassay through its ability to influence both diffusional properties and also speed of growth of the indicator organism (Tables 3.5 - 3.10). The optimum configuration for the CMS bioassay was found to be a base layer of Diagnostic Sensitivity Test agar (DSTA) with a seed layer of peptone water (PW) gelled with 0.4%w/v agar. These results confirmed the original supposition that the use of full strength agar hindered the diffusion of the CMS molecule.

All of the above investigations were repeated (such as agar diffusion method on single layer of agar, double layer of agar, different incubation conditions, various pre-diffusion time, choice of media, and size of the inoculum) using *B. bronchiseptica* NCTC 8344 as the indicator organism. The results confirmed that the disc diffusion assay on a single layer of agar produced small inhibition zones, while larger inhibition zones were produced using the double technique (DSTA as base layer and PW + technical agar 0.4% containing the indicator organism) with pre-diffusion for four hours at 4°C and then incubation at 37°C overnight.

The optimal CMS bioassay was performed by dispensing 200ml of the DSTA (pH 7.4) agar as a base layer followed by 50ml of the molten seeded agar (PW + technical agar 0.4%) in a random Latin square design bioassay plate. This molten agar was seeded with 200µL of the $10^8$ Cfu/ml of *B. bronchiseptica* (NCTC 8344). Volumes of 25µl of freshly prepared CMS solutions (water or serum) of different concentrations were pipetted onto sterile filter paper discs and placed onto the inoculated agar plates in a random Latin square design.
The assay plates were pre-diffused for four hours at 4\(^{\circ}\)C and then incubated at 37\(^{\circ}\)C overnight. A photograph of the optimised bioassay plate is shown in appendix. Calibration curves in water and serum were obtained using the optimized method; CMS sensitivity reached 2mg/L in water and serum (Figure 3.5 and 3.6). The blood levels of CMS in patients are likely to range from 10 to 15mg/L or 125-200units/ml (British National Formulary, 2010). The optimised bioassay is therefore sufficiently sensitive to be able to quantify the levels of CMS in patient’s serum.

This updated method overcomes the problem of poor CMS diffusion through agar, thus improving the bioassay sensitivity. Unfortunately it still requires incubation at 37\(^{\circ}\)C for 18 to 24 hours during which time the CMS will degrade to colistin base. In addition, it is unable to differentiate between colistin base and the colistin methylsulphonated derivatives.

### 9.2.2 Optimization of CLS bioassay

The same optimized conditions were applied to CLS but the limit of detection of CLS was shown to be reduced to 0.011mmol/L (16mg/L), compared to 0.0028mmol/L (5mg/L) for CMS in the same experiment (Figure 3.7). These results suggest impaired diffusion of CLS compared to CMS in agar, which agrees with the findings of Sande and Kaye (1970). The variation in the diffusion properties in agar between CLS and CMS has been discussed above and it has been shown that colistin base and CLS are both positively charged at neutral pH, while CMS is negatively charged. Agar has a negative charge and since CLS molecules have a positive charge this may contribute to the relatively poor diffusion through the agar.

In order to rectify this situation a range of different gelling agents were tested some of which carried alternative charges. Suitable gelling agents such as, Sodium alginate, Carrageenan type I and II, Gum guar, Gum xanthan Carbopol ultrez 21 and phytogel were employed in this study but not all of them were satisfactory in their gelling characteristics. Most of these gelling agents had the problem of relatively light bacterial growth (Table 3.13) when used alone as only low levels of nutrients were able to diffuse from the underlying base layer.
Of those polymers studied, phytogel at a concentration of 0.25% w/v was found to be the most suitable gelling agent. It can be used at concentrations up to 10% w/v but this concentration gave good results. It produced a clear, colourless, high strength gel which aided in detection of microbial contamination. When agar is used as a gelling agent it sets to form a gel automatically as it cools below about 45°C. Thus it retains the negative charges on its molecule. Phytogel is crossed linked using divalent cations such as calcium chloride and thus all the negative charges on the molecule are removed. As a result the essentially non-charged gel should facilitate the diffusion of the positively charged CLS.

Turbidity occurred when using the cross linking agent 1% CaCl$_2$ in the base layer (DSTA). In an effort to rectify this, the concentration of CaCl$_2$ was reduced from 1% to 0.5% but still turbidity occurred. Another approach was to prepare DSTA base agar free from CaCl$_2$ and to cross link the phytagel 0.25% by adding 0.1ml of 1% w/v CaCl$_2$ to the solidified DSTA on the plate (gel was added at the end), this approach was effective and eliminated the turbidity problem in base layer. In order to improve bacterial growth in the gelling agent, phytogel was made up with TS broth and NB as diluents; however, both diluents resulted in solutions which did not gel. Presumably this occurred because the monovalent cations present in the broth interfered with the binding sites for calcium on the phytogel molecule.

The optimal CLS bioassay conditions were thus obtained using the same procedure as developed for the CMS bioassay but the PW (0.4%w/v agar) seed layer was replaced by 50ml of 0.25% of phytogel with no nutrient supplementation. The nutrients absorbed from the DSTA base layer were sufficient to allow growth. CMS exhibited the same sensitivity (0.0014mmol/L or 2.5mg/L) in both the agar and phytogel systems. CLS on the other hand gave poorer results in the agar system with a sensitivity of 0.011mmol/L (16mg/L) and this was improved to 0.003mmol/L (4mg/L) with the phytogel system.
This optimized CLS bioassay overcame the problem of poor CLS diffusion into the agar, thus improving the sensitivity of the assay. Phytogel as a seed layer was satisfactory for CLS as well as CMS and produced bigger inhibition zones than agar. However, practical difficulties in spreading the gel and cross linking of the gel limited the use of this method to only CLS.

9.3 Optimizing chemical assays of colistin sulphate and colistin methanesulphonate

Many attempts for chemical analysis for CLS and CMS were carried out, these analytical procedures includes TLC and HPLC methods.

9.3.1 Optimizing the TLC methods

It was considered useful to develop TLC methods for CLS and CMS as rapid means to check on degradation and for identification. The BP (British Pharmacopeia, 2010, see appendix) has TLC methods for identification of these compounds, but these involve the prior complete hydrolysis to the constituent amino acids.

A starting point for this was the method of Ikai et al (1998a). Their method involves chemical derivatization to produce fluorescent compounds to enable visualisation. It was hoped to obviate this step, and work has been carried out to optimise the mobile and stationary phases and the visualisation procedure.

Usually fluorescence quenching is used to detect developed TLC plates (containing a fluorescent agent), but colistin base does not quench the fluorescence of the plate thus spraying with ninhydrin and heating was used as a visualisation procedure. CLS is very polar and preliminary experiments showed that it bound too strongly to normal phase TLC plates. With reversed phase plates (silica coated with octadecylsilane) better elution was observed using water / acetone mixtures. The presence of sodium chloride in the mobile phase was found to have a “salting out” effect with hydrophobic plates, as reported by Yoshimoto et al (1998), and much improved retention and decrease in peak tailing was observed. The optimum mobile phase was found to be 2M NaCl (60%) and acetone (40%) acidified with a drop of phosphoric acid (Figure 4.1).
With this system CLS produced three spots which were assigned to colistin A and B and one unknown compound at a higher Rf value suggesting a more polar constituent is present.

Preliminary experiments with CMS showed that normal phase silica plates were more appropriate than reversed phase, presumably because of the lower polarity of this compound.

Many different mobile phases were investigated for CMS. The most suitable was found to be methanol (90%) / acetone (10%) with a drop of added phosphoric acid (Figure 4.2). With this mobile phase, two spots appeared at Rf values of 0.63 and 0.56. These could be due to the A and B forms of colistin with 5 sulphomethyl groups or the lower one a degradation product, say CMS-4 (4 sulphomethyl groups). Some streaking from these spots may be due to degradation to CMS-3 and CMS-2 (with 3 and 2 sulphomethyl groups respectively) as these being more polar with have lower Rf values. A small faint spot, somewhat streaking, was observed based from the point of application of the sample. This was assigned to CLS as applying CLS to the plate using the same conditions gave a similar, but larger streak. This small spot was always observed with CMS samples, however rapidly they were prepared and applied, and suggests that the CMS material contained a small amount of CLS. It is however possible that some degradation does occur during elution.

As CMS is very temperature sensitive, some experiments were carried out with different methods of drying of the original spots when applied to the TLC plates. It is common practice to use a hot air blower to remove the solvent from an applied spot. This procedure had no effect on CLS samples. With CMS however, considerable degradation was apparent with hot air drying. Very much less was observed at room temperature, and with use of cold air. Room temperature drying was used in all subsequent experiments.

Experiments were carried out using both CLS and CMS in serum, to determine whether the TCL procedures would be useful for biological samples. In neither case did the samples move from the point of application, suggesting that both CLS and CMS are strongly serum bound, and prior extraction of the drugs would be necessary in order for TLC to be useful.
9.3.2 Optimizing the HPLC methods

The degradation of CMS-5 can give rise to 64 possible products (Li et al, 2003b). HPLC was considered to be the best potential method to attempt to monitor the degradation process. As a starting point the method of Li (Li et al, 2003b) was adopted and investigation carried out to try and optimise the method for the purposes of this work. Initial experiments were concerned with the appropriate concentrations necessary for convenient detection. CMS does not possess a good chromophore and only absorbs at very short wavelengths. Detection at 200 and 210nm was carried out. Higher absorbances are expected at shorter wavelengths but because mobile phase components also absorb more at 200nm, as do trace impurities in reagents used, problems with the background can be worse. It was found that 210nm was the preferred wavelength and a concentration of CMS of 1000mg/L (0.5667mmol/L) gave reasonable peak intensities (Figure 4.8).

In an attempt to optimize CMS analysis sodium sulphate was replaced with disodium hydrogen phosphate which offers buffering action in the mobile phase (Figure 4.10). A range of pH from 3 to 6.6 was tested to improve CMS separation as changing in pH may improve peak separation. These results showed that a range of pH3 to pH5.5 is suitable for CMS detection (Figure 4.12, 4.13, 4.14 and 4.15); while pH6 and pH6.6 (Figure 4.16 and 4.17) produced un-separated peaks and they are not suitable for CMS detection. Overall however there was no significant improvement in chromatographic separation using phosphate buffer in place of sodium sulphate.

Since the proportions of aqueous and organic components of the mobile phase are critical in HPLC systems with all types of column, different proportions of sodium sulphate to acetonitrile within the mobile phase were investigated. These results showed a considerable increase in the CMS retention times with the increase of the acetonitrile concentrations. Increasing acetonitrile by just 2% from 75% to 77% resulted in a delay in CMS retention time from 16 to 20 minutes (Figure 4.19), while increasing the acetonitrile from 75% to 80% resulted in a further delay in CMS retention time from 16 to 30 minutes (Figure 4.20). This contributes to the increase the binding capacity of the CMS with the material packed in the column.
Another attempt to improve the CMS assay was to increase the flow rate to keep peak widths narrow. The flow rate was changed from 1ml/min to 1.5ml/min; unfortunately no better resolution was achieved with a flow rate of 1.5 ml/min (Figure 4.21).

The previous experiments were conducted using solutions of CMS dissolved in the mobile phase to prevent additional peaks of solvent. However, future degradation studies will be performed on CMS in deionised water for HPLC; therefore the analytical process of CMS dissolved in deionised water for HPLC was performed in deionised water and results were identical to the one performed in mobile phase (Figure 4.23 and Figure 4.24).

The optimal conditions were: 20µl of the sample was injected into the HPLC system using 25% 1.2mM sodium sulphate: 75% acetonitrile as mobile phase, pH5. The flow rate was 1ml/min. and detection was at 210nm.

9.4 Studies into bacterial cell death and growth inhibition induced by colistin sulphate and colistin methanesulphonate

9.4.1 Determination of Minimum Inhibitory Concentrations (MICs)

Both forms of colistin: CLS and CMS were shown to have either bacteriostatic or bactericidal activity against most Gram-negative bacteria employed in this study depending on their concentrations.

A variety of methods for determining MICs are used such as dilution tests and diffusion techniques. In the dilution tests, the test organism is inoculated into serial dilutions of antibiotic in agar or broth while in diffusion tests the disk containing a fixed amount of antibiotic is placed onto an agar plate inoculated with the test organism and the sensitivity or the MICs are measured by the inhibition zones.
Since colistin has a poor diffusion characteristics in agar (Geles et al., 2001; Tan and Ng, 2006; Heijden et al., 2007), dilution techniques in agar and broth were used to determine MICs.

### 9.4.1.1 Agar dilution method

Bacterial growth inhibition studies appeared to show that both forms of colistin possessed good antimicrobial activity against all Gram-negative bacteria used in this study except *Serratia marcescens* (with inoculum sizes of 1 x 10^7 CFU/ml CMS had MIC values of ≤ 0.005mmol/L or ≤ 8mg/L while CLS had MIC values of ≤ 0.0023mmol/L or ≤ 3.2mg/L against most Gram-negative bacteria employed in this study) (Table 5.1 and 5.3).

CLS was considerably more effective, i.e. had a lower MIC values compared to CMS against all the bacteria employed in this study (Table 5.1 and 5.3), these results were in agreement with Li and co-workers (2001a) as results showed MICs (by dilution method) for the susceptible strains ranged from 1 to 4mg/L for CLS, while ranged from 4 to 16mg/L for CMS.

In this thesis, CLS and CMS had lower MICs against *B. bronchiseptica* (NCTC 8344) (MIC= 0.00007mmol/L or 0.1mg/L and 0.0003mmol/L or 0.5mg/L respectively) and *E. coli* (NCIB 8879) (MIC= 0.0006mmol/L or 0.8mg/L and 0.0023mmol/L or 4mg/L respectively).

The Gram-negative bacilli *Serratia marcescens* showed to be highly resistant to CLS and CMS even at high concentration such as ≥0.009mmol/L (≥ 13mg/L) or ≥0.0045mmol/L (≥ 8mg/L) respectively. These results were consistent with study done by Greenfield and Feingold (1970) and other study done by Catchpole and co-workers (1997) who reported that no useful activity was demonstrated against *Serratia* spp.
Infections due to *Serratia marcescens* such as septicaemia, endocarditis, meningitis, infections of wounds and of the respiratory and urinary tracts are difficult to treat because the organism is resistant to a variety of antibiotics. This bacterium acquires resistance to antibiotics by preventing the drug from reaching its target site either by alterations in the cell envelope or the drug itself is modified by inactivating enzymes (Douglas, 1983).

An inoculum size of $1 \times 10^7$ Cfu/ml gave higher MIC values than $1 \times 10^4$ Cfu/ml (**Table 5.1** and 5.2). Bulitta and co-workers (2010) found that the extent and rate of killing of *P. aeruginosa* by colistin were markedly decreased at high cell concentrations compared to those at low concentrations as the killing of the susceptible population was 23-fold slower at $10^9$ Cfu/ml and 6-fold slower at $10^8$ Cfu/ml than at the $10^6$ Cfu/ml.

A reason for the inoculum effect of colistin may be due to its antiendotoxin activity in neutralizing bacterial LPS. Since colistin forms mixed monolayers with phospholipids and is incorporated in micelles *in vitro*, binding of colistin to LPS fragments of killed bacteria might decrease the available free colistin concentration *in vitro* and contribute to the inoculum effect.

The mean MIC value of CMS against *P. aeruginosa* strains employed in this study was 0.005mmol/L or 8mg/L when used with an inoculum size of $10^7$Cfu/ml or ranged from 4-8mg/L when used with an inoculum size of $10^4$Cfu/ml which is higher than that described by Barnett (1964). In that study the MIC value was 2.3mg/L while Catchpole *et al* (1997) found a mean value of 4mg/L when used inoculum size of $10^4$Cfu/ml. However, the values found here were consistent with the results of Li *et al* (2001a) who demonstrated that CMS has a lower overall activity than CLS against 23 strains of *P. aeruginosa* with a mean MIC of 7.1mg/L for all susceptible strains tested. Any differences in these results may contribute to the differences in activity between different manufacturers as CMS are undefined mixtures of the mono-, di-, tri- tetra- and penta- substituted compounds. In addition, these may be due to dissimilarities in methodology, media employed in obtaining MIC values and different strains of the organism.
9.4.1.2 Broth dilution method

MIC values in agar dilution method were higher than in broth for both forms of colistin (Table 5.4 and 5.5), these results were consistent with those of Barnett et al (1964) (CMS against *P. aeruginosa*, MIC in broth was 1.97mg/L while in agar was 2.34mg/L). Since both experiments required the samples to be incubated overnight at 37°C it is unlikely that differences in degradation patterns might be responsible for these inconsistencies. More likely is the fact that CLS and to a lesser extent CMS bind to agar thus leaving fewer antibiotic molecules in the surrounding solution to bring about an antimicrobial effect. Indeed, CLS has been shown in this thesis to bind more extensively to agar than CMS and the differences between broth dilution and agar dilution are more pronounced for this antibiotic.

Disk diffusion method is widely used in microbiology laboratories but work done by Geles and co-workers (2001), Tan and Ng (2006) and Galani and co-workers (2008) revealed that the disk diffusion method is highly unreliable for colistin due to the poor agar diffusion characteristics of colistin. Agar dilution method is the alternative method for the disk diffusion method for colistin for either determining sensitivity test or the MICs of colistin.

9.4.2 Bacterial profiles

CLS and CMS are polypeptides that disrupt the bacterial cell membrane through a detergent like mechanism. High concentrations of CLS such as 0.045mmol/L caused *A. lwofii* R46383 to lyse (resulting in a 30% reduction in absorbance compared to the initial value) (Figure 5.1). The loss of absorbance observed in the presence of high concentrations of CLS can be due to lysis of the bacterial cells, caused by osmotic pressure following damage to the cytoplasmic membrane (Denyer and Hugo, 1991). In order to produce a marked reduction in absorbance values the cells must actually disintegrate due to dissolution of the cell walls. If the cells merely open up they will of course die and release all their contents but the optical density of the suspension may not reduce markedly as the cell debris will still cause significant light scattering.
CMS did not cause overt cell lysis even at higher concentrations such as 0.045mmol/L. The variation between the action of CLS and CMS may be attributed to the fact that CLS has a much stronger binding affinity to the bacterial cells than CMS. The availability of more sites on CLS to bind the cell wall might thus produce a greater lytic action (Figure 5.1).

**9.4.3 Time-survivor curves studies for CLS and CMS**

To study an antibiotic killing effect on bacteria, a suitable neutralizing agent to neutralize the antibiotic effect to recover viable microorganisms was required. This neutralization may be achieved by addition of a specific neutralizer, by dilution, by a combination of washing and dilution, or by any combination of these methods.

Many factors affect the choice of neutralizing agent activity such as the nature of the organism, the growth and preparation of the inoculum as it affects the physiological state of the cell, the specific conditions of the test such as the diluents and temperatures, and the conditions of recovery such as the recovery media (USP, 2011).

Lethicin was used in the first place as it is recommended for neutralizing quaternary ammonium compounds effect in USP 2011 and since their action is similar to the polymyxins, it was investigated but it was not effective alone. Combination of Tween 80 and lethicin in different concentrations was investigated.

Tween and lecithin in combination inactivate many antimicrobial agents. Many antibiotics are lipophilic and probably exert their effect by inserting themselves in the membranes of bacteria. Since Tween and lecithin have both polar and non-polar regions, they act as surfactants. The non-polar regions are attracted to the lipophilic molecules and the polar regions orient towards the solvent (i.e. water). So, the antibiotics they will work on are the lipophilic ones.

In order to ensure that 2% tween 80 with 0.2% lethicin was a suitable neutralizing agent for CLS and CMS in terms of efficacy, a control of cells plus antibiotic showed no growth, while there was a considerable growth on plates with the antibiotic and neutralizer (average counts were same as in HEPES control).
2% tween 80 with 0.2% lethicin was non-toxic to the bacterial cells; this was confirmed by comparing viable cell counts of the plates with neutralizing agent compared to HEPES control.

CLS showed a concentration dependent bactericidal activity (Figure 5.2). At the highest multiples of the MIC (0.0227mmol/L (32mg/L)) the bacterial killing was so rapid that a 3.8 log reduction in viable count was observed at 30 minutes time of contact with the antibiotic. Concentrations of 0.0113mmol/L (16mg/L) demonstrated rapid and extensive killing with just less than 2.5 log reduction in viable organism by 30 minutes. This indicates the immediate action of the CLS on the cell which may contribute to the increased sites of interactions between the CLS and the cell membrane (Figure 5.2). The same concentrations of CMS (0.0227 and 0.0113mmol/L) caused approximately 0.5 log reduction within one hours contact with the antibiotic (Figure 5.3), while higher concentrations of CMS such as 0.0453mmol/L (80mg/L) produced only about one log reduction within one hour contact with the antibiotic (Figure 5.3).

Results shown in 5.3 indicate the presence of a ‘shouldering’ effect for CMS prior to the onset of logarithmic kill against A. lwoffii. This often observed lag phase, before a noticeable decrease in viability occurs (Eddy, 1953; Lambert and Johnston, 2000) has been described as indicative of a number of events being required to take place before organisms begin to die. The phenomenon was particularly marked in the case of CMS where there was no reduction in viable cell numbers before 20 minutes even in the presence of concentrations up to 0.045mmol/L. Given the rapidity with which the CMS molecules bind to the bacterial cells this apparent lack of kill is somewhat surprising. This suggests that the presence of the neutralising agent can in some way reverse or negate any detrimental effects even after binding. Other reasons given for shoulders include clumping (Hanlon and Hodges, 2007) in which case the antibiotic may bind to the outer layer of cells in the time available but not manage to penetrate to those cells at the centre of the clump. When transferred to agar in the presence of neutraliser the surviving cells will thus be able to grow and form colonies.
Cerf O (1977) discussed the tailing effect observed in time survivor curves at the end of the curve which were also evident in these plots and concluded that tailing could be due to a variety of different phenomena including variation in resistance of individual cells in the bacterial population or physical differences in the delivery of antibiotic to individual cells caused by clumping or local variation in concentration or temperature (Figure 5.3). The concept of variable resistance within a population of cells has been widely discussed as the main mechanism by which tailing occurs but in this case the most likely explanation is that relating to the physiochemical action of the molecules with the cells. This has been discussed by Lambert and Johnston, (2000) as the “mechanistic theory” which attempts to explain a decrease in antibacterial concentration during the course of the reaction process. This decrease may be brought about by drug instability, microbial quenching or non-microbial quenching. In the context of this study the most likely explanation is again related to the rapid adsorption of the molecules to the bacterial cells. Following rapid adsorption of the antibiotic to the available cells those which receive a lethal dose ultimately die whereas those that do not will survive as molecules are not regenerated after adsorption.

9.5 Microbiological and chemical degradation of colistin sulphate and colistin methanesulphonate

When synthesizing CMS, the five amine groups present in CLS are sulphomethylated. During degradation of CMS in aqueous solution, it can be assumed that hydrolysis of the five sulphomethylated groups is random, and there could be up to 64 possible products including colistin itself (Li et al, 2003b and 2004; Bergen et al, 2006). Upon CMS hydrolysis, Colistin is formed in vivo (Li et al, 2003a, 2004 and 2005b) and in vitro (Li et al, 2003b). Studies into the antimicrobial activity of CMS has proven complicated due to this hydrolytic conversion of CMS to colistin (Li et al, 2003b and 2003c), particularly as colistin is markedly more biologically active than CMS.
In this study CLS and CMS were stored in aqueous solution and in human serum at various temperatures in order to investigate the degradation products and characteristics of decomposition. Previously optimised microbiological (chapter three) and chemical assays including TLC and HPLC methods (chapter four) were employed in this study but each is associated with specific problems which have been discussed previously.

**CLS stability at various temperatures**

Microbiological and chemical methods such as TLC were used to assay CLS activity in water and serum at various temperatures. CLS was stable in both water and serum when stored at -20, 4 and 25°C over nine days when assayed microbiologically (Figure 6.1, 6.2 and 6.3) and over eight days when assayed by TLC. However, storing CLS at 37°C resulted in a reduction of the CLS activity within three days when assayed microbiologically (Figure 6.4), while TLC plates showed clear merging of the two original spots (indicating the onset of degradation) when stored for more than four days at 37°C (Figure 6.5).

These results given here were consistent with those of Li et al (2003) who showed that an aqueous solution of CLS was stable for 60 days and five days upon storage at 4°C or 37°C respectively. However, CLS was stable at 37°C within five days according to Li’s results (Li et al, 2003) while in this study CLS lost about 30% of its activity within three days when assayed microbiologically and starts to degrade after four days when assayed by TLC method. This difference may be due to the incubation period at 37°C of 18-24 hours during the microbiological assay procedure. In addition this may have been due to the inaccuracy and insensitivity of the bioassays and TLC method compared to the HPLC method as the HPLC method provides better resolution, ease of quantification and operation.

**CMS stability at -20°C**

Microbiological assays, TLC and HPLC methods in this study indicated that CMS in aqueous solution or in serum was stable at -20°C over the storage period of eight days (Figure 6.1, 6.6 and 6.10).
The HPLC chromatogram of fresh aqueous solution of CMS showed a large number of peaks. This suggests that the sample contained colistin with a variable number of methylsulphonated groups. These results are with line with the TLC chromatograms as the fresh aqueous CMS solution contains un-eluted light spot around the original spot which suggests that original material is not a mixture of completely methylsulphonated colistin A and B.

![Diagram](image)

**Figure 9.1: Degradation of CMS in water or serum.**

**CMS stability at 4ºC**

Aqueous or serum solutions of CMS stored at 4ºC were shown to be stable for only two days. This was confirmed by testing CMS solution microbiologically and chemically (TLC and HPLC) (**Figure 6.2, 6.7 and 6.12**).

Barnet *et al* (1964) and Wallace *et al* (2008) observed increase in CMS activity within time and concluded the conversion of CMS to colistin when CMS was assayed microbiologically. When tested microbiologically using CMS system (agar), the CMS activity was reduced despite the theory of conversion to the more active compound colistin base, this contribute to the fact that hydrolysis of CMS to colistin base takes place rapidly, while the microbiological analysis frequency was performed over days, thus these may be the products of further degradation of the formed colistin base. In addition, the used optimized system for determination of CMS dose not allows proper diffusion of colistin base.
The hydrolysis which occurred in human plasma was faster than that in water at 25, 4 or 37°C, this may be contributed to the fact that this hydrolysis probably also takes place in the body to a greater extent. This may be due to the fact that plasma contained some components that have the ability to hydrolyse the sulphomethyl group with the liberation of free amino groups (Beveridge and Martin, 1966).

TLC chromatograms of CMS of two days were almost same as the fresh one (Figure 6.7). After three days both spots disappeared and a streak appeared from the original spot towards the top of the plate, this explains the conversion of CMS to the sulphomethate derivatives such as, CMS-5 to CMS-4, CMS-3, CMS-2, and colistin base. There are two forms of CMS, and for each of these there are 64 possible degradation products involving loss of methylsulphonate groups. With a total of 64 possible compounds of quite similar structure, it would be almost impossible to completely separate all of them, hence the apparent streaking observed as these are closely related compounds which cannot be separated on the TLC system. In addition, the fresh solution’s original spot intensity increased gradually as time passed; this intensity was considerably increased over time, this contributes to the conversion of CMS to the colistin base as applying CLS into the NP TLC plates obtain an un-eluted spot at the bottom of the plate.

HPLC chromatograms of CMS after two days were identical to the fresh one (Figure 6.11 and 6.12). After two days storage at 4ºC, the chromatogram peaks appeared earlier and their areas were changed (Figure 6.12), this indicates CMS degradation as the degraded products appear earlier. After two days of storage at 4ºC the HPLC chromatogram shows increase in the AUC of the shorter retention time peaks due to less methylsulphonated compounds, which suggests that this peak represents the conversion of 5,4,3,2 or 1-sulphomethylated group of CMS to CMS of 1-sulphomethylated group or colistin itself (multi-sulphonated CMS to lesser subsisted compounds). The peak assigned as CMS-1 or colistin appeared even in a fresh solution suggesting that CMS contained an amount of unsulphonated material. This was corroborated by the TLC results where fresh solutions always showed a spot of the colistin.
The results for CMS degradation shown here using TLC and HPLC methods were consistent with the results of Li et al (2003), as Li and co-workers reported that aqueous CMS was stable for two days at 4°C. 

**Figure 9.1** shows degradation of CMS in water or serum.

**CMS stability at 25ºC**

When CMS stored in water or serum at 25ºC, it showed quicker degradation than at 4ºC, as it was fairly stable for one day when assayed microbiologically (**Figure 6.3**), but TLC chromatograms shows that both eluted spots (which corresponds to the fresh solution) merged to become one spot within one day, two days later, the single spot disappeared and a streak appeared from the original spot towards the top of the plate (**Figure 6.8**).

HPLC chromatograms shows that the chromatogram of CMS peaks have eluted earlier and their areas have changed (**Figure 6.14 and 6.15**) after storage of 12 hours at 25°C. The chromatograms show an increase in the shorter retention time peaks due to less methylsulphonated compounds and a decrease in the AUC of the long retention time peaks due to more highly methylsulphonated material.

**CMS stability at 37ºC**

Greater accelerated degradation was obtained when storing CMS aqueous or serum solution at 37ºC as it starts to degrade within the first day when assayed microbiologically (**Figure 6.4**). TLC chromatograms showed CMS degradation within two hours, while degradation completed after one day (this complete degradation is **Figure 6.9**); similar results were shown in HPLC chromatograms as CMS started to degrade within one hour (**Figure 6.17**).

These results agree with the results of Li et al (Li et al, 2003) who reported that CMS in aqueous solution was completely degraded after 12 hours when stored at 37°C.
9.6 Studies into the mechanism of action of colistin sulphate and colistin methanesulphonate

The mechanisms of action of CLS and CMS were investigated against *A. lwaffii* R46383. Potassium leakage studies together with the release of 260nm absorbing materials were determined.

The most sensitive indicator of minor bacterial membrane damage is the leakage of small intracellular materials such as potassium ions into the surrounding environment, the level of damage inflicted at the membrane which may permit the recovery or lead to cell death can be determined by the rate and levels of the leakage (Lambert and Hammond, 1973). Another marker to detect more substantial bacterial membrane damage can be detected by the leakage of larger intracellular species such as pentose, purines, pyrimidines and even nucleotides.

Pentose is a substrate used for metabolism and macromolecular synthesis. It may exist within the cell in free form. It is possible to correlate pentose and 260nm-absorbing material leakage due to their similar leakage patterns. Purines and pyrimidines are essential for RNA/DNA synthesis and absorb at 260nm as well. The 260nm-absorbing materials detected upon leakage may include materials such as purines and pyrimidines or larger materials such as nucleosides and nucleotides. These two methods (leakage of potassium and 260nm-absorbing materials) were used as they detect the damage to the bacterial cytoplasmic membrane, which is the site of action of colistin."

The outer membrane of Gram negative bacteria acts as a highly effective barrier to the passage of many molecules including antibiotics into the cell. This barrier function is one of the main reasons for the intrinsic antibiotic resistance of Gram negative bacteria. Passage of hydrophobic molecules through this outer membrane is thought to be inhibited by the presence of anionic lipopolysaccharides (LPS), which are stabilised by divalent cations. The presence of the anionic LPS molecules may however, act as binding sites for cationic antibiotics such as the peptide antibiotics and the aminoglycosides. The initial action of
these antibiotics may thus be to disrupt the outer membrane and facilitate passage of further molecules into the cell. The result will be to cause damage to the cytoplasmic membrane and release of intracellular constituents.

Peterson et al., (1985) studied the relative abilities of polymyxin B and various aminoglycosides to displace divalent cations from Ps. aeruginosa LPS in solution thus indicating the strength of their binding affinities. The polymyxin molecule was shown to have increased binding affinity over gentamicin. Both polymyxin and the aminoglycosides are effective antibiotics against Gram negative bacteria probably as a result of this ability to bind to the anionic LPS. The lack of activity of the peptide antibiotic bacitracin against Gram negative bacteria may also be a reflection of the lack of overall positive charge at neutral pH. Similarly, the lesser antimicrobial activity of CMS compared to colistin could be attributed to the fact that the amino groups have been sulphomethylated thus reducing the positive charge on the molecule.

9.6. Intracellular potassium leakage

The classic method for detecting mechanism of action of the membrane active agents such as polymyxins involve the measurement of leakage of low molecular weight constituents from whole cell such as potassium ions (Denyer and Hugo, 1991). Leakage is usually rapid following exposure to membrane active agents so detecting the leakage of potassium ions may be an advantage.

Polymyxins including colistin kill bacteria by activating the initial interaction with the negatively charged cytoplasmic membrane of the bacteria. This interaction leads to permeability changes and leakage of intracellular material such as potassium ions.

To determine the intracellular potassium leakage and 260-nm-absorbing pool, cells were treated with 1mM Cetrimide which is known to cause the release of the intracellular potassium pool from the cells; it is a cationic surfactant and disrupts the cell membrane. The cells were also treated with chemicals which know to cause membrane damage such as: 1:10 Triton X100 (1%) and 1% trichloroacetic acid while others were subjected to
boiling for one hour. Mechanical disruption of the bacterial cells was carried out using a Bead Beater (as described in section 2.2.15).

In the following experiments, material that leaked following antibiotics treatment was recorded as a percentage of the total intracellular pool.

For potassium ions leakage, cetrimide 1% was used as it gave the highest leakage when assayed.

The results shown in chapter seven, section 7.1.1.7, Figures 7.4 and 7.5 demonstrate that CLS and CMS exert a damaging effect on the cytoplasmic membrane of the cells of *A. lwaffii* R46383. Potassium leakage occurred in a concentration–dependent manner both in the presence of bacteriostatic and to a greater extent, bactericidal concentrations of CLS and CMS.

Cells exposed to high concentrations (0.0227mmol/L or 32mg/L) of CLS lost all of their intracellular potassium within the first ten minutes of contact with the antibiotic, while lower concentrations (0.0113 or 16mg/L and 0.0057mmol/L or 8mg/L) displayed a more gradual leakage process, which was concentration dependent. Concentrations such as 0.0003mmol/L or 0.4mg/L (just above MIC concentration, MIC= 0.0023mmol/L or 3.2mg/L) leaked less than 30% intracellular potassium within one hour (Figure 7.4).

In contrast to the profile observed for CLS, cells exposed to CMS (Figure 7.5), lost their intracellular potassium in more gradual process, with almost the entire potassium pool being lost at the highest concentration (0.0227mmol/L or 40mg/L) after one hour. Lower concentrations such as 0.0113, 0.0057 and 0.0003mol/L (equivalent to 20, 10 and 0.5mg/L) leaked less than 40% of the total intracellular potassium after one hour contact with the antibiotic depending on the antibiotic concentrations.

Concentrations of CMS with low levels of lethal activity (CMS lethal levels below 0.011mmol/L), caused potassium. This was an indication that a subtle, reversible perturbation of the cytoplasmic membrane was occurring, which was not related to the
gross membrane damage induced by lethal drug concentrations (Gilbert et al, 1977). There was no correlation between potassium release and the death of cells. This suggested that these lesions were one of a number of lesions which integrated to cause cell death, rather than being the primary lesion themselves.

The release of a threshold amount of potassium induces the activation of latent ribonucleases leading to destruction of RNA and cell death. Nucleotide leakage from whole cells is biphasic, corresponding to an initial loss of low molecular weight pool metabolites (potassium ions) followed by slower efflux of rRNA breakdown products because of the drug-induced activation of latent RNase I activity (Lambert & Smith, 1976).

These studies indicate a difference in the mode of action of the two antibiotics on the cytoplasmic membrane. Cells exposed to CLS show evidence of substantial membrane damage, indicated by the immediate loss of the intracellular potassium pool. Although cells exposed to CMS also exhibited potassium leakage, this was not profound. A reason of why CMS displays little antimicrobial activity compared to colistin may be due to the difference in the mechanism of action of the cationic peptides, probably due to the difference in the structure. For gram-negative microorganisms, it is proposed that the antibacterial activity of polymyxins involves a two-step process that begins with the displacement of divalent cations such as Ca$^{2+}$ or Mg$^{2+}$ on cell surface lipopolysaccharides of the outer membrane, and then interaction with the negatively charged cytoplasmic membrane (Hancock and Chapple, 1999); the increasing net positive charge of the peptide promotes interaction with each membrane (Wu and Hancock, 1999). It has been shown that CMS is prodrug for colistin (Bergen et al, 2006) being relatively inactive itself, but degrading to the more potent colistin over time.

### 9.6.2 Release of 260nm absorbing materials

Measurements of specific cell leakage markers such as intracellular potassium ions and 260-nm-absorbing materials are indicative of membrane sensitivity to specific agents. Release of 260nm absorbing materials is due to the breakdown of RNA material.
CLS and CMS are membrane active agents as was evidenced by the immediate release of the two leakage markers (intracellular potassium ions and 260nm absorbing materials) when cells were exposed to a range of CLS and CMS concentrations.

On exposure of the cells to a high dose of CLS such as 0.0227mmol/L (32mg/L) and 0.0113 (16mg/L) irreversible cytoplasmic membrane damage was sustained by the general population. This was marked by the complete or high release of their intracellular pool (100% for potassium leakage within 45 minutes of exposure to the antibiotic (Figure 7.4) and 50-70% (Figure 7.8) for 260nm absorbing materials) corresponding to the rapid cell death, 3.8 and 2.5 log reduction of the population respectively (Figure 5.2). Exposure to concentrations of CLS such as 0.006mmol/L (8mg/L) represented a moderate leakage inducing concentration with the eventual complete loss (100%) of intracellular potassium pool after one hour to exposure, while the 260nm absorbing materials pool was only depleted by 40% corresponding to the gradual cell death of just less than 2.5 log reductions of the population within one hour. Low concentrations of CLS such as 0.003mmol/L (4mg/L) represented a moderate leakage inducing concentration with about 35% leakage of intracellular potassium pool after one hour to antibiotic exposure; corresponding to the gradual cell death of just less than 1.5 log reductions of the population within one hour.

On exposure of the cells to a high concentrations of CMS such as 0.045mmol/L (80mg/L), and 0.0227mol/L (40mg/L), no profound effect on the release of 260nm-absorbing materials was observed (less than 20% was released) (Figure 7.9), while concentration of 0.0227mmol/L (40mg/L) represented a gradual leakage inducing concentration with the eventual complete loss (100%) of intracellular potassium pool after 50 minutes of exposure to the antibiotic (Figure 7.5), this corresponds to gradual cell death of one log reduction (Figure 5.3).

Following exposure of cells to CMS concentrations of 0.0113 (20mg/L) and 0.0057mmol/L (10mg/L), no effect on the release of 260nm-absorbing materials was observed (less than 10% was released).
In conclusion both CLS and CMS are membrane active agents that interacted with the cytoplasmic membrane in A. lwoffii R46383, inducing the immediate leakage of intracellular constituents. Over the range of concentrations studied for leakage and MIC, CLS was marginally more potent than CMS.

The experimental design for the intracellular release of potassium and 260nm material was such that the time scales were relatively short, usually within 1 hour. For this reason it can be concluded that the results seen for CMS are, for the most part, attributable to the intact molecule. The stability studies reinforce this conclusion and showed that CMS degraded within 12 hours at 37°C but will be mostly intact within the first hour.

The microbiological assays on the other hand require between 18 to 24 hours before the plates are read and within this time period extensive degradation will have taken place. However, it should be borne in mind that the edge of the inhibition zone may be fixed long before the bacterial lawn becomes visible, perhaps after only a few hours. This suggests that the microbiological assays may not be quite as uninformative as first thought. It does, nevertheless, indicate disconnect between the events being predicted by the release experiments and the kill profiles.

9.6.3 Determination of adsorption isotherms

Uptake isotherms are essential parameters to understand the mode of action of antibiotics. At specific exposure time points, uptake isotherms record the amount of the antibiotic bound to the cells as a function of the residual level of antibiotic. Results from uptake studies are expressed as adsorption isotherms. The values of equilibrium concentrations are plotted on the horizontal axis against the amount of antibiotic absorbed to the cells.

The adsorption of both forms of Colistin, CLS and CMS, to cells was studied by adding A. lwoffii R46383 suspension to different concentrations of both antibiotics and the residual antibiotic in suspension was measured by UV spectroscopy at wavelength of 210nm (this wavelength showed reasonable absorbance for both antibiotics, table 7.4 and 7.5), also it was confirmed that the filtrate of the bacterial cells do not interfere with the antibiotic absorption at 210nm. However this method was found not to be sensitive enough to detect
lower concentrations employed in this investigation (Figure 7.10), therefore, it was decided to attempt to measure residual antibiotic using a microbiological assay.

Preliminary investigations showed that filtration does not affect the activity of CLS or CMS as aqueous solutions of both antibiotics were filtered through 0.45µm cellulose nitrate membrane filter (due to the concentrated bacterial culture three filters were used for each sample) and were compared with unfiltered samples, all samples were microbiologically assayed and found no difference in results (Table 7.7).

Also preliminary investigations showed that there was no change in CLS uptake after two minutes or CMS uptake after 60 minutes. This experiment was conducted by exposing CLS or CMS to A. lwoffii R46383 and then assaying both antibiotics after filtration through 0.45µm cellulose nitrate membrane filter and the antibiotic concentrations were determined by the updated microbiological assay at time interval of 2, 20, 30, 60 and 120 minutes.

The bacterial uptake of CLS was complete after two minutes even at high concentration such as 0.0907mmol/L (128mg/L), while CMS uptake was concentration dependent. When the data were plotted as adsorption isotherms, CLS gave results that were indicative of an H-shape high affinity isotherm.

This indicates that the solute (CLS) has a high affinity for the adsorbent (bacterial cells) even at high concentrations, so all used concentrations of the solute are fully adsorbed and that is why the equilibrium concentration is equal to zero.

This shows that the substantial positive charge on the CLS molecule had a rapid, high affinity interaction with the anionic LPS molecules in the outer membrane of the bacteria as at all the used concentrations all the molecules were adsorbed. This profile fits well with the data generated on potassium and 260nm material release and cell killing.

CLS possessed a very strong binding affinity for A. lwoffii R46383 cells, and no residual levels in the supernatant liquid (equilibrium concentration) were recorded. This could explain the greater levels of killing experienced using the CLS.
The data for CMS were quite different and the adsorption isotherm was an S-shape plot. This suggests a much lower affinity for binding sites on the cell than CLS which is in agreement with the data for potassium and 260nm release. It is suggested that the drug molecules binding primarily achieve partial penetration and disorganization of the bacterial walls, leaving available sites on the surface and at the same time creating new binding sites. With increasing concentration the walls are penetrated completely and the cytoplasm is reached.

9.7 Studies into the cytotoxicity of Colistin sulphate and Colistin methanesulphonate

CLS was introduced into clinical use for the treatment of serious Gram negative infections in the 1950s; however its toxicity (particularly nephrotoxicity and neurotoxicity) significantly limited its use (Kunin and Boggy, 1971; Beringer, 2001). As a consequence CMS was formulated with the purpose of having reduced toxicity (Barnett et al, 1964; Hancock and Chapple, 1999).

Cytotoxicity investigations were performed using the Proximal Tubular (PT) which is the main site of antibiotic-induced injury. The in vitro models of this segment of the nephron are important for the evaluation of the safety of antibiotics with regards to the kidney. The most commonly used PT cell line in nephrotoxicity studies includes the NRK-52E cells derived from the normal rat kidney.

Determining cell membrane integrity is one of the most common methods to determine cell viability. Compounds such as CLS and to less extent CMS have cytotoxic effects on cell membranes. Vital dyes, such as trypan blue are normally excluded from the inside of healthy cells as they freely cross the membrane of the injured cells and stain intracellular components (Riss and Moravec, 2004). Other methods to determine cell cytotoxicity are lactate dehydrogenase (LDH) or the-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay (MTT).
LDH is an intracellular enzyme which is impermeable to the plasma membrane. Damaged membrane leaks out intracellular contents of the cells such as LDH enzyme. Thus, the LDH assay measures the release of LDH marker from damaged cells and it is therefore used in cytotoxicity studies. A colorimetric method is used to determine the released LDH (Decker and Lohmann, 1988; Abe and Matsuki, 2000).

Cytotoxicity can also be monitored using the MTT assay. This assay determines the reducing potential of the healthy viable cell using a colorimetric reaction as the viable cells will reduce the MTT reagent to a water-insoluble purple or blue coloured formazan product with the aid of the mitochondrial dehydrogenase enzyme system. A similar redox-based assay has also been developed using the fluorescent dye, resazurin (Riss and Moravec, 2004).

In order to demonstrate the cytotoxic effect of CLS or CMS on renal cells, 85-90% confluent NRK-52E cells were exposed to increasing concentrations of CLS or CMS for two to four days (0.0011, 0.0023 or 0.023mmol/L equivalent to 2, 4, 40mg/L of CMS or 1.6, 3.2, 32mg/L of CLS). The toxic effect of CLS or CMS was evaluated by using MTT and LDH assays.

Also degraded CLS and CMS (stored at 37°C for five or two days respectively) were tested for the cytotoxicity using MTT and LDH assays.

The results obtained from in vitro study demonstrated that fresh or degraded CLS or CMS has no effect on levels of cell death or cell viability even in high doses such as 0.023mmol/L (40mg/L). This may be due to the fact that unlike the gram negative bacterial cells, the mammalian cells do not have a profound anionic surface charge. Hence the CLS and CMS will not have such a high affinity; this statement may be confirmed by looking into the adsorption isotherms of CLS and CMS on renal cells.

A study was performed on human fibroblast and keratinocytes cells to detect the cytotoxic effect of CMS on the skin. These cells were exposed to CMS for 48 hours. Cytotoxic effects of CMS on the skin were examined using MTT; no cytotoxicity was observed when
these cells were exposed to CMS for 48 hours at concentrations even higher than the therapeutic plasma concentrations (Damour et al, 1992).

9.8 Future work

Further work is necessary as CMS is often the last available recourse for multi-resistant Gram-negative bacteria (Linden et al, 2003; Berlana et al, 2005; Li et al, 2005b). Most of the Colistin kinetics data have been generated from microbiological assays of biological fluids which are not reliable since conversion of CMS to Colistin base takes place upon incubation. Updating pharmacokinetics information using the updated chemical analytical methods would achieve the harmonisation of dosage regimen, thus less toxicity will be associated with the administration of the CMS.

Since cytotoxicity studies (MTT and LDH assays) of fresh or degraded CLS and CMS has no effect on levels of cell death or cell viability, further work may include looking into the adsorption isotherms of CLS and CMS on renal cells.
References


Appendix

Arrangement in the large Latin square bioassay plate

<table>
<thead>
<tr>
<th>B</th>
<th>F</th>
<th>C</th>
<th>D</th>
<th>A</th>
<th>E</th>
</tr>
</thead>
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<td>C</td>
<td>A</td>
<td>B</td>
<td>E</td>
<td>D</td>
<td>F</td>
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<tr>
<td>F</td>
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<td>C</td>
<td>B</td>
<td>A</td>
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<tr>
<td>D</td>
<td>C</td>
<td>F</td>
<td>A</td>
<td>E</td>
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<tr>
<td>A</td>
<td>D</td>
<td>E</td>
<td>B</td>
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<td>C</td>
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<tr>
<td>E</td>
<td>B</td>
<td>A</td>
<td>F</td>
<td>C</td>
<td>D</td>
</tr>
</tbody>
</table>

These letters indicate the different concentrations of antibiotic used in the six point assay

Assay of CMS using optimised bioassay
British Pharmacopoeia
2010

Volume III

General Notices
Formulated Preparations
Blood-related Products
Immunological Products
Radiopharmaceutical Preparations
Surgical Materials
Herbal Drugs, Herbal Drug Preparations and Herbal Medicinal Products
Materials for use in the Manufacture of Homoeopathic Preparations

Incorporating the requirements of the 6th edition of the European Pharmacopoeia as amended by Supplements 6.1 to 6.5
Appendix XIV

Biological Assays and Tests

General guidance concerning biological assays and tests is provided in Supplementary Chapter I.

A. Microbiological Assay of Antibiotics

Even method 2.7.2).

The potency of an antibiotic is estimated by comparing the growth of a sensitive micro-organism produced in known concentrations of the antibiotic to be examined and a reference substance.

Reference substance use in the assays are substances whose activity has been precisely determined with reference to the corresponding international standard or international reference preparation.

The assay must be performed in a way that will permit the estimation of the validity of the mathematical model on the potency equation is based. If a parallel-line model is used, the 2-log dose-response (or transformed response) relationship must be parallel, but the reference concentration must be parallel, and it should be linear over the range of doses used in the calculation. These conditions must be met by the test for a given probability, usually 95%. Other mathematical models, such as the slope model, may be used providing that proof of validity is demonstrated.

Otherwise stated in the monograph, the confidence (P = 0.95) of the assay for potency is not less than 90% and not more than 105% of the estimated potency of the assay by method A or method B.

DIFFUSION METHOD

A medium suitable for the conditions of the assay is used at a suitable temperature, for example, 45°C for vegetative forms, with a known quantity of a suspension of a micro-organism sensitive to the antibiotic to be tested, such that clearly defined zones of inhibition of growth are produced with the concentrations of the antibiotic used for the assay. Immediately pour into Petri plates a large rectangular dishes a quantity of the inoculated medium to form a uniform layer 2-3 mm thick. Alternatively, the medium may consist of 2 layers, only the upper layer inoculated.

When dishes so that no appreciable growth or death of micro-organisms occurs before the plates are used and the surface of the medium is dry at the time of use.

Solutions and the buffer solution indicated in Table 2.7.2.2 prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations and presumed to be of equal activity. Apply the solution to the surface of the medium, for example, in rings of porcelain, stainless steel or other material as in cavities prepared in the agar. The same solution must be added to each cylinder or cavity.

Use sterile absorbent paper discs of suitable diameter, which absorb the discs with the solutions of the reference substance and the solutions of the antibiotic to be tested placed on the surface of the agar.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Reference substance</th>
<th>Solvent to be used in preparing the stock solution</th>
<th>Buffer solution (pH)</th>
<th>Micro-organism</th>
<th>Medium and final pH (± 0.1 pH unit)</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>Amphotericin B CRS</td>
<td>Dimethyl sulfoxide R</td>
<td>pH 10.5 (± 0.2 M)</td>
<td>Saccharomyces cerevisiae</td>
<td>F - pH 6.1</td>
<td>35.37 °C</td>
</tr>
<tr>
<td>Bacitracin zinc</td>
<td>Bacitracin zinc CRS</td>
<td>0.01 M hydrochloric acid</td>
<td>pH 7.0 (± 0.05 M)</td>
<td>Micrococcus luteus</td>
<td>A - pH 7.0</td>
<td>35.39 °C</td>
</tr>
<tr>
<td>Bleomycin sulphate</td>
<td>Bleomycin sulphate CRS</td>
<td>Water R</td>
<td>pH 6.8 (± 0.1 M)</td>
<td>Mycobacterium smegmatis</td>
<td>G - pH 7.0</td>
<td>35.37 °C</td>
</tr>
<tr>
<td>Colistimethate sodium</td>
<td>Colistimethate sodium CRS</td>
<td>Water R</td>
<td>pH 6.0 (± 0.05 M)</td>
<td>Bordetella bronchiseptica</td>
<td>B - pH 7.3</td>
<td>35.39 °C</td>
</tr>
<tr>
<td>Pramycetin sulphate</td>
<td>Pramycetin sulphate CRS</td>
<td>Water R</td>
<td>pH 8.0 (± 0.05 M)</td>
<td>Bacillus subtilis NCTC 10400</td>
<td>E - pH 7.9</td>
<td>30.37 °C</td>
</tr>
<tr>
<td>Gentamicin sulphate</td>
<td>Gentamicin sulphate CRS</td>
<td>Water R</td>
<td>pH 8.0 (± 0.05 M)</td>
<td>Bacillus subtilis NCTC 8241</td>
<td>A - pH 7.9</td>
<td>35.39 °C</td>
</tr>
<tr>
<td>Josamycin</td>
<td>Josamycin CRS</td>
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<td>pH 5.6</td>
<td>Bacillus subtilis NCTC 30400</td>
<td>A - pH 6.6</td>
<td>35.37 °C</td>
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<td>Josamycin propionate CRS</td>
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<td>pH 5.6</td>
<td>Bacillus subtilis NCTC 30400</td>
<td>A - pH 6.6</td>
<td>35.37 °C</td>
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<td>Water R</td>
<td>pH 8.0 (± 0.05 M)</td>
<td>Bacillus subtilis NCTC 10400</td>
<td>A - pH 7.9</td>
<td>30.37 °C</td>
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<td>Kanamycin acid sulphate CRS</td>
<td>Water R</td>
<td>pH 8.0 (± 0.05 M)</td>
<td>Bacillus subtilis NCTC 10400</td>
<td>A - pH 7.9</td>
<td>35.39 °C</td>
</tr>
<tr>
<td>Neomycin sulphate</td>
<td>Neomycin sulphate for microbiological assay CRS</td>
<td>Water R</td>
<td>pH 8.0 (± 0.05 M)</td>
<td>Bacillus subtilis NCTC 8241</td>
<td>E - pH 7.9</td>
<td>36.17 °C</td>
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<tr>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Antibiotic</td>
<td>Reference substance</td>
<td>Solvent to be used</td>
<td>Buffer solution (pH)</td>
<td>Micro-organism</td>
<td>Medium and final pH (±0.1 pH units)</td>
<td>Incubation temperature</td>
</tr>
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</tr>
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<td>Netilmicin sulphate</td>
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<td>S. iniae ATCC 6538P</td>
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<td>35-35 °C</td>
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<td>Nystatin</td>
<td>Upstain CRS</td>
<td>Dimethylformamide R</td>
<td>pH 4.6 (0.05 M)</td>
<td>Candida tropicalis ATCC 1263</td>
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<td>30-37 °C</td>
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<td>Rifampicin sodium</td>
<td>Rifampicin sodium CRS</td>
<td>Methanol R</td>
<td>pH 7.0 (0.05 M)</td>
<td>Micrococcus luteus ATCC 8340</td>
<td>A - pH 6.6</td>
<td>35-39 °C</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>Spiramycin CRS</td>
<td>Methanol R</td>
<td>pH 8.0 (0.05 M)</td>
<td>Bacillus subtilis ATCC 5493</td>
<td>A - pH 7.9</td>
<td>30-32 °C</td>
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<tr>
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<td>Streptomycin sulphate CRS</td>
<td>Water R</td>
<td>pH 8.0 (0.05 M)</td>
<td>Bacillus subtilis ATCC 8340</td>
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<td>30-37 °C</td>
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<tr>
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<td>Tetracycline CRS</td>
<td>pH 6.0 (0.05 M)</td>
<td></td>
<td>Bacillus subtilis ATCC 5493</td>
<td>A - pH 7.9</td>
<td>30-37 °C</td>
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<tr>
<td>Tobramycin sodium</td>
<td>Tobramycin sodium CRS</td>
<td>pH 6.0 (0.05 M)</td>
<td></td>
<td>Bacillus subtilis ATCC 5493</td>
<td>A - pH 7.9</td>
<td>30-37 °C</td>
</tr>
</tbody>
</table>

To test the optical apparatus used to measure the activity, the tubes, randomly distributed or in a Latin square block arrangement, in a water-bath or other apparatus fixed with a means of bringing all the tubes to the appropriate incubation temperature and in a test tube at that temperature for 3-4 h, taking care to ensure uniformity of temperature and identical time.

In addition, stop the growth of the micro-organisms by 5 ml of formaldehyde R to each tube or by heat and measure the opacity to 3 significant figures in the optical apparatus. Alternatively use a method that is applicable to each tube to be measured after the period of incubation.

Prepare using appropriate statistical methods.

The dose-response relationship, transformed to 4d, is often obtained only over a very limited range. It is this range which must be used in calculating the activity and it must include at least 3 consecutive doses in order to permit linearity to be verified. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay must be applied.

Use in each assay the number of replicates per case sufficient to ensure the required precision. The assay may be repeated and the results combined statistically to obtain the required precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

* Solution prepared by dissolving 16.73 g of dipotassium hydrogen oxalate and 0.523 g of potassium dibydrogen oxalate in about 750 ml of water, if necessary adjusting to pH 6.0 with 0.1N sodium hydroxide or 0.1N oxalic acid, and diluting to 1000 ml with water.
### Table: Additional section for monograph of the British Pharmacopoeia (Diffusion Method)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Reference substance</th>
<th>Solvent to be used in preparing the stock solution</th>
<th>Buffer solution (pH)</th>
<th>Micro-organism</th>
<th>Medium and final pH (± 0.1 pH unit)</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin Esolate</td>
<td>Erythromycin EPCRS</td>
<td>Methanol</td>
<td>pH 8.0 (0.05M)</td>
<td>Bacillus pumilus NCTC 8241 CIP 76.18</td>
<td>A: 7.9</td>
<td>30 - 37°C</td>
</tr>
<tr>
<td>Erythromycin Ethyl Succinate</td>
<td>Erythromycin EPCRS</td>
<td>Methanol</td>
<td>pH 8.0 (0.05M)</td>
<td>Bacillus subtilis NCTC 10400 CIP 52.62 ATCC 6633</td>
<td>A: 7.9</td>
<td>30 - 37°C</td>
</tr>
<tr>
<td>Erythromycin Stearate</td>
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<td>Methanol</td>
<td>pH 8.0 (0.05M)</td>
<td>Bacillus pumilus NCTC 8241 CIP 76.18</td>
<td>A: 7.9</td>
<td>30 - 37°C</td>
</tr>
<tr>
<td>Lymecycline</td>
<td>Lymecycline 2nd Int. Ref., 1971</td>
<td>Methanol</td>
<td>pH 5.8</td>
<td>Bacillus pumilus NCTC 8241 CIP 76.18</td>
<td>A: 6.6</td>
<td>31 - 39°C</td>
</tr>
<tr>
<td>Polymyxin B Sulphate</td>
<td>Polymyxin B Sulphate EPCRS</td>
<td>Water</td>
<td>pH 6.0 (0.05M)</td>
<td>Bordetella bronchiseptica NCTC 8344 CIP 53.157 ATCC 4617</td>
<td>B: 7.3</td>
<td>35 - 39°C</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>Tobramycin 2nd Int. Ref., 1985</td>
<td>Water</td>
<td>pH 8.0 (0.05M)</td>
<td>Bacillus subtilis NCTC 10400 CIP 52.62 ATCC 6633</td>
<td>A: 7.9</td>
<td>30 - 37°C</td>
</tr>
</tbody>
</table>

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The following section is published for information.

**Recommended micro-organisms**

The following text details the recommended micro-organisms and the conditions of use. Other micro-organisms may be used provided that they are shown to be sensitive to the antibiotic to be examined and are used in appropriate media and appropriate conditions of temperature and pH. The concentrations of the solutions used should be chosen so as to ensure that a linear relationship exists between the logarithm of the dose and the response in the conditions of the test.

**Preparation of inocula**  
*Bacillus subtilis*, *Bacillus pumilus*. Spore suspensions of the organisms used as inocula are prepared as follows.

Grow the organism at 35-37 °C for 7 days on the surface of a suitable medium to which has been added 0.001 g/l of manganese sulphate R. Using sterile water R, wash off the growth, which consists mainly of spores. Heat the suspension at 70 °C for 30 min and dilute to give an appropriate concentration of spores, usually 10 x 10² to 100 x 10⁶ per millilitre. The spore suspensions may be stored for long periods at a temperature not exceeding 4 °C.

Alternatively, spore suspensions may be prepared by cultivating the organisms in medium C at 20 °C for 4-6 days, then adding, spectrophotometrically, sufficient manganese sulphate R to give a concentration of 0.001 g/l and incubating for a further 48 h. Examine the suspension microscopically to ensure that adequate spore formation has taken place (about 80 per cent) and centrifuge. Re-suspend the sediment in sterile water R to give a concentration of 10 x 10⁶ to 100 x 10⁶ spores per millilitre, and then heat to 70 °C for 30 min. Store the suspension at a temperature not exceeding 4 °C.

*Burkholderia cepacia*  
Grow the test organism on medium B at 35-37 °C for 16-18 h. Wash off the bacterial growth with sterile water R and dilute to a suitable opacity. *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus epidermidis*. Prepare as described above for *B. cepacia* but using medium A and adjusting the opacity to one which has been shown to produce a satisfactory dose-response relationship in the turbidimetric assay, or to produce clearly defined zones of inhibition of convenient diameter in the diffusion assay, as appropriate.

*Saccharomyces cerevisiae*, *Candida tropicalis*. Grow the test organism on medium F at 30-37 °C for 24 h. Wash off the growth with a sterile 9 g/l solution of sodium chloride R. Dilute to a suitable opacity with the same solution.

**Buffer solutions**  
Buffer solutions having a pH between 5.8 and 8.0 are prepared by mixing 50.0 ml of 0.2 M potassium dihydrogen phosphate R with the quantity of 0.2 M sodium hydroxide indicated in Table 2.7.2.3. Dilute with freshly prepared distilled water R to produce 200.0 ml.

These buffer solutions are used for all microbiological assays shown in Table 2.7.2.3 with the exception of bleomycin sulphate and amphotericin B.

For bleomycin sulphate, prepare the buffer solution pH 6.8, as follows: dissolve 6.4 g of potassium dihydrogen phosphate R and 18.9 g of disodium hydrogen phosphate R in water R and dilute to 1000 ml with water R.

For amphotericin B, prepare the 0.2 M phosphate buffer solution pH 10.5 as follows: dissolve 35 g of dipotassium hydrogen phosphate R in 900 ml of water R, add 20 ml of 1 M sodium hydroxide and dilute to 1000.0 ml with water R.
dried residue, Appendix III A, is concordant with the reference specimen of colistin base (RS 266). TESTS
Styrene
Carry out the method for liquid chromatography, Appendix III D, using the following solutions. For solution (1) shake a quantity of the powder containing the equivalent of 2 g of anhydrous colistin base with 10 ml of water for 30 minutes, centrifuge and use the supernatant liquid. Solution (2) contains 0.0002% w/v of styrene in acetone.

The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm x 3.9 mm) packed with end-capped octadecylsilica gel for chromatography (10 μm) (μBondapak C18 is suitable), (b) a mixture of equal volumes of acetone and water at the mobile phase with a flow rate of 2 ml per minute and (c) a detection wavelength of 254 nm.

Inject separately 20 μl of each solution. In the chromatogram obtained with solution (1) the area of any peak corresponding to styrene is not greater than the area of the principal peak in the chromatogram obtained with solution (2) (1 ppm).

ASSAY
Prepare a solution of sodium glycolate by dissolving 1.5 g in 40 ml of hot water, cooling and diluting to 50 ml with water (solution A). To a quantity of the powder containing the equivalent of about 0.1 g of anhydrous colistin base add 25 ml of water and shake for 15 minutes. Centrifuge and carefully decant and discard the liquid layer.Repeat the procedure with a further 25 ml of water. Dry the washed residue at 100° for 2 hours. To the residue add 10 ml of solution A, shake mechanically for 2 hours and then centrifuge (solution B). Prepare solution C in the same manner using 0.11 g of colistamid BPCRS, beginning at the words ‘add 10.0 ml of solution A . . .’ Dilute, separately, 2 ml of solution A, 2 ml of solution B and 2 ml of solution C to 200 ml with water. To 1 ml of each solution in separate 10 ml graduated flasks add 4 ml of sulphuric acid (98%). Loosely stopper the flasks and heat at 60° for 15 minutes, cool and dilute to volume with sulphuric acid (98%). Allow the solutions to stand for 1 hour. Measure the absorbance of each solution at the maximum at about 318 nm, Appendix II B, using water in the reference cell. Calculate the quantity of colistin in the powder using the following expression:

$$M(A_1 - A_2)W_1W_5$$

where $M$ = stated value, in g, of sodium glycolate absorbed per g of colistin base BPCRS,

$A_1$ = absorbance of solution A,

$A_2$ = absorbance of solution B,

$A_5$ = absorbance of solution C,

$W_1$ = weight of colistin base BPCRS,

$W_5$ = weight of powder being examined,

$W_5$ = average weight of content of each unit.

LABELLING
The quantity of the active ingredient is stated in terms of the equivalent amount of anhydrous colistin base.

Colistimethate Injection

Action and use
Antibacterial.

DEFINITION
Colistimethate Injection is a sterile solution of Colistimethate Sodium in Sodium Chloride Intravenous Infusion. It is prepared by dissolving Colistimethate Sodium for Injection in the requisite amount of Sodium Chloride Intravenous Infusion.

The injection complies with the requirements stated under Parenteral Preparations.

STORAGE
Colistimethate Injection should be used immediately after preparation and, in any case, within the period recommended by the manufacturer when prepared and stored strictly in accordance with the manufacturer’s instructions.

COLISTIMETHATE SODIUM FOR INJECTION

Colistimethate Sodium for Injection is a sterile material consisting of Colistimethate Sodium with or without excipients. It is supplied in a sealed container.

The contents of the sealed container comply with the requirements for Powders for Injections stated under Parenteral Preparations and with the following requirements.

IDENTIFICATION
A. Carry out the method for thin-layer chromatography, Appendix III A, protected from light using a TLC silica gel G plate and a mixture of 25 parts of water and 75 parts of phenol as the mobile phase. Apply separately to the plate, at 10-mm bands, 5 μl of each of the following solutions. For solution (1) dissolve a quantity containing 62,500 IU in 1 ml of a mixture of equal volumes of hydrochloric acid and water, heat at 135° in a sealed tube for 5 hours, evaporate to dryness on a water-bath, continue the heating until any residual hydrochloric acid has evaporated and dissolve the residue in 0.5 ml of water. Solutions (2) to (5) contain (2) 0.2% w/v of esculin, (3) 0.2% w/v of thiorazone, (4) 0.2% w/v of phloroglucinol and (5) 0.2% w/v of ferric trichloride, respectively. Place the plate in the chromatographic tank so that it is not in contact with the mobile phase. Allow the plate to become impregnated with the vapour of the solvent for at least 12 hours and develop over a path of 12 cm using the same mobile phase. Dry the plate at 100° to 105°, spray with ninhydrin solution B1, and heat at 110° for 5 minutes. The chromatogram obtained with solution (1) shows zones corresponding to those in the chromatograms obtained with solutions (2) and (3) but shows no zones corresponding to those in the chromatograms obtained with solutions (4) and (5). The chromatogram obtained with solution (1) also shows a zone with a very low Rf value (2,4-diamino-6-chlorophenol).

B. Dissolve a quantity containing 125,000 IU in 5 ml of water. Heat 0.5 ml of the solution with 0.5 ml of chromotropic acid–sulphuric acid solution at 100° for 30 minutes. A purple colour is produced (distinction from colistin sulphate).

C. Dissolve a quantity containing 225,000 IU in 1 ml of 1% hydrochloric acid and add 0.5 ml of 0.01% thionin. The colour is discharged (distinction from colistin sulphate) and the resulting solution yields reaction A characteristic of sulphates, Appendix VI.
D. Yield reaction A characteristic of sodium salts, Appendix VI.

TESTS
Acidity or alkalinity
Dissolve a quantity in sufficient carbon dioxide-free water to produce a solution containing 125,000 IU per ml. The pH of the resulting solution, measured 30 minutes after preparation, is 6.5 to 8.5, Appendix V L.

Free colistin
Dissolve a quantity containing 1,000,000 IU in 3 ml of water, add 0.1 ml of 10% w/v solution of salicyclosalicic acid and allow to stand for 10 to 20 seconds. The resulting solution is not more opalescent than reference suspension II, Appendix IV A.

Loss on drying
When dried over phosphorus pentoxide at 60° at a pressure not exceeding 0.7 kPa for 3 hours, lose not more than 5.0% of their weight. Use 1 g.

Bacterial endotoxins
Carry out the test for bacterial endotoxins, Appendix XIV C, using method D. Dissolve the contents of the sealed container in water B.P. to give a solution containing 250,000 IU per ml (solution A). The endotoxin limit concentration of solution A is 43.75 IU of endotoxin per ml.

ASSAY
Determine the weight of the contents of each of 10 containers as described in the test for Uniformity of weight under Parenteral Preparations, Powders for Injections. Mix the contents of the 10 containers and carry out the microbiological assay of antibiotics, Appendix XIV A. The precision of the assay is such that the fiducial limits of error are not less than 95% and not more than 105% of the estimated potency. For a container of average content weight, the upper fiducial limit of error is not less than 95.0% and the lower fiducial limit of error is not more than 115.0% of the stated number of IU.

STORAGE
The sealed container should be protected from light.

LABELLING
The label of the sealed container states the total number of IU (units) contained in it.

Colistin Tablets

Action and use:
Antibacterial.

DEFINITION
Colistin Tablets contain Colistin Sulphate.
The tablets comply with the requirements stated under Tablets and with the following requirements.

Content of colistin sulphate, calculated as the sum of the contents of polymyxin E1, polymyxin E2, polymyxin E3, polymyxin E1-1 and polymyxin E1-7MOA
Not less than 77.0% of the stated amount of colistin sulphate.

Content of polymyxin E1-1
Not more than 10.0% of the stated amount of colistin sulphate.

Content of polymyxin E1-7MOA
Not more than 10.0% of the stated amount of colistin sulphate.

Content of polymyxin E3
Not more than 10.0% of the stated amount of colistin sulphate.

IDENTIFICATION
To a quantity of the powdered tablets containing 10 mg of Colistin Sulphate add 10 ml of water, shake and filter. Use the filtrate for the following tests.

A. Carry out the method for thin-layer chromatography, Appendix III A, protected from light using the following solutions.
(1) Add 0.5 ml of hydrochloric acid to 0.5 ml of the filtrate, heat in a sealed tube at 135° for 5 hours, evaporate to dryness on a water bath, continue to heat until any residual hydrogen chloride has been removed, dissolve the residue in 0.5 ml of water and centrifuge, if necessary.
(2) 0.25% w/v of L-leucine in water.
(3) 0.25% w/v of L-threonine in water.
(4) 0.25% w/v of L-phenylalanine in water.
(5) 0.25% w/v of L-serine in water.

CHROMATOGRAPHIC CONDITIONS
(a) Use silica gel G as the coating substance.
(b) Use the mobile phase described below.
(c) Apply 5 µl of each solution, as 10-mm bands. Place the plate in the tank so that it is not in contact with the mobile phase and expose it to the vapour of the mobile phase.
(d) After exposure of the plate to the mobile phase vapour for at least 12 hours, develop to 12 cm.
(e) Remove the plate, heat it at 100° to 105°, spray with ninhydrin solution R1 and heat at 110° for 5 minutes.

MOBILE PHASE
25 parts of water and 75 parts of phenol.

CONFIRMATION
The bands in the chromatogram obtained with solution (1) correspond to those in the chromatograms obtained with solutions (2) and (3) and do not correspond to those in the chromatograms obtained with solutions (4) and (5). The chromatogram obtained with solution (1) also shows a band with a very low Rf value (2.4-diaminobutanoic acid).

B. Heat 0.5 ml of the filtrate with 0.5 ml of chromatographic acid-sulphuric acid solution at 100° for 30 minutes. No purple colour is produced (distinction from colistin sulphamate).

C. The filtrate yields reaction A characteristic of sulphate, Appendix VI.

Related substances
Carry out the method for liquid chromatography, Appendix III D, using the following solutions.
(1) Shake a quantity of the powdered tablets containing 25 mg of Colistin Sulphate with 40 ml of water for 20 minutes, add sufficient acetonitrile to produce 50 ml and filter through a Whatman GFC filter and then through a 0.45-µm nylon filter.
(2) Dissolve the contents of a vial containing 25 mg of colistin sulphate EP<sup>RS</sup> in 40 ml of water and add sufficient acetonitrile to produce 50 ml.
(3) Dilute 1 volume of solution (2) to 100 volumes with a mixture of 20 volumes of acetonitrile and 80 volumes of water.
CHROMATOGRAPHIC CONDITIONS
(a) Use a stainless steel column (15 cm × 4.6 mm) packad
with end-capped octadecylsilica gel for chromatography
(3.5 µm) (SunFire C18 is suitable).
(b) Use isocratic elution and the mobile phase described
below.
(c) Use a flow rate of 1 ml per minute.
(d) Use a column temperature of 30°C.
(e) Use a detection wavelength of 215 nm.
(f) Inject 20 µl of each solution.
(g) For solution (1) allow the chromatography to proceed for
1.5 times the retention time of polymyxin E1.

MOBILE PHASE
22 volumes of acetonitrile and 78 volumes of a solution
prepared by dissolving 4.66 g of anhydrous sodium sulphate in
900 ml of water, adjusting the pH to 2.4 with dilute
orthophosphoric acid and adding sufficient water to produce
1000 ml.
When the chromatograms are recorded under the prescribed
conditions the retention time of polymyxin E1 is about
16 minutes. The ratio of relative to polymyxin E1 are:
about 0.45 (polymyxin E2); about 0.5 (polymyxin E3); about
0.8 (polymyxin E1-1); about 1.1 (polymyxin
E1-7MOA).

SYSTEM SUITABILITY
The test is not valid unless, in the chromatogram obtained
under solution (2) the retention factor between the peaks
due to polymyxins E2 and E3 is at least 8.0; the retention factor
between the peaks due to polymyxins E2 and E1-1 is at least
6.0; the retention factor between the peaks due to polymyxins
E1-1 and E1 is at least 2.3; the retention factor between the
peaks due to polymyxins E1 and E1-7MOA is at least 1.5.

LIMITS
In the chromatogram obtained with solution (1):
the area of any secondary peak is not greater than 4.0% by
normalisation;
the sum of the areas of all such peaks is not greater than
23.0% by normalisation.
Disregard any peak with an area less than the area of the
peak due to polymyxin E1 in the chromatogram obtained
with solution (3) (1%) and any peaks due to polymyxins E1,
E2, E3, E1-1 and E1-7MOA.

ASSAY
Carry out the method for liquid chromatography,
Appendix III D, using the following solutions.
(1) Shake a quantity of the powdered tablets containing
25 mg of Colistin Sulphate with 40 ml of water for
20 minutes, add sufficient acetonitrile to produce 50 ml and
filter through a Whatman GF/C filter and then though a
0.45-µm nylon filter.
(2) Dissolve the content of a vial containing 25 mg of
colistin sulphate EP CRS in 40 ml of water and add sufficient
acetonitrile to produce 50 ml.

CHROMATOGRAPHIC CONDITIONS
The chromatographic conditions described under Related
substances may be used.

DETERMINATION OF CONTENT
Calculate the content of polymyxins E3, E1-1 and E1-7MOA
and the sum of the contents of polymyxin E1, polymyxin E2,
polymyxin E3, polymyxin E1-1 and polymyxin E1-7MOA
using the declared contents of polymyxins E1, E2, E3, E1-1
and E1-7MOA in colistin sulphate EP CRS.

STORAGE
Colistin Tablets should be protected from light.

Flexible Collodion
Action and use
Skin protective.

DEFINITION
Flexible Collodion is a solution of Collophony in a mixture of
Virgin Castor Oil and Collodion.

Extemporaneous preparation
The following formula and directions apply.
Collophony
25 g
Virgin Castor Oil
25 g
Collodion
Sufficient to produce 1000 ml
Mix the ingredients and stir until the collophony has
dissolved; allow any deposit to settle and decant the clear
liquid.

Flexible collodion complies with the requirements stated under
Liquids for Cutaneous Application and with the following
requirements...

IDENTIFICATION
A. Expose a thin layer to the air. A thin, tenacious film is left
which, when ignited, burns rapidly with a yellow flame.
B. Mix with an equal volume of water. A white, viscous,
stringy mass is obtained.

Ethanol content
Carry out the method for gas chromatography, Appendix III B
using the following solutions in ether.
(1) 5% v/v of absolute ethanol and 5% v/v of acetonitrile
(internal standard).
(2) 20% v/v of the substance being examined.
(3) 20% v/v of the substance being examined and 5% v/v of
the internal standard.

CHROMATOGRAPHIC CONDITIONS
(a) Use a glass column (1.5 m x 4 mm) packed with porous
polymer beads (100 to 120 mesh) (Porapak Q is suitable).
(b) Use nitrogen as the carrier gas at 40 ml per minute.
(c) Use isothermal conditions maintained at 120°C.
(d) Use a flame ionisation detector.
(e) Inject 1 µl of each solution.

DETERMINATION OF CONTENT
Calculate the content of C22H40O from the areas of the peaks
due to ethanol and acetonitrile in the chromatograms
obtained with solution (1) and solution (3).

LIMIT
20 to 23% v/v.

For preparations in which industrial methylated spirit has been
used, determine the content of ethanol as described above.
Determine the concentration of methanol in the following
manner. Carry out the chromatographic procedure described
above but using the following solutions.
(1) 0.25% v/v of methanol and 0.25% v/v of acetonitrile
(internal standard):