CYCLODEXTRINS FOR ORAL LIQUID
PAEDIATRIC DRUG DELIVERY: APPLICATION
TO CORTICOSTEROIDS

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This thesis is submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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This thesis describes research conducted in the School of Pharmacy, University of London between 2005 and 2008 under the supervision of Dr. Catherine Tuleu. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature  
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Abstract

In the present research, the ability of cyclodextrins (CDs) to solubilise and taste mask poorly soluble bitter drugs was assessed by various analytical techniques, including a novel *in vitro* taste analyser, to propose CDs as excipients for oral liquid paediatric formulations. Corticosteroids were selected as model drugs because of their relevance in paediatrics, whilst model bitter drug quinine was also selected to provide comparative data.

Aside from various inclusion complex characteristics, such as stability constants and complexation efficiencies, phase solubility studies determined that: the CDs formed soluble 1:1 inclusion complexes with the corticosteroids studied, the CD derivatives were capable of providing adequate paediatric dose volumes, simulated media *in vivo* and pH did not to have a major influence on inclusion complexation. The presence of preservatives, however, reduced CD solubility efficiency, whilst reducing the preservative efficiency. ITC studies determined that inclusion complexation with corticosteroids was a spontaneous process and various interactions involved with complexation were proposed. $^1$H NMR spectroscopy studies indicated that CDs formed inclusion complexes with the corticosteroids, whereby the drug molecules entered the CD cavities via the wider cavity opening.

Human taste panel studies demonstrated that CDs were able to taste mask corticosteroids, often more efficiently than traditional taste masking agents, and with 10 times more drug being incorporated in the formulation. Excess CD was also observed to be more efficient at taste masking corticosteroids than molar equivalent concentrations. From these taste panel results, a mechanism of CD taste masking was proposed.

Preliminary electronic tongue analysis was found to be able to select concentrations of various taste masking agents for subsequent taste masking assessment. Nevertheless, little correlation was found between the assessment of the electronic tongue and the human taste panel, thus limiting the legitimacy of the instrument for this application.

This research has shown that CDs could be utilised in oral liquid paediatric formulations to provide dose uniformity, appropriate volume, and acceptable palatability for poorly soluble bitter drugs. Further work is required to examine the acceptability & bioavailability of these formulations in paediatric patients.
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## Table of Contents

### Chapter 1 - General Introduction

1.1 Paediatric medicine ................................................................. 26
   1.1.1 Introduction ................................................................. 26
   1.1.2 Unlicensed and “off label” drug use ............................... 26
   1.1.3 Current regulations in the European Union ................. 27
   1.1.4 Challenges and requirements for oral paediatric drug deliver ...
      1.1.4.1 Age, abilities and dosage form .......................... 28
      1.1.4.2 Excipients .......................................................... 29
      1.1.4.3 Taste ................................................................. 30
      1.1.4.4 Clinical trials .................................................... 31

1.2 Corticosteroids ........................................................................... 31
   1.2.1 Introduction ................................................................. 31
   1.2.2 Discovery, structure, and properties ............................ 33
   1.2.3 Corticosteroid drug therapy ........................................ 35
   1.2.4 Issues with oral corticosteroid formulations for paediatric patient ...

1.3 Taste Masking ............................................................................ 38
   1.3.1 Introduction ................................................................. 38
   1.3.2 The taste experience ..................................................... 38
   1.3.3 Maturation of taste ...................................................... 42
   1.3.4 Taste masking techniques .......................................... 43

1.4 Taste assessment ....................................................................... 44
   1.4.1 Introduction ................................................................. 44
   1.4.2 Taste assessment \textit{in vivo} ......................................... 45
   1.4.3 Taste assessment \textit{in vitro} ........................................ 46

1.5 Cyclodextrins ........................................................................... 47
   1.5.1 Introduction ................................................................. 47
   1.5.2 Discovery, history and development ............................ 48
   1.5.3 Structure and properties ............................................. 50
   1.5.4 Inclusion complexation .............................................. 53
   1.5.5 Factors influencing inclusion complex formation ......... 57
   1.5.6 Evidence of inclusion complex formation ................... 60
   1.5.7 Cyclodextrin influences on the properties of drugs ....... 64
1.5.7.1 Cyclodextrins and drug dissolution, solubility and bioavailability.................................64
1.5.7.2 Cyclodextrins and drug stability.................................................................65
1.5.7.3 Cyclodextrins and drug safety.................................................................66
1.5.7.4 Cyclodextrins and drug taste.................................................................66
1.5.8 Pharmaceutical application of cyclodextrins...............................................67
1.5.9 Drug absorption from cyclodextrin inclusion complexes in vivo.................69
1.5.10 Cyclodextrin limitations in research & development.................................70
1.5.11 Safety and fate of cyclodextrins in vivo.........................................................71
1.5.12 Cyclodextrins and corticosteroids...............................................................74

1.6 Aims and objectives.......................................................................................74

Chapter 2 - Characterisation of Inclusion Complex Formation

2.1 Introduction.......................................................................................................76
2.2 Section 1 - Phase solubility studies.................................................................76
2.2.1 Introduction.....................................................................................................76
2.2.2 Aims and objectives.......................................................................................79
2.2.3 Materials and methods..................................................................................81
2.2.3.1 Materials......................................................................................................81
2.2.3.2 Part 1.............................................................................................................82
2.2.3.2.1 Phase solubility study of prednisolone and hydrocortisone in cyclodextrin systems.................................82
2.2.3.2.2 Phase solubility method validation: a) degradation by heating..............83
2.2.3.2.3 Phase solubility method validation: b) loss through filtering...............83
2.2.3.2.4 Phase solubility method validation: c) assessment of supersaturation...83
2.2.3.2.5 Phase solubility method validation: d) HPLC method optimisation......84
2.2.3.3 Part 2 - Phase solubility study of prednisolone and hydrocortisone in cyclodextrin systems in the presence of parabens.................................84
2.2.3.4 Part 3 - Phase solubility study of hydrocortisone in cyclodextrin systems in various pH and in simulated media in vivo...............................84
2.2.3.5 Part 4 - Phase solubility study of quinine in cyclodextrin systems...........85
2.2.4 Results and discussion....................................................................................86
2.2.4.1 Part 1.............................................................................................................86
2.2.4.1.1 Phase solubility study of prednisolone and hydrocortisone in cyclodextrin systems..............................................86
2.2.4.1.2 Phase solubility method validation ...............................................................89
2.2.4.2 Part 2 - Phase solubility study of prednisolone and hydrocortisone in cyclodextrin systems in the presence of parabens ......................... 90
2.2.4.3 Part 3 - Phase solubility study of hydrocortisone in cyclodextrin systems in various pH and in simulated media in vivo ............................... 94
2.2.4.4 Part 4 - Phase solubility study of quinine in cyclodextrin systems ........ 98
2.2.5 Conclusions .................................................................................................................100

2.3 Section 2 - Isothermal titration calorimetry, and ¹H nuclear magnetic resonance spectroscopy studies ................................................................. 101
2.3.1 Introduction .................................................................................................................101
2.3.2 Aims and objectives .................................................................................................103
2.3.3 Materials and methods ............................................................................................103
   2.3.3.1 Materials ........................................................................................................103
   2.3.3.2 Isothermal titration calorimetry studies of the interaction between hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin with hydrocortisone and quinine ...................................................... 104
   2.3.3.3 ¹H Nuclear magnetic resonance spectroscopy studies of the interaction between hydroxypropyl-β-cyclodextrin with hydrocortisone ........ 105
2.3.4 Results .........................................................................................................................106
   2.3.4.1 Isothermal titration microcalorimetry ........................................................106
   2.3.4.2 ¹H Nuclear magnetic resonance spectroscopy .......................................... 107
2.3.5 Discussion ..................................................................................................................108
   2.3.5.1 Isothermal titration microcalorimetry ........................................................108
   2.3.5.2 ¹H Nuclear magnetic resonance spectroscopy .......................................... 112
2.3.6 Conclusions .................................................................................................................114

Chapter 3 - Microbiology Studies
3.1 Introduction ..............................................................................................................116
   3.1.1 Oral liquid formulation microbial preservation ............................................ 116
   3.1.2 Parabens .......................................................................................................... 117
   3.1.3 Interaction of Parabens with Cyclodextrin/Drug Inclusion Complex .......... 117
   3.1.4 Aims and objectives ...................................................................................... 118
3.2 Materials and methods ...........................................................................................118
   3.2.1 Materials ........................................................................................................ 118
   3.2.2 Efficacy of antimicrobial preservation ....................................................... 119
3.2.2.1 Preparation of Agar Plates .............................................................. 119
3.2.2.2 Culturing and harvesting of microorganisms .............................. 119
3.2.2.3 Formulation efficacy test ............................................................... 120
3.2.2.4 Serial dilution plate count ............................................................... 120
3.2.3 Determining the extent of parabens interaction with cyclodextrin/drug
inclusion complexes by turbidity testing ................................................... 122
3.2.3.1 Preparation of inocula ...................................................................... 120
3.2.3.2 Preparation of test samples ............................................................. 123
3.2.3.3 Turbidity testing .............................................................................. 123

3.3 Results and discussion .................................................................................... 124
3.3.1 Preliminary phase solubility study of hydrocortisone and HP-β-CD in the
presence of parabens ..................................................................................... 124
3.3.2 Efficacy of antimicrobial preservation test ................................................ 125
3.3.3 Turbidity testing ........................................................................................ 128

3.4 Conclusions ........................................................................................................ 133

Chapter 4 - Preliminary ASTREE electronic tongue studies
4.1 Introduction ......................................................................................................... 136
4.1.1 Taste masking assessment in vitro: ASTREE electronic tongue
studies ............................................................................................................. 136
4.1.2 ASTREE Electronic Tongue ....................................................................... 136
4.1.2.1 Introduction ...................................................................................... 136
4.1.2.2 Sensor technology ........................................................................... 137
4.1.2.3 Experimental procedure .................................................................. 139
4.1.3 Aims and objectives ..................................................................................... 147

4.2 Materials and methods ......................................................................................... 148
4.2.1 Materials ......................................................................................................... 148
4.2.2 Preliminary analysis - prednisolone, hydrocortisone and quinine
quantification by the ASTREE electronic tongue ........................................ 149
4.2.3 Preliminary taste masking analysis of hydrocortisone and quinine with
cyclodextrins, sweeteners, and flavouring agents ........................................ 150
4.2.4 Data processing, and replicate and sensor selection ................................... 151
4.2.5 Data analysis .................................................................................................. 151

4.3 Results .................................................................................................................. 152
6.2.3 Taste masking assessment in vivo: Selected adult taste panel assessment of preselected hydrocortisone and quinine formulations.................198

6.2.4 In vitro and in vivo correlation.................................................................198

6.3 Results and discussion................................................................................199

6.3.1 Taste masking assessment in vitro: ASTREE electronic tongue analysis of preselected hydrocortisone formulations.............................................199

6.3.2 Taste masking assessment in vitro: ASTREE electronic tongue analysis of preselected quinine formulations......................................................204

6.3.3 Taste masking assessment: in vitro and in vivo comparison of hydrocortisone and quinine formulations.......................................................209

6.4 Conclusion...................................................................................................218

Chapter 7 - General Discussion and Future Work

7.1 Introduction..................................................................................................220

7.2 Methodology considerations........................................................................221

7.2.1 Complex characterisation techniques.....................................................221

7.2.2 Efficacy of antimicrobial preservation.................................................222

7.2.3 Taste assessment in vitro.................................................................222

7.2.4 Taste assessment in vivo.................................................................224

7.3 Formulation considerations........................................................................225

7.3.1 Drug solubility......................................................................................225

7.3.2 Preservative efficacy............................................................................227

7.3.3 Taste masking.......................................................................................227

7.3.4 Industrial application.............................................................................229

7.3.5 Drug dissociation..................................................................................229

7.3.6 Safety....................................................................................................230

Appendix 1........................................................................................................231

Appendix 2........................................................................................................257

Appendix 3........................................................................................................261

Publications and Communications................................................................262

References.........................................................................................................263
List of Figures

Figure 1.1: The chemical structure of cortisol - the main endogenous glucocorticoid.32

Figure 1.2: The chemical structure of aldosterone - the main endogenous mineralocorticoid.32

Figure 1.3: The chemical structure of A) betamethasone, B) deflazacort, C) dexamethasone, D) fludrocortisone, E) hydrocortisone, F) methylprednisolone, G) prednisolone, H) prednisone, I) triamcinolone.34

Figure 1.4: The anatomy of a typical taste bud (Dowdey, 2007).39

Figure 1.5: Signaling transduction of bitter taste (Palmer, 2007).41

Figure 1.6: The chemical structure of a) α-CD, b) β-CD, c) γ-CD (Davis and Brewster, 2004).50

Figure 1.7: A schematic view of the orientation of a glucopyranose unit located within a cyclodextrin molecule, highlighting the six carbon atoms (taken from Mourtzis et al., 2007).51

Figure 1.8: The chemical structure of β-cyclodextrin and some of its derivatives (Loftsson et al., 2004).52

Figure 1.9: A schematic representation of formation of a CD/drug inclusion complex in aqueous solution (Loftsson and Stefansson, 1997).55

Figure 1.10: A schematic representation of the mode of drug absorption through biological membranes from CD inclusion complex (taken from Challa, 2005). Key: C = cyclodextrin, D = drug.69

Figure 2.1: Theoretical representations of A- and B- type phase solubility diagrams including applicable subtypes (A_P, A_L, A_N and B_S, B_I), according to Higuchi and Connors (1965).77

Figure 2.2: Phase solubility diagrams of prednisolone and hydrocortisone with various CDs in water at room temperature (23 ± 1 °C). Each point: mean ± SEM, n = 3. Note that some error bars are within the symbol. Significant difference (p = 0.05) between all CDs across whole concentration range.86
Figure 2.3: Supersaturation solubility equilibrium of free and complexed prednisolone at room temperature (23 ± 1 °C). Each point: mean ± SEM, n = 3. Note that some error bars are within data points.

Figure 2.4: Phase solubility diagrams of prednisolone with various CDs in aqueous parabens solutions at room temperature (23 ± 1 °C) (x-axis scale kept the same for all phase diagrams). Each point: mean ± SEM, n = 3. Note that some error bars are within data points.

Figure 2.5: Phase solubility diagrams of hydrocortisone with various CDs in aqueous parabens solutions at room temperature (23 ± 1 °C) (x-axis scale kept the same for all phase diagrams). Each point: mean ± SEM, n = 3. Note that some error bars are within data points. Significant difference (p = 0.05) between all concentrations of parabens for each CD, across the whole CD concentration range.

Figure 2.6: Phase solubility diagrams of hydrocortisone with HP-β-CD and Me-β-CD in various media at room temperature (23 ± 1 °C). Each point: mean ± SEM, n = 3. Note that some error bars are within data points. p = 0.05.

Figure 2.7: Phase solubility diagrams of quinine with HP-β-CD and Me-β-CD in water at room temperature (23 ± 1 °C). Each point: mean ± SEM, n = 3. Note that some error bars are within data points. Significant difference (p = 0.05) between CDs across the whole CD concentration range.

Figure 2.8: Typical ITC data obtained from the binding interaction of guest to a cyclodextrin - A) exothermic heat released upon injection of cyclodextrin into the guest solution; B) integrated heat data plotted against molar ratio of CD and guest to produce a differential binding curve (taken from Gaisford and O’Neil, 2006).

Figure 2.9: Titration curves of the binding interaction between cyclodextrin and drug at 298.2 ± 0.2 K. A) Titrant: 18.15 mM HP-β-CD; titrand: 0.91 mM hydrocortisone, B) Titrant: 11.61 mM HP-β-CD; titrand: 0.58 mM quinine, C) Titrant: 18.15 mM Me-β-CD; titrand: 0.91 mM hydrocortisone, D) Titrant: 11.61 mM Me-β-CD; titrand: 0.58 mM quinine.

Figure 2.10: Integrated heat data of cyclodextrin complexation as a function of molar ratio between: A) HP-β-CD and hydrocortisone, B) HP-β-CD and quinine, C) Me-β-CD and hydrocortisone, D) Me-β-CD and quinine, giving a binding curve fitted to a standard single-site binding model using a nonlinear least-squares method.
Figure 2.11: $^1$H NMR spectra of: A) 0.55 mM hydrocortisone, B) 7.74 mM HP-β-CD and C) HP-β-CD:HC (1:1) inclusion complex in D$_2$O at 298 K.

Figure 2.12: Schematic view of the average orientation of the most important protons on a HP-β-CD molecule (Adapted from Schneider, 1998).

Figure 3.1: Example of the serial dilution plate count process.

Figure 3.2: Flow diagram illustrating the principles behind the turbidity testing.

Figure 3.3: Phase solubility diagrams of hydrocortisone with HP-β-CD in aqueous parabens solutions at room temperature (23 ± 1°C). Each point: mean ± SEM, n = 3. Note that some error bars are within data points. Significant difference ($p = 0.05$) between all parabens concentrations, except 0 % and 0.05 % parabens, across whole CD concentration range.

Figure 3.4: Semi-logarithmic growth curve of *E. coli*, *P. aeruginosa*, and *S. aureus* (as determined by optical density), in tryptone soya broth after incubation at 37 °C in a Multitron II mixing oven at 200 rpm. Each point: mean ± SEM, n = 3. Note that some error bars are within data points.

Figure 3.5: A semi-logarithmic optical density plot (calibration curve), of *E. coli*, *P. aeruginosa*, and *S. aureus* in the presence of parabens after incubation at 37 °C in a Multitron II mixing oven at 200 rpm. Each point: mean ± SEM, n = 3. Note that some error bars are within data points.

Figure 4.1: ASTREE electronic tongue system. A) PC for data analysis and instrument control, B) associated interface electronics unit, C) 48-position autosampler and sensor array (Alpha M.O.S, 2004).

Figure 4.2: Schematic representation of an ASTREE CHEMFET sensor (Adapted from Alpha M.O.S, 2004).

Figure 4.3: An example of the raw data produced by the seven sensors of the ASTREE electronic tongue during taste analysis of a liquid sample.

Figure 4.4: Steps used during the ASTREE “start-up” procedure. Adapted from ASTREE user manual (Alpha M.O.S, 2004).

Figure 4.5: PCA map of an ASTREE electronic tongue sensor diagnostic illustrating the discrimination between HCl (purple), NaCl (green), MSG (orange).
Figure 4.6: An ASTREE electronic tongue analysis sequence used during quantification analysis. A - active sample, W - cleaning sample

Figure 4.7: ASTREE electronic tongues analysis sequences S1 & S2 used during taste masking analysis. P - placebo sample, A - active sample, W - cleaning sample

Figure 4.8: A bar graph taste print for sensor DA created during ASTREE electronic tongue taste masking analysis with all eight replicates present per sample

Figure 4.9: A bar graph taste print for sensor DA created during ASTREE electronic tongue taste masking analysis with the first three replicates omitted per sample

Figure 4.10: A PCA map of the ASTREE electronic tongue discrimination of prednisolone standards analysed with one cleaning dip after every sample measurement. Key: P00-water (blue), P01-0.01 mg/mL Pd (red), P02-0.02 mg/mL Pd (orange), P03-0.04 mg/mL Pd (yellow), P04-0.06 mg/mL Pd (light green), P05-0.08 mg/mL Pd (turquoise), P06-0.10 mg/mL Pd (violet), P07-0.12 mg/mL Pd (pink), P08-0.14 mg/mL Pd (peach), P09-0.16 mg/mL Pd (dark green), P10-0.18 mg/mL Pd (black), P11-0.20 mg/mL Pd (purple)

Figure 4.11: A PCA map of the ASTREE electronic tongue discrimination of prednisolone standards analysed with two cleaning dips after every sample measurement. Key: A1-water (blue), A2-0.01 mg/mL Pd (red), A3-0.02 mg/mL Pd (orange), A4-0.04 mg/mL Pd (yellow), A5-0.06 mg/mL Pd (light green), A6-0.08 mg/mL Pd (turquoise), A7-0.10 mg/mL Pd (violet), A8-0.12 mg/mL Pd (pink), A9-0.14 mg/mL Pd (peach), A10-0.16 mg/mL Pd (dark green), A11-0.18 mg/mL Pd (black), A12-0.20 mg/mL Pd (purple)

Figure 4.12: PLS of prednisolone standards analysed by the ASTREE electronic tongue with one cleaning dip (left) and two cleaning dips (right) after every sample measurement. Key: 0.01 mg/mL Pd (red), 0.02 mg/mL Pd (orange), 0.04 mg/mL Pd (yellow), 0.06 mg/mL Pd (light green), 0.08 mg/mL Pd (turquoise), 0.10 mg/mL Pd (violet), 0.12 mg/mL Pd (pink), 0.14 mg/mL Pd (peach), 0.16 mg/mL Pd (dark green), 0.18 mg/mL Pd (black), 0.20 mg/mL Pd (purple)

Figure 4.13: A PCA map of the ASTREE electronic tongue discrimination of hydrocortisone standards analysed with two cleaning dips after every sample measurement. Key: A1-water (blue), A2-0.01 mg/mL HC (red), A3-0.02 mg/mL HC (orange), A4-0.04 mg/mL HC (yellow), A5-0.06 mg/mL HC (light green), A6-0.08
mg/mL HC (turquoise), A7-0.10 mg/mL HC (violet), A8-0.12 mg/mL HC (pink), A9-0.14 mg/mL HC (peach), A10-0.16 mg/mL HC (dark green), A11-0.18 mg/mL HC (black), A12-0.20 mg/mL HC (purple).

Figure 4.14: PLS of hydrocortisone standards analysed by the ASTREE electronic tongue with two cleaning dips after every sample measurement. Key: A2-0.01 mg/mL HC (red), A3-0.02 mg/mL HC (orange), A4-0.04 mg/mL HC (yellow), A5-0.06 mg/mL HC (light green), A6-0.08 mg/mL HC (turquoise), A7-0.10 mg/mL HC (violet), A8-0.12 mg/mL HC (pink), A9-0.14 mg/mL HC (peach), A10-0.16 mg/mL HC (dark green), A11-0.18 mg/mL HC (black), A12-0.20 mg/mL HC (purple).

Figure 4.15: A PCA map of the ASTREE electronic tongue discrimination of quinine standards analysed with two cleaning dips after every sample measurement. Key: Q1-water (blue), Q2-0.01 mg/mL Qu (red), Q3-0.02 mg/mL Qu (orange), Q4-0.04 mg/mL Qu (yellow), Q5-0.08 mg/mL Qu (light green), Q6-0.10 mg/mL Qu (turquoise), Q7-0.12 mg/mL Qu (violet), Q8-0.14 mg/mL Qu (pink), Q9-0.16 mg/mL Qu (peach).

Figure 4.16: PLS of quinine standards analysed by the ASTREE electronic tongue with two cleaning dips after every sample measurement. Key: Q1-0.01 mg/mL Qu (red), Q2-0.02 mg/mL Qu (orange), Q3-0.04 mg/mL Qu (yellow), Q4-0.08 mg/mL Qu (light green), Q5-0.10 mg/mL Qu (turquoise), Q6-0.12 mg/mL Qu (violet), Q7-0.14 mg/mL Qu (pink), Q8-0.16 mg/mL Qu (peach).

Figure 4.17: Sinusoidal raw data response of the ASTREE electronic tongue sensors when analyzing prednisolone standards.

Figure 4.18: Proposed application of the ASTREE electronic tongue and sensory panel in formulation development (Lorenz et al., 2009).

Figure 5.1: An example of taste station used during the adult taste panel study. Key: A) desk, B) stool, C) taste assessment score sheet, D) spittle bowl, E) distilled water, F) tissues, G) divisional screen.

Figure 5.2: Initial bitterness intensity 100 mm visual analogue scale used during the adult taste panel study (not to scale).

Figure 5.3: PCA map of the hydrocortisone bitterness intensity scores from the 16 taste assessors who scored the all standards in the correct order. Taste assessors selected for omission were vol 1, vol 6, vol 7 and vol 15.
Figure 5.4: Mean bitterness taste intensities (+/- SEM) of hydrocortisone and quinine standards assessed by the selected 12 adult taste assessors. Each point: mean ± SEM, n = 3. Note that some error bars are within data points.

Figure 5.5: Mean bitterness intensity (+/- SEM) of 17 hydrocortisone formulations assessed by a selected adult human taste panel. Each point: mean ± SEM, n = 3. Note that some error bars are within data points. * Significant difference between F1 & F2, # significant difference between F3 & F4, ** significant difference between F5 & F6, ## significant difference between F7 & F8, ### significant difference between F9 & F10, #### significant difference between F14 & F15, **** significant difference between F16 & F17. P = 0.05.

Figure 5.6: Mean bitterness intensity (+/- SEM) of 17 quinine formulations assessed by a selected adult taste panel. Each point: mean ± SEM, n ≥ 3. Note that some error bars are within data points. * Significant difference between FI & F2, # significant difference between F3 & F4, ** significant difference between F5 & F6, ## significant difference between F7 & F8, ### significant difference between F9 & F10, #### significant difference between F14 & F15, **** significant difference between F16 & F17. P = 0.05.

Figure 5.7: An illustration of the proposed hypothesis of cyclodextrin taste masking.

Figure 6.1: An example of the analysis sequence used during the ASTREE electronic tongue analysis of preselected quinine formulations. Key: W – Water/cleaning, P – Placebo, F – Formulation (Active).

Figure 6.2: A PCA map of the ASTREE electronic tongue discrimination of hydrocortisone formulations (F1-F17), and their respective placebo formulations (P3-P17), including reference formulations 12 (R1) and 15 (R2). Key: See Table 6.1.

Figure 6.3: Pn-An PCA Distances (+/- 73 (95 % CI)), of selected hydrocortisone formulations analysed by ASTREE electronic tongue.

Figure 6.4: A0-An PCA Distances (+/- 73 (95 % CI)), of selected hydrocortisone formulations analysed by ASTREE electronic tongue. * Significant difference between F3 & F4, # significant difference between F7 & F8, ** significant difference between
F11 & F12 and F11 & F13, # # significant difference between F12 & F13, ***
significant difference between F14 & F15. $p = 0.05$......................................................203

**Figure 6.5:** A PCA map of the ASTREE electronic tongue discrimination of quinine formulations (F1-F17) and their respective placebo formulations (P3-P17), including reference formulations 7 (R1) and 2 (R2). Key: See Table 6.2.................................205

**Figure 6.6:** Pn-An PCA Distances (+/- 73 (95 % CI)), of selected quinine formulations analysed by ASTREE electronic tongue. * Significant difference between F3 & F4, #
significant difference between F7 & F8, ** significant difference between F9 & F10, ##
significant difference between F14 & F15, *** significant difference between F16 &
F17. $p = 0.05$............................................................................................................................206

**Figure 6.7:** A0-An PCA Distances (+/- 73 (95 % CI)), of selected quinine formulations analysed by ASTREE electronic tongue. * Significant difference between F3 & F4, #
significant difference between F5 & F6, ** significant difference between F7 & F8, ##
significant difference between F11 & F13 and F12 & F13, *** significant difference
between F14 & F15, ### significant difference F16 & F17. $p = 0.05$............................208

**Figure 6.8:** Pn-An and A0-An PCA Distances (+/- 73 (95 % CI)) from the ASTREE electronic tongue and corrected mean bitterness intensity (90 % CI) from the human adult taste panel from assessment of selected hydrocortisone formulations (F3 - F17). Key: See Table 6.1. $p = 0.05$.................................................................210

**Figure 6.9:** Linear Pn-An and A0-An PCA Distances (+/- 73 (95 % CI)), from the ASTREE electronic tongue and corrected mean bitterness scores (+/- 90 % CI), from the human adult taste panel of the assessment of selected hydrocortisone formulations (A-2 mg/mL, B-0.2 mg/mL).................................................................211

**Figure 6.10:** Pn-An and A0-An PCA Distances (+/- 73 (95 % CI)) from the ASTREE electronic tongue and corrected mean bitterness intensity (90 % CI) from the human adult taste panel from assessment of selected quinine formulations (F3 - F17). Key: See Table 6.2. $p = 0.05$.................................................................212

**Figure 6.11:** Linear Pn-An PCA Distances (+/- 73 (95 % CI)), A0-An PCA Distances
(+/- 73 (95 % CI)), and corrected mean bitterness scores (+/- 90 % CI), with grouping
and ranking of selected quinine formulations (A-1.6 mg/mL, B-0.16 mg/mL), analysed
by the ASTREE electronic tongue and selected adult taste panel.........................213
## List of Tables

**Table 1.1:** Age categorisation of paediatric patients (adapted from EMEA, 2006).....28

**Table 1.2:** Excipients and their potential side-effects/adverse reactions in paediatric patients (adapted from Paware and Kumar, 2002; Breitkreutz and Boos, 2007)...........30

**Table 1.3:** Characteristics of frequently used corticosteroids in paediatric patients (Martindale, 2004)....................................................................................................................34

**Table 1.4:** Oral corticosteroids formulations administered to paediatric patients in the UK (BNF-C, 2008)...................................................................................................................36

**Table 1.5:** Flavouring agents and sweeteners listed in commercially available paediatric formulations in the United States of America (Strickely et al., 2008)......................44

**Table 1.6:** Physicochemical characteristics of natural cyclodextrins and two derivatives (Loftsson and Brewster, 1996; Connors, 1997; Brewster and Loftsson, 2007)............53

**Table 1.7:** Pharmaceutical products that contain cyclodextrins (Loftsson et al., 2004; Davis and Brewster, 2004)........................................................................................................68

**Table 2.1:** HPLC parameters used during optimisation of the HPLC method.............84

**Table 2.2:** Constituents of various buffer solutions and simulated media in vivo used during phase solubility studies of hydrocortisone.........................................................85

**Table 2.3:** Inclusion complex data of prednisolone and hydrocortisone with various cyclodextrins at room temperature (23 ± 1 °C).................................................................88

**Table 2.4:** Phase solubility method validation: degradation via heating and drug loss via filtering.................................................................................................................................89

**Table 2.5:** Retention times obtained during the phase solubility HPLC optimisation...90

**Table 2.6:** Inclusion complex data of prednisolone and various cyclodextrins in aqueous parabens solution at room temperature (23 ± 1 °C).................................92

**Table 2.7:** Inclusion complex data of hydrocortisone and various cyclodextrins in aqueous parabens solution at room temperature (23 ± 1 °C).................................94

**Table 2.8:** Inclusion complex data of hydrocortisone with HP-β-CD and Me-β-CD in various aqueous media at room temperature (23 ± 1 °C).................................96
Table 2.9: Inclusion complex data for quinine with HP-β-CD and Me-β-CD in water at room temperature (23 ± 1°C)........................................................................................................................................99

Table 2.10: 'H NMR spectroscopy samples used during the 'H NMR spectroscopy study..................................................................................................................................................105

Table 2.11: Thermodynamic parameters for the complexation of HP-β-CD and Me-β-CD with hydrocortisone and quinine in aqueous solution at 298.2 ± 0.2 K..............107

Table 2.12: 'H Chemical shifts corresponding to HP-β-CD in the presence and absence of hydrocortisone........................................................................................................108

Table 2.13: Association constants calculated from phase solubility diagrams and ITC.........................................................................................................................................................110

Table 3.1: An example of the preservatives used in pharmaceutical formulations (adapted from Clontz, 1997 and Rabiu et al., 2004)........................................................................116

Table 3.2: Formulations prepared for the efficacy for antimicrobial preservation study..................................................................................................................................................120

Table 3.3: British Pharmacopoeia (2008), total viable microbial count requirements for oral preparations during the efficacy of antimicrobial preservation test..............122

Table 3.4: Test formulations used in the turbidity study.................................................123

Table 3.5: Inclusion complex data of hydrocortisone and HP-β-CD in aqueous parabens solution at room temperature (23 ± 1° C).................................................................................125

Table 3.6: Antimicrobial preservation effectiveness of hydrocortisone formulations containing HP-β-CD and various amounts of parabens.......................................................127

Table 3.7: Optical density measurements of E. coli, P. aeruginosa, and S. aureus within the study formulations........................................................................................................130

Table 3.8: Free parabens concentrations of formulations 1-12 as determined from the calibration curves (Figure 3.5), of E. coli, P. aeruginosa, and S. aureus...............131

Table 3.9: Free parabens concentration in various HP-β-CD formulations containing 2 mg/mL hydrocortisone as determined by phase solubility and turbidity studies.......133
Table 4.1: An example of part of an ASTREE electronic tongue data library with eight replicates (sample measurements) for samples A0 and A1, per sensors (ZZ, AB, BA, BB, CA, DA, JE)....................................................................................................................144

Table 4.2: Sample concentrations used during preliminary taste masking analysis....150

Table 4.3: Molar ratio of CD used during the preliminary taste masking analysis.....151

Table 4.4: A0-An PCA Distances calculated during ASTREE electronic tongue analysis of prednisolone standards....................................................................................................................153

Table 4.5: A0-An PCA Distances calculated during ASTREE electronic tongue analysis of hydrocortisone standards....................................................................................................................157

Table 4.6: A0-An PCA Distances calculated during ASTREE electronic tongue analysis of quinine standards....................................................................................................................160

Table 4.7: Selected taste masking concentrations determined by Pn-An PCA Distances and A0-An PCA Distances from preliminary taste masking analysis of 0.20 mg/mL hydrocortisone (and 2.0 mg/mL hydrocortisone for the cyclodextrins)..................163

Table 4.8: Selected taste masking concentrations determined by P0-A0 PCA Distances and A0-An PCA Distances from preliminary taste masking analysis of 0.16 mg/mL quinine (and 1.6 mg/mL quinine for the cyclodextrins)..........................................................163

Table 4.9: Possible reasons for the differing preliminary taste masking results........164

Table 5.1: Hydrocortisone formulations assessed during the adult taste panel study..179

Table 5.2: Quinine formulations assessed during the adult taste panel study.........180

Table 6.1: Preselected hydrocortisone formulations analysed during ASTREE electronic tongue analysis.................................................................................................................................196

Table 6.2: Preselected quinine formulations analysed during ASTREE electronic tongue analysis.................................................................................................................................196
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>Ångstrom</td>
</tr>
<tr>
<td>A</td>
<td>Active formulation</td>
</tr>
<tr>
<td>A. niger</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADI</td>
<td>Acceptable Daily Intake</td>
</tr>
<tr>
<td>Ag/AgCl</td>
<td>Silver/Silver Chloride</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ASTREE</td>
<td>ASTREE electronic tongue</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BNF-C</td>
<td>British National Formulary for Children</td>
</tr>
<tr>
<td>C1</td>
<td>First principle component in PCA analysis</td>
</tr>
<tr>
<td>C2</td>
<td>Second principle component in PCA analysis</td>
</tr>
<tr>
<td>C. albicans</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>Calcium Chloride Dihydrate</td>
</tr>
<tr>
<td>CD(s)</td>
<td>Cyclodextrin(s)</td>
</tr>
<tr>
<td>CE</td>
<td>Complexation Efficiency</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CGTase</td>
<td>Cyclodextrin Glucosyl Transferase</td>
</tr>
<tr>
<td>CHEMFET</td>
<td>Chemically Modified Field-Effect-Transistor</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlated Spectroscopy</td>
</tr>
<tr>
<td>(D)</td>
<td>Drug</td>
</tr>
<tr>
<td>D₀</td>
<td>Intrinsic Solubility</td>
</tr>
<tr>
<td>D₂O</td>
<td>Deuterium Oxide</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DCD</td>
<td>Drug and Cyclodextrin Inclusion Complex</td>
</tr>
<tr>
<td>DI</td>
<td>Discrimination Index</td>
</tr>
<tr>
<td>DS</td>
<td>Degree of Substitution</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EC</td>
<td>European Community</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
</tr>
<tr>
<td>EMEA</td>
<td>European Medicines Agency</td>
</tr>
</tbody>
</table>
EU - European Union
FDA - Food and Drug Administration
GI - Gastrointestinal
HC - Hydrocortisone
HCl - Hydrogen Chloride
HP - Hewlett & Packard
HP-β-CD - Hydroxypropyl-β-Cyclodextrin
HPLC - High Performance Liquid Chromatography
HPMC - Hydroxy Propyl Methyl Cellulose
IP3R3 - Inositol Phosphate type III Receptor
IP3 - Inositol trisphosphate
ITC - Isothermal Titration Calorimetry
JP - Japanese Pharmacopeia
K - Kelvin
K_{1:1} - Stability Constant for a 1:1 (drug:cyclodextrin) inclusion complex
K_{C} - Stability Constant
K_{2}HPO_{4} - Dipotassium Hydrogen Phosphate
KCl - Potassium Chloride
KH_{2}PO_{4} - Potassium Dihydrogen Phosphate
KSCN - Potassium Thiocyanate
LR-CDs - Large Ring Cyclodextrins
M - Molar
Me-β-CD - Methyl-β-Cyclodextrin
MES - 2-(n-Morpholino) Ethanesulfonic Acid
MgSO_{4}.7H_{2}O - Magnesium Sulphate Septahydrate
MHRA - Medicines and Healthcare Products Regulatory Agency
MS - Molar Degree of Substitution
MSG - Monosodium Glutamate
MW - Molecular Weight
NaCl - Sodium Chloride
NaHCO_{3} - Sodium Bicarbonate
Na_{2}HPO_{4}.2H_{2}O - Disodium Hydrogen Phosphate Dihydrate
Na_{2}SO_{4}.10H_{2}O - Sodium Sulphate Decahydrate
NHS - National Health Service
NH_{4}Cl - Ammonium Chloride
(NH₂)₂CO - Urea
NMR - Nuclear Magnetic Resonance
NOE - Nuclear Overhauser Effects
P - Placebo formulation

*P. aeruginosa* - *Pseudomonas aeruginosa*

Parabens - *p*-hydroxybenzoic Acid Esters
PC - Personal Computer
PCA - Principle Component Analysis
Pd - Prednisolone

PIP - Paediatric Investigation Plan
PIP2 - Phosphatidylinositol bisphosphate
Ph.Eur - European Pharmacopeia
PLCβ2 - Phospholipase C–β2

PLS - Partial Least Squares

PUMA - Paediatric Use Marketing Authorisation
Qu - Quinine

R - Gas Constant (8.314 J K⁻¹ mol⁻¹)
R1 - ASTREE Reference Formulation 1
R2 - ASTREE Reference Formulation 2

RPM - Revolutions Per Minute
ROE - Nuclear Overhauser Effects in the Rotating Frame
RSD - Relative Standard Deviation

T - Absolute Temperature
TAM - Thermal Activity Monitor
THF - Tetrahyrdofuran

TRPM5 - Non-selective cation channel transient receptor potential melastatin 5

TSA - Tryptone Soya Agar
TSB - Tryptone Soya Broth

S1 - ASTREE analysis Sequence 1 (P0, P1...Pn, A0, A1...An)
S2 - ASTREE analysis Sequence 2 (P0, A0, P1, A1... Pn, An)

*S. aureus* - *Staphylococcus aureus*

SD - Standard Deviation
SDA - Sabouraud Dextrose Agar

SEM - Standard Error of the Mean

TSD - Total Degree of Substitution
USP/NF - USA Pharmacopeia
UV - Ultraviolet
VAS - Visual Analogue Scale
Vis - Visible
W - Water/cleaning sample
w/v - Weight/Volume
Z. Rouxii - Zygosaccharomyces Rouxii
2D ROESY - 2 Dimensional Rotating Frame Overhause Effect
5-HT - Serotonin
° C - Degrees Celsius
ΔH - Standard Enthalpy Change
ΔS - Standard Entropy Change
ΔG - Gibbs Free Energy
Δδ - Induced Chemical Shift
Chapter 1 - General Introduction
Chapter 1 - General Introduction

1.1 Paediatric medicine

1.1.1 Introduction

Numerous oral medicines prescribed to paediatric patients are unlicensed or prescribed “off label” (Pandolfini and Bonati, 2005). This has given rise to potentially poor quality formulations prescribed to paediatric patients with no evidence in areas such as safety, efficacy or bioavailability, as well as formulations with inadequate dose uniformity and stability, and intolerable palatability (Breitkreutz and Boos, 2007; Standing and Tuleu, 2005). Recent European Union (EU) legislation, however, enforcing paediatric clinical trials and drug development (Regulation [EC] No. 1901/2006), preceded by a European Medicines Agency (EMEA) reflection paper on the formulations of choice for the paediatric population (EMEA, 2006), were implemented so new paediatric medicines developed by pharmaceutical companies within the EU have appropriate quality, safety, and efficacy, whilst being adhered to by paediatric patients.

1.1.2 Unlicensed and “off-label” drug use

In the UK, before a medicinal product can be used in the treatment of specific medical conditions it is scrutinised by the Medicines and Healthcare Products Regulatory Agency (MHRA). Once various criteria regarding safety, efficacy and quality are ascertained successfully, a marketing authorisation (formally product license), is granted to the pharmaceutical company enabling licensed use of the medicine. The marketing authorisation includes the indication, dose, route of administration, and age group of patient for which the drug may be used. This licensing process was introduced in response to drug-related tragedies in paediatric patients, such as chloramphenicol-induced grey baby syndrome (Mulhall et al., 1983), thalidomide-induced phocomelia (Newman, 1986), and deaths following diethylene glycol poisoning (MacDonald et al., 1987). The process is, therefore, in place to protect patients and maintain confidence in medicinal products. Nevertheless, pharmaceutical companies typically applied to the MHRA for marketing authorisation for use of medicines in adults and did not focus on applying for the use of the medicines in children, frequently due to cost and resources. Consequently paediatric patients are commonly exposed to medicines that are not specifically licensed for use in children (unlicensed) or are used in a different manner to that recommended in the license (“off-label”). This is often the case when there is no suitable alternative or the unlicensed/”off-label” drug is the preferred method of treatment (Pandolfini and Bonati, 2005).
Chapter 1 - General Introduction

The use of unlicensed or “off-label” drugs by paediatric patients has been well documented by various researchers worldwide (Pandolfini and Bonati, 2005). From this body of research approximately 6 - 62 %, 11 - 37 %, and 55 - 80 % of medicines used in general paediatric wards, community setting, and neonatal intensive care units, respectively, were found to be used unlicensed or “off-label”, whilst approximately 11 % of medicines in UK community practice were found to be unlicensed or “off-label”. These figures are alarming since evidence indicates adverse drug reactions (Neubert et al., 2004; Turner et al., 1999; Choonara and Conroy, 2002; and Horen et al., 2002), dosing errors (Wong et al., 2004), and non-compliance (Ramgoolam and Steele, 2002), were linked to unlicensed or “off-label” drug use of adult licensed medications in paediatric patients.

1.1.3 Current regulations in the European Union

In the past, pharmaceutical companies carried out very little clinical trials in paediatric populations and commonly developed formulations without paediatric patients in mind. This was typically because of time constraints during development and little or no incentives to develop paediatric medicines. Nevertheless, the pharmaceutical industry is now obliged to develop paediatric medicines because newly approved legislation within the EU governing the development and authorisation of medicines for paediatric use came into effect January 2007 (Regulation [EC] No. 1901/2006). The legislation encompasses the following areas: to increase development of paediatric medicines, to avoid subjecting children to unnecessary clinical investigations, to ensure and encourage the research into paediatric medicines, to ensure and encourage the development of paediatric dosage forms, and to improve labeling of paediatric medicines (Ernest et al., 2007). The legislation sets out requirements, incentives, and rewards so pharmaceutical companies produce high quality research leading to the development of medicines that are safe and effective in children and are specifically authorised for such use. Incentives include a six-month extension to the supplementary protection certificate for developing formulations for children and eight plus two years of data exclusivity on paediatric use of an off-patent drug via a Paediatric Use Marketing Authorisation (PUMA). Assistance will also be provided so that obtaining a PUMA does not delay the licensing of the medicine in adults. Requirements companies have to meet include: compulsory submission of existing drug studies in children, marketing of the paediatric medicine within 12 months of being granted a PUMA, and...
Chapter 1 - General Introduction

submission of a paediatric investigation plan (PIP) detailing planned paediatric clinical studies that are in agreement with the EMEA’s Paediatric Committee.

1.1.4 Challenges and requirements for oral paediatric drug delivery

1.1.4.1 Age, abilities and dosage form

One of the first aspects of paediatric drug formulation highlighted in the formulations of choice for the paediatric population reflection paper (EMEA, 2006), was the age range of paediatric patients that medicines have to treat. In the past, too much bearing was held on the belief that if a medicine were safe and effective in adults, then it would be safe and effective in paediatric patients. Moreover, a large emphasis was held on the belief that a drug response from a child would be the same as the response of an adult. Children are not, however, “small adults”, having differing drug absorption, distribution, metabolism, and elimination to adults (Kearns et al., 2003; de Zwart et al., 2004; Stephenson, 2005). Paediatric patients encompass a wide age range categorised into subpopulations based on their physiological and pharmacokinetic differences and should not be classed as one group with one group of needs (Table 1.1). The formulations used during medical treatment should, therefore, relate to the age of the patient and not stem from the adult-based formulation. Ideally, drugs should be available in a range of dosage forms that are acceptable at different ages and abilities, and a range of strengths or concentrations allowing administration of the correct age-related dose (Nunn and Williams, 2005).

Table 1.1: Age categorisation of paediatric patients (adapted from EMEA, 2006)

<table>
<thead>
<tr>
<th>Category</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-term newborn infants (&quot;premature&quot;)</td>
<td>&lt; 37 - weeks gestation</td>
</tr>
<tr>
<td>Full-term newborn infants (&quot;neonates&quot;)</td>
<td>0 - 27 days</td>
</tr>
<tr>
<td>Infants and toddlers</td>
<td>28 days - 23 months</td>
</tr>
<tr>
<td>Pre-school children</td>
<td>2 - 5 years</td>
</tr>
<tr>
<td>School children</td>
<td>6 - 11 years</td>
</tr>
<tr>
<td>Adolescents</td>
<td>12 - 17 years</td>
</tr>
</tbody>
</table>

The oral route of drug administration is the preferred route for paediatric patients. Children over six years of age can usually swallow a tablet/capsule and younger children can potentially be taught how to swallow solid dosage forms, especially when the medication regime is long term (Ernest et al., 2007). Nonetheless, the EMEA suggests liquid formulations are typically the dosage form of choice for children under eight years of age and should, therefore, be considered the favoured dosage form.
administered (EMEA, 2006). Because, however, drugs were previously formulated without considering the age and abilities of paediatric patients, the majority of oral formulations are usually only commercially available as solid dosage forms (Schirm et al., 2003), and manipulation of the formulation are required often, so patients receive the right type of medication that they are capable of taking (Standing and Tuleu, 2005).

Manipulation of dosage forms is also commonplace because the dose of the formulation is not appropriate for the paediatric patient. Adult formulations contain concentrations/quantities of drug, which are only appropriate for administration in adult patients and, therefore, need to be manipulated so the correct dose can be administered to the paediatric patient. This might include splitting tablets, opening of capsules, or diluting liquids to obtain a concentration of drug suitable for the patient. Splitting of tablets, however, was associated with dose inaccuracy (van der Steen et al., 2002; Teng et al; 2002), commercial tablet splitters do not always improve the tablet segment uniformity (Breitkreutz et al., 1999), and diluting oral liquids was shown to be inaccurate (Berman et al., 1978). In addition, extemporaneous formulations were associated with production errors, have no compatibility study back up, have little evidence to demonstrate they have adequate chemical, physical, and microbial stability, and may not be bioequivalent to the original preparation (Woods, 1997; Standing and Tuleu, 2005). Thus, to meet the demands of paediatric patients, new oral paediatric formulations are required to be developed at age appropriate concentrations, where the dose of the formulation can be administered easily, accurately and safely.

Whilst liquids are the preferred oral dosage form for children under eight years of age (EMEA, 2006), other types of oral dosage form other than conventional tablets and capsules can be developed to meet the demand of paediatric patients. These include oral effervescent dosage forms, oral powders and multiparticulate systems, orodispersible dosage forms, chewable tablets, chewing gum, pellets, melting tablets/films/wafers and mini tablets (EMEA, 2006; Krause and Breitkreutz, 2008). These innovative dosage forms are favoured in a wider age range than just conventional tablets and capsules (Krause and Breitkreutz, 2008), and illustrate that pharmaceutical companies are acting on the demands of paediatric patients.

### 1.1.4.2 Excipients

When formulating oral medicines for the paediatric population, especially liquid formulations, excipients are added to enhance drug solubility, maintain chemical,
physical, and microbiological stability, enhance bulk and drug dispersion, and improve palatability of the formulation, amongst other reasons (EMEA, 2006). Nevertheless, various excipients added to formulations were shown to be unsafe in certain groups of paediatric patients (Pawar and Kumar, 2002) (Table 1.2), and formulators are required consequently to be cautious of the excipients they use within a formulation, especially the excipients highlighted bold in Table 1.2, most of which are used frequently in liquid formulations and are used commonly in formulations to treat chronic illness.

Table 1.2: Excipients and their potential side-effects/adverse reactions in paediatric patients (adapted from Paware and Kumar, 2002; Breitkreutz and Boos, 2007)

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Side-effect/adverse reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>Good medium for potential microorganism growth; Gluten-induced celiac disease</td>
</tr>
<tr>
<td>Lactose</td>
<td>Lactose intolerance - diarrhoea, abdominal pain, flatulence.</td>
</tr>
<tr>
<td>Calcium salts</td>
<td>Potential influence of bioavailability of active ingredient</td>
</tr>
<tr>
<td>Citric acid</td>
<td>Hypercalcemia and hypercalciuria for patients with certain metabolic conditions and nephrocalcinosis</td>
</tr>
<tr>
<td>Dextrose</td>
<td>Tooth erosion; Copper deficiency in patients with renal tubular acidosis</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Neurotoxicity</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Hereditary fructose intolerance - hypoglycemia; Dental caries; Hyperglycemia</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Mucositis of the stomach; Diarrhoea, electrolyte disturbance</td>
</tr>
<tr>
<td>Flavouring agents</td>
<td>Allergic &amp; pseudoallergic reactions; Hypersensitivity reactions</td>
</tr>
<tr>
<td>Polyols (Mannitol)</td>
<td>Hereditary fructose intolerance; Osmotic diarrhoea</td>
</tr>
<tr>
<td>Saccharin</td>
<td>Irritability, hypertonia, insomnia, opisthotonus, strabismus</td>
</tr>
<tr>
<td>Aspartame</td>
<td>Phenylketonuria, headaches, panic attacks; Granulomatous panniculitis</td>
</tr>
<tr>
<td>Dyes</td>
<td>Anaphylactoid reactions, angioedema, asthma, urticaria, hyperkinesis</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>Bronchoconstriction, erythema multiforme-like skin rash</td>
</tr>
<tr>
<td>Benzalkonium chloride</td>
<td>Bronchoconstriction</td>
</tr>
<tr>
<td>Sulfites</td>
<td>Asthma attacks, rashes, abdominal upset, dyspnea</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>Cardiac arrhythmias, seizures, respiratory depression, severe hyperosmolarity, lactic acidosis</td>
</tr>
<tr>
<td>Aluminium salts</td>
<td>Encephalopathy, microcytic anaemia, osteodystrophy</td>
</tr>
<tr>
<td>Parabens</td>
<td>Allergies, contact dermatitis</td>
</tr>
<tr>
<td>Fructose</td>
<td>Hereditary fructose intolerance</td>
</tr>
</tbody>
</table>

1.1.4.3 Taste

Drugs are typically bitter tasting molecules and consequently make oral administration of medicine to children very difficult, especially oral liquid formulations (Sohi et al., 2004). Regime adherence and successful therapeutic outcomes through compliance of a palatable tasting formulation is an important feature of paediatric pharmacy (Matsui, 2007). Excipients such as sugars, sweeteners and flavouring agents, are added frequently to formulations to enhance palatability and to achieve good compliance. Whereas in the past the taste of a formulation was commonly an afterthought of dosage form development, taste masking of bitter/unwanted tastes is now fast becoming a major part of formulation development and plays a significant role in how a medicine is administered (Cram et al., 2009). Taste masking of bitter drugs and taste assessment of formulations is discussed in further detail in Section 1.3 and Chapters 4, 5 and 6.
Chapter 1 - General Introduction

1.1.4.4 Clinical Trials

Ethical dilemmas inevitably exist when the topic of paediatric clinical trials is brought to light during the development of paediatric medicines. Paediatric patients have the right to well researched and regulated drugs (Davies, 2004), yet experimentation in children is still believed to be unethical by some campaigners. Pharmaceutical companies previously shunned clinical trials in children, principally because they were fraught with technical, practical and ethical problems (Sutcliffe, 2003). Moreover, pediatric drug trials often provided insufficient information on the formulation and method of administration, thus impairing their validity and reliability (Standing et al., 2005). With the new paediatric regulation in place since January 2007 (Regulation [EC] No. 1901/2006), pharmaceutical companies are now obliged to produce paediatric clinical data in each application for a marketing authorisation of a new/not licensed drug. These clinical trials have to be outlined in the PIP, submitted no later than the date of completion of the relevant adult pharmacokinetic studies, and accepted by the EMEA’s Paediatric Committee. In addition, there are paediatric networks aimed at facilitating clinical trials in children - the UK Medicines for Children Research Network (part of the European network), was set up to monitor the running of clinical trials in children, further adding to the infrastructure in place to ensure new paediatric dosage forms are evaluated in paediatric populations in a suitable, safe and efficient manner.

1.2 Corticosteroids

1.2.1 Introduction

The term “corticosteroid” is the collective name for a class of steroid hormones produced endogenously by the adrenal cortex, or are synthetically produced compounds with corticosteroid-like effects. The endogenously produced corticosteroids are categorised into two sub-groups known as glucocorticoids and mineralocorticoids.

Glucocorticoids, such as cortisol (Figure 1.1), bind to cytosolic glucocorticoid receptors expressed in almost every cell in the body. Receptor activation by glucocorticoids evokes a wide range of effects, namely glucose metabolism, anti-inflammation and immunosupression. Because glucocorticoids have a potent anti-inflammatory effect and depress the immune response of the body, the synthetic versions are widely used as medicinal products.
Chapter 1 - General Introduction

Mineralocorticoids, such as aldosterone (Figure 1.2), bind to mineralocorticoid receptors located on numerous tissues throughout the body, e.g. the kidney, colon, heart, central nervous system (hippocampus), brown adipose tissue and sweat glands. The most influential action of mineralocorticoids is reabsorption of sodium and subsequently passive reabsorption of water. Other effects include increasing blood pressure and blood volume via active secretion of potassium in the principal cells of the cortical collecting tubule and active secretion of protons via proton ATPases in the luminal membrane of the intercalated cells of the collecting tubule. The mineralocorticoid receptor also has affinity for some glucocorticoids and as such the enzyme 11-β hydroxysteroid dehydrogenase type II is present in mineralocorticoid target tissues to catalyse the deactivation of glucocorticoids, thus preventing overstimulation of the receptor by glucocorticoids.

In pharmacy and related fields, the term corticosteroid refers generally to the synthetically produced drugs structurally related to endogenous corticosteroids, which have affinity for the glucocorticoid and mineralocorticoid receptors. Synthetic corticosteroids are able consequently to mimic the effects of endogenous corticosteroids.
Chapter 1 - General Introduction

and are, therefore, utilised in medicine for their glucocorticoid and mineralocorticoid action. To avoid confusion, in this thesis the term corticosteroid will refer to synthetically produced drugs, unless otherwise stated.

1.2.2 Discovery, structure, and properties

The glucocorticoid cortisone was the first of the endogenous corticosteroids to be isolated during the work of Edward Kendall, Phillip Hench and Tadeus Reichstein on bovine adrenal glands in 1936 (Mason et al., 1936; Reichstein, 1936). It was during the isolation of cortisone and the discovery of its biological effects, after promising animal studies, that the therapeutic value of the steroid hormone became apparent (Mason et al., 1936; Reichstein, 1936). Research by collaborators of Kendall et al., led to the synthesis of cortisone by Lewis Sarett, an organic chemist at Merck Research Labs in the USA (Sarett, 1946). Sarett’s initial synthesis of cortisone originated with desoxycholic acid, which was extracted from ox bile and followed a complicated 36-step process, resulting in a yield of approximately 18 mg of cortisone. Following on from the initial synthesis extensive research brought about improved production and in 1948 allowed for pioneering use of the drug when an intramuscular injection of cortisone was given to a female patient with rheumatoid arthritis (Kendall, 1953). The vast improvements in the patient’s health resulted in cortisone being hailed as a “wonder drug” and led to further clinical trials, commercial availability of cortisone, and further research into corticosteroid analogues.

In 1950, the 11β-hydroxy analogue of cortisone (hydrocortisone) was synthesised by Wendler et al., and this was followed by the synthesis of prednisone and prednisolone in 1955 by scientists at the Schering Corporation. During this period, the mineralocorticoid aldosterone was also discovered (Simpson et al., 1953). The increased medical use of corticosteroids in the 1950s led to increased awareness of their adverse effects, such as excessive salt and water retention, increased gastric acidity and psychosis and consequently other corticosteroids were developed by various drugs companies to create “side-effect free” alternatives (Feiser and Feiser, 1959). Corticosteroids remain frontline drugs for numerous conditions such as asthma, and continue to be developed. For instance, a new corticosteroid called ciclesonide was recently developed for the treatment of asthma and was reported to be a good alternative to other corticosteroids, although further study is required, especially in paediatric patients (Manning et al., 2008).
Chapter 1 - General Introduction

Corticosteroids are generally white to practically white, crystalline powders and are notorious for having very poor aqueous solubility. They are typically hydrophobic molecules with a partition coefficient (log P (octanol/water)) of approximately 1.5 (Table 1.3). Like other steroid hormones, corticosteroids have a carbon skeleton with four fused rings, which acts as a structural backbone (also known as steroid backbone), and are direct synthetic congeners or derivatives of the endogenous corticosteroids. Variation in the properties of corticosteroids, e.g. affinity for the glucocorticoid receptor, is a result of the various functional groups that are attached at various points on the steroid backbone (Figure 1.3).

Table 1.3: Characteristics of frequently used corticosteroids in paediatric patients

(Martindale, 2004)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Formula</th>
<th>Mw (g/mol)</th>
<th>Solubility in water (mg/L)</th>
<th>Melting Point (°C)</th>
<th>logP (octanol/water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betamethasone</td>
<td>C22H29F05</td>
<td>392.45</td>
<td>60</td>
<td>231-234</td>
<td>1.9</td>
</tr>
<tr>
<td>Deflazacort</td>
<td>C25H31N06</td>
<td>441.50</td>
<td>*</td>
<td>255-257</td>
<td>1.3</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>C22H29F05</td>
<td>392.45</td>
<td>90</td>
<td>268-271</td>
<td>1.8</td>
</tr>
<tr>
<td>Fludrocortisone</td>
<td>C21H29F05</td>
<td>380.45</td>
<td>140</td>
<td>233-234</td>
<td>1.5</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>C21H30O5</td>
<td>362.50</td>
<td>280</td>
<td>214</td>
<td>1.6</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>C22H30O5</td>
<td>374.47</td>
<td>90</td>
<td>228-237</td>
<td>1.8</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>C21H28O5</td>
<td>360.44</td>
<td>220</td>
<td>240-241</td>
<td>1.6</td>
</tr>
<tr>
<td>Prednisone</td>
<td>C21H26O5</td>
<td>358.43</td>
<td>310</td>
<td>226-232</td>
<td>1.5</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>C21H27F06</td>
<td>394.43</td>
<td>80</td>
<td>269-271</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* No data available - practically insoluble in water; soluble in chloroform

Figure 1.3: The chemical structure of A) betamethasone, B) deflazacort, C) dexamethasone, D) fludrocortisone, E) hydrocortisone, F) methylprednisolone, G) prednisolone, H) prednisone, I) triamcinolone
Chapter 1 - General Introduction

1.2.3 Corticosteroids drug therapy

The use of corticosteroids in paediatric medicine is focused on two types of drug therapy, known as glucocorticoid therapy and replacement therapy. Replacement therapy refers to the deficiency of the adrenal cortex to secrete the endogenous corticosteroids resulting in a low level of natural corticosteroids in the body. This can occur following adrenalectomy, hypopituitarism, acute adrenal insufficiency, or chronic adrenal insufficiency (Addison's disease). Corticosteroid drugs are consequently administered to supplement sufficient glucocorticoid and mineralocorticoid activity. The front-line corticosteroids in adrenal replacement therapy are hydrocortisone and fludrocortisone acetate for their glucocorticoid and mineralocorticoid activity, respectively (BNF-C, 2008). Glucocorticoid therapy refers to the use of corticosteroids that have a potent glucocorticoid effect and are used typically for their anti-inflammatory and immunosuppressant effects. They are, therefore, administered to paediatric patients for a whole host of disease states and ailments such as: exfoliative dermatitis, acute leukaemia, rheumatoid arthritis, asthma, and anaphylactic shock.

Previously termed “wonder drugs”, corticosteroids are not without their adverse effects in pediatric patients. Over dosage or prolonged use can potentiate some of the normal physiological actions of corticosteroids resulting in mineralocorticoid and glucocorticoid side effects. The main mineralocorticoid side effects include hypertension, sodium and water retention, and depletion of potassium and calcium. Glucocorticoid side effects include diabetes and osteoporosis, as well as high doses being associated with avascular necrosis of the femoral head.

The differing formulations and routes of drug administration depend generally on the illness. Local treatment with creams, inhalations, and eye-drops are preferred to systemic treatment. Oral, intramuscular, intravenous, or slow infusion formulations are, however, often necessary and are commonly the only method of treatment, as well as frequently administered long term. Oral formulations make up a large portion of the corticosteroids administered to paediatric patients in the UK, providing a wide range of treatment (Table 1.4).
Chapter 1 - General Introduction

Table 1.4: Oral corticosteroids formulations administered to paediatric patients in the UK (BNF-C, 2008)

<table>
<thead>
<tr>
<th>Indication</th>
<th>Formulation</th>
<th>Excipients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Betamethasone</strong></td>
<td>Oral ulceration</td>
<td>Soluble tablets (5 ug) (as betamethasone sodium phosphate)</td>
</tr>
<tr>
<td><strong>Deflazacort</strong></td>
<td>Inflammatory and allergic disorders; Nephrotic syndrome</td>
<td>Oral tablet (6 mg) Microcrystalline cellulose, lactose, sucrose, maize starch and magnesium stearate</td>
</tr>
<tr>
<td><strong>Dexamethasone</strong></td>
<td>Inflammatory and allergic disorders; Physiological replacement; Croup</td>
<td>Oral tablet (500 mg) or oral solution (2 mg/5 mL) (as dexamethasone sodium phosphate)</td>
</tr>
<tr>
<td><strong>Fludrocortisone</strong></td>
<td>Mineralocorticoid replacement in adrenocortical insufficiency</td>
<td>Oral tablet (100 ug) (as fludrocortisone acetate) Maize starch, dibasic calcium phosphate, lactose anhydrous and monohydrate, t alc, sodium benzoate, magnesium stearate</td>
</tr>
<tr>
<td><strong>Hydrocortisone</strong></td>
<td>Congenital adrenal hyperplasia; Adrenal hypoplasia, Addison's disease, chronic maintenance or replacement therapy</td>
<td>Oral tablet (10 mg) Maize starch, lactose monohydrate, povidone K90, silica colloidal anhydrous, talc, magnesium stearate</td>
</tr>
<tr>
<td><strong>Methylprednisolone</strong></td>
<td>Inflammatory and allergic disorders</td>
<td>Oral tablet (2 mg) Lactose, rose colour, sucrose, maize starch and calcium stearate</td>
</tr>
<tr>
<td><strong>Prednisolone</strong></td>
<td>Autoimmune inflammatory disorders (including juvenile idiopathic arthritis, connective tissue disorders and systemic lupus erythematosus); Autoimmune hepatitis; Corticosteroid replacement therapy; Infantile spasms; Idiopathic thrombocytopenic purpura; Nephrotic syndrome; Asthma; Pneumocystis infections</td>
<td>Oral tablets (1 mg, 2.5 mg (E/C)) or oral soluble tablet (5 mg) (as sodium phosphate) Oral tablet - Lactose, maize starch, stearic acid, purified talc, magnesium stearate. Oral tablet (E/C) - Core: calcium carbonate, lactose, magnesium stearate, maize starch. Coating: polyvinyl alcohol, titanium dioxide, purified talc, lecithin, xanthan gum, polyvinyl acetate phthalate, polyethylene glycol, sodium hydrogen carbonate, triethyl citrate, purified stearic acid, sodium alginate, colloidal sodium dioxide, lactose, methylcellulose, sodium carboxymethyl cellulose, iron oxide, beeswax, carnauba wax, polysorbate 20 and sorbic acid. Oral soluble tablet - sodium acid citrate, sodium bicarbonate, saccharin sodium, povidone, erythrosine, sodium benzoate</td>
</tr>
<tr>
<td><strong>Triamcinolone</strong></td>
<td>Oral ulceration</td>
<td>Oral paste (0.1 %) (as acetone) Gelatin, pectin, sodium carboxymethylcellulose, polyethylene resin and liquid paraffin.</td>
</tr>
</tbody>
</table>

1.2.4 Issues with oral corticosteroid formulations for paediatric patients

Of the 12 different corticosteroid drugs listed in Table 1.4, only one is formulated as an oral liquid, whilst nine are formulated as oral tablets (six tablets, two soluble tablets and one oromucosal tablet). This illustrates oral corticosteroids are not available in age-appropriate dosage forms for paediatric patients. Although the tablets are considered for children over six years of age these particular corticosteroid formulations are not suitable for children who are incapable of swallowing tablets, do not want to swallow tablets, or patients who require a fraction of the dose of the tablet. Corticosteroid formulations are often manipulated so the patient can receive a formulation that they are
capable of taking, or receive the drug at a relevant dose. In a recent study of extemporaneous preparations in various NHS trusts throughout the UK, hydrocortisone was placed 13th and dexamethasone placed 32nd for total units of extemporaneous formulations made (Lowey and Jackson, 2008). Whilst according to the MHRA, hydrocortisone and dexamethasone were both placed in the top 20 extemporaneously prepared formulations for paediatric patients (MHRA Medicines for Children, 2006). It is clear from these findings that the needs of paediatric patients are not being met fully by the current corticosteroid formulations and more age-appropriate formulations need to be developed.

The excipients used in the oral corticosteroids listed in Table 1.4 are not all age-appropriate for paediatric patients. Some of the dosage forms contain excipients shown to cause adverse effects to some paediatric populations (Pawar and Kumar, 2002), and, depending on age and exposure, are not recommended to some age groups/patients (EMEA, 2006). For instance, the dexamethasone oral solution contains propylene glycol, liquid maltitol, liquid sorbitol and isopropanol. Although these particular excipients are present to aid the formulation in various ways, and certain excipients are less harmful than others, more tolerable excipients should be used in these corticosteroid formulations for paediatric patients.

When oral corticosteroid formulations are administered to paediatric patients they are continually associated with having poor palatability and tasting extremely bitter, often causing vomiting and nausea, and consequently poor adherence and slow resolution of the illness. A study reported 21 % of hospitalised paediatric patient receiving oral prednisolone for acute asthma vomited (Dawson et al., 1992), whilst another study reported that 23 % of children vomited when given crushed prednisolone tablets with custard or lemonade, compared to no vomiting when given a banana flavoured liquid prednisolone formulation (Lucas-Bouwman et al., 2001). In similar research, 17.7 % of children with asthma vomited when given a generic oral liquid prednisolone formulation but only 5.4 % vomited when given a new oral solution form of prednisolone sodium phosphate that was developed to address the problem of poor palatability (Kim et al., 2006). From these three studies, it can, therefore, be suggested that a fifth of children will vomit when they take a prednisolone formulation that has not had its palatability optimised.
Chapter 1 - General Introduction

1.3 Taste Masking

1.3.1 Introduction

Attenuating or eliminating unwanted tastes from oral medicines is an important part of paediatric formulation development. Active pharmaceutical ingredients, as well as some excipients, can be extremely bitter and since bitterness is an exceedingly aversive taste to children of all ages (Ramgoolam and Steele, 2002), an unpalatable formulation can result in compliance issues amongst paediatric patients (Steele et al., 2000; Kim et al., 2006). The taste of a formulation can play a significant role in whether a medication is ingested fully and ultimately leads towards successful treatment (Matsui, 2007). Taste masking via traditional or novel techniques is currently, therefore, of high interest in paediatric pharmaceutics (EMEA, 2006; Cram et al., 2009). Taste assessments in vivo are consequently very topical, especially since taste studies in children has to be considered during the development of paediatric medicine (Davies and Tuleu, 2008). In addition, the development of in vitro taste analysers is also expanding now that palatability is more at the forefront of formulation development (Anand et al., 2007).

Before any taste masking techniques are applied to a formulation it is essential to gain an understanding of the anatomy of the tongue and the taste transduction process. Understanding how bitter tastes are evoked is of particular importance.

1.3.2 The taste experience

The superior surface of the human tongue bears epithelial projections known as lingual papillae, of which there are three types: fungiform papillae, foliate papillae and circumvallate papillae. The fungiform papillae are located on the anterior two-thirds of the tongue; the foliate papillae are found on the sides of the posterior one-third; the circumvallate papillae form a “V-shape” near the posterior margin of the tongue. Some papillae are also located on the pharynx, larynx and epiglottis. Each papilla has from one to several hundred taste buds (Miller, 1995).

Each taste bud has 50 - 150 taste receptor cells recessed into the surrounding epithelium. The taste receptor cells are made up of gustatory cells, transitional cells and supportive basal cells and exhibit a rapid turnover; their average lifespan is about 10 days (Beidler and Smallman, 1965). The gustatory cells are categorised further into three types, designated I, II, and III, distinguished unequivocally by specific markers.
and physiological characteristics (Palmer, 2007). The apical end of the gustatory cells, near the surface of the tongue, have thin extensions called microvilli that project into the taste pore, a narrow opening exposed to the contents of the mouth. On each microvillus are various taste receptors. At the opposing end the gustatory cells synapse with the endings of the gustatory afferent axons that relay taste signals into the brain stem after activating cranial nerves VII (fungiform papillae & palate), IX (foliate & circumvallate papillae) and X (epiglottis & larynx) (Figure 1.4). Taste information from these cranial nerves passes through the gustatory division of the nucleus tractus solitarius in the medulla oblongata, where basic somatic acceptance and rejection reflexes in response to taste stimulation are generated (Smith and Scott, 2003). From the medulla oblongata taste signals are projected towards the ventroposteromedial nucleus of the thalamus before the sensory information is relayed to the primary gustatory cortex and finally the orbito-frontal cortex, the secondary gustatory cortex.

![Figure 1.4: The anatomy of a typical taste bud (Dowdey, 2007)](image)

The identities of the particular molecules that stimulate different taste receptors are not all known. Studies have revealed various genes that encode for numerous taste receptors located on the gustatory cells (Roper, 2007). Chemical interaction with these receptors to produce a taste sensation is categorised into the five primary taste sensations (stimuli) - the overall taste of a substance is based on a unique blend of these primary taste sensations, much like colour perception being based on three primary colours. Each taste bud usually responds to one of the five primary taste stimuli when the substance is in low concentration, and can be excited by two or more of the primary taste stimuli.
Chapter 1 - General Introduction

when the substances are in high concentration. The primary taste stimuli are recognised as being: sweet, salty, sour, bitter and umami. Taste stimuli evoke their sensation by passing directly through ion channels (salt and sour); binding to and blocking ion channels (sour and bitter); binding to membrane receptors that activate second messenger systems, that, in turn, open or close ion channels (sweet, bitter, umami) (Roper, 2007). Interaction with the receptors consequently leads to membrane depolarisation of the host gustatory cell and subsequent release of synaptic vesicles that interact with the gustatory afferent axon, which relay the taste stimuli to the brain.

Bitter taste issues predominate with pharmaceuticals. They are transduced by G-protein-coupled receptors and at present 25 bitter-taste receptor genes and 11 pseudogenes have been identified in humans (Matsunami et al., 2000). These bitter receptors were named T2Rs (also termed Tas2Rs). At present a focused search for ligands that activate the 25 human T2Rs is underway, with at least five bitter-taste receptors and their ligands identified to date (Roper, 2007):

- Receptor - hTas2R16, ligand - salicin
- Receptor - hTas2R38, ligand - phenylthiocarbamide and propylthiouracil
- Receptor - hTas2R43 and hTas2R44, ligand - high concentrations of saccharin and acesulpham K
- Receptor - hTas2R46, ligand - absinthin, strychnine and denatonium

The precise signal transduction pathway that results in a bitter taste sensation is not known fully. Studies on the subject so far allude to the following second-messenger signal transduction process that involves typical second-messenger molecules and enzymes: tastant molecules, acting as agonist ligands, bind to and stabilise the active conformation of T2Rs in the apical microvilli of type II gustatory cells. The active receptor interacts with G-proteins, such as gustducin, that consequently release their β subunits to stimulate intracellular phospholipase C-β2 (PLCβ2). Hydrolysis of membrane phosphatidylinositol bisphosphate (PIP2) by PLCβ2 generates inositol trisphosphate (IP3) that, in turn, interacts with and opens the inositol phosphate type III receptor (IP3R3) channel on internal calcium stores. The increase in $[\text{Ca}^{2+}]_i$ activates non-selective cation channel transient receptor potential–melastatin 5 (TRPM5)-mediated changes in membrane potential, and subsequently adenosine triphosphate (ATP) is released through the pannexin-1 hemichannel. Secreted ATP acts on P2Y purinergic receptors on type III taste cells, causing the exocytotic release of serotonin (5-HT) into the synapse between type III cells and afferent sensory neurons. ATP from
Chapter 1 - General Introduction

type II cells is also believed to stimulate afferent sensory neurons that relay neuronal taste signals to the brain (Figure 1.5) (Palmer, 2007).

Bitter sensations do not originate from one single type of chemical agent but a diverse range of substances. The majority of bitter tastes are, however, almost entirely from organic substances. These are commonly long-chain organic substances that contain nitrogen, and alkaloids. It is considered that bitterness sensations are part of an evolutionary defense mechanism (Garcia and Hankins, 1975). When a bitter taste is evoked at high intensity it usually causes an involuntary rejection of the substances and subsequent vomiting. The bitter taste of toxins and poisons is not coincidence but considered an evolutionary trait that has enabled humans to avoid ingesting dangerous substances. In relation, numerous drugs used in medicine are derived from plant toxins, or are alkaloids, and as such they often evoke a very bitter taste. Moreover, compared to the other taste sensations the sensitivity for bitter tastes is much higher, enabling detection of bitter substances at very low concentrations. Bitter molecules are also diverse, sometimes evoking more than one taste sensation. For instance, sweeteners
Chapter 1 - General Introduction

acesulpham K and saccharin are predominantly sweet molecules but can evoke bitter tastes at high concentrations (Roper, 2007).

1.3.3 Maturation of taste

The human fetus starts to develop specialised taste cells at approximately the seventh or eighth week of gestation and is seen to have structurally mature taste buds at 13 to 15 weeks (Matsui, 2007). The peripheral gustatory system undergoes further anatomical and neural changes after birth. Various studies to determine whether children are more sensitive to taste than adults have contrasting results. For instance, it was reported that papilla density decreased with age (Moses et al., 1967), whereas Arvidson (1979) found no age-related differences in the number of taste buds in fungiform papillae in cadavers from infants aged two days to six months when compared with adults. Whilst these anatomical studies have contrasting results they may not ultimately relate to taste sensitivity. It was found that children aged nine years old were more sensitive than adults to sucrose (Stein et al., 1994). Nevertheless, psychophysical studies of taste thresholds with nine year-old males indicate the system is not mature fully at this age (James et al., 1997). In addition, it was found that nine year-old children have difficulty analysing taste mixtures into their components, due to their attentional and possibly gustatory shortcomings when compared adults (Oram et al., 2001). Overall there is a paucity of research on the ontogeny of bitter taste sensitivity; the extent of the differences in perception between adults and children is unknown. Furthermore, the maturation of various tastes, especially bitterness has yet to be categorised fully and cover the age categorisation of paediatric patients.

Aside from sensitivity differences between adults and children, major differences are seen in taste preferences between the ages. Sour taste appears repudiated by infants (Steiner, 1979), whereas sour-tasting substances are preferred to blander tastes by children aged eight and over (Liem and Mennella, 2003). Sweet taste sensations are innately liked and remain enjoyable throughout life but with marked preference in children compared to adults; salty taste is neutral in infants but a preference for salty foods develops during the first months of life (Cowart, 1981). Children prefer significantly higher concentrations of sweeteners and salt compared to adults (Desor et al., 1975). Bitter taste rejection is present in neonates (Kajiura et al., 1992), but evidence on further developmental changes in bitter taste acceptance is lacking. Nonetheless, increasing preference for bitter-tasting foods such as coffee, tea or beer (Zellner, 1991) suggests possible learning through social pressure or caloric conditioning.
1.3.4 Taste masking techniques

Taste-related sensations such as texture, viscosity, temperature, and smell all provide added sensory information that can enhance the flavour of a substance without being related to taste transduction. In this particular research taste masking refers to the masking of taste sensations, bitter taste in particular, and not other aspects associated with flavour enhancement.

Bitterness reduction and inhibition is an important characteristic when formulating an oral dosage form, especially oral liquids. Whilst bitter taste masked solid dosage forms might be beneficial for adults, adolescents and older children, they are not so beneficial to younger children and those who cannot swallow them. It is, therefore, important, that taste masked oral liquid formulations are available, although this is often hard to achieve. Taste masking techniques for oral liquid formulations are achieved by two main methods, termed in this research as taste deception and taste obstruction.

Taste deception is the simplest and most traditional approach to taste masking, whereby sweeteners and flavouring agents, or a combination of both, are added to a formulation to “over-ride” the bitter taste of the drug and evoke a sweet/flavoured taste. Considering the target population is an important factor when selecting flavours to be used in paediatric formulations - certain sweeteners and flavouring agents may not be suitable for all paediatric subpopulations (Pawar and Kumar, 2002), and cultural differences can affect patient adherence to a medicine (EMEA, 2006). Market research has demonstrated that “bubble gum” and “grape” flavours are preferred in the USA, whilst “liquorice” is a Scandinavian preference. In a recent review of the commercially available paediatric formulations in the USA, the following flavouring agents and sweeteners were listed as being incorporated into the formulations (Table 1.5).

Although the addition of sweeteners and flavouring agents is used commonly in paediatric liquid preparations, high concentrations of sweeteners may be unsuccessful in masking the taste of highly bitter/highly soluble drugs (Barra et al., 1999). Thus, other taste masking techniques, frequently in addition to the use of flavours, need to be used to achieve a palatable formulation (Matsui, 2007).
Chapter 1 - General Introduction

**Table 1.5**: Flavouring agents and sweeteners listed in commercially available paediatric formulations in the United States of America (Strickely *et al.*, 2008)

<table>
<thead>
<tr>
<th>Flavouring agents</th>
<th>Sweeteners</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anise</td>
<td>Mandarin orange</td>
</tr>
<tr>
<td>Banana</td>
<td>Mint</td>
</tr>
<tr>
<td>Blackcurrent</td>
<td>Mixed fruit</td>
</tr>
<tr>
<td>Bubble gum</td>
<td>Orange</td>
</tr>
<tr>
<td>Cherry</td>
<td>Peach</td>
</tr>
<tr>
<td>Cotton Candy</td>
<td>Peppermint</td>
</tr>
<tr>
<td>Creamy Caramel</td>
<td>Strawberry &amp; banana</td>
</tr>
<tr>
<td>Crème de vanilla</td>
<td>Strawberry &amp; cream</td>
</tr>
<tr>
<td>Fruit punch</td>
<td>Strawberry &amp; mint</td>
</tr>
<tr>
<td>Grape</td>
<td>Tutti-fruity</td>
</tr>
<tr>
<td>Lemon crème</td>
<td>Vanilla</td>
</tr>
</tbody>
</table>

Taste obstruction refers to taste masking techniques that alleviate bitterness by obstructing bitter drug molecules from interacting with the taste receptors that evoke bitter taste sensations. Put simply, a barrier of some kind is used to prevent drug molecules from reaching the receptor interface. Taste obstruction methods include **physical barriers** such as polymer coatings, lipids, including lipid emulsions and liposomes, and nano- and/or micro-particulate systems, as well as **molecular barriers**, including resins and cyclodextrins (CDs) (Ernest *et al.*, 2007). It is CDs that are of particular interest in this thesis and will be the main focus of research. Much like other taste obstruction methods, CDs provide taste masking of bitter drug molecules by forming a protective barrier between the drug molecules and taste receptors. This technique was used recently to mask bitter taste of various drugs in solution (Szejtli and Szente, 2005; Section 1.5.6.4). The additional benefits for the use of CDs as a taste masking agent is that they were also reported to improve the solubility and stability of drugs in solution (Section 1.5).

**1.4 Taste assessment**

**1.4.1 Introduction**

Since the Paediatric Regulation came into force in the EU in January 2007 (Regulation [EC] No. 1901/2006) the palatability of paediatric medicines is now, more than ever, a crucial part of formulation development (EMEA, 2006). Thus, taste assessment of formulations is fast becoming a pivotal and important stage in evaluating the taste of a formulation and/or taste masking ability of various excipients (Anand *et al.*, 2007; Matsui, 2007; Cram *et al.*, 2009; Davies and Tuleu, 2008). The most widely used method for measuring the taste of a pharmaceutical formulation is psychophysical
evaluation by a human taste panel, although other \textit{in vivo} approaches, such as electrophysiological methods and animal preference studies can be performed. Recent advances in sensor technology has led to novel \textit{in vitro} taste assessment apparatus to be developed and this field of taste assessment is gradually becoming an appealing tool for formulation scientists looking to develop palatable medicines. Additional \textit{in vitro} taste assessment can be carried out via drug release studies, although this predominantly applies to solid dosage forms.

1.4.2 Taste assessment \textit{in vivo}

\textbf{Human taste panel studies}, also known as gustatory sensation studies, sensory panel studies and taste trials, are still the "gold standard" in evaluating the taste of a formulation during development. The International Organization for Standardization provides a series of standards that safeguard the running of efficient and safe taste panel studies. Trained healthy adult volunteers are used typically to evaluate the taste of formulations in statistically designed analysis. Evaluation of the formulation is usually performed on an adjective scale, such as a visual analogue scale (VAS), which panelists use to evaluate a particular property of the formulations. These properties range from intensity of bitterness, sweetness, etc..., to intensity scaling of perceptual parameters, such as astringency, spiciness and irritation (Meilgaard \textit{et al.}, 2006).

Although human taste panel studies have well-established methodologies, they are not without their limitations. Panelists are expensive to train and the overall cost of running a human taste panel can be very expensive. Whilst studies are designed to reduce bias panelists remain subjective. Ethical and/or safety approval can often limit taste trials, especially when formulations contain novel drugs that have limited toxicological data or may be toxic to healthy subjects, e.g. cytotoxics. This leads to a concern regarding exposing panelists to medicines that may cause an array of potentially harmful side effects. Taste panel studies may also be time-consuming and laborious, which could lead to inaccurate taste evaluation from fatigued assessors.

Taste assessment of paediatric formulations by human taste panels should ideally be carried out by children and not adults, and should, therefore, be designed for non-adult assessors. Evidence illustrates that infant assessors can produce reliable results similar to adults. For instance, Mennella \textit{et al.}, (2003), during taste masking evaluation of bitter drugs reported that children assessors came to the same evaluation regarding the bitter taste of formulations as their adult counterparts. It was reported, however, that mid-
Chapter 1 - General Introduction

Childhood humans have difficulty analysing taste mixtures into their components, due to attention and possibly gustatory shortcomings (Oram et al., 2001). Taste assessment by children, therefore, needs to be tailored to the age and ability of the assessors.

Methodologies for formulation taste assessment in paediatric populations are varied and may require a different approach than adult taste studies (Davies and Tuleu, 2008). Taste trials in children need to be short and intrinsically motivating, as well as easy to follow for the young assessors. The number of variants also needs to be kept to a minimum so the children provide reliable taste assessment. Trained assessors may provide higher quality data but are subjected to more samples and, therefore, susceptible to fatigue. Factors that need to be considered when carrying out formulation taste assessment studies include: environment and timing of the study, using trained or untrained assessors, using a multi-dose or single dose approach, using healthy or ill taste assessors, the taste assessment scale used, the statistics performed on the data, and the size of the study population. There are also ethical dilemmas surrounding taste analysis in paediatric subjects since taste analysis may increase drug exposure to children during clinical trials.

Taste preference studies of medicines and bitterness detection of drugs in vivo can be performed successfully in animals. The ability of animals, such as rats, to identify bitter-tasting compounds in behavioral avoidance studies may serve as a potential screening tool for new drug molecules, especially drugs with limited toxicological data in humans (Anand et al., 2007). Limited studies, however, have been carried out on the relationship between animal bitterness sensation and human bitterness sensation to ensure data produced from animal models is reliable. Attempts are being made to develop robust animal behavioral tests that are capable of being extrapolated to humans (Anand et al., 2007). Electrophysiological recordings in animals, as well as humans, can also provide taste data of medicines in vivo by directly measuring taste stimuli from the nerves that innervate the tongue. Although this particular method can provide dose response curves of taste stimuli, data analysis and interpretation of results are often troublesome, as well as limited industrial investment and development (Anand et al., 2007).

1.4.3 Taste assessment in vitro

Taste assessment of pharmaceutical formulations via analytical instrumentation was inspired by the biological gustatory system and was developed to provide rapid taste
assessment of formulations and taste masking agents. Varying systems were researched and developed by numerous scientists across the world; the majority of literature available in this field stems from three groups: Toko and co-workers in Japan, Winquist and colleagues in Sweden, and Vlasov and co-workers in Russia. Significant developments and reviews on the application of “electronic tongues” were reported (Toko, 1998, 2000; Vlasov et al., 2002; Legin et al., 2002; 2004; Zheng and Keeney 2005; Funasaki et al., 2006; and Anand et al., 2007).

Whilst the methodology of the various “electronic tongues” may differ, the principles of analysis for each apparatus are essentially the same. *In vitro* multicomponent analysis of liquids is conducted typically with a sensor array made up of multiple cross-sensitive or sparingly selective potentiometric chemical sensors. Located on each sensor are a semiconductor and/or transistor with an exposed lipid and/or polymer membrane, often called the detecting membrane. Chemicals in the test solution interact with the membrane and elicit a membrane potential. Different potentials are produced from different chemicals and/or species of chemical, in the test solution, as well as from the different membranes. The signal output for a particular sensor is the potential difference, in mV, between the detecting membrane and a reference electrode, which is generally an Ag/AgCl reference electrode. The potential-difference-signal from each sensor is subsequently amplified and recorded by a computer. In a multicomponent environment the sensor array produces complex signals, known as patterns, which contain information about the different compounds in solution. Finally, pattern-recognition/ multivariate statistical analysis is conducted on the acquired data, frequently after data treatment, to provide quantitative and qualitative interpretation of the information held within the signal pattern. *Further details regarding in vitro taste masking analysis with a commercially available “electronic tongue” will be discussed in Chapters 4, 5 and 6.*

### 1.5 Cyclodextrins

#### 1.5.1 Introduction

It is apparent from Section 1.2.4 that there are limited age-appropriate corticosteroids formulations available for paediatric patients. The need for palatable oral liquid formulations with non-hazardous excipients requires further research so that alternative formulations can be developed. One particular class of excipient that may be beneficial
Chapter 1 - General Introduction

in producing a palatable oral liquid corticosteroid formulation and potentially other oral liquid formulations, specifically for children, are cyclodextrins.

1.5.2 Discovery, history and development

Cyclodextrins (CDs) were first documented in the published work of French scientist A. Villiers in 1891, where he described a small amount of crystalline material produced from the Bacillus amylobacter digestion of starch (Villiers, 1891). His experimental results determined that the crystalline substance was a dextrin with similar properties to cellulose; thus Villiers aptly named the crystalline substance “cellulosine”. Twelve years later the Austrian microbiologist Franz Schardinger published an article describing the isolation of two crystalline compounds from the bacterial digests of potato starch, naming them α-dextrin and β-dextrin (Schardinger, 1903). Subsequently Schardinger identified the β-dextrin as Villiers’ “cellulosine” but was unable to maintain the bacterial strain capable of producing the crystalline compounds. Continuing his work, in 1911 Schardinger published findings that the bacteria Bacillus macerans could produce the crystalline dextrins and determined that they were cyclic oligosaccharides (Schardinger, 1911). It was at this point that the nomenclature for the crystalline dextrins changed to cyclodextrin (CD), although sometimes they were referred to as cyclomaltodextrins, cycloamyloses or Schardinger dextrins.

In 1935 γ-CD was first isolated and indentified by Freudenberg and Jacobi (Freudenberg and Jacobi, 1935), and in the following 20 years vast advancements in the development and understanding of CDs were achieved. Freudenberg and his colleagues determined that CDs consisted of cyclic oligosaccharides with α(1→4)-linked D-glucopyranose units, a central cavity, and deduced that methylation of CD molecules was possible (Freudenberg and Meyer-Delius, 1938). The molecular weight of the natural CDs was determined consequently and it was suggested that CDs larger than γ-CD could exist (Freudenberg and Cramer, 1948). By 1954 the ability of CDs to solubilise and stabilise drug molecules through inclusion complexation was known (Cramer, 1954). In 1953 the first CD related patent was issued to Freudenberg, Cramer and Plieniger (Freudenberg et al., 1953), although the patent was never utilised for industrial use (Cramer 1987). This was likely attributed to insufficient yields and purity of CDs that were created by the various strains of bacteria used to produce laboratory scale CDs, causing the novel oligosaccharides to be too expensive and too hazardous for the pharmaceutical industry (Oguma and Kawamoto, 2003).
Chapter 1 - General Introduction

Continuing the development and potential pharmaceutical application of CDs, in 1965 French et al., reported the first definitive evidence for the existence of large-ring CDs (LR-CDs), with nine to 13 glucopyranose units per CD molecule (French et al, 1965). As with the natural CDs, LR-CDs were difficult to purify and their yields were deemed too low for pharmaceutical application (Ueda, 2002). CDs only started to become considered viable commodities for the pharmaceutical industry after the advancement of the biotechnology industry in the late 1970’s, leading to the discovery of cyclodextrin glucosyl transferase (CGTase), the enzyme produced by Bacillus macerans, (Saenger, 1980). In recent years advancements in genetic engineering made possible the isolation of various CGTases from different strains of Bacilli, Micrococcus and Klebsiella (Sicard and Saniez, 1987), enabling the production of large quantities of highly pure natural CDs. This resulted in cheaper manufacturing of these once expensive chemicals (Oguma and Kawamoto, 2003), ultimately enabling more research and subsequently greater interest of CDs in the pharmaceutical domain (Sicard and Saniez, 1987).

In 1976 the first pharmaceutical product containing CD, prostaglandin E2/β-CD (Prostarmon E™ sublingual tablets), was released onto the Japanese market, and 11 years later the first European pharmaceutical product containing CD, piroxicam/β-CD (Brexin® tablets), was released (Szejtli, 2004). In the early years of pharmaceutical CD application, β-CD was the preferred CD of choice because of its greater abundance compared to the other natural CDs. Furthermore, β-CD provided pharmaceutically useful complexation characteristics with the broadest range of drugs (Szejtli, 1988). It became apparent, however, that once utilisation of natural CDs as potential pharmaceutical excipients evolved, limitations in their application might occur. For instance, natural CDs have a low aqueous solubility than comparable linear dextrins, especially β-CD, in addition to various toxicological issues, such as β-CD’s demonstrated nephrotoxicity (Antlsperger and Schmid, 1996). Factors like these led to the development of modified CDs, the CD derivatives, in the 1990’s (Albers and Müller, 1995). CD derivatives are produced usually by random alkylations, aminations, or esterifications of primary and secondary hydroxyl groups on the glucopyranose units of CD molecules, and depending on the substituted moiety, usually perform better than their parent CD (Szente and Szejtli, 1999).
1.5.3 Structure and properties

The natural CDs, α-, β-, and γ- CD are crystalline, hydrophilic, homogeneous, and nonhygroscopic substances composed of six, seven and eight glucopyranose units, respectively (Szejtli, 1997) (Figure. 1.6). All glucopyranose units are linked by glycosidic α(1→4) bonds, and are positioned in the $^4C_1$ chair conformation, as determined by NMR and X-ray diffraction studies (Rao and Foster, 1963). This attributes CDs to have a hollow truncated cone structure, often referenced in the literature as being a “doughnut” or “lobster pot” shape.

![Figure 1.6: The chemical structure of a) α-CD, b) β-CD, c) γ-CD (Davis and Brewster, 2004)](image)

The CD cavity is lined with two rings of CH groups and by lone pairs of glycosidic oxygen-bridge atoms (Saenger, 1982; Szejtli, 2004), endowing the internal cavity with a hydrophobic character and allowing for a lipophilic microenvironment. The polarity of the cavity was estimated to be similar to that of aqueous ethanolic solution (Fromming and Szejtli, 1994), where included water molecules are present and in direct contact with the apolar wall of the cavity, both in solid state and in aqueous solution. The hydroxyl groups of the glucopyranose units are orientated to the cone exterior with the primary hydroxyl groups on the C-6 atoms located near the narrow part of the torus, directed “away” from the cavity, whilst the secondary hydroxyl groups on the C-2 and C-3 atoms are positioned near the wide edge of the torus (Figure 1.7). More CH groups are situated on the exterior of the molecule, in addition to the polar sugar hydroxyl groups. These groups, to the exterior of the CD torus, bestow a hydrophilic character and, therefore, provide the aqueous solubility for these molecules.
Figure 1.7: A schematic view of the orientation of a glucopyranose unit located within a cyclodextrin molecule, highlighting the six carbon atoms (taken from Mourtzis et al., 2007)

In all CD molecules, a hydroxyl group on the C-2 of a glucopyranose unit can form a hydrogen bond with a hydroxyl group on the C-3 of an adjacent glucopyranose unit (Szejtli, 2004). This ring of hydrogen bonds is in general established fully in β-CD and γ-CD but in α-CD one glucopyranose unit is in an imprecise location. Thus, instead of the 6 possible hydrogen bonds, only four can be established simultaneously. In β-CD, a complete ring of hydrogen bonds is established between secondary hydroxyl groups. These molecules are consequently rigid in aqueous solution and the extent of intramolecular hydrogen bonding detracts from hydrogen bond formation with surrounding water molecules, resulting in less negative heats of hydration (Fromming and Szejtli, 1994; Miyazawa et al., 1995). Thus, these CDs have a low aqueous solubility and relatively unfavourable enthalpies of solution. Furthermore, because of the limited aqueous solubility, the utilisation of β-CD as a solubiliser might be limited as the potential to form a precipitate is plausible. To appease this problem, modification of the CD structure to create CD derivatives enables CDs to have increased aqueous solubility (Szente and Szejtli, 1999).

To produce CD derivatives from the natural CDs, the hydroxyl groups on C-2, C-3 and C-6 are available as suitable functional groups for modification without hindering the vicinity of the central cavity. Functional groups that can replace the protons of the hydroxyl groups are numerous; CD derivatives that are of particular pharmaceutical interest include hydroxypropyl derivatives of β-CD, the randomly methylated β-CD, sulfobutyl ether β-CD, and branched CDs such as glucosyl-β-CD (Figure 1.8) (Szejtli, 1988; Loftsson and Brewster, 1996; Rajewski and Stella, 1996; Irie and Uekama, 1997; Uekama et al., 1998; Thompson, 1997). These CD derivatives are amorphous and
heterogeneous, as apposed to being crystalline and homogenous like their parent CD (Szejtli, 1997).

The number and position of the substituents on the parent CD molecule greatly affects the physicochemical properties of the molecule. The average degree of substitution (DS) gives the average number of substituted hydroxys of a glucopyranose unit and can be zero, one, two or three (Blanchard and Proniuk, 1999). It is, therefore, common for a CD derivative to have a DS value that is not a whole number because the degree of substitution per glucopyranose unit is different for every unit throughout the whole molecule. In addition, the DS of a derivatised CD does not uniquely characterise that particular CD derivative. For instance, when produced under the same conditions CD derivatives with the same DS may not be identical in terms of physiochemical characteristics. Great consideration, therefore, needs to be taken when using CD derivatives from differently manufactured batches (Blanchard and Stefan, 1999). The average molar degree of substitution (MS), confused often with the DS, is the average number of moles of the substituting agent per mole of glucopyranose and is used frequently as a marker for purity.

\[
\text{Cyclodextrin} \\ \begin{array}{l}
\beta\text{-Cyclodextrin} \\
2\text{-Hydroxypropyl}\beta\text{-cyclodextrin} \\
\text{Sulfobutylether } \beta\text{-cyclodextrin sodium salt} \\
\text{Randomly methylated } \beta\text{-cyclodextrin} \\
\text{Branched } \beta\text{-cyclodextrin}
\end{array} \\
\begin{array}{l}
R = H \text{ or} \\
-CH_2\text{CHOHCH}_3 \\
-(\text{CH}_2)_4\text{SO}_3^- \text{Na}^+ \\
-\text{CH}_3 \\
\text{Glucosyl or maltosyl group}
\end{array}
\]

**Figure 1.8:** The chemical structure of $\beta$-cyclodextrin and some of its derivatives (Loftsson et al., 2004a)

To represent the average number of substituted groups per CD molecule the total degree of substitution (TSD) is often quoted and represents the average number of substituted
groups per CD molecule. Thus, for a CD derivative of β-CD, which has seven glucopyranose units, the TSD value can theoretically range from 0 to >21. The TSD is also used to avoid confusion between DS and MS and to calculate the molecular weight of a CD derivative. The important physicochemical characteristics of the natural CDs, and the CD derivatives to be utilised by this current research are outlined in Table 1.6.

Table 1.6: Physicochemical characteristics of natural cyclodextrins and two derivatives

(Loftsson and Brewster, 1996; Connors, 1997; Brewster and Loftsson, 2007)

<table>
<thead>
<tr>
<th>Physicochemical characteristic</th>
<th>α-CD</th>
<th>β-CD</th>
<th>γ-CD</th>
<th>HP-β-CD</th>
<th>Me-β-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of glucopyranose units</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>MW (Da)</td>
<td>972</td>
<td>1135</td>
<td>1297</td>
<td>1447</td>
<td>1310</td>
</tr>
<tr>
<td>Solubility in pure water at 25°C (% w/v)</td>
<td>14.5</td>
<td>1.85</td>
<td>23.2</td>
<td>&gt;60</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Central cavity diameter (Å)</td>
<td>4.7-5.3</td>
<td>6.0-6.5</td>
<td>7.5-8.3</td>
<td>6.0-6.5</td>
<td>6.0-6.5</td>
</tr>
<tr>
<td>Outer diameter (Å)</td>
<td>14.6</td>
<td>15.4</td>
<td>17.5</td>
<td>15.4</td>
<td>15.4</td>
</tr>
<tr>
<td>Inner diameter (Å)</td>
<td>5.7</td>
<td>7.8</td>
<td>9.5</td>
<td>7.8</td>
<td>7.8</td>
</tr>
<tr>
<td>Height of torus (Å)</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
</tr>
<tr>
<td>Cavity volume (Å³)</td>
<td>174</td>
<td>262</td>
<td>427</td>
<td>262</td>
<td>262</td>
</tr>
<tr>
<td>Melting onset (°C)</td>
<td>~275</td>
<td>~280</td>
<td>~275</td>
<td>~278</td>
<td>~280</td>
</tr>
</tbody>
</table>

1.5.4 Inclusion Complexation

The most important characteristic of CDs is the ability to form non-covalently bonded inclusion complexes with various guest molecules (Saenger, 1980; Szejtli, 1982). Freudenberg recognised that CDs could form inclusion compounds (Freudenberg and Cramer, 1948), and the formation of CD-guest complexes was studied methodically by Cramer and co-workers throughout the developmental years of CD research (Cramer, 1954; Cramer and Henglein, 1957; Cramer and Hettler, 1967). The degree of CD inclusion complex formation with different sized guest molecules varies as a result of the different internal cavity diameters of the parent CDs (α-CD, β-CD, γ-CD) (Bender and Komiyama, 1978).

An inclusion complex is a unique form of chemical complex in which one molecule is enclosed within another molecule or aggregation of molecules. They were observed first by Mylius in 1886, where his work concluded that hydroquinone and several volatile compounds were interacting in the absence of ordinary chemical bonds and suggested that one molecule was enclosing the other (Frank, 1975). The essential criterion of inclusion complexation is simply that the enclosed molecule or “guest” be of a suitable size and shape to fit into the cavity of the “host” (Powell, 1948, 1954). Guest molecules
Chapter 1 - General Introduction

capable of fitting inside the cavity of CD molecules are, therefore, ideally suited for inclusion complexation. The stereochemistry, and to some extent the polarity, of both the CD and guest molecules determines whether inclusion can occur. Aside from CDs including whole guest molecules into their cavity, it is also possible for CDs to partially include guest molecules, where one particular region of the guest (typically some lipophilic moiety), is included whilst the remaining part of the molecule is exposed (Loftsson et al., 2004a).

From a pharmaceutical point of view, CD inclusion complex formation is equivalent to molecular encapsulation, as the drug molecules are isolated from each other and are dispersed on a molecular level in an oligosaccharide matrix, i.e. a process similar to the encapsulation of the drug in a hydrophilic matrix, e.g. polyvinylpyrrolidone or cellulose (Pitha et al., 1983 and Szejtli, 1985). Drug molecules of a suitable size and shape can, therefore, be held within the cavity of a particular CD. In general, the smaller the drug molecule, the greater the complexing activity. Similarly, the degree of activity for large drug molecules is dependant on the presence of a suitable group or moiety capable of fitting into the CD cavity. Of the natural CDs, β-CD is deemed the most useful complexing agent, as its cavity is the ideal size for the majority of drug molecules; α-CD is too small, whilst γ-CD is too big (Szejtli, 1988).

Inclusion complex formation between CD and drug molecules typically takes place in an aqueous environment, although inclusion complexation can be evoked in the solid state. The structure of solid CD complexes is not always identical to the complexes in solution. In the dissolved state, all or part of the guest molecule is located within the cavity of the CD, whilst a multilayer hydrate shell surrounds the whole complex. In the solid state, however, guest molecules are located not only inside the CD cavity but also between CD rings as crystal-lattice inclusions (Szejtli, 1985). CD derivatives being less crystalline than their parent CDs create inclusion complexes that are amorphous. Thus, CD derivative inclusion complexes are more soluble than inclusion complex made with natural CDs (Szente and Szejtli, 1999).

CD inclusion complexes in aqueous solution are not static species. A dynamic equilibrium is established between the complexed and free drug molecules of the surrounding solution (Friedman et al., 1987), with drug molecules included within the CD cavity rapidly exchanging with free drug molecules (Figure 1.9). The driving forces leading to inclusion complex formation evolves from the release of the enthalpy-rich
water molecules that are included within the CD cavity and are exchanged with guest molecules of less polarity.

**Figure 1.9:** A schematic representation of formation of a CD/drug inclusion complex in aqueous solution (Loftsson and Stefansson, 1997)

The process of guest inclusion firstly involves the included water molecules being released from the CD cavity into the bulk aqueous environment, once a guest molecule is in the immediate vicinity of the cavity. Simultaneously, as the guest molecule approaches the CD and gradually enters the cavity there is a breakdown of the water structure surrounding the molecule. The guest molecule sheds its hydrate shell and once positioned inside the CD cavity, the complex is stabilised by van der Waals forces and occasionally by hydrogen bonding between the guest and the CD. The water structure is then restored around the exposed part of the guest molecule after complexation has occurred (Liu and Guo, 2002).

The majority of drug molecules form 1:1 complexes with CD molecules: the extent of complexation is characterised by the association constant (also called stability constant) of the inclusion complex and depends on how well the guest molecule fits into the CD cavity (Loftsson and Bodor, 1989 and Pitha et al., 1983). The rate of inclusion complexation is rapid ($10^{-2} - 10^{-8}$ s mol$^{-1}$), with equilibrium binding usually establishing with half-lives of much less than one second; the kinetics of dissociation are, therefore, generally expected to be much faster than many physiological processes (Hersey et al., 1986; Cramer et al., 1967). The greater the magnitude of the association compared to the dissociation: the greater the stability of the complex (Lach and Chin, 1964).

The value of the stability constant ($K_c$) is frequently between 50 and 2000 M$^{-1}$ with a mean value of 129, 400 and 355 M$^{-1}$ for $\alpha$-CD, $\beta$-CD, and $\gamma$-CD, respectively (Equation 1.1) (Stella and Rajewski, 1997; Connors, 1995; Connors, 1997; Rao and
Chapter 1 - General Introduction

Stella, 2003). Very labile inclusion complexes result in premature release of the drug, because to the weak interaction between CD and drug (D). Very stable inclusion complexes result in a retarded or an incomplete release of the drug, and thus absorption is hindered. There are examples, however, where CD inclusion complexes with low \( K_C \) have still provided some benefit to the drug molecule. For instance, Artemisinin and \( \beta \)-CD were found to have a \( K_C \) of 63 M\(^{-1} \) yet improved oral bioavailability compared to a commercial tablet was demonstrated (Wong and Yuen, 2001).

\[
D + CD \underset{k} \rightarrow DCD
\]

\[
K_c = \frac{[DCD]}{[D][CD]} \tag{1.1}
\]

Several methods have been reported for the determination of \( K_C \):

- Phase solubility (Cohen and Lach, 1963; Higuchi and Connors, 1965; Hamada et al., 1975; Szejtli, 1982; Imai et al., 1984) (See Chapter 2).
- Polarography (Szejtli, 1982; Choi et al., 1992; Yanez et al., 2004).
- Potentiometry (Miyaji et al., 1976; Kahle and Holzgrabe, 2004; Beni et al., 2007).
- Circular dichroism (Otagiri et al., 1978; Szejtli, 1982; Bekers et al., 1990; Jadhav and Vavia, 2008).
- Optical rotation dispersion (Bergeron et al., 1977; Zhang and Polavarapu, 2007).
- Electrospray ionization mass spectrometry (Zhang et al., 2006; Beni et al., 2007).
- Fluorescence (Otagiri et al., 1978; Shen et al., 2006; Fernández et al., 2008).
- Conductometry (Sheehy and Ramstad, 2005; Cabaleiro-Lago et al., 2006).
- High-pressure liquid chromatography (HPLC) (Uekama et al., 1978; de Melo et al., 2008).
- Ultraviolet/visible spectroscopy (Connors and Mollica, 1966; Otagiri et al., 1978; Bekers et al., 1990; Funasaki et al., 1999).
- Microcalorimetry (Otagiri et al., 1978; Aki et al., 2004; Perry et al., 2006) (See Chapter 2).
- Nuclear magnetic resonance spectroscopy (Bergeron et al., 1977; Szejtli, 1982; Schneider et al., 1998; Fielding, 2000) (See Chapter 2).

Complexes do exist with other CD:drug ratios (Uekama and Otagiri, 1987). For instance, 2:1 (\( \beta \)-CD:progesterone), 3:2 (\( \beta \)-CD:testosterone), and 4:1 (\( \gamma \)-CD:digoxin) complexes have been demonstrated. Aside from direct inclusion complexation, it was also demonstrated that CDs can form non-inclusion complexes with drug molecules,
much like their non-cyclic counterparts (Tomasik and Schilling, 1998). It is also possible for inclusion and non-inclusion complexes to be in unison within a solution creating complexes of higher order than 1:1 (Gabelica et al., 2002; Gabelica et al., 2003; Andronati et al., 1996; Amato et al., 1996). For instance, α-CD was reported to form inclusion and non-inclusion complexes with α,ω-dicarboxylic acids, where 1:1 complexes were found to be inclusion complexes, and 2:1 (α,ω-dicarboxylic acid/α-CD) non-inclusion complexes were found to be formed between an acid molecule and a 1:1 inclusion complex (Gabelica et al., 2002; Gabelica et al., 2003).

It was also demonstrated that both CDs and CD inclusion complexes self-associate to form aggregates of two or more CD molecules or complexes (Loftsson et al., 2002; Loftsson et al., 2004c; He et al., 2008). Research on these aggregates has shown that they are capable of forming non-inclusion complexes with poorly water-soluble drugs, in addition to being capable of solubilising drugs via micelle-like structures (Loftsson et al., 2002, Magnusdottir et al., 2002). This might explain why the value for the $K_C$ is sometimes concentration dependant and why the numerical value deduced is dependant often on the method used to determine the value (Loftsson et al., 2002; Loftsson et al., 2004c). Awareness of complexation with CDs, other than the classical 1:1 CD-drug inclusion complex, therefore, needs to be taken into consideration when research is carried out with CDs.

### 1.5.5 Factors influencing inclusion complex formation

Numerous factors can affect the formation of CD inclusion complexes, driving either association or dissociation of the CDs and guest molecules. These factors need to be considered when utilising CDs within a pharmaceutical formulation (Loftsson and Brewster, 1996). Numerous methods of preparing CD-drug complexes are, therefore, employed to combat or utilise these factors (Hirayama and Uekama, 1987).

The most influential factor for CD inclusion complex formation is the type of CD that is employed to form the complex. The CD cavity needs to be a sufficient area to host drug molecules of a particular size and shape (Mura et al., 1999). For example, sufficient drug binding affinity to cocaine was seen only with β-CD compared to α-CD and γ-CD (Nesna et al., 2000). The type of substituent on a CD derivative (e.g. hydroxypropyl, methyl, sulfobutyl ether) can also influence the affinity of a drug molecule to a CD (Diaz et al., 1999), in addition to the DS influencing inclusion complex formation (Blanchard and Proniuk, 1999). For instance, it was illustrated that
Chapter 1 - General Introduction

CD derivatives of β-CD showed enhanced solubility over β-CD when complexed to albendazole (Castillo et al., 1999).

The charge of the CD or guest molecule can influence CD inclusion complex formation (Zia, 2001). The pH of the localised environment and the ionisation state of the CDs and guest molecule will affect complex formation (Loftsson and Brewster, 1996). CDs and drug molecules carrying opposite charge often have better stability constants than when complexed with neutral CDs, or CDs and drug molecules carrying the same charge (Nagase et al., 2001). Furthermore, the stability constant of inclusion complexes formed between un-ionized or neutral drug molecules and CDs was found to be much larger when compared to ionized drug molecules, especially when complexed with neutral CDs (Liu et al., 1992; Boudeville and Burgot, 1995). It is often the case, however, that ionisation of the drug increases the intrinsic solubility resulting in enhancing complexation and thus less need for CD complexation. This is also associated with inclusion complex formation of hydroxy acids/salts of basic drug molecules (Loftsson et al., 2004b; Redenti et al., 2001).

CD inclusion complex formation is an exothermic process (Inoue et al. 1993). The enthalpy of the system decreases when the temperature of the system is lowered, resulting in increased complexation (Loftsson and Brewster, 1996). It was, therefore, demonstrated that increasing the temperature decreases the magnitude of CD inclusion complexation with guest molecules (Zarzycki and Lamparczyk, 1998). Since CD inclusion complexes are usually prepared and stored at room temperature the change in binding affinity needs to be considered if the complexes are subjected to conditions in vivo.

There are numerous methods to prepare CD inclusion complexes, each capable of yielding complexes with different stability constants. The method of inclusion complex preparation is, therefore, a crucial factor that can influence complex formation (Miller et al., 2007). The most common techniques to produce solid CD inclusion complexes are: kneading, co-precipitation, solid dispersion, solvent evaporation, spray-drying, or freeze-drying - carried out usually in aqueous systems. These various manufacturing techniques have a significant affect on the stability constant of the complex that can be formed and was shown to produce complexes of varying characteristics. For example, when β-CD and vitamin D₂ were prepared via spray-drying, kneading, and solid dispersion; the kneading method produced a good
yield of complex, whilst the spray-drying resulted in complete complexation and produced the best dissolution profile (Palmieri et al., 1993). When solutions of CD inclusion complex need to be prepared, i.e. for parental or oral formulations, where there is no need to produce a dry complex, the techniques for complexation are fewer and simpler. The method of complex formation does, however, still influences CD inclusion complex formation (Holvoet et al., 2007). The main complex forming techniques to produce CD inclusion complexes in solution are co-solvent mixing (Li et al., 1999), and wet mixing of solid drug into CD solution - insoluble complexes are simply removed from the solution via filtration.

The ability of water-soluble polymers to enhance the solubilising effect of CDs has been demonstrated (Savolainen et al., 1998; Loftsson et al., 1998). For instance, when an array of water-soluble polymers were added to solutions containing β-CD and various drugs, it was found that the polymers not only enhanced solubilisation of β-CD and its complexes, but was also able to enhance formation of complexes between the drugs and β-CD (Loftsson and Fridriksdóttir, 1996). This may have been attributed to the formation of more soluble ternary complexes or the ability of the polymers to enhance the solubility of the CD and its complexes. In contrast, formation of polymer/CD inclusion complexes has been shown frequently to reduce the ability of the CD molecules to solubilised drugs through complexation (Brewster and Loftsson, 2007).

As suggested earlier, the inclusion complexation can be enhanced via ionisation, in addition to salt formation, formation of metal complexes and addition of organic co-solvents to the aqueous complexation media. On the other hand, however, within a formulation there are also numerous excipients that have the potential to compete with the drug molecules for the CD cavities, and thus decrease the amount of CD-drug inclusion complex and apparent stability constant (Stella et al., 1999). These additives include salts, surfactants, preservatives, and organic solvents, which also have a strong affinity to the CD cavity and, therefore, influence and reduce the efficiency of binding between drug and CD often. This phenomenon is known as competitive displacement.

There are additional factors that can affect the dissociation of a CD-drug inclusion complex and, therefore, need consideration during formulation development and clinical trials. The dilution of the inclusion complex media has a major influence on the drug affinity to a CD, especially weakly complexed drug molecules. When CD inclusion
complex solutions are administered orally, ophthalmically, or intravenously, the dilution of the solution varies as a function of the volume of physiological fluids the solution is exposed to. The degree of inclusion complexation is, therefore, altered as a result of the inclusion complexes being reconstituted in a greater aqueous volume (Stella et al., 1999). In addition, further dissociation occurs via the protein binding of complexed drug molecules, competing actively with the CDs for the drug molecules (Frijlink et al., 1991). Protein binding is typically applicable to strongly bound drug molecules that are not influenced by dilution. When the dilution influence is minimal, i.e. when administered by ocular, nasal, rectal, or dermal routes, another potential mechanism of drug release from a CD inclusion complex is preferential drug uptake by tissue (Rajewski and Stella, 1996). When the drug is lipophilic and has access to tissue, and is not available to the CD or the inclusion complex, the tissue then acts as a sink, causing dissociation of the complex based on simple mass action principles.

1.5.6 Evidence of inclusion complex formation

To confirm the formation of CD inclusion complexes, the interaction between a CD and a guest molecule can be studied by a number of methods (Szejtli, 1982). The most direct method used to determine the inclusion of a guest molecule into a CD cavity and for structural elucidation of a CD inclusion complex in solution is NMR spectroscopy (Schneider et al., 1998; Fielding, 2000). The first detailed report regarding the use of NMR spectroscopy during CD inclusion complex formation research was by Demarco and Thakkar (1970), and Thakkar and Demarco (1971). Their workings described a $^1$H NMR spectroscopy method for examining the mode of interaction of β-CD with a variety of aromatic substances and paved the way for use of NMR spectroscopy in CD inclusion complex confirmation. $^1$H NMR spectroscopy and $^{13}$C NMR spectroscopy methods with relaxation and correction time measurements, nuclear Overhauser effects (NOE) (Neuhaus and Williamson, 1989), and NOE in the rotating frame (ROE) have now become methods of choice in CD inclusion complex studies, providing information about molecular conformation of CD inclusion complex structures, in addition to distances and relative orientation and geometry of the host and guest molecules within the complex (Schneider et al., 1998). The complexing ability of CDs, complex stoichiometry, stability constant, and chiral recognition are all complex parameters that can be determined by NMR spectroscopy. Recently two-dimensional NMR spectroscopy, such as Correlated spectroscopy, 2-Dimentional NOE and ROE, and double quantum filtered-correlation spectroscopy has also been applied to CD inclusion
complex studies to provide additional information regarding inclusion complexation. For example, $^1$H NMR spectra have been used to characterise the inclusion complexes formed between β-CD and hydrocortisone (Frank and Kavaliunas, 1983). In addition, double quantum filtered-correlation spectroscopy and 2-Dimentional ROE spectra were used to characterise the inclusion complexes formed between β-CD and prednisolone, and β-CD and methylprednisolone (Larsen et al., 2005). NMR spectroscopy analysis of CD complexation is presented in Chapter 2.

UV-Vis spectroscopy is a valuable tool in monitoring formation of CD inclusion complexes by their influence on measured spectra, and is comparable with other techniques in the quantitative study of complex stability constants, since the measured absorbances are proportional to the respective concentrations by virtue of the Lambert-Beer law (Connors, 1997). The technique is widely applicable to CD complex formation with guest molecules that are absorbing strongly, with the shift of the UV absorption maximum on inclusion complex formation most likely being caused by a partial shielding of the excitable electrons in the CD cavity (Szejtli, 1982). These effects of CD inclusion on the absorption spectra of guest molecules are frequently weak, characterised by peak shifts of the order of a few nm, and changes of the extinction coefficients rarely exceed 10 percent. In despite of the minimal effects encountered in the absorption of CD inclusion complexes, the technique of UV-Vis spectroscopy is nevertheless employed frequently as an easily performed preliminary-test for the occurrence of CD inclusion complexation. For instance, UV-Vis spectroscopy was used in the characterisation of dexamethasone/hydroxypropyl-β-CD inclusion complexes and dexamethasone acetate/hydroxypropyl-β-CD inclusion complexes (Usayapant et al., 1991).

Fluorescence emission spectroscopy is a potentially useful method for determining CD inclusion complex geometry of fluorescent guest molecules, since the fluorescent properties of many drugs are dependant largely on their local environment (Cox and Turro, 1984; Cox et al 1984). Fluorescing guests frequently show a significant increase in fluorescence quantum yield and lifetime with increasing CD concentration, thus signifying the formation of CD inclusion complexes (Connors, 1997). In addition, the emission maximum of guest molecules is shifted towards shorter wavelengths upon CD inclusion complexation. The sensitivity of the measurements in fluorescence emission analysis is such that typically a very diverse range of CD and drug concentrations is accessible. Recent research utilising fluorescence emission spectroscopy in
characterising CD inclusion complexation include analysis of the intermolecular interactions of \( \alpha \)-CD, \( \beta \)-CD, \( \gamma \)-CD and heptakis(2,6-di-O-methyl)- \( \beta \)-CD with syringic acid in aqueous solution (Song et al., 2008) and inclusion complexes of \( \beta \)-CD with L-tyrosine (Shanmugam et al., 2007).

**Circular dichroism** is a spectroscopic technique that can also provide useful information about CD inclusion complexation (Connors, 1997). Circular dichroism is a technique that detects the differential absorption of circularly polarised light passing through a chiral substance. The natural CDs do not absorb light in the visible or ultraviolet wavelength range (220 - 700 nm) and, therefore, show no circular dichroism, despite being chiral compounds. These CDs can, however, induce intermolecular circular dichroism (induced circular dichroism), if they form inclusion complexes with achiral compounds bearing observable chromophoric groups (Connors, 1997). This induced chirality is a direct measure of the formation of a complex and allows determination of the complex stability constant. Szegvári (2008) recently utilised circular dichroism to analyse the inclusion complex formation between steroids and hydroxypropyl-\( \gamma \)-CD and \( \gamma \)-CD, where circular dichroism spectra provide information about the examination of their cyclodextrin-mediated enantioselective solubility (Szegvári, 2008).

**Differential scanning calorimetry** (DSC) is a complementary tool for the confirmation of CD inclusion complex formation with drug molecules, especially when used in combination with other experimental techniques, such as NMR spectroscopy (Szejtli, 1982). In DSC, the sample under examination is heated/-cooled gradually at a certain rate, and the molecular events caused by the temperature change (phase transitions, melting, decomposition, etc...), are recorded as heat released or absorbed. When guest molecules are included in CD cavities, their melting, boiling, or sublimation points shift to different temperatures or disappear. Typically, during DSC analysis of CD inclusion complexes with amorphous CD derivatives, an endothermic peak is observed in the DSC thermogram of the guest molecule or physical mixture, located at the melting point temperature for the crystalline drug. This endothermic peak disappears when a complex is formed as a result of the inclusion complex being an amorphous material (Yan et al., 2008). Evidence of CD inclusion complex formation using DSC has been used for hydrocortisone/hydroxypropyl-\( \beta \)-CD complexation (Filipović-Grdić et al., 2000), hydrocortisone/\( \beta \)-CD complexation (Cavalli et al., 1999), prednisolone/\( \beta \)-CD...
complexation and prednisolone/hydroxypropyl-β-CD complexation (Fatouros et al., 2001).

**Isothermal titration calorimetry** (ITC) is often employed to confirm CD inclusion complexation, as well as provide thermodynamic binding data, and information regarding the stability constant and stoichiometry of the inclusion complex (Loftsson and Brewster, 1996). During ITC, one component (CD or drug) is titrated in a stepwise fashion into a solution of the other component, with all the molecular binding events that occur upon this addition being registered in a thermogram. As ITC is a highly sensitive technique the associated energy changes of complexation can be recorded successfully for even very weak interactions (Buckton and Beezer, 1991). Examples of ITC used in CD/steroid inclusion complex analysis include research by Cooper et al., (2005) and Liu et al., (2004). **ITC analysis of CD complexation in presented in Chapter 2.**

The **phase-solubility method** developed from the research of Higuchi and Connors (1965), is a typically applied method for the analysis of CD inclusion complex formation. Phase-solubility analysis is based on CDs acting as solubilising agents to poorly water-soluble guest molecules and examines the solubilising effect CDs have on guest molecules by producing plots of drug solubility versus CD concentration, known as phase-solubility profiles or diagrams (Loftsson et al., 2005b). The phase-solubility method is used often to obtain the stoichiometry of an inclusion complex and values for the stability constant. Recent examples of the phase-solubility method being used in CD inclusion complex studies include analysis of dexamethasone acetate/β-CD complexes (Doile et al., 2008), and inclusion complexes formed between prednisolone and methylprednisolone with α-, β-, and γ-CDD as well as hydroxypropyl-, sulfobutyl ether-, glucosyl-, and maltosyl-β-CD, and sulfobutyl ether γ-CD (Larsen et al., 2005), to name a few. **Further details on the use of the phase-solubility method in CD analysis are located in Chapter 2.**

In addition to the methods described above CD inclusion complexes have been analysed by **polarography** (Yanez et al., 2004; Ziémons et al., 2007), **infrared spectroscopy** (Aleem et al., 2008; Shivakumar et al., 2007), **Raman spectroscopy** (Yang et al., 2008; Rossi et al., 2006), **electron spin resonance spectroscopy** (Jullian et al., 2007), **thin-layer chromatography** (Guo et al., 2004), **mass-spectrometry** (Frycák and Schug, 2008; Short and Syage, 2008), **differential thermal analysis** (Shen et al., 2005),
thermal gravimetric analysis (Zoppetti et al., 2007), and X-ray crystallography (Reddy et al., 2004).

1.5.7 Cyclodextrin influences on the properties of drugs

1.5.7.1 Cyclodextrins and drug dissolution, solubility and bioavailability

When a drug is presented to the body (via the various routes of administration), it typically needs to be in a molecularly dispersed form, i.e. in solution, before it can be absorbed across biological membranes (Florence and Attwood, 1998). The dissolution process of solid drug must precede drug absorption to allow pharmacological affect to take place. Drugs of poor aqueous solubility, such as corticosteroids, present consistent problems regarding their formulation and bioavailability (Bittner and Mountfield, 2002). For instance, drug dissolution of solid drug particles in a tablet dosage form, can potentially result in drug absorption into the blood circulation to be dissolution rate-limited, as a direct consequence of the poor aqueous solubility of the drug (Loftsson et al., 2004a).

Various drug delivery strategies exist for poorly water-soluble drugs (Fahr and Liu, 2007). CDs can enhance the apparent aqueous solubility of drugs by forming water-soluble inclusion complexes with the poorly soluble compounds (Loftsson and Brewster, 1996; Loftsson et al., 2005b). CDs, therefore, enable solution-based formulations such as parenteral and oral liquids to be made with drugs of poor aqueous solubility (Szejtli, 2004). Furthermore, in the solid-state, CDs enhance dissolution through the formation of solid dispersions, whereby the drug of interest (usually crystalline), is converted into a dispersion of the amorphous drug within the CD (Leuner, 2000; Londhe and Nagarsenker, 1999). Drugs with a lower intrinsic aqueous solubility typically obtain a greater solubility enhancement than drugs with a higher intrinsic aqueous solubility when they are complexed to CDs. According to the US Food and Drug Administration (FDA) Biopharmaceutics Classification System (Amidon et al., 1995), drugs can be classified as Class I to Class IV:

- Class I includes highly soluble and highly permeable drugs.
- Class II includes poorly soluble and highly permeable drugs.
- Class III includes highly soluble and poorly permeable drugs.
- Class IV includes poorly soluble and poorly permeable drugs.

Loftsson et al., (2004a) proposed that CDs can enhance absorption of Class II and Class IV drugs, but only modify oral absorption and/or reduce local irritation by drugs of
Chapter 1 - General Introduction

Classes I and III. The majority of studies of CDs with corticosteroids have demonstrated that CDs can enhance the water solubility of poorly soluble drugs. Examples include α-, β-, and γ-CD, as well as hydroxypropyl-, sulfobutyl ether-, glucosyl-, and maltosyl-β-CD, and sulfobutyl ether γ-CD enhancing the solubility of prednisolone and methylprednisolone, although the derivatised CDs enhanced the solubility of these drugs much more than the natural CDs (Larsen et al., 2005).

Since CDs increase the apparent solubility and enhance dissolution kinetics of poorly water-soluble drugs, CDs frequently enhance the bioavailability of hydrophobic drugs by making the drug available at the surface of the biological barrier, e.g., gastrointestinal mucosa, eye cornea, or skin (Loftsson et al., 2005a; Loftsson et al., 2007a). CDs have, therefore, been seen to enhance the bioavailability of drugs, such as corticosteroids, at various biological barriers. Reviews of CDs and their bioavailability enhancement include: Carrier et al., (2007) for oral bioavailability, Kaur et al., (2004) for ophthalmic bioavailability, Loftsson and Olafsson, (1998) for subcutaneous/transdermal bioavailability, whilst Gudmundsdottir et al., (2001) demonstrated increased bioavailability via the nasal route, and Jalalipour et al., (2008) showed enhanced bioavailability via the pulmonary route. In some circumstances, when present in excess, CDs were found to reduce drug bioavailability in topical drug delivery to the eye because the equilibrium was driven towards CD complexation in the absence of adequate dilution media (Loftsson and Stefansson, 1997; Van Dorne, 1993).

1.5.7.2 Cyclodextrins and drug stability

A drug must be stable during storage and whilst in biological fluids, since production of molecules that are pharmaceutically less active than the parent drug will often reduce the therapeutic effectiveness (Florence and Attwood, 1998). Stability issues, therefore, hinder the formulation of some drugs. A major benefit of CD inclusion complexation is the influence it has on the chemical and physical stabilities of drugs in solution and in the solid state (Loftsson and Brewster, 1996). CDs have the ability to accelerate or retard various degradation reactions that affect labile compounds (Loftsson et al., 2005a), and the effects are more apparent when the whole drug or labile moiety is deep inside the CD cavity. At the molecular level, CD inclusion complexation of a labile drug is akin to molecular encapsulation; the CD shields the included drug molecule from various reactive species or hostile environments. In contrast to the stabilising effect CDs have on drugs, CDs have also been shown to produce a destabilising effect on drugs as a result of inclusion complexation. For instance, moisture-promoted solid-
Chapter 1 - General Introduction

State decomposition of carmofur, an anticancer agent, was accelerated upon complexation with β-CD since the solid drug-CD complexes were more water-soluble than the pure lipophilic drug (Loftsson and Brewster, 1996).

1.5.7.3 Cyclodextrins and drug safety

As a consequence of increased drug efficacy and potency from CD-increased solubility, dissolution, stability and bioavailability, CDs can be used to ameliorate the tissue irritation and toxicity caused by drugs (Rajewski and Stella, 1996). For instance, Piao et al. (2008) reported that naproxen tissue damage in the colon could be reduced with complexation with hydroxypropyl-β-CD. β-CD also reduced the drug toxicity of ganciclovir whilst enhancing its antiviral activity (Nicolazzi et al., 2002). Crystallisation of poorly water-soluble drugs in parenteral formulations can lead to various toxicity issues after injection. This can be reduced by CD complexation (Brewster et al., 1989). This was illustrated by phenytoin/hydroxypropyl-β-CD complexes producing a significant decreased tendency to precipitate in vitro, with tissue irritation reduced considerably compared to the commercial injection in a BALB/c mouse model (Blanchard et al., 2000). CDs can, however, enhance toxicity and irritation of drugs by enabling more drug to be in solution at vulnerable or susceptible locations. For example, the hepatotoxicity of clotrimazole was enhanced by inclusion complexation with β-CD (Yong et al., 2007).

1.5.7.4 Cyclodextrins and drug taste

Since 1953 formulation scientists have been aware of the taste masking characteristics of CDs. In the research that led to the very first CD/drug patent were details regarding the taste masking of bromoisovaleryl urea by CDs (Freudenberg et al., 1953). The taste masking ability of CDs is believed to be via taste deception and taste obstruction methods. Firstly, and predominantly, CDs prevent bitter molecules from interacting with the taste receptor cells on the tongue by forming an inclusion complex with the molecule. As the molecule is “entrapped” by the CD it is unable to evoke its usual taste and is taste masked accordingly. As CDs are sugar molecules they elicit a sweet taste, which can potentially “over-ride” the bitter taste of various compounds and produce a sweet taste. This is supported by evidence that a 2.5 % β-CD solution was as sweet as 1.71 % sucrose solution (Toda et al., 1985). CDs have been consequently utilised to modify the taste of numerous varieties of food and drink, ranging from coffee and tea (Hamilton and Heady, 1970), to soy-bean products (Norinsuisansho et al., 2000).
Similarly, CDs have been shown to eliminate the bitter tastes of various drugs in solid and liquid oral formulations (Szejtli and Szente, 2005). Only dissolved drug molecules can elicit taste and since a certain concentration of drug does not complex with the CD, but resides in the solution uncomplexed, the concentration of CD in solution is frequently in excess to ensure that a maximum amount of drug is being complexed by the CD. For instance, the bitterness of ibuprofen solutions was reduced using 11 and 15 molar excess of hydroxypropyl-β-CD in a 1:1 complex (Motola et al., 1991). Nevertheless, excess amounts of CD may not enhance taste masking compared to a molar amount of CD equal to the concentration of the drug and, therefore, the excess amount may be unnecessary. The addition of 11 and 15 molar excess of CD to the solution may drive the equilibrium to greater complexation but a certain concentration of drug (typically equal to the intrinsic solubility of the drug) will remain uncomplexed and detectable by the taste receptors. Thus, lower concentrations of CD may have equivalent taste masking. Furthermore, whilst adding excess CD may prove to enhance taste masking of some bitter drugs it may not successfully work for others. For example, if the complexed drug molecules are not encapsulated fully within the cavities of the CD molecules, and part of the drug molecules are exposed, there might be a chance that the drug molecules will still be able to interact with the taste receptors of the tongue, and evoke a bitter taste. Bitterness taste-masking of drugs in liquid formulations with CDs will be evaluated/investigated during part of this PhD research.

1.5.8 Pharmaceutical application of cyclodextrins

CDs have been incorporated into 37 pharmaceutical products (as of 2004) (Loftsson et al., 2004a; Davis and Brewster, 2004), where they are mainly used to increase aqueous solubility, increase stability and bioavailability of drug molecules (Avdeef et al., 2007; Kim and Park, 2004; Loftsson et al., 2005a). The products encompass a wide array of administration routes, namely: oral, parenteral, ophthalmic, nasal, and rectal, and in a variety of dosage forms (Loftsson et al., 2004a; Davis and Brewster, 2004). Few are, however, licensed for persons under 18 years of age, let alone neonates or infants. From the vast amount of research literature involving the use of CDs in drug delivery there are little data available with regard to paediatrics. Table 1.7 lists the products that have been identified as being marketed with CDs in the formulation and focuses on the type of formulation in which the CD is used.
In addition to the marketed pharmaceutical formulations, continuing research is leading towards the development of CDs in dermal and transdermal drug delivery, brain drug delivery and brain targeting, controlled release delivery, colon-specific drug delivery, peptide and protein delivery, and gene and oligonucleotide delivery (Loftsson et al., 2005a). Aside from these more conventional delivery methods there was recent...
development in the design of novel delivery systems that incorporate the use of CDs. These novel delivery systems include liposomes, microspheres, microcapsules, nanoparticles, CD grafted cellulosic fabric, hydrogels, nanosponges, beads, nanogels/nanoassemblies, and CD containing polymers (Vyas et al., 2008). As illustrated in Table 1.7 the only marketed oral solutions that contain CDs are an intraconazole formulation and an iodine gargle. Nevertheless, many articles can be found investigating clinical, intestinal perfusion and/or bioavailability of various CD/drug complexes in oral liquid formulations.

1.5.9 Drug absorption from cyclodextrin inclusion complexes in vivo

CDs act primarily as carrier molecules to the site of absorption, transporting the drug molecule through an aqueous milieu to the lipophilic biological membrane. Since the biological membrane has a higher affinity for the lipophilic guest molecule compared to the CD cavity, the guest molecule dissociates from the CD and passes across the membrane into the general blood circulation (Fig 1.10) (Loftsson et al., 2007). Nonetheless, if the CD/drug complex is very stable (i.e. large stability constant), or the CD concentration is high, the inclusion complex equilibrium is shifted greatly towards complexation and absorption is considerably retarded regardless on the class of drug. (Pitha et al., 1983; Szejtli 1985; Rajewski and Stella, 1996).

![Figure 1.10: A schematic representation of the mode of drug absorption through biological membranes from CD inclusion complex (taken from Challa, 2005). Key: C = cyclodextrin, D = drug](image-url)

In general CDs do not enhance permeability of hydrophilic water-soluble drugs through lipophilic biological membranes (Loftsson and Masson, 2001). Since CDs posses characteristics that typically do not allow for biological membrane permeation i.e. molecular weight > 970 Da, large number of hydrogen donors and accepters, very low octanol/water partition coefficient (approximately log $P_{ow}$ between -3 and 0 (Moldenhauer, 2004)), CDs only permeate lipophilic membranes in small amounts, thus
limiting their role as membrane permeating enhancers (Irie and Uekama, 1997; Matsuda and Arima, 1999). Nevertheless, lipophilic CDs, such as methylated-β-CD, are capable of permeating biological membranes by reducing barrier function and could act potentially as active transporters (Marttin et al., 1998).

1.5.10 Cyclodextrin limitations in research & development

During formulation development the role of every excipient has to be carefully considered; the addition of CDs is no different. The benefits and risks of making use of CD inclusion complexes must be made apparent and thorough exploration of these characteristics needs to be conducted. A formulator must be aware of the limitations an excipient possesses, so that they do not become a hindrance later on in development.

Points to be addressed when preparing a CD/drug inclusion complex include:

- The ability of a compound to form an inclusion complex with CDs - some compounds do not form inclusion complexes with CDs as they do not have the correct geometry in relation to the CD cavity.
- Required drug dose in relation to drug molecular weight and stoichiometry of the complex - these factors determine the feasibility of administration of a CD inclusion complex, especially in tablet form. If a high dosage is required, large amounts of CD complex would be required that may make the dosage form size/volume unfeasible, e.g. too much bulk to make a conventional tablet.
- The stability of the CD inclusion complex in solution characterised by the stability constant - if the stability constant is too high then drug release will be slow: if the stability constant is too low then the guest will not stay complexed for very long and the CD may, therefore, be disadvantageous.
- CD interaction with other excipients in the formulation - other excipients can form inclusion complexes with CDs, thus competing with drug molecules for the CD cavity. This was demonstrated by CDs interacting with preservatives in a formulation (Lofthsson et al., 1992).
- CD interaction with other substances in the gastrointestinal tract, e.g. fat soluble vitamins, polyunsaturated fatty acids, and bile salts (Munro et al., 2004)

The natural CDs are no longer under patent protection; patents for certain methods of manufacture on the other hand are still in place. There are also many unexpired patents claiming specific complexes with natural CDs. The situation varies for CD derivatives and their complexes. For example, the hydroxyethylated β-CD derivatives, such as hydroxypropyl-β-CD, have been established for almost 20 years and initial patents have
expired. Nevertheless, Johnson & Johnson has a patent on pharmaceutical application of hydroxypropyl-\(\beta\)-CD in the USA. Patents are also in place on methods to enhance the performance of CDs, such as the addition of water-soluble polymers to improve CD-solubilising effects. In Europe, Jansen Pharmaceutical Co of Belgium has patents relating to pharmaceutical compositions containing hydroxypropyl-\(\beta\)-CD complexed to drugs that are unstable or sparingly soluble in water. The majority of CD-related patent applies to specific drug/CD complexes. These patents limit the freedom of other pharmaceutical companies to operate and use the patent protected CDs.

The regulatory status of CDs continues to develop with numerous pharmaceutical formulations containing CDs now on the market (Table 1.7). In terms of pharmacopoeia monographs, as of 2006, \(\alpha\)-CD appears in the European pharmacopeia (Ph.Eur) and Japanese pharmacopeia (JP), \(\beta\)-CD appears in the USA pharmacopeia (USP/NF), Ph.Eur and JP, \(\gamma\)-CD is in the process of being in the Ph.Eur and is in the USP/NF and JP, and hydroxypropyl-\(\beta\)-CD appears in Ph.Eur and USP/NF.

### 1.5.11 Safety and fate of cyclodextrins in vivo

Safety is paramount when considering new excipients intended for use in pharmaceutical formulations, especially formulations developed for the paediatric population. The toxicological issues together with the biological fates of CDs must be investigated thoroughly before practical use can be considered. Safety profiles and toxicological evaluations for the natural CDs and some of their derivatives were conducted from numerous animal studies and human clinical trials *in vivo* (Irie and Uekama 1997; Thompson 1997; Munro *et al.*, 2004; Gould and Scott 2005; and Rajewski *et al.*, 1995). The majority of natural CDs and their hydrophilic derivative do not permeate biological membranes, because the chemical and physical attributes they possess do not warrant permeation across lipophilic membranes. These CDs are considered practically non-toxic when administered orally, topically and ophthalmically. In addition, \(\gamma\)-CD, sulphobutyl ether-\(\beta\)-CD, sulphated-\(\beta\)-CD, maltosyl-\(\beta\)-CD, and hydroxypropyl-\(\beta\)-CD all appear to be harmless when administered parenterally (Irie and Uekama 1997). These parameters have been carried into the marketed products with the natural CDs and their hydrophilic derivative making up the majority of the CD-containing formulations. Lipophilic methylated-\(\beta\)-CDs are capable of permeating biological membranes and as such, the use of various methylated-\(\beta\)-CDs has been limited in oral formulations. The use of methylated-\(\beta\)-CDs in parenteral
formulations is also deemed unsuitable, whilst α-CD has to be limited and the use of β-CD is not permitted parenterally.

When administered orally the fate of the natural CDs varies between digestion by microflora of the colon and small intestine, and endogenous enzymatic degradation of the gastrointestinal secretions. Natural CDs are degraded generally by gastrointestinal bacterial flora into glucose and its oligomers, and excreted subsequently as \( \text{CO}_2 \) and \( \text{H}_2\text{O} \), thus producing no toxic degradents (Pitha and Szente, 1982). Antenucci and Palmer (1985) reported that 24 of the 30 bacteroid strains isolated from the human colon were capable of degrading CDs, indicating that colonic bacteria may play a role in the digestion of CDs. Furthermore, when a daily dose of 10 g β-CD was given orally to adult humans the faecal \textit{Bifidobacterium} content increased 10 to 100 fold within two weeks, further supporting the involvement of colon microflora in the metabolism of β-CD (Szejtli, 1987). A typical transit time through the colon is sufficient for the bacterial enzymes to hydrolyse the natural CDs partially or completely. Aside from microflora degradation, various starch-degrading enzymes can cleave the glycosidic bonds of CDs with the necessary substrate specificity, having a reaction rate much slower than that of the comparative linear sugars (Szejtli, 1988). For instance, it was found that human salivary and pancreatic α-amylase can hydrolyse γ-CD, although α-CD and β-CD are practically resistant (Marshall and Miwa, 1981). CD derivatives take longer to degrade via these enzymes than parent CDs because of a reduced conformational fit between the enzymes and CD molecules. When CD derivatives are administered orally, they are particularly resistant to the degradation of microflora and the amylases, and are excreted intact in the faeces.

A very small percentage of CDs administered orally reach the systemic blood circulation and join the fate of CDs that are administered parenterally. Methylated-β-CDs can, however, pass successfully through the mucus membranes of the gastrointestinal tract. Once in the systemic circulation CDs are removed rapidly via the urine and a small percentage in the faeces. The elimination of CDs is, therefore, strongly dependant on renal function. α-CD and β-CD are excreted typically as whole molecules in the urine, whilst γ-CD undergoes some breakdown (Irie and Uekama, 1997). As such, a high intake of β-CD may prove troublesome, since β-CD has demonstrated nephrotoxicity (Antlsperger and Schmid, 1996). Nevertheless, an acceptable daily intake (ADI) for CDs has not been established fully. The World
Chapter 1 - General Introduction

Health Organisation has only appointed a 5 mg/kg/day ADI for β-CD whilst no other ADI has been established for the remaining CDs.

There are very few studies involving the safety of CDs in the paediatric population. A study regarding the repeated-dose pharmacokinetics of an oral solution of itraconazole in infants and children (six months - 12 years), reported less than 1 % of the hydroxypropyl-β-CD incorporated in the formulation was excreted in the urine by the majority (11 out of 14) of the paediatric patients (six months - 12 years) (de Repentigny et al., 1998). This falls in line with evidence that demonstrates that the majority of CDs are not absorbed by the biological membranes of the gastrointestinal tract (Irie and Uekama, 1997). In a similar study Abdel-Rahman et al., (2007), found that the concentration of hydroxypropyl-β-CD in an intravenous intraconazole formulation fell below quantification limits 12 hours after administration to infants, children and adolescents, and that total plasma clearance approximated estimates of the glomerular filtration rate. Both studies concluded that hydroxypropyl-β-CD had no adverse effects to paediatric patients, thus indicating that the CDs, which are safe via oral and parenteral routes in adults, are likely to be safe in children.

The adverse effects of dimethyl-β-CD, hydroxypropyl-β-CD, and sulfobutyl ether- β-CD on human intestinal epithelial Caco-2 cells were investigated during development of paediatric oral solutions of spironolactone, where dimethyl-β-CD showed clear cytotoxic effects on human intestinal epithelial Caco-2 cells (Totterman et al., 1997). Continuing this work Kaukonen et al., (1998), recommended a reduction of spironolactone dosage during clinical studies with premature infants. Their results also indicate that sulfobutyl ether- β-CD could be a safe and suitable excipient for the solubilisation of spironolactone in paediatric formulations.

From the 37 marketed products listed in Table 1.7 it was found that only six of the formulations are licensed for paediatric patients. The alprostadil, piroxicam and nimesulide formulations have guidance for use in paediatric patients according to their patient information leaflets; the voriconazole preparation can be used by infants two years and above; the iodine gargle can be used by infants six years and above; the cephalosporin tablets can only be used by persons 12 years and above. Interestingly, according to the patient information the itraconazole intravenous and oral solution use in children is not recommended unless the potential benefit outweighs the potential risks, since clinical data on the use in paediatric patients is limited.
1.5.12 Cyclodextrins and corticosteroids

As yet there are only two corticosteroid formulations on the market that contain CDs: a dexamethasone dermal ointment in Japan and a hydrocortisone mouthwash in Iceland. As per all other classes of drug there has been plenty of research focused on the use of CDs in various corticosteroid preparations, where CDs are reported to enhance corticosteroid solubility, dissolution, stability and bioavailability. For instance, β-CD, γ-CD, dimethyl-β-CD, trimethyl-β-CD, methyl-β-CD, randomly methylated-β-CD, hydroxypropyl-β-CD, and hydroxypropyl-γ-CD were studied for their influence on aqueous solubility of betamethasone by phase solubility studies and on the encapsulation efficiency in liposomes (Piel et al., 2006). In addition, γ-CD was complexed with budesonide to aid pulmonary delivery of the drug (Kinnarinen et al., 2003). There is, however, very limited research focused on the application of CDs in oral liquid corticosteroid formulations, especially as a taste masking agent, as well as being focused on paediatric patients.

1.6 Aims and objectives

The main dilemmas concerning paediatric pharmaceutics and administration of corticosteroids to paediatric patients have been discussed and provide some background for the work in this thesis. The gustatory system, taste masking of bitter drugs and taste assessment of formulations were also addressed. The use of CDs as an excipient in an oral liquid corticosteroid formulation was put forward and consequently a brief review of CDs was presented. Thus, the overall aims of this thesis were to: 1) explore whether, and how, CDs could be used in oral liquid paediatric corticosteroid formulations to provide an accurate and reproducible dose through improved solubility, and palatability via bitterness taste masking; 2) explore the use of a novel in vitro taste analyser for taste masking assessment of liquid formulations.

The objectives were:

- To characterise aqueous inclusion complex formation between corticosteroids and CDs, via phase solubility studies, ITC studies and NMR studies.
- To determine the influence CD on the preservative efficacy of preservatives.
- To assess the taste masking ability of CDs in comparison with a traditional taste masking technique.
- To compare and contrast traditional taste assessment in vivo and a novel taste assessment in vitro technique.
Chapter 2 - Characterisation of Inclusion Complex Formation
Chapter 2 - Characterisation of Inclusion Complex Formation

2.1 Introduction

Chapter 1 discussed the problems encountered with paediatric medicine and the necessity for paediatric formulation development. CDs were proposed as an excipient in oral liquid paediatric formulations because of their ability to provide solubility enhancement and bitterness taste masking to poorly soluble bitter drugs. In this chapter an attempt is made to characterise the inclusion complex formation between CD and poorly soluble bitter drugs using analytical techniques used commonly for inclusion complex characterisation. The chapter is divided into two sections; the first deals with phase solubility studies while the second deals with isothermal titration calorimetry (ITC) and $^1$H nuclear magnetic resonance spectroscopy ($^1$H NMR spectroscopy) studies.

2.2. Section 1 - Phase solubility studies

2.2.1 Introduction

As explained in Chapter 1, the most frequently used technique to study inclusion complex formation is the phase solubility method as described by Higuchi and Connors (1965), and involves solubility determinations of drug (D) in the presence of CD. Phase solubility studies can be used to characterise inclusion complex formation, such as deducing the value of the complex stability constant ($K_c$) and provide data to determine the equilibrium stoichiometry. By carrying out phase solubility studies with various drugs and CDs conclusions can be made regarding the affinity of differing drugs to a particular CD, or differing CDs to a particular drug, under various experimental conditions.

Phase solubility studies are carried out by adding a constant volume of aqueous CD solution to an excess of drug contained in several vials - each solution having a successively higher concentration of CD. The vials are heated/sonicated to produce a supersaturated suspension and subsequently agitated at constant temperature until equilibrium is established. Excess solid drug is filtered away and the drug concentration is quantified by analytical means. Plotting the total molar concentration of drug on the $y$-axis and the total concentration of CD on the $x$-axis generates a phase solubility diagram. Initial characterisation regarding whether a particular CD and drug form soluble inclusion complexes begins with assigning a phase solubility profile to the phase solubility diagram. Phase solubility profiles fall into two main categories: A-type and B-type (Figure 2.1).
Chapter 2 - Characterisation of Inclusion Complex Formation

Figure 2.1: Theoretical representations of A- and B-type phase solubility diagrams including applicable subtypes (A_P, A_L, A_N, and B_S, B_I), according to Higuchi and Connors (1965)

An A-type profile illustrates soluble inclusion complexes were formed and the apparent solubility of the drug increases as a function of CD concentration. There are three subtypes of A-type profile: A_L profiles represent linear increases of drug solubility, as a function of CD concentration and are first order with respect to CD (i.e. DCD, D_2CD, D_3CD,...D_nCD). A_P profiles possess a positively deviating isotherm and indicate the formation of higher order inclusion complexes with respect to CD at higher CD concentrations (i.e. DCD, DCD_2, DCD_3,...DCD_n). A_N profiles have a negatively deviating isotherm and are likely due to an alteration in the effective nature of the solvent in the presence of a high CD concentration, thus leading to a change in the stability constant, or can be caused by a self-association of CD molecules at high concentration, thus reducing the ability of CDs to form inclusion complexes. A solubility plateau may also be seen with A_N profiles, which can be caused by the disappearance of solid drug from the system (i.e. the drug was not in excess), or saturation of the system with respect to CD.

A B-type profile is observed when insoluble complexes are formed between CD and drug, and can be a result of numerous processes. There are two subclasses of B-type profile: B_S profiles possess the initial solubility trend similar to an A-type profile, where the drug concentration increases as a function of CD. At a certain point the solubility limit of the inclusion complex is reached and the linear increase in drug
Chapter 2 - Characterisation of Inclusion Complex Formation

Solubility is halted. Addition of further CD results in formation of more inclusion complex that precipitates resulting in a drug solubility plateau. The drug concentration is maintained by constant dissolution of solid drug or solid inclusion complex, but ceases when the excess solid drug depletes, and further addition of CD causes inclusion complex formation and concomitant precipitation of insoluble complex. Bi profiles are similar to Bs profiles with a drug solubility plateau and decrease, except the inclusion complexes are so insoluble that the initial rise in drug concentration is not detectable.

By utilising the initial linear segment of the phase solubility diagram, the stability constant of inclusion complex formation (Kc or K_m:n), the characteristic that describes the strength of interaction between a CD and a drug, can be determined (Higuchi and Connors, 1965).

The Kc of a typical 1:1 inclusion complex (D1CD1), where one drug molecule (D) forms a complex with one CD molecule (CD), can be represented by:

\[ K_{1:1} = \frac{[DCD]}{[D][CD]} \]  

Equation 2.1

Under such conditions an A1-type phase-solubility diagram, with slope less than unity, would be observed, and the K_{1:1} of the complex can be calculated from the slope and the intrinsic solubility (D0) of the drug in the aqueous complexation media (i.e. the equilibrium solubility of the drug when no CD is present), by Equation 2.2 (Loftsson et al., 2005a):

\[ K_{1:1} = \frac{\text{Slope}}{D_0[1-\text{Slope}]} \]  

Equation 2.2

In addition to the K_{1:1}, the complexation efficiency (CE) can be determined from the phase solubility diagram and provides an alternative measurement of the ability of a particular CD to form an inclusion complex with a particular drug (Loftsson et al., 2007b). Also known as the solubilising efficiency, the CE is equal to the complex to free CD concentration ratio (DCD/CD) and can be deduced from the slope of the phase solubility diagram:

\[ CE = \frac{\text{DCD}}{\text{CD}} = \frac{\text{Slope}}{(1 - \text{Slope})} \]  

Equation 2.3
Chapter 2 - Characterisation of Inclusion Complex Formation

Where DCD and CD represent the concentration of dissolved complex and free CD, respectively. The CE can also be used in an alternative means to determine the exact molar ratio of the inclusion complex (Loftsson et al., 2007b):

\[
DCD = \frac{1}{1 + \frac{1}{CE}}
\]

Equation 2.4

It should be noted that during the data analysis of a phase solubility diagram, a 1:1 DCD inclusion complex is assumed unless otherwise stated. The molar ratio still assumes a 1:1 complex, but states out of how many CD molecules it takes to form a 1:1 inclusion complex. For instance, a molar ratio 1:3 (drug:CD) means that on average only one out of every three CD molecules in solution are forming water-soluble complexes with the drug (Loftsson et al., 2005). In addition, when calculating the number of CD molecules the number of molecules is always rounded up to the nearest whole molecule.

2.2.2 Aims

Part 1 - Phase solubility study of prednisolone and hydrocortisone in cyclodextrin systems

The first aim of this chapter was to determine, by phase solubility studies, whether corticosteroids form soluble inclusion complexes with CDs. To achieve this aim prednisolone (Pd) and hydrocortisone (HC) were chosen as model corticosteroid drugs. Pd was selected because the literature has highlighted a great deal of concern regarding Pd administration to paediatric patients (Lucas-Bouwman et al., 2001; Dawson et al., 1992). For example, a fully taste masked oral liquid preparation of Pd was better tolerated than a simplistic oral liquid formulation (Kim et al., 2006). HC, being the synthetic version of cortisol and chemically very similar to Pd, was selected to provide comparative data to Pd. In addition, in a recent study of extemporaneous preparations in various NHS trusts throughout the UK, HC was the highest placed corticosteroid for total units of extemporaneous formulations made (Lowey and Jackson, 2008), and, therefore, makes an ideal model corticosteroid for this research.

To provide comparative data on the ability of CDs to form inclusion complexes with corticosteroids, four CDs were selected. It was deemed applicable that two of the CDs should be natural CDs and, therefore, β-CD and γ-CD were chosen: β-CD being deemed the most useful complexing agent (Szejtli, 1988), and γ-CD having the largest
Chapter 2 - Characterisation of Inclusion Complex Formation

internal cavity. In addition to the natural CDs, two CD derivatives (hydroxypropyl-\(\beta\)-cyclodextrin (HP-\(\beta\)-CD) and methyl-\(\beta\)-cyclodextrin (Me-\(\beta\)-CD)) were selected, because of the physicochemical enhancements that they have over the natural CDs.

To gain assurance in the results that were produced, an aim of this chapter was to perform a method validation on the phase solubility methods that were carried out. As the phase solubility samples were taken to supersaturation via heating, the degradation of drug by the heating process was monitored. In addition, as all of the samples contained excess drug and required filtering, the loss of drug through filtering was determined. The time necessary for solubility equilibration was also examined because the time stated in the literature varies for different drugs and CDs. Finally, because the concentration of drug in the phase solubility samples was quantified via HPLC, an accurate and efficient HPLC method was required and, therefore, various parameters were optimised.

Part 2 - Phase solubility study of prednisolone and hydrocortisone in cyclodextrin systems in the presence of parabens

As mentioned in Chapter 1, various additives can affect inclusion complex formation between a particular CD and drug. Within any oral liquid formulation there is a need for preservatives. Previous research has shown that CDs increase preservative solubility (Matsuda et al., 1993), as well as reduce antimicrobial efficacy because of CD/preservative inclusion complex formation (Loftsson et al., 1992). From the preservatives that were shown to form inclusion complexes with CDs, the \(p\)-hydroxybenzoic acid esters (parabens) readily form complexes with various CDs (Matsuda et al., 1993; Tanaka et al., 1995; Zughul et al., 1998; Chan et al., 2000). As parabens are often included in oral liquid formulations, the aim of this part of the chapter was to investigate the affect that dissolved parabens had on the ability of the selected CDs to form inclusion complexes with the selected corticosteroids. This particular research has yet to be published in the literature and represents a novel aspect of this thesis. It was hypothesised that the parabens would not disrupt inclusion complex formation between the CDs and corticosteroids, because the parabens were already dissolved in solution and, therefore, would not require the solubilising aid of the CDs to become solubilised.
Chapter 2 - Characterisation of Inclusion Complex Formation

Part 3 - Phase solubility study of hydrocortisone in cyclodextrin systems in various pH and simulated media in vivo

The environment that a formulation will be subject to upon oral administration, namely the saliva, gastric fluid, and intestinal fluid, has the potential to influence CD inclusion complexes because the varied pH, and various soluble components might affect the CD, drug, or complex and, therefore, alter typical inclusion complex equilibrium (Loftsson and Brewster, 1996). As the majority of phase solubility studies are conducted in water, the aim of this part of the chapter was to investigate the influence of various pH and simulated media in vivo on the inclusion complex formation between HC and HP-β-CD and Me-β-CD. It was hypothesised that because HC and the CDs do not have a pKa and are unionizable, they will not be affected by the pH of the various media. In addition, because all of the various compounds within the media are in solution they will not require solubilisation by the CDs and, therefore, will not effect inclusion complex formation.

Part 4 - Phase solubility study of quinine in cyclodextrin systems

In the latter stages of this PhD research the focus of study progressed from phase solubility studies to the ability of CDs to act as taste masking agents for corticosteroids (Chapter 4, 5 and 6). To provide comparative data regarding the ability of CDs to taste mask bitter drug molecules, the model bitter drug quinine (Qu) was selected. Qu not only has two distinct functional groups (quinoline ring and aliphatic ring), and three chiral centers but also plenty of binding sites that can form one or more hydrogen bonds with the hydroxyl group of the CDs. It was, therefore, necessary to carry out phase solubility studies with Qu and the CDs used for the taste masking analysis, so that the inclusion complex formation could be characterised and CD concentrations required for the taste masking analysis could be determined.

2.2.3 Materials and methods

2.2.3.1 Materials

Hydrocortisone (HC) (> 98 % purity), methyl 4-hydroxybenzoate (methylparaben), propyl 4-hydroxybenzoate (propylparaben), urea, potassium thiocyanate, sodium sulphate decahydrate, MES (2-(n-morpholino) ethanesulfonic acid), hydrochloric acid, magnesium sulphate septahydrate, disodium hydrogen phosphate dihydrate, dipotassium hydrogen phosphate, disodium hydrogen decahydrate and calcium chloride were all purchased from Sigma Aldrich (Poole, UK). Prednisolone (Pd) was obtained
Chapter 2 - Characterisation of Inclusion Complex Formation
from Sanofi-Aventis (Romainville, France). Ammonium chloride and anhydrous
quinine (Qu) were acquired from Acros Organics (Geel, Belgium). Pepsin from hog
stomach (Ph Eur) was obtained from Fluka Biochemika (Buchs, Switzerland). Sodium
chloride, potassium chloride, potassium dihydrogen phosphate and calcium chloride
dihydrate were all purchased from BDH Laboratory Supplies (UK). β-cyclodextrin (β-
CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD, MW: 1447) (pharmaceutical grade,
Cavitron™ 82005) were kindly donated by Cargill Inc., (Cedar Rapids IA, USA). γ-
cyclodextrin (γ-CD) was kindly donated by Wacker Biochem Corp (Munich, Germany).
Methyl-β-cyclodextrin (Me-β-CD, MW: 1310) (Kleptose® Crymeb) was kindly
donated from Roquette (Lestrem, France). Acetonitrile (ACN), acetic acid and methanol
were acquired from Fisher Scientific (Loughborough, UK), and were HPLC grade.
Tetrahyrdofuran (THF) (HPLC grade), was purchased from VWR Limited (UK). All
materials were used as received and solutions were prepared using deionised water
produced in the laboratory.

2.2.3.2 Part 1
2.2.3.2.1 Phase solubility study of prednisolone and hydrocortisone in
cyclodextrin systems
The phase solubility experiments for Pd and HC were based on the methods of Larsen
et al., (2005), and Loftsson and Hreinsdottir (2006). To a number of 1.5 mL Safe-Lock
micro test tubes (Eppendorf AG, Germany), containing excess Pd or HC (~50 mg), 1
mL of 0.01 - 0.10 M aqueous HP-β-CD, Me-β-CD, or γ-CD solution (0.0016 - 0.0163
M in the case of β-CD), was added. Triplicates of six different concentrations of CD
were used. Each Safe-Lock micro test tube was sealed and vortexed briefly on a
rotormixer (Hook & Tucker Instruments Ltd), until the drug was dispersed. The
suspensions formed were heated at 60 °C for 60 min in a fan-assisted oven (Termaks
Series TS 800).

After cooling to ambient temperature the Safe-Lock micro test tubes were opened and a
small amount of solid drug (5 mg ± 0.2), was added to the sample to promote drug
precipitation. The samples were resealed and agitated constantly (100 rpm), at room
temperature (23 ± 1 °C), for 6 days using a fixed angle rotator (Baird & Tatlock Ltd), to
allow equilibrium of the supersaturated solution to occur. The samples were filtered
through a 0.45 μm membrane filter (Millipore Corp, Bedford MA, USA), and the drug
concentration quantified by HPLC (Section 2.2.3.2.5). The intrinsic solubility of Pd and
Chapter 2 - Characterisation of Inclusion Complex Formation

HC was determined by following the phase solubility methods with 5 aqueous samples containing no CD. Significant results were expressed as $K_{1:1}$ (Equation 2.2), CE (Equation 2.3), molar ratio (Equation 2.4), and percent complexed CD (derived from phase diagram slope).

2.2.3.2.2 Phase solubility method validation: a) degradation by heating

To a number of Safe-Lock micro test tubes containing excess Pd (~50 mg), 1 mL of 0.04 M HP-β-CD was added. In addition, to a number of Safe-Lock micro test tubes containing 5 mg Pd, 1 mL of deionised water was added. Each Safe-Lock micro test tube was sealed and vortexed briefly on a rotormixer until the drug was dispersed. Half (n = 3), of the 50 mg/mL and 5 mg/mL samples were heated at 60 °C for 60 min in a fan-assisted oven, whilst the other half (n = 3), of the samples were left at room temperature (23 ± 1 °C). Once heating was completed, the samples were left overnight to cool to room temperature (23 ± 1 °C), and dissolved in 10 mL of methanol before being quantified by HPLC analysis.

2.2.3.2.3 Phase solubility method validation: b) loss through filtering

To a number of Safe-Lock micro test tubes containing 1 mL of 0.10 mg/mL Pd in deionised water, half (n = 3), were passed through a 0.45 μm membrane filter and quantified by UV/Vis spectrometry at a wavelength of 247 nm - whilst the other half (n = 3), of the samples were quantified directly by UV/Vis spectrometry without the filtering step. The drug loss was determined from the difference in Pd concentration between the two sets of samples. To deduce the drug loss from filtering for a solution containing solubilised CD/drug complexes, the same process was applied to premixed solutions of 0.04 M HPβCD and 5 mg/mL Pd.

2.2.3.2.4 Phase solubility method validation: c) assessment of supersaturation

To a number of Safe-Lock micro test tubes containing excess Pd (~50 mg), 1 mL of 0.04 M HPβCD was added. In addition, to a number of Safe-Lock micro test tubes containing 5 mg of Pd, 1 mL of deionised water was added. Each Safe-Lock micro test tube was sealed and vortexed briefly on a rotormixer until the drug was dispersed. The samples were heated at 60 °C for 60 min in a fan-assisted oven and left to cool for 6 hrs, after which the Safe-Lock micro test tubes were opened and a small amount of the solid drug was added (5 mg ± 0.2), to the sample. The samples were resealed and agitated constantly (100 rpm), until they were filtered through a 0.45 μm membrane filter and prepared for HPLC. Each time point after heating was analysed in triplicate at the
Chapter 2 - Characterisation of Inclusion Complex Formation

following time points: 0, 12, 24, 48, 72, 96, 120, 144, 168, 336 and 504 hrs (21 days), after cooling.

2.2.3.2.5 Phase solubility method validation: d) HPLC method optimisation

Reference samples of Pd, HC, propylparaben and methylparaben were analysed individually, and as a mixture, using an integrated Hewlett & Packard (HP) 1050 HPLC system comprising an HP 1050 autosampler, an HP 1050 pump and an HP 1050 multiple wavelength detector system, set at a wavelength of 247 nm. The detector was interfaced with a PC with PC/Chrom+ Software (H & A Scientific Inc., USA), whilst the column temperature was 40 °C. Table 2.1 lists the HPLC conditions used during the method optimisation. The samples were diluted accordingly so that absorbance readings were within the limit of detection by HPLC under conditions of Trial 5 (Table 2.1).

Table 2.1: HPLC parameters used during optimisation of the HPLC method

<table>
<thead>
<tr>
<th>HPLC Parameter</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
<th>Trial 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>ACN:water (35:65)</td>
<td>ACN:water (35:65)</td>
<td>ACN:water (35:65) + 1 % acetic acid</td>
<td>ACN:water (25:75) + 1 % acetic acid</td>
<td>ACN:water (25:75) + 1 % acetic acid</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>4 μm 60 Å C18 silica</td>
<td>4 μm 80 Å C18 silica</td>
<td>4 μm 80 Å C18 silica</td>
<td>4 μm 80 Å C18 silica</td>
<td>4 μm 80 Å C18 silica</td>
</tr>
<tr>
<td>Column length</td>
<td>250 x 4.6</td>
<td>100 x 4.6</td>
<td>150 x 4.6</td>
<td>150 x 4.6</td>
<td>150 x 4.6</td>
</tr>
<tr>
<td>Flow rate (mL/min)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Injection volume (µL)</td>
<td>20</td>
<td>40</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

2.2.3.3 Part 2 - Phase solubility study of prednisolone and hydrocortisone in cyclodextrin systems in the presence of parabens

The phase solubility methods as mentioned in Section 2.2.3.2.1 were repeated with CD solutions containing 0.10 and 0.20 % w/v parabens. The intrinsic solubility for Pd and HC was deduced with aqueous solutions containing 0.10 or 0.20 % w/v parabens with no CD. Parabens concentrations were based on a propylparaben: methylparaben ratio of 1:9.

2.2.3.4 Part 3 - Phase solubility study of hydrocortisone in cyclodextrin systems in various pH and simulated media in vivo

The phase solubility methods as mentioned in Section 2.2.3.2.1 were conducted with HP-β-CD and Me-β-CD solutions containing: a) phosphate buffer pH 4.5, b) phosphate
buffer pH 6.8, c) simulated saliva medium pH 6.7, d) simulated gastric fluid pH 1.2, e) simulated intestinal fluid pH 6.8 (Table 2.2).

### Table 2.2: Constituents of various buffer solutions and simulated media in vivo used during phase solubility studies of hydrocortisone

<table>
<thead>
<tr>
<th>Buffer component</th>
<th>Phosphate buffer</th>
<th>Phosphate buffer</th>
<th>Simulated saliva medium</th>
<th>Simulated gastric fluid</th>
<th>Simulated intestinal fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>6.8 g</td>
<td>1.0 g</td>
<td>655 mg</td>
<td>12 mg</td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>6.8 g</td>
<td>1.0 g</td>
<td>655 mg</td>
<td>12 mg</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>8.5 g</td>
<td>2.0 g</td>
<td>126 mg</td>
<td>2.0 g</td>
<td>1.6 g</td>
</tr>
<tr>
<td>KCl</td>
<td>964 mg</td>
<td>189 mg</td>
<td>2.0 g</td>
<td>1.6 g</td>
<td>80 mg</td>
</tr>
<tr>
<td>KSCN</td>
<td>12 mg</td>
<td>326 mg</td>
<td>189 mg</td>
<td>2.0 g</td>
<td>80 mg</td>
</tr>
<tr>
<td>(NH₂)₂CO</td>
<td>200 mg</td>
<td>200 mg</td>
<td>2.0 g</td>
<td>1.6 g</td>
<td>80 mg</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>228 mg</td>
<td>228 mg</td>
<td>2.0 g</td>
<td>1.6 g</td>
<td>80 mg</td>
</tr>
<tr>
<td>Na₂SO₄.10H₂O</td>
<td>763 mg</td>
<td>763 mg</td>
<td>2.0 g</td>
<td>1.6 g</td>
<td>80 mg</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>178 mg</td>
<td>178 mg</td>
<td>2.0 g</td>
<td>1.6 g</td>
<td>80 mg</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>631 mg</td>
<td>631 mg</td>
<td>2.0 g</td>
<td>1.6 g</td>
<td>80 mg</td>
</tr>
<tr>
<td>Pepsin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCl</td>
<td>80 mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄.2H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>4.5</td>
<td>6.8</td>
<td>6.7</td>
<td>1.2</td>
<td>6.8</td>
</tr>
<tr>
<td>Volume</td>
<td>1 L</td>
<td>1 L</td>
<td>1 L</td>
<td>1 L</td>
<td>1 L</td>
</tr>
</tbody>
</table>

The intrinsic solubility of HC in the various media was also determined by following the phase solubility methods with the various media, without any CD. The pH of each solution was determined using a pre-calibrated pH 211 Microprocessor pH Meter (Hanna Instruments). The HC concentrations were quantified by a UV/Vis spectrophotometer (Carey 3E UV-Visible Spectrophotometer), at a wavelength of 247 nm.

### 2.2.3.5 Part 4 - Phase solubility study of quinine in cyclodextrin systems

Phase solubility profiles of Qu were deduced by following the phase solubility methods as mentioned in Section 2.2.3.2.1, this time CD solutions contained HP-β-CD and Me-β-CD at 0.01 - 0.10 M were added to 1.5 mL Safe-Lock micro test tubes containing excess Qu (~50 mg). The intrinsic solubility for Qu was determined by following the
**2.2.4 Results and discussion**

**2.2.4.1 Part 1**

**2.2.4.1.1 Phase solubility study of prednisolone and hydrocortisone in cyclodextrin systems**

A linear standard curve by peak area versus drug concentration was obtained in the concentration range [10 to 200 μg/mL], for both steroids, during every HPLC analysis and was used for accurate drug quantification (mean \( R^2 = 0.999 \)). Phase solubility diagrams of Pd and HC with various CDs at 23 ± 1 °C were subsequently obtained (Figure 2.2).

![Figure 2.2: Phase solubility diagrams of prednisolone and hydrocortisone with various CDs in water at room temperature (23 ± 1 °C). Each point: mean ± SEM, n = 3. Note that some error bars are within the symbol. Significant difference (\( p = 0.05 \)) between all CDs across whole concentration range.](image)

With the exception of both \( \gamma \)-CD phase diagrams and the HC/\( \beta \)-CD phase diagram, all CDs exhibited \( A_1 \)-type profiles in the selected concentration range. In contrast, \( \gamma \)-CD and HC/\( \beta \)-CD exhibited \( B_3 \)-type diagrams indicating a limited solubility of the formed inclusion complex. From the phase solubility diagrams, straight lines could be fitted to the \( A_1 \)-type isotherms with regression coefficients (\( R^2 \)) > 0.998, indicating the
formation of soluble inclusion complexes between the substrate (Pd or HC), and the ligand (β-CD, HP-β-CD, and Me-β-CD), and a first order dependency with respect to the CDs. These findings are consistent with reported phase solubility studies and illustrated the reproducibility of the phase solubility method. For example, Larsen et al., (2005), described A_L-type profiles between HP-β-CD and β-CD with Pd, while a B_S-type profile was reported between γ-CD and Pd. A_L-type profiles were also illustrated between HP-β-CD and HC (Loftsson et al., 2002), whereas a B_S-type profile was reported between β-CD and HC (Frank and Kavaliunas, 1983; Loftsson et al., 2003).

The intrinsic aqueous solubility (D_0) of Pd and HC was determined to be 0.2 ± 0.003 mg/mL and 0.2 ± 0.002 mg/mL, respectively. The D_0 was, however, slightly lower than determined previously; 0.4 mg/mL (Larsen et al., 2005; Loftsson and Hreinsdóttir, 2006), and 0.3 mg/mL (Stella et al., 1995), for Pd, and 0.4 mg/mL (Loftsson and Hreinsdóttir, 2006), for HC. This may be attributed to slightly higher temperatures being used by the other researchers.

The maximum solubility of the Pd complexes increased in the order Pd/ Me-β-CD > HP-β-CD > β-CD > γ-CD, whilst the solubility of the HC complexes increased in the order HC/ Me-β-CD > HP-β-CD > γ-CD > β-CD, indicating that the CD derivatives can obtain a higher corticosteroid solubility than the natural CDs. Remaining consistent with the literature, all the A_L-type profiles and the initial linear slope of the B_S-type profiles had a slope less than unity, denoting the probable formation of 1:1 inclusion complexes in aqueous solution between the CDs and corticosteroids (Larsen et al., 2005; Loftsson et al., 2002).

All of the phase solubility profiles (including the B_S-type profiles) signified that CDs were capable of forming soluble inclusion complexes with corticosteroids. Considering the Pd and HC formulations mentioned in Table 1.4, and utilising the phase solubility profiles in Figure 2.2, it is possible to calculate the minimum concentration of CD required for a specific dose of Pd or HC in solution. A 5 mg/mL Pd solution provides a clinically relevant paediatric dose volume. To achieve a 5 mg/mL Pd solution it would require a minimum of 6.0 % w/v HP-β-CD and 2.8 % w/v Me-β-CD. β-CD and γ-CD cannot, however, ascertain a 5 mg/mL Pd solution because this concentration is above the solubility of the inclusion complexes that these two CDs form with Pd. In contrast, to achieve a clinically relevant HC solution of 2 mg/mL it would require 1.1, 0.5 and
Chapter 2 - Characterisation of Inclusion Complex Formation

1.2 % w/v of HP-β-CD, Me-β-CD and γ-CD, respectively, and once again β-CD would be unable to ascertain the desired drug concentration due to its limited solubility.

Assuming a 1:1 complex stoichiometry, the $K_{1:1}$ and the CE for Pd and HC with various CDs were calculated from the slope of the fitted straight line or linear section of the $B_s$-type diagrams. In addition, based on the Equation 2.4, the ratio of the number CD molecules in solution that were forming a water-soluble inclusion complex with the corticosteroids were calculated, as well as the percentage of CD that was complexed with the drug molecules (Table 2.3).

Table 2.3: Inclusion complex data of prednisolone and hydrocortisone with various cyclodextrins at room temperature (23 ± 1 °C)

<table>
<thead>
<tr>
<th>Cyclodextrin</th>
<th>Phase solubility profile</th>
<th>Intrinsic solubility, $D_s$ (mg/mL)</th>
<th>$K_{1:1}$ (M$^{-1}$)</th>
<th>Molar ratio (Drug : CD)</th>
<th>Maximum solubility increase (Fold)</th>
<th>Complexed cyclodextrin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisolone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP-β-CD</td>
<td>Al</td>
<td>0.2</td>
<td>1105</td>
<td>0.48</td>
<td>1 : 3</td>
<td>74</td>
</tr>
<tr>
<td>Me-β-CD</td>
<td>Al</td>
<td>0.2</td>
<td>3577</td>
<td>1.54</td>
<td>1 : 2</td>
<td>140</td>
</tr>
<tr>
<td>β-CD</td>
<td>Al</td>
<td>0.2</td>
<td>2373</td>
<td>1.02</td>
<td>1 : 2</td>
<td>20</td>
</tr>
<tr>
<td>γ-CD$^1$</td>
<td>Bs</td>
<td>0.2</td>
<td>1597</td>
<td>0.69</td>
<td>1 : 3</td>
<td>19</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP-β-CD</td>
<td>Al</td>
<td>0.2</td>
<td>1725</td>
<td>1.14</td>
<td>1 : 2</td>
<td>82</td>
</tr>
<tr>
<td>Me-β-CD</td>
<td>Al</td>
<td>0.2</td>
<td>9851</td>
<td>6.50</td>
<td>1 : 1</td>
<td>130</td>
</tr>
<tr>
<td>β-CD$^2$</td>
<td>Bs</td>
<td>0.2</td>
<td>3557</td>
<td>2.35</td>
<td>1 : 1</td>
<td>8</td>
</tr>
<tr>
<td>γ-CD$^1$</td>
<td>Bs</td>
<td>0.2</td>
<td>1482</td>
<td>0.98</td>
<td>1 : 2</td>
<td>14</td>
</tr>
</tbody>
</table>

$^1$ taken from the linear portion 0 - 20 mM; $^2$ taken from the linear portion 0 - 7 mM

As illustrated by Table 2.3, the $K_{1:1}$ determined for both Pd and HC falls between the predicted range for pharmaceutical agents (0 - 100,000 M$^{-1}$) (Rajewski and Stella, 1996). Me-β-CD gave the strongest $K_{1:1}$ for both Pd and HC with a higher percentage of CD molecules complexing with the drug molecules compared to HP-β-CD and the natural CDs. This enhanced solubilising efficiency is reiterated with Me-β-CD having the highest CE for both corticosteroids compared to the other CDs. Despite β-CD having a limited solubility compared to HP-β-CD and γ-CD, $K_{1:1}$ of β-CD with Pd and HC were greater than HP-β-CD and γ-CD. These trends were also reported by Larsen et al., (2005), therefore, providing support that the phase solubility methods used during this research were appropriate.

All CDs, except γ-CD, had a greater $K_{1:1}$ with HC than Pd indicating the slightly larger corticosteroid had a better conformational fit in the cavity of these CDs than the Pd. Sadlej-Sosnowska (1997), found structural features of the A-ring or nearby the A-ring
Chapter 2 - Characterisation of Inclusion Complex Formation

of steroids had the largest influence on their $K_{i;1}$ with CDs. HC has two extra hydrogen molecules on the A-ring compared to Pd and, therefore, this difference could have aided inclusion complexation by providing extra molecules for non-covalent bonding without hindering cavity entry. Out of the natural CDs that were studied, β-CD was shown to complex the corticosteroids more strongly and efficiently than the γ-CD, because approximately only half of the γ-CD molecules were forming complexes with the drug molecules. This demonstrates the cavity of the γ-CD was too large for the corticosteroids to provide a stable complex. Similar observations were made by Uekama et al., (1982), who reported that γ-CD complexed HC more loosely compared with β-CD.

2.2.4.1.2 Phase solubility method validation

To examine whether the heating stage of the phase solubility method caused degradation of free and complexed drug, the concentration of Pd with and without heating was determined (Table 2.4). In addition, the effect of the filtering section of the phase solubility method for free and complexed Pd was determined (Table 2.4).

Table 2.4: Phase solubility method validation: degradation via heating and drug loss via filtering

<table>
<thead>
<tr>
<th>Drug degradation via heating (%)</th>
<th>Drug loss via filtering (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Prednisolone</td>
<td>0.6</td>
</tr>
<tr>
<td>Complexed Prednisolone</td>
<td>0.6</td>
</tr>
</tbody>
</table>

In their technical note regarding the determination of aqueous solubility by heating and equilibrium, Loftsson and Hreinsdóttir (2006), suggested drug degradation should be no greater than 1 % during the heating cycle. Thus, the method in this particular research was sufficient for accurate solubility analysis without error associated with degraded drug. In contrast there is no guidance on the accepted level of drug loss via filtering. Drug loss from free Pd samples was slightly higher than complexed Pd, yet both percentage drug losses were minimal in terms of the amount actually lost via filtering. The filtering step in the phase solubility method was, therefore, deemed sufficient.

The time for solubility equilibrium after supersaturation for free and complexed Pd was assessed (Figure 2.3). Initial free and complexed drug solubility over the first five days illustrated supersaturated solutions until solubility equilibrium was established at day
six. This compares well with reports from other authors who stated that solubility equilibrium was established between three to seven days (Loftsson and Hreinsdóttir, 2006), and in particular, Loftsson et al., (2003), and Masson et al., (2005), who found that it took six days to reach solubility equilibrium during phase solubility analysis.

![Figure 2.3: Supersaturation solubility equilibrium of free and complexed prednisolone at room temperature (23 ± 1 °C). Each point: mean ± SEM, n = 3. Note that some error bars are within data points.](image)

To obtain an accurate quantification of the phase solubility samples, various HPLC parameters were explored until accurate and efficient analysis was achieved. Table 2.5 highlights the retention times for each drug and shows the HPLC parameters of Trial 5 were best suited for the remainder of the phase solubility study, with retention times allowing successful resolution of all analytes.

| Table 2.5: Retention times obtained during the phase solubility HPLC optimisation |
|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 |
| **Prednisolone** | 5.7 | 5.2 | 6.6 | 20.7 | 10.8 |
| **Hydrocortisone** | 6.0 | 5.4 | 6.9 | 21.8 | 11.4 |
| **Methylparaben** | 6.9 | 13.4 | 17.5 | 43.0 | 31.0 |
| **Propylparaben** | 6.9 | 13.4 | 5.0 | 14.4 | 7.1 |

2.2.4.2 Part 2 - Phase solubility study of prednisolone and hydrocortisone in cyclodextrin systems in the presence of parabens

Phase solubility diagrams of Pd and HC with various CDs at 23 ± 1 °C in the presence of 0, 0.1 and 0.2 % w/v parabens were obtained (Figure 2.4 and Figure 2.5), and
Chapter 2 - Characterisation of Inclusion Complex Formation assuming a 1:1 complex stoichiometry phase solubility characteristics were calculated (Table 2.6 and Table 2.7).

Figure 2.4: Phase solubility diagrams of **prednisolone** with various CDs in aqueous parabens solutions at room temperature (23 ± 1 °C) (*x-axis scale kept the same for all phase diagrams*). Each point: mean ± SEM, \( n = 3 \). Note that some error bars are within data points.

Figure 2.4 shows that the presence of parabens affected the inclusion complex formation between Pd and CDs and remains consistent with the findings of Loftsson *et al.*, (1992), who found that preservative molecules reduced the solubilising effects of CDs. Figure 2.4 also illustrates that when more parabens was present in solution less Pd was solubilised, and this was likely to be attributed to more parabens molecules competing with the Pd for the CD cavities. The \( K_{1:1} \) and CE for Pd/CD complexation also decreased as a function of increasing parabens concentration (Table 2.6). These
findings disprove the hypothesis that the parabens would not disrupt inclusion complex formation between the CDs and the corticosteroids because the parabens was already in solution.

All CDs expressed the same phase solubility profile regardless whether parabens was present, illustrating that the parabens did not alter the solubility of the formed Pd/CD inclusion complexes. The only apparent influence of the presence of parabens was the reduction in the amount of free CD available for the Pd. For instance, it was calculated that 51 % of β-CD was complexing with the Pd when no parabens was in solution, but 23 % was complexing when 0.2 % parabens was present. It could be assumed, therefore, that 28 % (51 -23 %), of the β-CD was no longer complexing with the Pd but complexing with the parabens instead. Because of the reduction in CD molecules complexing with the Pd the molar ratio was also seen to increase as a function of parabens concentration.

Table 2.6: Inclusion complex data of prednisolone and various cyclodextrins in aqueous parabens solution at room temperature (23 ± 1 °C)

<table>
<thead>
<tr>
<th>Parabens (%)</th>
<th>Phase solubility profile</th>
<th>Intrinsic solubility, D₀ (mg/mL)</th>
<th>Kᵢ₁ (M⁻¹)</th>
<th>CE</th>
<th>Molar ratio (Drug : CD)</th>
<th>Maximum solubility increase (Fold)</th>
<th>Complexed cyclodextrin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-β-CD</td>
<td>0</td>
<td>A₁</td>
<td>0.2</td>
<td>1105</td>
<td>0.48</td>
<td>1 : 4</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>A₁</td>
<td>0.2</td>
<td>816</td>
<td>0.45</td>
<td>1 : 4</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>A₁</td>
<td>0.2</td>
<td>711</td>
<td>0.38</td>
<td>1 : 4</td>
<td>55</td>
</tr>
<tr>
<td>Me-β-CD</td>
<td>0</td>
<td>A₁</td>
<td>0.2</td>
<td>3577</td>
<td>1.54</td>
<td>1 : 2</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>A₁</td>
<td>0.2</td>
<td>2196</td>
<td>1.21</td>
<td>1 : 2</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>A₁</td>
<td>0.2</td>
<td>1983</td>
<td>1.07</td>
<td>1 : 2</td>
<td>100</td>
</tr>
<tr>
<td>β-CD</td>
<td>0</td>
<td>A₁</td>
<td>0.2</td>
<td>2373</td>
<td>1.02</td>
<td>1 : 2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>A₁</td>
<td>0.2</td>
<td>865</td>
<td>0.48</td>
<td>1 : 4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>A₁</td>
<td>0.2</td>
<td>549</td>
<td>0.16</td>
<td>1 : 5</td>
<td>9</td>
</tr>
<tr>
<td>γ-CD</td>
<td>0 ¹</td>
<td>B₃</td>
<td>0.2</td>
<td>1597</td>
<td>0.69</td>
<td>1 : 3</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>0.1 ²</td>
<td>B₃</td>
<td>0.2</td>
<td>374</td>
<td>0.21</td>
<td>1 : 6</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>0.2 ²</td>
<td>B₃</td>
<td>0.2</td>
<td>293</td>
<td>0.30</td>
<td>1 : 8</td>
<td>11</td>
</tr>
</tbody>
</table>

¹ taken from the linear portion 0 - 20 mM; ² taken from the linear portion 0 - 40 mM

Due to these findings, if parabens were used to preserve a Pd/CD formulation, a higher CD concentration would have to be used to ensure adequate solubilisation of Pd to reach desired dose volume. Referring to the illustration in Part 1 of the phase solubility study where a minimum of 2.8 % w/v Me-β-CD would be required for a 5 mg/mL Pd concentration, if 0.1 % or 0.2 % parabens was present then 3.3 or 3.7 % w/v Me-β-CD
Chapter 2 - Characterisation of Inclusion Complex Formation

would be required, respectively, to ensure a 5 mg/mL Pd concentration. Clearly this
would be vital knowledge when formulating an oral liquid corticosteroid formulation
that included drug, CD and preservative. The antimicrobial activity of the preservative
might also be effected if it is no longer free in solution but complexed with CD
molecules. *This aspect was studied in Chapter 3.*

![Figure 2.5: Phase solubility diagrams of hydrocortisone with various CDs in aqueous parabens solutions at room temperature (23 ± 1 °C) (x-axis scale kept the same for all phase diagrams). Each point: mean ± SEM, n = 3. Note that some error bars are within data points. Significant difference (p = 0.05) between all concentrations of parabens for each CD, across the whole CD concentration range.*

The influence of parabens on the ability of CDs to solubilise HC differed in comparison
to the Pd solubilisation. Whilst HP-β-CD and β-CD solubilised less HC as a function of
increasing parabens concentration and, therefore, had a reduction in \( K_{1:1} \) and CE as a
Chapter 2 - Characterisation of Inclusion Complex Formation

function of increasing parabens concentration, the parabens showed enhancing effects on the HC solubilising powers of Me-β-CDs and, to a lesser extent, γ-CD (Figure 2.5). For instance, the maximum HC concentration increased 25 fold when 0.2 % w/v parabens was present compared to when no parabens was present. This resulted in an Ap type phase solubility profile, a slope greater than unity and, therefore, the possible formation of 2:1 (HC:Me-β-CD), inclusion complexes or the formation of water-soluble microaggregates of HC and HC/Me-β-CD (Loftsson et al., 2003; Loftsson et al., 2004c). The minimal action of parabens on the HC solubilising effect of γ-CD is unlikely due to 2:1 inclusion complexes but the formation of water-soluble microaggregates of HC and HC/γ-CD in presence of parabens is possible. It is more likely, however, the parabens enhanced the solubility of the virtually insoluble HC/γ-CD inclusion complexes, thus increasing the overall HC concentration.

Table 2.7: Inclusion complex data of hydrocortisone and various cyclodextrins in aqueous parabens solution at room temperature (23 ± 1 °C)

<table>
<thead>
<tr>
<th>Parabens (%)</th>
<th>Phase solubility profile</th>
<th>Intrinsic solubility, D₀ (mg/mL)</th>
<th>Kst (M⁻¹)</th>
<th>Molar ratio (Drug : CD)</th>
<th>Maximum solubility increase (Fold)</th>
<th>Complexed cyclodextrin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-β-CD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>A₁</td>
<td>0.2</td>
<td>1725</td>
<td>1.14</td>
<td>1:2</td>
<td>82</td>
</tr>
<tr>
<td>0.1</td>
<td>A₁</td>
<td>0.3</td>
<td>1316</td>
<td>1.01</td>
<td>1:2</td>
<td>68</td>
</tr>
<tr>
<td>0.2</td>
<td>A₁</td>
<td>0.3</td>
<td>1094</td>
<td>0.89</td>
<td>1:2</td>
<td>62</td>
</tr>
<tr>
<td>Me-β-CD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>A₁</td>
<td>0.2</td>
<td>9851</td>
<td>6.50</td>
<td>1:1</td>
<td>130</td>
</tr>
<tr>
<td>0.1</td>
<td>A₁</td>
<td>0.3</td>
<td>4522</td>
<td>3.48</td>
<td>1:1</td>
<td>103</td>
</tr>
<tr>
<td>0.2</td>
<td>Ap</td>
<td>0.3</td>
<td>8000*</td>
<td>-</td>
<td>-</td>
<td>155</td>
</tr>
<tr>
<td>β-CD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 ³</td>
<td>B₃</td>
<td>0.2</td>
<td>3557</td>
<td>2.35</td>
<td>1:1</td>
<td>8</td>
</tr>
<tr>
<td>0.1 ³</td>
<td>B₃</td>
<td>0.3</td>
<td>1011</td>
<td>0.85</td>
<td>1:2</td>
<td>7</td>
</tr>
<tr>
<td>0.2 ³</td>
<td>B₃</td>
<td>0.3</td>
<td>716</td>
<td>0.58</td>
<td>1:3</td>
<td>6</td>
</tr>
<tr>
<td>γ-CD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 ³</td>
<td>B₃</td>
<td>0.2</td>
<td>1482</td>
<td>0.98</td>
<td>1:2</td>
<td>14</td>
</tr>
<tr>
<td>0.1 ³</td>
<td>B₃</td>
<td>0.3</td>
<td>257</td>
<td>0.20</td>
<td>1:6</td>
<td>9</td>
</tr>
<tr>
<td>0.2 ³</td>
<td>B₃</td>
<td>0.3</td>
<td>337</td>
<td>0.27</td>
<td>1:5</td>
<td>12</td>
</tr>
</tbody>
</table>

³ taken from the linear portion 0 - 7 mM; ⁴ taken from the linear portion 0 - 10 mM; ⁵ taken from the linear portion 0 - 20 mM; ⁶ taken from the linear portion 0 - 40 mM; * calculated based on an Ap phase solubility profile (Brewster and Loftsson, 2007). CE and molar ratio calculated for 1:1 complexes.

2.2.4.3 Part 3 - Phase solubility study of hydrocortisone in cyclodextrin systems in various pH and simulated media in vivo

Phase solubility diagrams of HC with HP-β-CD and Me-β-CD at 23 ± 1 °C in the presence of phosphate buffer pH 4.5, phosphate buffer pH 6.8, simulated saliva medium pH 6.7, simulated gastric fluid pH 1.2, and simulated intestinal fluid pH 6.8 were
Chapter 2 - Characterisation of Inclusion Complex Formation

obtained (Figure 2.6), and assuming a 1:1 complex stoichiometry, phase solubility characteristics were calculated (Table 2.8).

Significant difference (p < 0.05) between simulated saliva and other media across whole CD concentration range.

Significant difference (p < 0.05) between simulated saliva and other media across whole CD concentration range.

**Figure 2.6:** Phase solubility diagrams of hydrocortisone with HP-β-CD and Me-β-CD in various media at room temperature (23 ± 1 °C). Each point: mean ± SEM, n = 3.

Note that some error bars are within data points, p = 0.05

With the exception of HP-β-CD in simulated gastric fluid and Me-β-CD in simulated saliva, all CDs exhibited A\(_L\)-type profiles and HC solubilisation similar to the phase solubility of HC in water. These findings support the hypothesis that the pH and simulated media would not influence the solubilising capacity of the CDs. In contrast, however, HP-β-CD in simulated gastric fluid exhibited an A\(_N\)-type diagram indicating that simulated gastric fluid altered the solubilising effect of HP-β-CD at high HP-β-CD concentration, whilst Me-β-CD in simulated saliva exhibited an A\(_P\)-type diagram indicating an enhanced complex solubility, the possible formation of 2:1 (HC:Me-β-CD) inclusion complexes or the formation of water-soluble microaggregates at high Me-β-CD concentration. In practical terms, the results indicated that none of these media would likely to cause precipitation of HC from CD inclusion complexes if they were to be exposed to these particular environments during oral administration. This
Chapter 2 - Characterisation of Inclusion Complex Formation

would be extremely important to know if, for instance, the inclusion complexes became insoluble in saliva and a precipitate was formed in the mouth.

Table 2.8: Inclusion complex data of hydrocortisone with HP-β-CD and Me-β-CD in various aqueous media at room temperature (23 ± 1 °C)

<table>
<thead>
<tr>
<th>Media</th>
<th>Phase Solubility Profile</th>
<th>Intrinsc Solubility, $D_o$ (mg/mL)</th>
<th>$K_{1:1}$ (M⁻¹)</th>
<th>CE</th>
<th>Molar Ratio (Drug : CD)</th>
<th>Maximum solubility increase (Fold)</th>
<th>Complexed cyclodextrin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HPβCD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>$A_L$</td>
<td>0.2</td>
<td>1863</td>
<td>1.23</td>
<td>1 : 2</td>
<td>82</td>
<td>55</td>
</tr>
<tr>
<td>Phosphate</td>
<td>$A_L$</td>
<td>0.4</td>
<td>1087</td>
<td>1.24</td>
<td>1 : 2</td>
<td>49</td>
<td>55</td>
</tr>
<tr>
<td>Phosphate buffer pH 4.5</td>
<td>$A_L$</td>
<td>0.1</td>
<td>3741</td>
<td>1.05</td>
<td>1 : 2</td>
<td>183</td>
<td>51</td>
</tr>
<tr>
<td>Phosphate buffer pH 6.8</td>
<td>$A_L$</td>
<td>0.3</td>
<td>1343</td>
<td>1.12</td>
<td>1 : 2</td>
<td>64</td>
<td>53</td>
</tr>
<tr>
<td>Simulated Intestinal Fluid</td>
<td>$A_L$</td>
<td>0.4</td>
<td>2540</td>
<td>3.05</td>
<td>1 : 2</td>
<td>62</td>
<td>75</td>
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<tr>
<td>Simulated Saliva</td>
<td>$A_L$</td>
<td>0.3</td>
<td>2409</td>
<td>1.86</td>
<td>1 : 2</td>
<td>60</td>
<td>65</td>
</tr>
<tr>
<td>Simulated Gastric Fluid</td>
<td>$A_L$</td>
<td>0.2</td>
<td>5431</td>
<td>3.58</td>
<td>1 : 2</td>
<td>118</td>
<td>78</td>
</tr>
<tr>
<td>Phosphate</td>
<td>$A_L$</td>
<td>0.4</td>
<td>4477</td>
<td>4.43</td>
<td>1 : 2</td>
<td>85</td>
<td>82</td>
</tr>
<tr>
<td>Phosphate buffer pH 6.8</td>
<td>$A_L$</td>
<td>0.4</td>
<td>10429</td>
<td>10.33</td>
<td>1 : 2</td>
<td>93</td>
<td>91</td>
</tr>
<tr>
<td>Simulated Intestinal Fluid</td>
<td>$A_L$</td>
<td>0.3</td>
<td>5380</td>
<td>3.87</td>
<td>1 : 2</td>
<td>110</td>
<td>79</td>
</tr>
<tr>
<td>Simulated Saliva</td>
<td>$A_L$</td>
<td>0.5</td>
<td>7000</td>
<td>-</td>
<td>-</td>
<td>106</td>
<td>131</td>
</tr>
<tr>
<td>Simulated Gastric Fluid</td>
<td>$A_L$</td>
<td>0.4</td>
<td>6184</td>
<td>6.12</td>
<td>1 : 2</td>
<td>96</td>
<td>86</td>
</tr>
</tbody>
</table>

1 Calculated based on an $A_r$ phase solubility profile. CE and molar ratio calculated for 1:1 complexes.

A major influence on the $K_{1:1}$ is the intrinsic solubility ($D_o$) of the drug (Equation 2.2). During the phase solubility studies with various pH and simulated media the $D_o$ of HC differed as a result of the differing media but also varied in the same media during differing analysis. For example, the $D_o$ of HC ranged from 0.1 mg/mL in phosphate buffer pH 6.8 to 0.5 mg/mL in simulated saliva, but the $D_o$ for HC in phosphate buffer pH 6.8 also varied from 0.1 mg/mL in the HP-β-CD part of the analysis to 0.4 mg/mL for the Me-β-CD part of the analysis. Whilst the varying $D_o$ of HC in differing media is an understandable characteristic the variation of $D_o$ in the same media makes comparing the $K_{1:1}$ for similar phase solubility diagrams virtually impossible. For instance, the phase solubility profiles between HC and HP-β-CD in phosphate buffer pH 6.8 and simulated intestinal fluid were virtually identical apart from the differing $D_o$ of HC in each media. The $K_{1:1}$ for HC/HP-β-CD in phosphate buffer pH 6.8 was consequently 3741 M⁻¹, whilst the $K_{1:1}$ for HC/HP-β-CD in simulated intestinal fluid was 1343 M⁻¹. It
Chapter 2 - Characterisation of Inclusion Complex Formation

would, therefore, be concluded that HC has a stronger affinity for HP-β-CD in phosphate buffer pH 6.8 than simulated intestinal fluid when, although this might be the case, the affinity may not be so great. For this particular reason Loftsson et al., (2007) suggests the use of the CE to compare phase solubility profiles of CDs because this avoids the variation in $D_0$.

The CE between HP-β-CD and HC in phosphate buffer pH 4.5, 6.8, and simulated intestinal fluid was very similar to the CE in water, illustrating that these media did not influence inclusion complexation and the solubilisation efficiency of HP-β-CD remained same. These findings remain consistent with the CEs determined by Loftsson et al., (2007). In contrast, the CE between HP-β-CD and HC in simulated saliva and, to a lesser extent, simulated gastric fluid was greater than in water. These results showed that simulated saliva and simulated gastric fluid did alter the solubilising efficiency of HP-β-CD for HC. Regardless of the media, the CE, and assuming 1:1 inclusion complexation, approximately one out of every two HP-β-CD molecules complexed with the HC, highlighting that the amount of HP-β-CD molecules complexesing with the HC remained approximately the same.

Compared to the CEs between HP-β-CD and HC, the CEs between Me-β-CD and HC was greater, as was the percentage of Me-β-CD that was complexing with the HP. This emulates the findings in Part 1 that Me-β-CD was a better solubilising agent than HP-β-CD. The CE between Me-β-CD and HC in the various media followed a similar trend to the HP-β-CD; phosphate buffer pH 4.5 and simulated intestinal fluid had no particular effect on the solubilising effect of Me-β-CD and produced CEs similar to water, whilst the Me-β-CD had a greater efficiency for HC in simulated gastric fluid. Phosphate buffer pH 6.8, on the other hand, enhanced the solubilising effect of Me-β-CD on HC as illustrated by a greater CE than what was produced in water. This was also seen by the inclusion complexation in the simulated saliva but because inclusion complexation was so great in this particular media that there was possible formation of 2:1 (HC:Me-β-CD) inclusion complexes or the formation of water-soluble microaggregates, thus preventing the accurate calculation of a CE based on the assumption of 1:1 complex stoichiometry.

The results produced in this particular study provided a good preliminary insight to the influences of pH and various media in vivo on the HP-β-CD and Me-β-CD complexation of HC. Nonetheless, the biological environments that the complexes
would be exposed to would also have extra features that have not been investigated in this particular study and may be likely to further influence complexation. For instance, various enzymes would be present in the differing simulated media; the temperature that the formulation would be exposed to after initial administration will vary; the volumes of the various media in vivo would also differ. All these aspects have been shown to greatly influence complexation (Irie and Uekama, 1997), and would, therefore, require investigating before full assurances can be given that the pH and biological media has little influence on the HP-β-CD and Me-β-CD complexation of HC.

2.2.4.4. Part 4 - Phase solubility study of quinine in cyclodextrin systems
Phase solubility diagrams of Qu with HP-β-CD and Me-β-CD at 23 ± 1 °C were obtained (Figure 2.7), and assuming a 1:1 complex stoichiometry, phase solubility characteristics were calculated (Table 2.9).

![Figure 2.7](image_url)

**Figure 2.7:** Phase solubility diagrams of quinine with HP-β-CD and Me-β-CD in water at room temperature (23 ± 1 °C). Each point: mean ± SEM, n = 3. Note that some error bars are within data points. Significant difference (p = 0.05) between CDs across the whole CD concentration range.
Chapter 2 - Characterisation of Inclusion Complex Formation

Table 2.9: Inclusion complex data for quinine with HP-β-CD and Me-β-CD in water at room temperature (23 ± 1 °C)

<table>
<thead>
<tr>
<th>CD</th>
<th>Phase solubility profile</th>
<th>Intrinsic solubility, D_s (mg/mL)</th>
<th>K_{1:1} (M^{-1})</th>
<th>CE</th>
<th>Molar ratio (Drug : CD)</th>
<th>Maximum solubility increase (Fold)</th>
<th>Complexed cyclodextrin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-β-CD</td>
<td>A_l</td>
<td>0.2</td>
<td>738</td>
<td>0.36</td>
<td>1:4</td>
<td>56</td>
<td>26</td>
</tr>
<tr>
<td>Me-β-CD</td>
<td>A_l</td>
<td>0.2</td>
<td>37772</td>
<td>18.42</td>
<td>1:1</td>
<td>189</td>
<td>95</td>
</tr>
</tbody>
</table>

Both CDs exhibited A_l-type profiles in the selected concentration range with regression coefficients (R^2) > 0.996, indicating the formation of soluble inclusion complexes between Qu and the HP-β-CD or Me-β-CD. The profiles exhibited a first order dependency with respect to the CDs and both had a slope less than unity. Me-β-CD was determined to be a better solubilising agent for Qu than HP-β-CD; the intrinsic solubility of Qu was determined to be 0.2 mg/mL and was increased a maximum 56 fold with HP-β-CD but 189 fold with Me-β-CD. This was also made evident by the affinity of HC for the Me-β-CD cavity - the K_{1:1} between Me-β-CD and HC was far greater than HP-β-CD and HC, as was the CE (Table 2.9). In addition, approximately 70 % more Me-β-CD was complexing with the HC than the HP-β-CD.

The aim of this particular part of the study was to determine how much HP-β-CD or Me-β-CD was required to solubilise a certain concentration of Qu, so that sufficient comparative concentrations could be used during the taste masking analysis. It was decided that 1.6 mg/mL Qu would be the concentration to be taste masked during the taste masking studies as it was approximately 10 times the intrinsic solubility concentration and coincided with the HC concentrations used during the human taste panel studies. From these phase solubility studies it was calculated that a minimum of 2.9 % w/v HP-β-CD and 1.0 % w/v Me-β-CD was required to ascertain a 1.6 mg/mL Qu concentration in solution.

Comparing phase solubilities of HP-β-CD and Me-β-CD with Pd, HC, and Qu it can be firstly concluded that Me-β-CD was better at solubilising all three of these drugs than HP-β-CD. Although their cavity has the same dimensions, clearly the type of functional group on the β-CD molecule plays a vital role in the efficiency of the CD molecule to solubilise various drugs. Out of the three drugs HP-β-CD was better at solubilising the corticosteroids than the Qu and in particular, the HC, whilst Me-β-CD was immensely better at solubilising the Qu than the corticosteroids. These results illustrated that the structural differences between the corticosteroids and Qu, and the HP-β-CD and Me-β-
Chapter 2 - Characterisation of Inclusion Complex Formation

CD played a pivotal role in the conformational fit that occurred during CD inclusion complexation.

### 2.2.5 Conclusions

These phase solubility studies demonstrated that CDs formed soluble inclusion complexes with corticosteroids, thus increasing the solubility of these poorly soluble drugs. The CD derivatives (HP-β-CD and Me-β-CD), were found to be better solubilising agents than the natural CDs, (β-CD and γ-CD), over a greater concentration range. Subtle changes in guest molecule structure were shown to affect inclusion complex formation, where HC formed stronger inclusion complexes with the majority of CDs. Parabens molecules were found to displace Pd molecules from the CD cavity reducing the solubilising effect of the CD, as well as displace HC molecules from the HP-β-CD cavity and β-CD. Parabens was shown, however, to enhance the solubility of HC/Me-β-CD and HC/γ-CD via possible 2:1 inclusion complexes or formation of water-soluble microaggregates. The pH or type of simulated media in vivo was found not to have a major influence on inclusion complex formation, except simulated saliva with HC and Me-β-CD. In this particular instance enhanced complex solubility was demonstrated and attributed to the possible formation of 2:1 (HC:Me-β-CD) inclusion complexes or the formation of water-soluble microaggregates at high Me-β-CD concentration. Overall these phase solubility studies illustrated that CDs could be a viable solubilising agent in an oral liquid corticosteroid formulation, and in particular HP-β-CD and Me-β-CD.
Chapter 2 - Characterisation of Inclusion Complex Formation

2.3 Section 2 - Isothermal titration calorimetry, and $^1$H nuclear magnetic resonance spectroscopy studies

2.3.1 Introduction

It was discovered previously during the phase solubility studies that HC and Pd were able to form water-soluble inclusion complexes with various CDs with a 1:1 stoichiometry. Moreover, it was shown that HP-β-CD and Me-β-CD were greater solubilising agents than natural CDs, β-CD and γ-CD. Furthermore, other CD inclusion complexation characteristics, such as $K_c$, CE, molar ratio and percentage of complexed CD were generated to help to develop further the understanding of CD inclusion complexation with corticosteroids. Aside from phase solubility studies, ITC and NMR spectroscopy can provide CD inclusion complexation characteristics (Loftsson and Brewster, 1996; Schneider et al., 1998).

Isothermal Titration Microcalorimetry (ITC): ITC is an exceedingly powerful and highly sensitive calorimetric technique, particularly useful for measuring the interaction of reacting species in solution. It has been used to provide thermodynamic information on the mechanisms involved in the complexation of CD and guest (Loftsson and Brewster, 1996). Knowledge of the complexation thermodynamics is crucial for understanding the phenomena of molecular interaction between guest and CD. Aside from the binding stoichiometry and $K_c$, ITC allows for the calculation of the standard enthalpy change ($\Delta H$) and standard entropy change ($\Delta S$) of CD/drug complexation, from which the Gibbs free energy of the process ($\Delta G$) can be derived. The $K_c$ provides an overall view of the inclusion complexation, its value encompassing all of the events that have occurred upon complexation (including changes in structural configuration, the loss or formation of van der Waals’ forces, hydrogen bonds and electrostatic interactions, and changes in the properties of the solvent). $\Delta H$, $\Delta S$ and $\Delta G$ of CD/drug complexation provide further characterisation of the complexation. For instance, the more negative the $\Delta H$ and $\Delta G$, the stronger and more spontaneous the interaction between drug and CD, respectively. The relationship between the $\Delta H$ and $\Delta S$ of CD/drug complexation allows for the proposal of various driving forces for inclusion complexation, such as hydrophobic interactions and van der Waals’ forces, to be deduced (Loftsson and Brewster, 1996).

In a typical CD ITC experiment, small aliquots of a concentrated solution of CD (titrant), held in a syringe external to the instrument, are added in a time-controlled
Chapter 2 - Characterisation of Inclusion Complex Formation

manner to a drug solution ampoule (titrand), held within the calorimetric vessel, which is maintained at constant temperature. During injections of CD to the drug solution, the CD and drug interact (complex), and the observed heat change per injection is recorded (Figure 2.8A). Since the population of complexing drug molecules in the drug solution ampoule becomes more saturated with CD molecules, the heat change diminishes until only the background heat of dilution is observed. In a conventional experiment the concentration of CD far exceeds that of the drug to assure complete saturation of the drug solution ampoule and to assure an end point in complexation is reached. Concentrations of the CD and drug are calculated typically to enable up to 30 injections of CD (~ 5 - 15 µL each), to be made into the drug solution ampoule (~ 200 - 500 µL), with the time delay between injections dependant on the heat change returning to baseline.

Data analysis is carried out by measuring the area under each peak, equal to the heat change per injection of CD, and plotting the areas against the number of moles of CD injected to yield a binding isotherm (binding curve) (Figure 2.8B). After integrating the heat as a function of molar ratio between the CD and drug, it is possible to fit the binding curve to various reaction models making it possible to determine \( K_c \), the stoichiometry and thermodynamic profile of the CD to drug interaction (Gaisford and O’Neil, 2006).

![Figure 2.8](image)

**Figure 2.8**: Typical ITC data obtained from the binding interaction of guest to a cyclodextrin - A) exothermic heat released upon injection of cyclodextrin into the guest solution; B) integrated heat data plotted against molar ratio of CD and guest to produce a differential binding curve (taken from Gaisford and O’Neil, 2006)
Chapter 2 - Characterisation of Inclusion Complex Formation

**Nuclear Magnetic Resonance Spectroscopy (NMR spectroscopy):** As previously discussed in Chapter 1, NMR spectroscopy is the most direct method to determine the inclusion of a guest molecule into a CD cavity and for structural elucidation of a CD inclusion complex in solution (Schneider *et al.*, 1998; Fielding, 2000). The NMR spectroscopy technique provides direct and detailed observations of individual nuclei relevant to the structure and dynamics of the system. In a typical CD $^1$H NMR spectroscopy experiment, $^1$H NMR spectra are produced for solutions containing CD, drug, and CD/drug inclusion complexes. The $^1$H protons from each molecule are assigned subsequently to the peaks of each spectra. $^1$H chemical shifts corresponding to the protons of the complexed CD and/or complexed drug are then calculated to evaluate the inclusion complexation that has taken place.

### 2.3.2 Aims and objectives

The aims of this part of the chapter were to characterise the inclusion complex formation between CDs and corticosteroids by determining the $K_c$ and stoichiometry of inclusion complexation, as well as the $\Delta H$, $\Delta S$, and $\Delta G$ of CD/drug complexation. This was achieved by carrying out ITC with HP-$\beta$-CD and Me-$\beta$-CD as titrant and HC as titrand. To provide comparative data for the HC, the bitter model drug Qu was also analysed throughout the ITC study.

Further aims of this part of the chapter were to determine whether:

- CDs form inclusion complexes with corticosteroids and determine whether the whole corticosteroid molecule is included into the CD cavity.
- Excess CD influences complexation of corticosteroids compared to molar equivalent concentrations of CD (or produces differing complexation compared to molar equivalent concentrations of CD).
- CDs are capable of complexing low concentrations of corticosteroids, which were capable of existing in solution without the presence of the CD.

These aims were achieved by carry out $^1$H NMR spectroscopy on various concentrations of uncomplexed and complexed HP-$\beta$-CD and HC.

### 2.3.3 Materials and methods

#### 2.3.3.1 Materials

As per Section 2.2.3.1, as well as $D_2O$ purchased from Sigma Aldrich (Poole, UK).
2.3.3.2 Isothermal titration calorimetry studies of the interaction between hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin with hydrocortisone and quinine

Experiments were conducted using a 2277 Thermal Activity Monitor (TAM, ThermoMetric AB, Jarfalla, Sweden), controlled by Digitam 4.1 software, at a fixed temperature of 298.2 ± 0.2 K and repeated in triplicate. Prior to analysis, the instrument was dynamically calibrated by electrical substitution. For the measurement of host (HP-β-CD and Me-β-CD), to guest (HC or Qu), interaction, a 4 mL stainless steel sample ampoule and reference ampoule were loaded initially with 3 mL of 0.91 mM (0.33 mg/mL), HC or 0.58 mM (0.19 mg/mL), for Qu and 3 mL deionised water, respectively. The drug concentrations corresponded to the maximum intrinsic solubility of the HC and Qu. The ampoules were placed in the thermal equilibration position of the TAM for 1 h, before being lowered into the measurement position.

Titrations were performed by titrating 7.5 μL aliquots of 18.15 mM (2.62 % w/v HP-β-CD or 2.38 % w/v Me-β-CD), CD solution for the HC analysis and 11.67 mM (1.69 % w/v HP-β-CD or 1.53 % w/v Me-β-CD), CD solution for the Qu analysis into the stirred (50 rpm), drug sample ampoule using a 500 μL Hamilton syringe controlled by a syringe pump (6110 Lund Syringe Pump, ThermoMetric AB, Jarfalla, Sweden). The concentration of CD corresponded to the concentration required to achieve double the drug concentration at the end of the experiment, thus ensuring a complexation end point was reached. The interval between two CD injections was 15 min, which was sufficient time for the signal to return to baseline. To deduct the dilution heats of CD and drug solutions, titration experiments were also performed for deionised water titrated into drug solution and CD solution titrated into deionised water, respectively, and acted as control titrations.

Integrated areas were extracted from the titration curves to create binding isotherms using the Ligand Binding Analysis program supplied by the Digitam 4.1 software. Triplicate data were corrected with the heat of dilution for CD and drug. By convention the areas were multiplied by -1 to correct the integrated values. The integrated heats were divided by the number of moles of CD injected and the resulting values were plotted as a function of the molar ratio between CD and drug. The resultant data were best fit according to a model for one binding site, using a nonlinear least-squares method. The Ligand Binding Analysis program automatically calculated the $K_C$ and $\Delta H$.
Chapter 2 - Characterisation of Inclusion Complex Formation

values for the CD complexation from the binding isotherms. The complexation stoichiometry was deduced from the inflection point (also known as stoichiometric point) of the binding isotherm. The change in ΔG for the formation of a CD complex was expressed by the relationship ΔG = RT ln K, where R equals the gas constant (8.314 J K⁻¹ mol⁻¹), and T equals the absolute temperature of the interaction in Kelvin (298.2 ± 0.2). The change in TΔS was determined from ΔG = ΔH - TΔS.

7.3.3.4 ¹H nuclear magnetic resonance spectroscopy studies of the interaction between hydroxypropyl-β-cyclodextrin with hydrocortisone

The HP-β-CD and HC solutions used for ¹H NMR spectroscopy analysis were produced in D₂O and were transferred to 5 mm NMR spectroscopy tubes to give a sample total volume of 500 μL (Table 2.10).

Table 2.10: ¹H NMR spectroscopy samples used during the ¹H NMR spectroscopy study

<table>
<thead>
<tr>
<th>¹H NMR sample</th>
<th>¹H NMR sample constituents</th>
<th>Concentration (mM)</th>
<th>Concentration corresponding to taste assessment studies</th>
<th>Molar Ratio</th>
<th>Adult taste panel formulation No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hydrocortisone</td>
<td>0.55</td>
<td>0.20 mg/mL</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>HP-β-CD</td>
<td>7.74</td>
<td>1.12% w/v</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>HP-β-CD</td>
<td>77.4</td>
<td>11.2% w/v</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Hydrocortisone + HP-β-CD</td>
<td>0.55 + 7.74</td>
<td>0.20 mg/mL + 1.12% w/v</td>
<td>1:14</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Hydrocortisone + HP-β-CD</td>
<td>5.50 + 7.74</td>
<td>2.0 mg/mL + 1.12% w/v</td>
<td>1:1</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>Hydrocortisone + HP-β-CD</td>
<td>0.55 + 77.4</td>
<td>0.20 mg/mL + 11.2% w/v</td>
<td>1:140</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>Hydrocortisone + HP-β-CD</td>
<td>5.50 + 77.4</td>
<td>2.0 mg/mL + 11.2% w/v</td>
<td>1:14</td>
<td>4</td>
</tr>
</tbody>
</table>

¹H NMR spectroscopy spectra of HC, HP-β-CD, and their complexes were acquired using a Bruker Avance spectrometer operating at nominal ¹H frequency of 500 MHz and equipped with a 5 mm BBO probe, including Z-axis pulse field gradients. Spectra of samples in D₂O were acquired at 298 K. All spectra were processed and ¹H HP-β-CD protons were assigned using the NMR spectroscopy software, TOPSPIN 1.3. The resonance at approximately 4.72 ppm due to the residual solvent was used as internal reference. The induced chemical shifts (Δδ) of the HP-β-CD protons were calculated to determine the influence of HC on HP-β-CD (Equation 2.5).

\[ \Delta \delta = \delta(\text{complex}) - \delta(\text{free}) \]  

Equation 2.5
Chapter 2 - Characterisation of Inclusion Complex Formation

2.3.4 Results

2.3.4.1 Isothermal titration microcalorimetry

Titration curves for the variation of heat-flow associated with the addition of successive aliquots of HP-β-CD or Me-β-CD solution to HC or Qu solution, as a function of time were produced from the ITC analysis (Figure 2.9). Differential binding isotherms were produced by plotting the integrated heat data from the titration curves against the molar ratio of CD and drug (Figure 2.10). Thermodynamic parameters were calculated from the binding isotherms (Table 2.11).

Figure 2.9: Titration curves of the binding interaction between cyclodextrin and drug at 298.2 ± 0.2 K. A) Titrant: 18.15 mM HP-β-CD; titrand: 0.91 mM hydrocortisone, B) Titrant: 11.61 mM HP-β-CD; titrand: 0.58 mM quinine, C) Titrant: 18.15 mM Me-β-CD; titrand: 0.91 mM hydrocortisone, D) Titrant: 11.61 mM Me-β-CD; titrand: 0.58 mM quinine
Chapter 2 - Characterisation of Inclusion Complex Formation

Figure 2.10: Integrated heat data of cyclodextrin complexation as a function of molar ratio between: A) HP-β-CD and hydrocortisone, B) HP-β-CD and quinine, C) Me-β-CD and hydrocortisone, D) Me-β-CD and quinine, giving a binding curve fitted to a standard single-site binding model using a nonlinear least-squares method.

Table 2.11: Thermodynamic parameters for the complexation of HP-β-CD and Me-β-CD with hydrocortisone and quinine in aqueous solution at 298.2 ± 0.2 K.

<table>
<thead>
<tr>
<th>Reaction model</th>
<th>$K_c$ (M$^{-1}$)</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
<th>$\Delta S$ (J K mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-β-CD + HC = HP-β-CD:HC</td>
<td>2396440</td>
<td>-5.5 ± 1.8$^a$</td>
<td>-35.7</td>
<td>101</td>
</tr>
<tr>
<td>Me-β-CD + HC = Me-β-CD:HC</td>
<td>175119</td>
<td>-20.4 ± 11.1$^a$</td>
<td>-29.3</td>
<td>30</td>
</tr>
<tr>
<td>HP-β-CD + Qu = HP-β-CD:Qu</td>
<td>0.014</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Me-β-CD + Qu = Me-β-CD:Qu</td>
<td>167106000</td>
<td>-4.7 ± 1.4$^a$</td>
<td>-46.0</td>
<td>139</td>
</tr>
</tbody>
</table>

$^a$ The error of each $\Delta H$ was calculated according to the results of three replicates.

2.3.4.2 $^1$H Nuclear magnetic resonance spectroscopy

The possibility of inclusion complex formation between HC and HP-β-CD at various concentrations was examined using $^1$H NMR spectroscopy (Figure 2.11). Induced chemical shifts ($\Delta\delta$) of the HP-β-CD protons were calculated via Equation 2.5 to determine the influence of HC on HP-β-CD (Table 2.12).
Chapter 2 - Characterisation of Inclusion Complex Formation

Figure 2.11: $^1$H NMR spectra of: A) 0.55 mM hydrocortisone, B) 7.74 mM HP-β-CD and C) HP-β-CD:HC (1:1) inclusion complex in D$_2$O at 298 K

Table 2.12: $^1$H Chemical shifts corresponding to HP-β-CD in the presence and absence of hydrocortisone

<table>
<thead>
<tr>
<th>HP-β-CD Proton</th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
<th>H4</th>
<th>H5</th>
<th>H6</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ(free) 7.74 mM HP-β-CD</td>
<td>5.052</td>
<td>3.608</td>
<td>3.994</td>
<td>3.485</td>
<td>3.685</td>
<td>3.848</td>
</tr>
<tr>
<td>δ(free) 77.4 mM HP-β-CD</td>
<td>5.056</td>
<td>3.581</td>
<td>4.000</td>
<td>3.490</td>
<td>3.705</td>
<td>3.850</td>
</tr>
<tr>
<td>Δδ 0.55 mM HC + 7.74 mM HP-β-CD</td>
<td>0.001</td>
<td>0.002</td>
<td>0.019</td>
<td>0.002</td>
<td>0.010</td>
<td>0.005</td>
</tr>
<tr>
<td>Δδ 5.55 mM HC + 7.74 mM HP-β-CD</td>
<td>0.003</td>
<td>0.002</td>
<td>0.013</td>
<td>0.003</td>
<td>0.013</td>
<td>0.010</td>
</tr>
<tr>
<td>Δδ 5.55 mM HC + 77.4 mM HP-β-CD</td>
<td>0.003</td>
<td>0.003</td>
<td>0.015</td>
<td>0.000</td>
<td>0.010</td>
<td>0.004</td>
</tr>
<tr>
<td>Δδ 5.5 mM HC + 77.4 mM HP-β-CD</td>
<td>0.003</td>
<td>0.002</td>
<td>0.013</td>
<td>0.002</td>
<td>0.007</td>
<td>0.006</td>
</tr>
</tbody>
</table>

$\Delta \delta = \Delta \delta$(complex) - $\Delta \delta$(free)

2.3.5 Discussion

2.3.5.1 Isothermal titration microcalorimetry

All four experiments demonstrated positive power peaks and by convention indicated that exothermic heat (release of energy), was produced from the interaction between CD and drug molecules (Figure 2.9). The largest exothermic peaks from each experiment
were produced from the initial CD injections, illustrating that the interaction between CD and drug was greatest when the concentration of free drug within the ampoule was at its highest and the addition of CD was immediately followed by complexation. The magnitude of each exothermic peak gradually decreased after each injection of CD into the drug solution until the generated exothermic heat was indistinguishable from the heats of dilution (control titrations). These results were indicative of the added CD molecules progressively binding with the diminishing free drug molecules until the stoichiometric point was reached. After the stoichiometric point, the amount of free drug remaining in the ampoule was rapidly reduced and hence the magnitude of the heat effect similarly decreased even upon addition of an excess amount of CD. Decreasing exothermic peaks were produced for the titrations between HP-β-CD and HC (Figure 2.9A), indicating that complexation was likely to have occurred between the two types of molecule. In contrast, minimal interaction occurred between the HP-β-CD and Qu, as illustrated by the similar magnitude in size from each exothermic peak (Figure 2.9B). Comparatively decreasing exothermic peaks were produced from the titrations between Me-β-CD and HC (Figure 2.9C), and Me-β-CD and Qu (Figure 2.9D), signifying complexation occurred during both these experiments.

Stoichiometry: The binding isotherms of HP-β-CD and HC, Me-β-CD and HC, and Me-β-CD and Qu demonstrated inflection points located at the molar ratio of the complexation binding stoichiometry (Figure 2.10A, 2.10C and 2.10D, respectively). These particular binding isotherms, illustrated steep gradients at the inflection point, which were indicative of strong binding affinities and indicated 1:1 stoichiometry between the CD and the drug. The binding isotherm of HP-β-CD and Qu showed a lack of inflection point, implying weaker affinity between the two species and the likelihood that few inclusion complexes were formed between HP-β-CD and Qu. These results compare well with the phase solubility results, which demonstrated that Qu had a far stronger affinity for Me-β-CD than HP-β-CD and confirm that 1:1 inclusion complexes were formed between the CDs and corticosteroids.

Association constant: The $K_C$ values demonstrated that HC had a stronger affinity to HP-β-CD compared to Me-β-CD (Table 2.11). In contrast, Qu had an extremely strong affinity to Me-β-CD but virtually no affinity to HP-β-CD. These observations were likely to have been caused by the substitutions of the CD hydroxyls (methyl groups/hydroxypropyl groups) producing altered cavity dimensions, such as modifying the size of the cavity opening, which may have varied the accessibility of the HC and Qu.
Chapter 2 - Characterisation of Inclusion Complex Formation

molecules to the cavity. Alternatively, substitution of the CD hydroxyls may have transformed the geometry and properties of the hydrophobic cavity or varied the hydrogen bonding and hydrophobic binding sites.

The values of the $K_C$ determined by ITC were notably different to those obtained by phase solubility (Table 2.13). This may be due to the differing concentrations of CD and drug used in each method. The phase solubility profile utilised a large concentration range of CD and excess drug, whilst the drug was already in solution for the ITC experiments. Various authors have reported errors with determining the $K_C$ from phase solubility studies and poor fitting of the binding isotherm, respectively (Loftsson, et al., 2005b) (Wiseman et al., 1989). Differences between the $K_C$ determined from ITC studies and phase solubility studies has been reported (Rodriguez-Perez et al, 2006). In this research the $K_C$ of the inclusion complexation between sertaconazole and HP-β-CD was different when calculated by ITC and phase solubility studies, and was attributed to the different composition of the solvent medium (methanol was absolutely necessary for having enough drug concentration for ITC measurements), and the concentration range evaluated.

Table 2.13: Association constants calculated from phase solubility diagrams and ITC

<table>
<thead>
<tr>
<th>Association constant (M$^{-1}$)</th>
<th>Phase solubility diagram</th>
<th>ITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-β-CD/HC</td>
<td>1725</td>
<td>2396440</td>
</tr>
<tr>
<td>Me-β-CD/HC</td>
<td>9851</td>
<td>175119</td>
</tr>
<tr>
<td>HP-β-CD/Qu</td>
<td>738</td>
<td>0.014</td>
</tr>
<tr>
<td>Me-β-CD/Qu</td>
<td>37772</td>
<td>167106000</td>
</tr>
</tbody>
</table>

Standard enthalpy change, standard entropy change and Gibbs free energy of CD/drug complexation: The thermodynamic parameters obtained for the inclusion complexation between HP-β-CD and HC compares well with that obtained by Loftsson et al., (1994), whilst the thermodynamic values of the other complexes are similar to the thermodynamic parameters documented in the literature (Loftsson and Brewster, 1996) (Table 2.11). Various binding mechanisms have been proposed to help explain the rational behind the thermodynamic parameters produced during CD complexation with drugs. The principle driving force behind CD complexation is believed to be the release of enthalpy rich water molecules from the CD cavity (Loftsson and Brewster, 1996). More suitable drug molecules replace these water molecules, lowering the energy of the
Chapter 2 - Characterisation of Inclusion Complex Formation

system and resulting in a more favourable (negative $\Delta H$) enthalpy. Van der Waals’ forces between the CD and drug also enhance the favourable enthalpy, predominantly when the drug molecules are sufficiently close to the walls of the CD cavity.

The $\Delta H$ values of the CD:HC inclusion complexes produced in this particular research were both negative, confirming that the inclusion complexation was an exothermic process. Because the Me-β-CD:HC complex had a more negative $\Delta H$ than the HP-β-CD:HC complex, the HC molecules may have expelled more water molecules from the cavity of the Me-β-CD than the HP-β-CD or had more contact with the walls of the cavity to produce more van der Waals’ interactions with the Me-β-CD than the HP-β-CD. In contrast, only the $\Delta H$ for the Me-β-CD:Qu complexation was negative, whilst the $\Delta H$ for the interaction HP-β-CD to Qu was not observed due to virtually no complexation being measured. Similarly no $\Delta G$ was observed for the interaction between HP-β-CD and Qu because of the minimal complexation. On the other hand, negative values for $\Delta G$ were seen from the complexation between HP-β-CD and HC, Me-β-CD and HC and Me-β-CD and Qu, indicating that the formation of inclusion complexes in aqueous solution from these molecules was spontaneous.

To account for the entropy values produced during CD inclusion complexation both classical hydrophobic interactions and “non classical interactions” are believed to take place (Loftsson and Brewster, 1996). Classical hydrophobic interactions are characterised by large positive $\Delta S$, predominantly due to the release of structured water molecules surrounding the drug molecules before complexation and are entropy driven (Jencks, 1969). “Nonclassical hydrophobic interactions” are associated with smaller $\Delta S$ values because of the balance in entropy from the release of structured water molecules surrounding the drug molecules and gaining in order from the increase in complexation and are enthalpy driven (Griffiths and Bender, 1973). Aside from the HP-β-CD:Qu complex, the $\Delta S$ values produced from this research were all positive, indicating favourable entropy. The complexation between HP-β-CD and HC had a $\Delta S$ greater than the complexation between Me-β-CD and HC, indicating that the HP-β-CD:HC inclusion complex was more associated with classical hydrophobic interactions than the Me-β-CD:HC complex, which was more associated with nonclassical hydrophobic interactions and increased van der Waals’ forces. Because no complexation was observed between HP-β-CD and Qu no entropy of complexation was calculated. The $\Delta S$ of complexation for Me-β-CD and Qu was, however, particularly large in comparison to the Me-β-CD complexation of the HC, therefore, signifying that the Me-β-CD:Qu
complexation was more associated with classical hydrophobic interactions than non-classical hydrophobic interactions and produced less van der Waals’ forces than the HC complexes.

2.3.5.2 Nuclear magnetic resonance spectroscopy

The $^1$H NMR spectrum of 0.55 mM HC shows poor resolution of peaks, overlapping of signals and low resolution from the baseline from the HC protons located from approximately 1 - 2.7 ppm. This prevented successful assignment of the HC protons and was likely due to the low concentration of HC in the NMR spectroscopy sample. The $^1$H NMR spectrum of 7.74 mM HP-β-CD (Figure 2.11B), shows adequate signals from the HP-β-CD protons, which compares well with the literature and allowed for the successful assignment of the HP-β-CD protons (Schneider, 1998). These protons corresponded with six protons located on the glucopyranose units of the HP-β-CD molecule (Figure 2.12). The H1, H2, and H4 protons were positioned outside the HP-β-CD cavity, whilst the H3 and H5 protons were located inside the HP-β-CD cavity and the H6 proton located on the cavity rim at the narrow end of the molecule (Figure 2.12) (Schneider, 1998). In the presence of HP-β-CD there was little enhancement or improved resolution of the peaks from the HC protons, thus preventing information regarding the orientation of the HC in the interaction with HP-β-CD. The induced chemical shifts of the protons of 7.74 mM HP-β-CD and 77.4 mM HP-β-CD were slightly different owing to the enhanced definition of peaks from the 77.4 mM HP-β-CD.

![Figure 2.12: Schematic view of the average orientation of the most important protons on a HP-β-CD molecule (Adapted from Schneider, 1998)](image)

In the presence of HC, the HP-β-CD spectrum (Figure 2.11C), was shifted upfield, producing induced chemical shifts for some of the HP-β-CD protons. The induced chemical shifts of H1, H2, and H4 were similar and not pronounced. In contrast, the induced chemical shifts of H3, H5, and H6 were appreciably shifted upfield and confirm that inclusion complexes were formed between the HP-β-CD and HC, since significant
Chapter 2 - Characterisation of Inclusion Complex Formation

changes in the internal microenvironment had occurred (Schneider, 1998). These results also confirm that inclusion complexes were produced during the phase solubility and ITC studies.

The upfield shift of the H3 proton lying on the inner surface of the cavity of the secondary hydroxyl group side of the HP-β-CD was the most prominent, followed by the H5 proton located on the inner surface at the primary hydroxyl group side, and by the H6 proton. Because of the higher shielding effect on the H3 proton with respect to the H5 and H6, it can be hypothesised that the HC molecules preferentially penetrate the cavity from the more accessible wider side of the cavity, where the secondary hydroxyl groups are located. Frank and Kavaliunas, (1981), suggested that HC enters the torus from the H5 side of the cavity since greater shielding was observed from the H5 proton. These observations, however, were seen with β-CD complexes as apposed to HP-β-CD used during this research.

Without the induced chemical shifts of the HC protons being detectable, the exact positioning of the HC molecules within the HP-β-CD cavity at differing concentrations is unknown, and the aims of the 1H NMR spectroscopy study were not fulfilled fully. Nevertheless, these 1H NMR spectroscopy results did help confirm that HP-β-CD formed inclusion complexes with HC at a concentration capable of existing freely in solution without the aid of the HP-β-CD (i.e. 0.20 mg/mL HC), since significant induced chemical shifts were demonstrated from the H3, H5 and H6 HP-β-CD protons in the presence of 0.55 mM HC. This result compares well with the phase solubility studies, confirming already dissolved drug complexes with CD. Furthermore, this result illustrated that all the drug molecules in solution were in continual equilibrium with the HP-β-CD cavity and not just the excess amount.

The inclusion complexes formed from the 5.50 mM HC and 7.74 mM HP-β-CD sample produced a larger induced chemical shift from the H6 proton compared to all the other complexes. This indicated an increased presence of HC near the cavity rim at the narrow end of the molecule for this particular scenario and may elude that the HC molecule fitted deeper into the cavity of the CD or even protruded though the narrow end of the cavity. The induced chemical shifts of the internal protons being similar for all samples analysed, showed that excess HP-β-CD had no influence on the inclusion complexes that were formed. In addition, the induced chemical shifts of the internal protons were not altered due to the presence of more HC to the system.
Chapter 2 - Characterisation of Inclusion Complex Formation

To improve on the NMR analysis that was conducted, optimisation of the method is required to enhance the resolution of the HC signals and avoid overlapping. Altering the solvent used and increasing the HC concentration may achieve this. Performing 2D ROESY spectroscopy on the samples would also enhance the evaluation of the complexes by provide further data on the orientation of HC within the HC cavity.

2.3.6 Conclusions

It was demonstrated by the ITC experiments that HC forms 1:1 complexes with HP-β-CD and Me-β-CD, and that Qu forms 1:1 complexes with Me-β-CD. The ITC results indicated that minimal complexation occurred between HP-β-CD and Qu. The association of Me-β-CD with Qu was observed to be stronger than both the HC complexes. These results compared well with the inclusion complex data determined in the phase solubility studies. Nevertheless, HC was found to have a stronger association to HP-β-CD than Me-β-CD, which apposes the results of the phase solubility studies. The inclusion complex formation between HP-β-CD and Me-β-CD with HC and Me-β-CD with Qu was found to be a spontaneous process with a negative ΔG. The negative values of ΔH signified that release of water molecules from the CD cavity and van der Waals’ forces were involved in complexation. The ΔS values observed indicated that both classical and nonclassical hydrophobic interactions were also involved in complexation.

The $^1$H NMR spectroscopy experiments demonstrated that HP-β-CD formed inclusions complexes with HC, whereby the HC molecules entered the HP-β-CD cavity via the wider cavity opening. The $^1$H NMR spectroscopy confirmed HP-β-CD was capable of forming inclusion complexes with HC even when the HC was at a concentration below its intrinsic solubility. Excess HP-β-CD was observed to have no influence of inclusion complex formation. An increased presence of HC also had no influence of inclusion complex formation. Nonetheless, because the proton signals of the HC were to small or overlapping to be assigned, a full evaluation on the inclusion complexes that were formed was not achieved by the $^1$H NMR spectroscopy as initially expected. Further optimisation of the $^1$H NMR spectroscopy methods are required, as well as the performance of 2D ROESY spectroscopy.
Chapter 3 - Microbiology Studies

3.1 Introduction

3.1.1 Microbial preservation of oral liquid formulations

Pharmaceutical formulations, especially oral liquids, are exposed frequently to microbial contamination that can pose potential health risks to the patient and cause product spoilage, aesthetic changes, and possible loss of drug efficacy (Clontz, 1997). Microbial contamination can originate from numerous sources during product manufacture and use, whilst contamination in multidose containers, non-sterile liquid formulations, and extemporaneous formulation is a common occurrence (Ghulam et al., 2007). Products most prone to microbial contamination include those with a moderate to high water content; contain sweeteners or sugars, and those administered from multidose containers (Clontz, 1997). Numerous formulations are consequently manufactured with antimicrobial preservatives that prevent proliferation or limit microbial contamination throughout the shelf life of the product, thus limiting the potential problems associated with spoilage.

If an oral liquid corticosteroid formulation were to be formulated it would require an adequate preservative system to maintain microbial stability. There are numerous chemical agents that are used to provide antimicrobial preservation in pharmaceutical formulations (Table 3.1). It should be noted, however, that some of these preservatives are mentioned in Chapter 1 as being potentially dangerous to certain paediatric patients and may, therefore, be unsuitable in paediatric formulations (Rabiu et al., 2004).

<table>
<thead>
<tr>
<th>Preservatives</th>
<th>Usual Concentration (%)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzalkonium chloride</td>
<td>0.004-0.02</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Benzoic acid and salts</td>
<td>0.05-0.1</td>
<td>Antifungal agent</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>0.5-5.0</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Boric acid and salts</td>
<td>0.5-1.0</td>
<td>Antifungal agent</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>0.0025-0.01</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.25-0.5</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Cresol</td>
<td>0.1-0.5</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Dowicil 200</td>
<td>0.02-0.3</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Ethanol</td>
<td>15.0-20.0</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Parabens</td>
<td>0.05-0.2</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.2-0.5</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Phenyl mercuric nitrate</td>
<td>0.004</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Potassium sorbate</td>
<td>0.01</td>
<td>molds &amp; Yeasts</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>2.0-5.0</td>
<td>Broad Spectrum</td>
</tr>
<tr>
<td>Sorbic acid and salts</td>
<td>0.05-0.2</td>
<td>Antifungal agent</td>
</tr>
<tr>
<td>Thiomersal</td>
<td>0.001-0.1</td>
<td>Broad Spectrum</td>
</tr>
</tbody>
</table>

Table 3.1: An example of the preservatives used in pharmaceutical formulations (adapted from Clontz, 1997 and Rabiu et al., 2004)
Chapter 3 - Microbiology Studies

3.1.2 Parabens

The 4-hydroxybenzoic acid esters, collectively known as parabens, are a type of antimicrobial preservative used commonly in pharmaceutical formulations. Their antimicrobial action stems from an inhibitory effect on membrane transport and mitochondrial function within microorganisms whereby the antimicrobial effect increases as the chain length of the parabens ester group increases (Elder, 1984). Typically used parabens are: methyl-, ethyl-, propyl-, butyl-, heptyl-, and benzylparaben. The main benefits of parabens make them an ideal candidate for an oral liquid corticosteroid formulation and include:

- Being able to be used singly or in combination
- Possessing a broad spectrum of antimicrobial activity
- Being effective over a broad pH range (pH 4 - 8)
- Being potent against yeasts, molds and Gram-positive bacteria

Combinations of parabens are more active than individual parabens (Boehm and Maddox, 1972), and the most commonly used parabens in pharmaceutical products are methylparaben and propylparaben (Soni et al., 2005). When used in combination the typical concentrations used range from 0.01 % w/v and 0.09 % w/v propylparaben and methylparaben, respectively, to 0.02 % w/v and 0.18 % w/v propylparaben and methylparaben, respectively (Rowe et al., 2005). Various safety assessments of parabens have been carried out and, in general, they are deemed practically non-toxic at the concentrations used in pharmaceutical products (Soni et al., 2005). It is suggested, however, that formulations containing parabens should be avoided as much as possible in acutely ill neonates presenting jaundice, kernicterus or hyperbilirubinaemia (Rabiu et al., 2004). In addition and although not a medicines regulatory body, the European Food Safety Authority (EFSA) has set a lowest observed adverse effect level for propylparaben at 10 mg /kg body weight/day (EFSA, 2004) after research showed that propylparaben reduced the daily sperm production in the testis of juvenile male rats (Oishi, 2002).

3.1.3 Interaction of Parabens with Cyclodextrin/Drug Inclusion Complex

The aqueous solubility of methylparaben and propylparaben at 25 °C is approximately 2.5 and 0.4 mg/mL, respectively (Kibble, 2000). This has been shown to be enhanced by inclusion complex formation with CDs (Loftsson et al., 1992; Matsuda et al., 1993;
Chapter 3 - Microbiology Studies

Tanaka et al., 1995; Zughul et al., 1997; Chan et al., 2000). In addition, various parabens were reported to displace drug molecules from the cavity of CDs, thus reducing the drug solubilising capacity of the CDs (Loftsson et al., 1992; Chapter 2). It was also determined that because of the inclusion complex formation with CDs, the antimicrobial activity of parabens was found to decrease as a function of CD concentration, as only freely solubilised parabens has an antimicrobial action (Loftsson et al., 1992; Matsuda et al., 1993). If a combination of parabens are to be used in an oral liquid formulation that contain CDs it is, therefore, necessary to thoroughly monitor the influence of parabens on the solubilising effect of CDs (Chapter 2), and determine the efficacy of antimicrobial preservation within the formulation. Evaluation of antimicrobial efficacy is carried out typically by “challenge tests”, where the formulation is inoculated with microorganisms and total viable counts, over certain periods of time, are set against pharmacopoeia requirements to determine whether the preservative system is adequate (British Pharmacopoeia, 2008). Determining the amount of free preservative within the formulation would also be informative, since only the free preservative can provide antimicrobial activity.

3.1.4 Aims and objectives

The aims of this chapter were to:

- Determine the efficacy of antimicrobial preservation of various concentrations of parabens in an oral liquid corticosteroid formulation that contained CD as a solubilising agent for the corticosteroid.
- Determine the concentration of free parabens within HC formulations. This was achieved by carrying out turbidity testing (via optical density measurements), on inoculated HC formulations that contained various concentrations of parabens, and HP-β-CD concentrations determined from the phase solubility studies reported in Chapter 2.

3.2 Materials and methods

3.2.1 Materials

Hydrocortisone (HC) (> 98 % purity), methyl 4-hydroxybenzoate (methylparaben), and propyl 4-hydroxybenzoate (propylparaben) were all purchased from Sigma Aldrich (Poole, UK), and were > 99 % purity or analytical grade unless otherwise stated. Hydroxypropyl-β-Cyclodextrin (HP-β-CD, MW: 1447) (Pharmaceutical grade, Cavitron™ 82005) was kindly donated by Cargill Inc., (Cedar Rapids IA, USA).
Chapter 3 - Microbiology Studies

Tryptone soya agar (TSA), tryptone soya broth (TSB), sabouraud dextrose agar (SDA), buffered sodium chloride peptone solution, phosphate buffered saline (PBS), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Aspergillus niger* (ATCC 9642), *Candida albicans* (ATCC 10231), *Staphylococcus aureus* (ATCC 6538), and *Zygosaccharomyces Rouxii* were all purchased from Oxoid Ltd (Basingstoke UK). Sodium chloride was purchased from BDH Laboratory Supplies (UK) and polysorbate 80 was purchased from Fluka Biochemika (Buchs, Switzerland) - both were analytical grade. All materials were used as received and solutions were prepared using deionised water produced in the laboratory.

3.2.2 Efficacy of antimicrobial preservation

The test for efficacy of antimicrobial preservation described in the British Pharmacopoeia (2008), was carried out on HP-p-CD solutions containing 2.0 mg/mL HC and 0, 0.05, 0.10, and 0.20 % w/v parabens (propylparaben: methylparaben ratio of 1:9) (Chapter 2).

3.2.2.1 Preparation of Agar Plates

TSA and SDA plates were made by dissolving 16 g and 26 g of agar powder into 400 mL of deionised water, respectively. The solutions were autoclaved at 121 °C for 20 minutes and cooled to 55 °C before being poured into 90 mm Petri dishes under aseptic conditions. The agar plates were left to solidify at 5 °C until they were required for use.

3.2.2.2 Culturing and harvesting of microorganisms

TSA plates were inoculated with *E. coli*, *P. aeruginosa*, and *S. aureus* whilst SDA plates were inoculated with *A. niger*, *C. albicans*, and *Z. Rouxii*. The plates were incubated for adequate colony growth - bacterial cultures incubated at 37 °C for 24 hrs, *C. albicans*, and *Z. Rouxii* culture incubated at 25 °C for 48 hrs, whilst the *A. niger* was incubated at 25 °C for 7 days. The bacterial, *C. albicans*, and *Z. Rouxii* cultures were harvested and suspended in sterile 5 mL buffered sodium chloride peptone solution and the *A. niger* was harvested and suspended in an 5 mL aqueous solution of 0.90 % w/v sodium chloride and 0.05 % v/v polysorbate 80, to create an inoculum for each microorganism. The number of colony forming units per mL (CFU/mL) for each inoculum was subsequently determined by serial dilution plate count (Section 3.2.2.4), to deduce that an adequate microorganism concentration was present for the remainder of the study.
Chapter 3 - Microbiology Studies

3.2.2.3 Formulation efficacy test

HP-β-CD solutions (20 mL), containing 2.0 mg/mL HC and 0, 0.05, 0.10, and 0.20 % w/v parabens were prepared based on the phase solubility profile of HC in HP-β-CD, in the presence of parabens determined in Chapter 2 (Table 3.2).

**Table 3.2:** Formulations prepared for the efficacy for antimicrobial preservation study

<table>
<thead>
<tr>
<th>Formulation Component</th>
<th>0 % w/v Paraben</th>
<th>0.05 % w/v Paraben</th>
<th>0.10 % w/v Paraben</th>
<th>0.20 % w/v Paraben</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl 4-Hydroxybenzoate (% w/v)</td>
<td>-</td>
<td>0.045</td>
<td>0.09</td>
<td>0.18</td>
</tr>
<tr>
<td>Propyl 4-Hydroxybenzoate (% w/v)</td>
<td>-</td>
<td>0.005</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Hydroxypropyl-β-Cyclodextrin (% w/v)</td>
<td>1.12</td>
<td>1.31</td>
<td>1.84</td>
<td>2.27</td>
</tr>
<tr>
<td>Hydrocortisone (mg/mL)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Individual solutions were “spiked” with 0.1 mL of microbial inoculum, mixed thoroughly, and the CFU/mL concentration determined by serial dilution plate count (Section 3.2.2.4), to obtain a baseline microbial count at 0 hour. The samples were incubated at 25 °C until the CFU/mL concentration was determined via serial dilution plate count (Section 3.2.2.4), at 14 days and 28 days post spiking. Control formulations were also tested - these formulations were not inoculated with microorganisms but contained the various concentrations of HP-β-CD, parabens and HC, and were exposed to the air in the same manner as the “spiked” samples during the inoculation process.

3.2.2.4 Serial dilution plate count

Plate counts by serial dilution were performed on day 0, 14 and 28 under aseptic conditions in duplicate. The day 0 serial dilution plate count was carried out on the original microbial inoculum, the test formulations, and the non-spiked control test samples - whilst the test formulations and non-spiked control test samples were also analysed on day 14 and 28.

The serial dilution (Figure 3.1), was performed by transferring 1 mL of solution into either 9 mL buffered sodium chloride peptone for solutions containing bacteria, *C. albicans*, and *Z. Rouxii*, or an 9 mL aqueous solution of 0.90 % w/v sodium chloride and 0.05 % v/v polysorbate 80 for solutions containing *A. niger*. The non-spiked control samples were diluted into both media. The solutions were mixed gently and the dilutions repeated down to the $10^{-8}$ level.
Chapter 3 - Microbiology Studies

Figure 3.1: Example of the serial dilution plate count process

The concentration of viable microorganisms in the serial dilution samples was determined in quadruplet, by smearing 0.1 mL of the solution onto TSA plates for bacteria samples or SDA for fungi and yeast samples. The non-spiked control samples were analysed on both plates depending on which media they were diluted with. The plates were incubated upside down until adequate microbial growth was present. Bacteria, _C. albicans_, and _Z. rouxii_ plates were incubated for 24 hrs at 37 °C, and _A. niger_ plates were incubated at 25 °C. Once adequate growth was present, colony counting was performed on the readable plates. The CFU/mL was determined by:

\[
\text{CFU/mL} = \text{No. colonies on agar plate} \times \text{reciprocal of dilution of sample}
\]

_Equation 3.1_

For example, if 32 colonies were on a plate of 1/10,000 dilution, then the microbial count would be 32 x 10,000 = 320,000 CFU/mL in the sample. As the plate count was performed in quadruplet and the serial dilution in duplicate, there were 8 plates per formulation. Mean (± SEM) CFU/mL were, therefore, determined for each sample and used to determine the efficacy of the parabens, by comparing the CFU/mL concentration in the formulations with the accepted limits depicted by the British Pharmacopoeia (2008), test for efficacy of antimicrobial preservation (Table 3.3).
Table 3.3: British Pharmacopoeia (2008), total viable microbial count requirements for oral preparations during the efficacy of antimicrobial preservation test

<table>
<thead>
<tr>
<th></th>
<th>Log Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 14</td>
</tr>
<tr>
<td>Bacteria</td>
<td>3</td>
</tr>
<tr>
<td>Fungi</td>
<td>1</td>
</tr>
</tbody>
</table>

3.2.3 Determining the extent of parabens interaction with cyclodextrin/drug inclusion complexes by turbidity testing

To determine the extent in which paraben interacts with the CD/drug inclusion complex, turbidity testing was carried out on inoculated HP-β-CD solutions containing 2.0 mg/mL HC and 0, 0.05, 0.10, and 0.20 % w/v paraben (propylparaben: methylparaben ratio of 1:9), at a time point when exponential microbial growth was occurring, as determined from a microbial growth curve. Optical density measurements were used to correspond to the turbidity of the samples, being directly related to the number of microorganisms in the solution (Figure 3.2) (Srivastava and Srivastava, 2003). The optical density of the test formulations was subsequently compared to calibration samples to deduce the free and complexed paraben concentration within the test formulations.

**Turbidity testing**

- Low parabens/CD complex → High free parabens → Low microorganism growth → Low turbidity – low optical density
- High parabens/CD complex → low free parabens → High microorganism growth → High turbidity – high optical density

*Figure 3.2: Flow diagram illustrating the principles behind the turbidity testing*

3.2.3.1 Preparation of inocula

Preparatory to the turbidity testing, microbial inocula of *E. coli*, *P. aeruginosa*, and *S. aureus* in PBS were prepared from harvesting colonies grown on TSA plates, as mentioned in Section 3.2.2.1. To obtain inocula with a concentration of approximately $10^8$ CFU/mL, the microbial suspensions were quantified via serial dilution plate count as mentioned in Section 3.2.2.4, with PBS as the diluting media.
Chapter 3 - Microbiology Studies

3.2.3.2 Preparation of test samples

HP-β-CD solutions (10 mL), containing 2.0 mg/mL HC and 0, 0.05, 0.10, and 0.20 % w/v parabens (Formulation 1 to 4 (Table 3.4)), were prepared based on the phase solubility profile of HC in HP-β-CD in the presence of parabens, determined in Chapter 2. In addition, control formulations were prepared without HC (Formulation 5 to 8 (Table 3.4)), and without parabens (Formulation 9 to 12 (Table 3.4)). The parabens concentration was based on a propylparaben: methylparaben ratio of 1:9.

Table 3.4: Test formulations used in the turbidity study

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Paraben (% w/v)</th>
<th>Cyclodextrin (% w/v)</th>
<th>Hydrocortisone (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1.12</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>1.31</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>1.84</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
<td>2.27</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>1.12</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0.05</td>
<td>1.31</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0.10</td>
<td>1.84</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0.20</td>
<td>2.27</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>1.12</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>1.31</td>
<td>2.0</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>1.84</td>
<td>2.0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>2.27</td>
<td>2.0</td>
</tr>
</tbody>
</table>

To create viable and sterile test solutions, 5 mL of each formulation was added to 150 mg TSB and autoclaved at 121 °C for 20 min and left to cool to 37 °C in a Multitron II mixing oven at 200 rpm. The same process was carried on the calibration solutions containing 5 mL 0 - 0.30 % w/v parabens (propylparaben: methylparaben ratio of 1:9).

3.2.3.3 Turbidity testing

Test formulations and calibration solutions were inoculated with 5 μL of microbial inoculum, creating an approximate microbial concentration of 10^5 to 10^6 CFU/mL. Once inoculated the test formulations and calibration solutions were incubated at 37 °C in a Multitron II mixing oven at 200 rpm, until the time duration for exponential microbial growth occurred. The time duration for exponential microbial growth was assessed from a growth curve produced from the optical density measurements of autoclaved solutions containing 150 mg TSB and 5 μL spiked microbe only, over a time period of 400 min, with measurements at 30 min intervals. These measurements were conducted in triplicate. The optical density was measured by UV/Vis spectroscopy at a wavelength of 600 nm. Once the exponential microbial growth was assured, the test formulations and calibration solutions were taken out of incubation and analysed in...
Chapter 3 - Microbiology Studies

triplicate by UV/Vis spectroscopy at a wavelength of 600 nm. The resultant optical density measurements of the calibration samples were used to form a calibration curve that was in turn used to determine the free (and consequently complexed), parabens concentration in the test formulations.

3.3 Results and discussion

3.3.1 Preliminary phase solubility study of hydrocortisone and HP-β-CD in the presence of parabens

As mentioned in Chapter 2, phase solubility diagrams of HC with HP-β-CD in the presence of parabens were obtained, and assuming a 1:1 complex stoichiometry, phase solubility characteristics were calculated and discussed. To gain further insight into the antimicrobial efficacy of parabens in the presence of CDs, an extra parabens concentration was included in the study and, therefore, a phase solubility diagram of HC and HP-β-CD in the presence of 0.05 % w/v parabens was obtained and phase solubility characteristics calculated (Figure 3.3 and Table 3.5).

![Figure 3.3: Phase solubility diagrams of hydrocortisone with HP-β-CD in aqueous parabens solutions at room temperature (23 ± 1°C). Each point: mean ± SEM, n = 3. Note that some error bars are within data points. Significant difference (p = 0.05) between all parabens concentrations, except 0 % and 0.05 % parabens, across whole CD concentration range.](image-url)
Chapter 3 - Microbiology Studies

Table 3.5: Inclusion complex data of hydrocortisone and HP-β-CD in aqueous parabens solution at room temperature (23 ± 1°C)

<table>
<thead>
<tr>
<th>Parabens (%)</th>
<th>Phase solubility profile</th>
<th>Intrinsic solubility, ( D_s ) (mg/mL)</th>
<th>( K_{11} ) (M⁻¹)</th>
<th>CE</th>
<th>Molar ratio (Drug:CD)</th>
<th>Maximum solubility increase (Fold)</th>
<th>Complexed cyclodextrin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-β-CD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Aₜ</td>
<td>0.2</td>
<td>1725</td>
<td>1.14</td>
<td>1:1.9</td>
<td>82</td>
<td>53</td>
</tr>
<tr>
<td>0.05</td>
<td>Aₜ</td>
<td>0.3</td>
<td>1599</td>
<td>1.23</td>
<td>1:1.8</td>
<td>73</td>
<td>55</td>
</tr>
<tr>
<td>0.10</td>
<td>Aₜ</td>
<td>0.3</td>
<td>1316</td>
<td>1.01</td>
<td>1:2.0</td>
<td>68</td>
<td>50</td>
</tr>
<tr>
<td>0.20</td>
<td>Aₜ</td>
<td>0.3</td>
<td>1094</td>
<td>0.89</td>
<td>1:2.1</td>
<td>62</td>
<td>47</td>
</tr>
</tbody>
</table>

The phase solubility profile of HC/HP-β-CD in the presence of 0.05 % w/v parabens was similar to the solution containing 0 % w/v parabens indicating that 0.05 % w/v parabens did not compete with the HC for the HP-β-CD cavity unlike 0.10 and 0.20 % w/v parabens. Nevertheless, after calculating the minimum HP-β-CD concentrations required to obtain 2 mg/mL HC in the presence of parabens, it was apparent that an increasing amount of HP-β-CD was required as a function of increasing parabens concentration. This demonstrates that the parabens did compete with the HC for the HP-β-CD cavity even at a concentration of 0.05 % w/v, when already in solution.

The minimum HP-β-CD concentrations to obtain 2 mg/mL HC were calculated as 1.12, 1.31, 1.84 and 2.27 % w/v HP-β-CD when the solutions contained 0, 0.05, 0.10 and 0.20 % w/v parabens, respectively. In other words, double the HP-β-CD was required when 0.20 % w/v parabens was present compared to 0 % w/v parabens. If all the extra HP-β-CD complexed with the parabens, and assuming a 1:1 complex was formed between the HP-β-CD and the parabens, it was calculated that:

- 0.030 % w/v parabens was actually free from the 0.05 % w/v parabens - (60 %)
- 0.023 % w/v parabens was actually free from the 0.10 % w/v parabens - (23 %)
- 0.057 % w/v parabens was actually free from the 0.20 % w/v parabens - (29 %)

Based on the free parabens concentrations it was, therefore, hypothesised that the formulations containing 0.05 and 0.10 % w/v parabens would not have adequate antimicrobial preservation because the level of free parabens was too low (limit of affect) but the formulation containing 0.20 % w/v parabens would possess adequate preservative characteristics as the free level of parabens was sufficient.

3.3.2 Efficacy of antimicrobial preservation test

To gain assurance that no microorganisms were already present in the samples, “non-spiked” formulations of HP-β-CD (20 mL), containing 2.0 mg/mL HC and 0, 0.05, 0.10,
and 0.20 % w/v parabens (propylparaben: methylparaben ratio of 1:9), were monitored for microbial growth over the 28-day study period. These samples were not inoculated with microorganisms but were opened for a brief period of time in the same aseptic conditions to simulate that they were inoculated. From the control experiment all formulations were shown to produce no microbial growth over the 28-day test period, illustrating that there was no pre-contamination of the formulations that may have influenced the inoculated formulations.

The test for efficacy of antimicrobial preservation described in the British Pharmacopoeia (2008) was subsequently carried out on HP-β-CD solutions containing 2.0 mg/mL HC and 0, 0.05, 0.10, and 0.20 % w/v parabens (Table 3.6). This type of study has yet to be published in the literature and illustrates part of the novelty of this thesis. This test essentially challenged the parabens within the formulations with *E. coli*, *P. aeruginosa*, *S. aureus*, *A. niger*, *C. albicans*, and *Z. Rouxii*, and monitored the preservative properties of the preparation against the requirements of the test (Table 3.3). To pass the pharmacopoeia guidelines the parabens had to firstly ascertain a total viable microbial count log reduction of 1 (fungi), or 3 (bacteria), from the day 0 total viable microbial count and secondly occur no microbial increase from the day 14 count after 28 days. In other words, the parabens had to kill of an initial amount of microorganisms and then prevent proliferation of the surviving microorganisms.

The results of the antimicrobial efficacy test show that the control formulation (0 % w/v parabens), had no antimicrobial preservation against the microorganisms, except against *A. niger* and, therefore, showed that HC and HP-β-CD had no antimicrobial properties. These findings are consistent with Loftsson et al., (1992), who found that HP-β-CD aqueous solutions did not possess any antimicrobial activity when challenged with microorganisms and that addition of HC did not enhance the antimicrobial effectiveness of the preservative. The formulations containing 0.05 and 0.10 % w/v parabens only had antimicrobial preservation against *A. niger* and *C. albicans* for the 0.05 and 0.10 % w/v parabens, respectively, and had no antimicrobial preservation against any of the bacteria. Thus, it can be suggested that because a fraction of the parabens in these formulations was likely to complexed with some of the HP-β-CD, as depicted by the phase solubility studies, there was not enough free parabens to provide adequate antimicrobial preservation (Loftsson et al., 1992; Matsuda et al., 1993). Since parabens has greater antimicrobial activity against yeasts and molds than bacteria the
concentration of free parabens was adequate to preserve the formulations against these microorganisms (Soni et al., 2005).

Table 3.6: Antimicrobial preservation effectiveness of hydrocortisone formulations containing HP-β-CD and various amounts of parabens

<table>
<thead>
<tr>
<th></th>
<th>Log Reduction</th>
<th></th>
<th>Result: Pass/Fail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 14</td>
<td>Day 28</td>
<td></td>
</tr>
<tr>
<td><strong>Aspergillus niger</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 % parabens</td>
<td>1</td>
<td>No increase</td>
<td>Pass</td>
</tr>
<tr>
<td>0.05 % parabens</td>
<td>1</td>
<td>No increase</td>
<td>Pass</td>
</tr>
<tr>
<td>0.10 % parabens</td>
<td>No reduction</td>
<td>No increase</td>
<td>Fail</td>
</tr>
<tr>
<td>0.20 % parabens</td>
<td>1</td>
<td>No increase</td>
<td>Pass</td>
</tr>
<tr>
<td><strong>Candida albicans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 % parabens</td>
<td>No reduction</td>
<td>No increase</td>
<td>Fail</td>
</tr>
<tr>
<td>0.05 % parabens</td>
<td>1</td>
<td>No increase</td>
<td>Pass</td>
</tr>
<tr>
<td>0.10 % parabens</td>
<td>1</td>
<td>No increase</td>
<td>Pass</td>
</tr>
<tr>
<td>0.20 % parabens</td>
<td>2</td>
<td>No increase</td>
<td>Pass</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 % parabens</td>
<td>No reduction</td>
<td>No increase</td>
<td>Fail</td>
</tr>
<tr>
<td>0.05 % parabens</td>
<td>No reduction</td>
<td>No increase</td>
<td>Fail</td>
</tr>
<tr>
<td>0.10 % parabens</td>
<td>2</td>
<td>Increase</td>
<td>Fail</td>
</tr>
<tr>
<td>0.20 % parabens</td>
<td>3</td>
<td>Increase</td>
<td>Fail</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 % parabens</td>
<td>1</td>
<td>No increase</td>
<td>Fail</td>
</tr>
<tr>
<td>0.05 % parabens</td>
<td>1</td>
<td>Decrease</td>
<td>Fail</td>
</tr>
<tr>
<td>0.10 % parabens</td>
<td>1</td>
<td>Decrease</td>
<td>Fail</td>
</tr>
<tr>
<td>0.20 % parabens</td>
<td>3</td>
<td>No increase</td>
<td>Pass</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 % parabens</td>
<td>1</td>
<td>Decrease</td>
<td>Fail</td>
</tr>
<tr>
<td>0.05 % parabens</td>
<td>2</td>
<td>No increase</td>
<td>Fail</td>
</tr>
<tr>
<td>0.10 % parabens</td>
<td>3</td>
<td>Increase</td>
<td>Fail</td>
</tr>
<tr>
<td>0.20 % parabens</td>
<td>3</td>
<td>No increase</td>
<td>Pass</td>
</tr>
<tr>
<td><strong>Zygosaccharomyces Rouxii</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP criteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 % parabens</td>
<td>2</td>
<td>3</td>
<td>Fail</td>
</tr>
<tr>
<td>0.05 % parabens</td>
<td>Log increase of 2</td>
<td>Log increase of 0.1</td>
<td>Fail</td>
</tr>
<tr>
<td>0.10 % parabens</td>
<td>Log increase of 1</td>
<td>No growth</td>
<td>Fail</td>
</tr>
<tr>
<td>0.20 % parabens</td>
<td>No growth</td>
<td>No growth</td>
<td>Pass</td>
</tr>
</tbody>
</table>

The formulation containing 0.20 % w/v parabens reduced sufficiently the total viable counts at day 14 for all microorganisms and prevented the growth of all the microorganisms tested at day 28, apart from the *S. aureus*. This particular failure was unexpected, since the formulations containing 0.20 % w/v parabens worked sufficiently with all the other microorganisms. Because all the other parabens concentrations failed to meet the BP criteria in relation to the *S. aureus* it is reasonable to say that perhaps...
Chapter 3 - Microbiology Studies

this particular bacterial strain was resistant to the parabens. It is more likely, however, an experimental error occurred. For instance, it is possible that the concentration of the S. aureus inoculation was too high at the beginning of the experiment, thus being too high for sufficient antimicrobial action. Aside from the S. aureus result, it would appear that the concentration of free parabens (approximately calculated at 0.06 % w/v), was adequate enough to combat the microorganisms and meet the antimicrobial criteria set by the pharmacopoeia, despite there being more parabens complexing with the HP-β-CD when 0.20 % w/v parabens was present than when 0.05 or 0.10 % w/v parabens was present.

The overall results of the efficacy of antimicrobial preservation study show that the formulation containing 0.20 % w/v parabens had greater antimicrobial preservation than the formulations containing 0.05 and 0.10 % w/v parabens. The formulations, however, failed the meet the Pharmacopoeia’s full requirements, despite the formulation containing 0.20 % w/v parabens failing only one aspect of the study. The hypothesis that the formulations containing 0.05 and 0.10 % w/v parabens would fail the test because they contained not enough free parabens was confirmed but the formulation containing 0.20 % w/v parabens also failed the test due to insufficient free parabens and, therefore, conveys that the initial hypothesis was incorrect.

Based on these results if a 2.0 mg/mL HC formulation containing HP-β-CD were to have parabens as a preservative, it would be necessary to carry out further phase solubility studies with HC and HP-β-CD in the presence of parabens greater than 0.20 % w/v. It would then be necessary to determine the HP-β-CD concentration required to achieve 2 mg/mL HC in the presence of the parabens, and then carry out the efficacy of antimicrobial preservation monograph on the formulations to ensure that the parabens have sufficient preservative properties.

3.3.3 Turbidity testing

During the phase solubility studies of HC with HP-β-CD in the presence of 0.05, 0.10, and 0.20 % w/v parabens, the theoretical concentrations of free parabens within the solutions were calculated. These concentrations were 0.030, 0.023, and 0.057 % w/v parabens for the solutions containing 0.05, 0.10, and 0.20 w/v parabens. To confirm whether the free parabens concentrations were accurately determined from the phase solubility studies and to gain assurance of the concentration of free parabens within the
formulations a turbidity test study (via optical density measurements), using *E. coli*, *P. aeruginosa*, and *S. aureus* was carried out (Figure 3.2).

Firstly, the time taken for exponential microbial growth was monitored via optical density measurements to ensure that the microorganisms were growing at an exponential rate (Figure 3.4). As exponential microbial growth was observed the incubation time of 400 minutes was used during the remainder of the study to ensure that the formulations were analysed at a time point when microbial proliferation was occurring. Following on from monitoring the time for exponential microbial growth, a calibration curve of parabens activity against *E. coli*, *P. aeruginosa* and *S. aureus* was determined via optical density measurements from inoculated TSB solutions, which were incubated at 37 °C in a Multitron II mixing oven at 200 rpm for 400 minutes (Figure 3.5). The presence of *E. coli*, *P. aeruginosa*, and *S. aureus* (as determined by optical density), within the formulations (Table 3.4), was then determined (Table 3.7), and subsequently the concentration of free parabens within the formulations was calculated from the calibration curves (Table 3.8).

![Figure 3.4: Semi-logarithmic growth curve of *E. coli*, *P. aeruginosa*, and *S. aureus* (as determined by optical density), in tryptone soya broth after incubation at 37 °C in a Multitron II mixing oven at 200 rpm. Each point: mean ± SEM, $n = 3$. Note that some error bars are within data points.](image-url)
Figure 3.5: A semi-logarithmic optical density plot (calibration curve), of *E. coli*, *P. aeruginosa*, and *S. aureus* in the presence of parabens after incubation at 37 °C in a Multitron II mixing oven at 200 rpm. Each point: mean ± SEM, *n* = 3. Note that some error bars are within data points.

Table 3.7: Optical density measurements of *E. coli*, *P. aeruginosa*, and *S. aureus* within the study formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th><em>E. coli</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>1.1</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>0.9</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>1.3</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>6</td>
<td>1.3</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>7</td>
<td>1.2</td>
<td>0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>0.5</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>9</td>
<td>1.3</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>10</td>
<td>1.3</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>11</td>
<td>1.3</td>
<td>0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>12</td>
<td>1.3</td>
<td>0.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Table 3.8: Free parabens concentrations of formulations 1-12 as determined from the calibration curves (Figure 3.5), of E. coli, P. aeruginosa, and S. aureus

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Total Parabens (% w/v)</th>
<th>Free parabens (% w/v) - measured</th>
<th>Free parabens (% w/v) - calculated from phase diagram</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>E. coli: 0, P. aeruginosa: 0, S. aureus: 0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>E. coli: 0.02, P. aeruginosa: 0.02, S. aureus: 0.02</td>
<td>0.030</td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>E. coli: 0.04, P. aeruginosa: 0.05, S. aureus: 0.04</td>
<td>0.023</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
<td>E. coli: 0.08, P. aeruginosa: 0.10, S. aureus: 0.08</td>
<td>0.057</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>E. coli: 0, P. aeruginosa: 0, S. aureus: 0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.05</td>
<td>E. coli: 0, P. aeruginosa: 0, S. aureus: 0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.10</td>
<td>E. coli: 0.02, P. aeruginosa: 0.01, S. aureus: 0</td>
<td>0.01 (0.006)</td>
</tr>
<tr>
<td>8</td>
<td>0.20</td>
<td>E. coli: 0.06, P. aeruginosa: 0.07, S. aureus: 0.08</td>
<td>0.07 (0.006)</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>E. coli: 0, P. aeruginosa: 0, S. aureus: 0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>E. coli: 0, P. aeruginosa: 0, S. aureus: 0</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>E. coli: 0, P. aeruginosa: 0, S. aureus: 0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>E. coli: 0, P. aeruginosa: 0, S. aureus: 0</td>
<td></td>
</tr>
</tbody>
</table>

All three microbial calibration curves calculated similar values for the free parabens within Formulations 1-12. This provided assurance that the average free parabens concentrations were assessed appropriately by the optical density measurements and that the results were reproducible using different microorganisms. Formulations 9-12 were control formulations containing no parabens. These formulations showed large optical densities that corresponded to no preservative action within the formulations, thus the concentration of microorganisms within the formulations were equal to when no parabens was present on the calibration curves. This illustrated that HC and HP-β-CD had no preservative action and coincided with the results determined during the efficacy of antimicrobial preservation study and previously reported studies of the antimicrobial preservation of CD formulations (Loftsson et al., 1992).

Formulations 1-4, which represented the same formulations used during the efficacy of antimicrobial preservation study, showed increasing antimicrobial activity as a function of increasing parabens concentration. Formulation 1 and 5, containing no parabens, did not have any antimicrobial preservation and produced identical results to Formulation 9-12, demonstrating reproducibility of the experiment. The increasing concentration of parabens in Formulation 2, 3, and 4, produced increasing antimicrobial activity against the test microorganisms. The level of antimicrobial preservation was, however, not at the concentration of parabens that was added initially to the formulation (i.e. 0.05, 0.10, 0.20).
Chapter 3 - Microbiology Studies
and 0.20 % w/v parabens), but at much lower level corresponding to the concentration
that was not complexed with the HP-β-CD but free in solution. This further supports the
findings from the efficacy of antimicrobial preservation study and previously reported
studies of the antimicrobial preservation of CD formulations, in that parabens interacts
with CD molecules in solution, which ultimately reduces the free parabens in solution
and antimicrobial activity of the parabens (Loftsson et al., 1992; Matsuda et al., 1993).
Formulations 5-8 consisted of HP-β-CD solutions containing 0, 0.05, 0.10, and 0.20 %
w/v parabens, respectively, but without the presence of HC. As expected Formulation 5,
which contained no parabens, showed no antimicrobial activity and further illustrated
that HP-β-CD had no preservative effect. Formulations 6-8 showed on average an
increase in antimicrobial preservation as a function of increasing parabens
concentration. Nevertheless, the level of antimicrobial preservation did not correlate
with the corresponding solutions, Formulations 2-4, where the antimicrobial activity
was lower in Formulations 6-8 than Formulations 2-4. Formulation 6, which contained
0.05 % parabens, showed no preservative effect had taken place. It is, therefore,
assumed that all of the parabens was interacting with the HP-β-CD in the absence of the
HC, thus no parabens was free in solution capable of antimicrobial action. Formulations
7 and 8 produced similar results, each showing that more parabens was complexed with
the HP-β-CD in the absence of HC. These results highlighted the hydrophobic nature of
parabens and were likely to be caused by the increased concentration of free HP-β-CD,
which the parabens could complex with when no HC was present, compared to when
HC was present.

The main aim of the turbidity test study was to determine the concentration of actual
free parabens within the various HC formulations. This was calculated as 0.02, 0.04,
and 0.09 % w/v parabens for the formulations containing 0.05, 0.10, and 0.20 % w/v
parabens. These results further illustrate that the parabens complexes with the HP-β-CD
as a function of increasing parabens concentration and that the level of free parabens
was less than half the original amount. This is extremely important to know when
assessing the likelihood of a formulation having adequate microbial preservation.

Comparing the free parabens concentrations from the turbidity study with the theoretical
values from the phase solubility study of HP-β-CD and HC in the presence of parabens,
it is clear to see the assumptions put forward when calculating the theoretical values
were inaccurate (Table 3.9).
Chapter 3 - Microbiology Studies

Table 3.9: Free parabens concentration in various HP-β-CD formulations containing 2 mg/mL hydrocortisone as determined by phase solubility and turbidity studies

<table>
<thead>
<tr>
<th>HP-β-CD/HC Formulation containing parabens (% w/v)</th>
<th>Free parabens (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phase solubility study</td>
</tr>
<tr>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>0.10</td>
<td>0.02</td>
</tr>
<tr>
<td>0.20</td>
<td>0.06</td>
</tr>
</tbody>
</table>

The free parabens determined from the phase solubility study was calculated based on the assumption that the extra HP-β-CD required to provide 2 mg/mL HC was all complexing in a 1:1 fashion with the parabens. With the exception, however, of the lower concentration of parabens, the turbidity study illustrated that there was less parabens complexing with the HP-β-CD than assumed and more parabens was free in solution, capable of providing antimicrobial preservation. These results, therefore, imply that not all the extra HP-β-CD required in the formulations was complexing with the parabens. Thus, the equilibrium dynamics between the HP-β-CD, HC and parabens is not as straightforward as first assumed.

Based on the results of the turbidity study, it may be necessary to carry out further studies to determine how much initial parabens (complexed and uncomplexed), is required to ascertain a level of free parabens sufficient enough for antimicrobial action. At some point, however, the initial level of parabens required to achieve a sufficient antimicrobial action may be unachievable in terms of maximum solubility and unacceptable in terms of concentrations for a paediatric formulation. High levels of parabens are deemed unacceptable in paediatric formulations, especially propylparaben. If the initial levels of parabens (complexed and uncomplexed), within the formulation became too high just to achieve a sufficient free parabens concentration then an alternative preservative may be required. It would also be necessary to see if the toxic effects of parabens remained the same when in complexed form. If CD complexation reduced the adverse effects of parabens then more could be incorporated into the formulation.

3.4 Conclusions

It was illustrated in this chapter that the complexation of the parabens with HP-β-CD reduced the antimicrobial efficacy of the preservative, thus rendering the formulations in which they were incorporated unpreserved to microbial contamination. The 2 mg/mL HC formulations containing HP-β-CD and 0, 0.05, and 0.10 % w/v parabens produced
Chapter 3 - Microbiology Studies

vastly inadequate preservative activity and failed the British Pharmacopoeia efficacy of antimicrobial preservation test, whilst the 2 mg/mL HC formulation containing HP-β-CD and 0.20 % w/v parabens failed the test narrowly. By carrying out a turbidity study, the amount of free parabens that was present within these formulations was also demonstrated. In addition, the turbidity study illustrated that more free parabens was contained in the solutions than previously determined by the phase solubility diagrams signifying that not all the extra HP-β-CD required in the formulations was complexed with the parabens. Thus, the data obtained here indicates that parabens, up to a concentration of 0.20 % w/v, was not a suitable preservative for an oral liquid paediatric formulation that contained CDs, as the concentration of free parabens within the formulation was too low. Further phase solubility, antimicrobial efficacy, and turbidity studies are also required to research the possible toxicity of parabens complexed with CD.
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

4.1 Introduction

4.1.1. Taste masking assessment *in vitro*: ASTREE electronic tongue studies

Evaluating the taste of a medication is a crucial part of paediatric dosage form development. Palatability studies with human taste panels are carried out commonly at the latter stages of formulation development. Recent advances in sensor technology have allowed the development of *in vitro* taste analysers, termed commonly “electronic tongues”, for the taste assessment of medicines (Legin *et al.*, 2004). Electronic tongue systems consist typically of multi-sensor systems with a variety of selectivity/ cross-sensitivity and sophisticated data processing techniques to interpret their outputs. Electronic tongues have advances over human taste panels by providing rapid-throughput screening of tastants and taste masking agents, as well as being able to be implemented at an earlier stage of formulation optimisation, often when toxicological data has yet to be established (Anand *et al.*, 2007).

4.1.2. ASTREE electronic tongue

4.1.2.1 Introduction

The ASTREE electronic tongue (ASTREE) (Figure 4.1) is a commercially available *in vitro* taste analyser that was developed for the qualitative and quantitative taste assessment of liquid samples (Kataoka *et al.*, 2005; Sadrieh *et al.*, 2005; Zheng and Keeney, 2006; Kayumba *et al.*, 2007; Li *et al.*, 2007; Lorenz *et al.*, 2009). The system has been developed to emulate biological taste reception at the receptor level, the circuit level and the perceptual level. *In vitro taste analysers are a relatively new concept to pharmaceutics. Since the ASTREE was used extensively throughout the taste masking research of this thesis it was deemed appropriate to describe how the ASTREE works, give an outline of typical analysis methods performed with the ASTREE, and explain the data processing techniques that are used, as well as familiarise the terminology associated with in vitro taste analysers. This was also deemed appropriate because published ASTREE literature is minimal and the extensive novel ASTREE research in this thesis is particularly innovative to drug formulation.*
Figure 4.1: ASTREE electronic tongue system. A) PC for data analysis and instrument control, B) associated interface electronics unit, C) 48-position autosampler and sensor array (Alpha M.O.S, 2004)

4.1.2.2 Sensor technology

At the “receptor level” the ASTREE has seven cross-sensitive and cross-selective potentiometric chemical sensors based on chemically modified field-effect-transistor (CHEMFET) technology (Bergveld, 2003). The exact details regarding the make up of each sensor is limited due to current patent applications by the manufacturer, Alpha M.O.S. Each sensor is composed of two highly conducting silicone semiconductor transistor regions: a source and a drain that are surrounded by an insulator and an encapsulating polymer coating. Covalently bonded above the insulator between the source and the drain is an organic coating that is exposed to the outside environment (i.e. the various molecules within the liquid samples). The composition of the organic coating varies between the sensors and defines its selectivity and sensitivity to various compounds. In addition to the seven sensors is an Ag/AgCl reference electrode, which has a fixed voltage applied to it during sample measurements (Figure 4.2). The seven sensors used during this particular research form a “pharmaceutical sensor array” that is specifically designed for taste analysis with pharmaceutical formulations (Alpha M.O.S, 2004). The seven sensors are designated ZZ, AB, BA, BB, CA, DA, and JE. It is uncertain whether the designated sensor names have any relevance to the molecules that they are capable of interacting with.
Measurements of compounds within a sample are conducted in a potentiometric fashion - interactions between molecules present in the sample and the organic membrane affects the voltage of the membrane of each particular sensor and thus, the source and drain lying underneath. As the composition of each organic membrane varies the voltage produced by each sensor is different. Voltage measurements from the sensors are amplified by the ASTREE associated interface electronics unit, which in turn relays on-line voltage measurements to the ASTREE software on the PC. The recorded on-line sample measurement for a particular sensor corresponds to the voltage difference between the sensor and the Ag/AgCl reference electrode. The length of time for an on-line sample measurement is predetermined before analysis commences - a typical sample measurement is 120 seconds (long enough to obtain a linear response to the sample), although some researchers have reported longer measurement times for more complex formulations (Sadrieh et al., 2005). The ASTREE produces and records seven voltage measurements per sample over the designated time period, i.e. a multicomponent measurement. These measurements are otherwise known as “taste patterns” (Legin et al., 2002). The sensors produce different taste patterns for chemical substances with different tastes, whereas similar patterns are obtained for substances with similar tastes (Vlasov et al., 2002). In this research the taste patterns recorded by the ASTREE software are collectively termed “raw data” (Figure 4.3).
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

Figure 4.3: An example of the raw data produced by the seven sensors of the ASTREE electronic tongue during taste analysis of a liquid sample

Various methods of multivariate calibration and pattern recognition can be used for raw data processing. The type of data processing depends on the type of analysis conducted and the aims of the analysis. The ASTREE may be applied in principle to two main tasks: quantitative determination of the content of components and classification (recognition, identification, discrimination).

4.1.2.3 Experimental procedures

The ASTREE “start-up” procedure - The ASTREE “start-up” procedure (Figure 4.4), is carried out before each study, on a weekly basis, when the sensors need reconditioning/ recalibrating, or when the sensors are left in the air for any substantial time (minimum 48 hrs), that would cause sensor dehydration. Each step is automated by the ASTREE software and conducted at room temperature (Alpha M.O.S, 2004).

A system auto-test is performed to monitor that all parts of the equipment are configured and communicating. Sensors are conditioned in three solutions of 80 mL 0.01 M HCl for 1 h to obtain fully hydrated sensors and obtain a linear signal output - if poor sensor readings are recorded, the problem is investigated and the sensor conditioning procedure is repeated until satisfactory sensor recordings are produced. After sensor conditioning, the sensors undertake the chemical calibration process in 80 mL 0.01 M HCl, under the following standard calibration parameters:
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

- Sensor conditioning check trial No: n = 3
- Sensor calibration check trial No: n = 3
- Sensor stability criteria: 0.9 mV
- Sensor dispersion: 5.5 mV
- Conditioning criteria 1 - overall dispersion: 30 mV
- Conditioning criteria 2 - overall stability: 0.55 mV
- Sensor calibration adjustment: 10 mV

**Figure 4.4:** Steps used during the ASTREE “start-up” procedure. Adapted from ASTREE user manual (Alpha M.O.S, 2004)

Once the sensor calibration process is successful (approximately 1 h), the sensors’ discriminatory power is analysed via the sensor diagnostic process, whereby 80 mL solutions of 0.01 M HCl, NaCl, and MSG are analysed by the ASTREE. The diagnostic results are monitored for sample discrimination by the sensors via sensor mV recordings, and principle component analysis (PCA). The ASTREE software automatically carries out the whole diagnostic process, including PCA. A successful diagnostic is achieved when all sensors discriminate the three diagnostic solutions, and the PCA discrimination index (DI) is greater than 94 (Alpha M.O.S, 2004) (Figure 4.5). Once the “start-up” procedure is complete the conditioning and calibration steps are repeated with a sample containing the drug/compounds that are to be assessed. This process allows the sensors to be conditioned and calibrated to the molecules within the analysis samples, readjusts the potentiometric voltage measurement of the ASTREE, and reduces the likelihood of sensor drift.
Sample preparation - Samples used during analysis are required to be liquid samples made from distilled water that are free from particles and are prepared at room temperature. Solids can interfere with the sensors resulting in inaccurate voltage measurement. Samples are, therefore, filtered before they are analysed. The sample volume is required to be 80 mL for an ASTREE fitted with a 16-position autosampler so that the organic coating on each sensor is fully submerged in the sample. Less volume of sample is required for an ASTREE fitted with a 48-position autosampler.

Analysis sequences - The experimental procedures conducted during this research utilised two main analytical methods: quantification of sample components (preliminary taste analysis), and sample discrimination (taste masking analysis). The analysis sequence for quantification of sample components follows a series of drug concentrations from low to high (Figure 4.6). The drug samples are termed A1, A2, A3...An, whilst the cleaning samples (distilled water), are termed W1, W2, W3...Wn. In this instance there is also a drug sample containing distilled water and no drug termed A0. This is different for the preliminary taste masking analysis.
The analysis sequence for **preliminary taste masking analysis** involves the use of two types of sample:

- **Active** - Samples that contain the drug (at a fixed concentration) and an increasing concentration of taste masking agent, starting from the A0 sample containing just the drug in distilled water with no taste masking agent. These samples are known as “active” samples and are given the prefix A0, A1, A2, A3…An.

- **Placebo** - Samples that contain NO drug but the corresponding concentration of taste masking agent to the active samples. These samples are known as “placebo” samples and are given the prefix P0, P1, P2, P3…Pn. The P0 sample, corresponding to the A0 sample, is just a plain distilled water sample.

The analysis sequence used to analyse the taste masking samples follows one of two patterns. The first sequence (S1) is formed on the basis of P0, P1…Pn, A0, A1…An and consists of two concentration gradients in terms of taste masking agent concentration throughout the sequence. The second sequence (S2) follows a P0, A0, P1, A1…Pn, An pattern and consists of one concentration gradient (Figure 4.7). Both sequences incorporate cleaning samples that, although interspersed on the autosampler, are used after every sample measurement.
Sample measurement - Samples are placed on the autosampler before analysis commences. Sample measurements are conducted typically over 120 seconds and samples are measured one-after-the-other in the sequence order that was programmed into the ASTREE software. Sensors are cleaned in distilled water, termed “cleaning samples”, for 10 seconds in between each sample measurement to ensure no contamination of the samples occurs. One full sequence of sample measurements is called a “repeat” and each sequences is replicated typically eight times to produce eight sample measurements, or “replicates”, of 120 seconds for each sample.

Data processing, and replicate and sensor selection - The raw data of eight replicates is condensed into a data library using the ASTREE software. The library consists of average potentiometric voltage measurements for each sample over the last 20 seconds of the 120 second recorded measurement, per sensor (Table 4.1). These parameters can be adapted if the sensors require longer to equilibrate to the samples but have been demonstrated to be optimal for most types of analysis. For instance, Sadrieh et al., (2005), took potentiometric voltage measurements for 180 seconds and built data libraries of average potentiometric voltage measurements for each sample over the last 120 seconds, whilst Lorenz et al., (2009), took potentiometric voltage measurements for 120 seconds and built data libraries of average potentiometric voltage measurements for each sample over the last 90 seconds.
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

Table 4.1: An example of part of an ASTREE electronic tongue data library with eight replicates (sample measurements) for samples A0 and A1, per sensors (ZZ, AB, BA, BB, CA, DA, JE)

<table>
<thead>
<tr>
<th>Sample Names</th>
<th>ZZ</th>
<th>AB</th>
<th>BA</th>
<th>BB</th>
<th>CA</th>
<th>DA</th>
<th>JE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0 020.ast</td>
<td>746.99</td>
<td>175.85</td>
<td>997.42</td>
<td>778.47</td>
<td>1212.15</td>
<td>815.85</td>
<td>1468.94</td>
</tr>
<tr>
<td>A0 057.ast</td>
<td>695.16</td>
<td>367.51</td>
<td>962.56</td>
<td>699.49</td>
<td>847.55</td>
<td>608.43</td>
<td>1457.49</td>
</tr>
<tr>
<td>A0 094.ast</td>
<td>717.44</td>
<td>386.81</td>
<td>1011.16</td>
<td>719.31</td>
<td>702.27</td>
<td>574.66</td>
<td>1450.33</td>
</tr>
<tr>
<td>A0 131.ast</td>
<td>688.21</td>
<td>428.19</td>
<td>1013.12</td>
<td>700.63</td>
<td>486.21</td>
<td>563.68</td>
<td>1438.56</td>
</tr>
<tr>
<td>A0 168.ast</td>
<td>695.83</td>
<td>435.45</td>
<td>1037.24</td>
<td>690.15</td>
<td>434.48</td>
<td>538.73</td>
<td>1426.54</td>
</tr>
<tr>
<td>A0 205.ast</td>
<td>684.12</td>
<td>434.19</td>
<td>1022.62</td>
<td>678.31</td>
<td>402.67</td>
<td>526.44</td>
<td>1363.48</td>
</tr>
<tr>
<td>A0 242.ast</td>
<td>678.02</td>
<td>194.06</td>
<td>1103.01</td>
<td>668.62</td>
<td>395.1</td>
<td>517.23</td>
<td>1364.2</td>
</tr>
<tr>
<td>A0 279.ast</td>
<td>691.92</td>
<td>147.07</td>
<td>1010.16</td>
<td>681.85</td>
<td>402.96</td>
<td>530.53</td>
<td>1371.18</td>
</tr>
<tr>
<td>A1 023.ast</td>
<td>4.16</td>
<td>239.27</td>
<td>383.06</td>
<td>102.66</td>
<td>604.78</td>
<td>463.43</td>
<td>842.53</td>
</tr>
<tr>
<td>A1 060.ast</td>
<td>13.98</td>
<td>428.75</td>
<td>390.76</td>
<td>68.17</td>
<td>258.75</td>
<td>297.41</td>
<td>848.42</td>
</tr>
<tr>
<td>A1 097.ast</td>
<td>6.9</td>
<td>457.19</td>
<td>440.29</td>
<td>72.57</td>
<td>108.91</td>
<td>251.33</td>
<td>846.82</td>
</tr>
<tr>
<td>A1 134.ast</td>
<td>5.77</td>
<td>494.42</td>
<td>458.56</td>
<td>73.54</td>
<td>22.3</td>
<td>245.95</td>
<td>841.98</td>
</tr>
<tr>
<td>A1 171.ast</td>
<td>7.54</td>
<td>498.59</td>
<td>493.68</td>
<td>70.8</td>
<td>60.34</td>
<td>236.1</td>
<td>823.97</td>
</tr>
<tr>
<td>A1 208.ast</td>
<td>4.74</td>
<td>497.48</td>
<td>489.31</td>
<td>72.58</td>
<td>103.24</td>
<td>227.72</td>
<td>808.54</td>
</tr>
<tr>
<td>A1 245.ast</td>
<td>2.18</td>
<td>221.15</td>
<td>521.18</td>
<td>76.51</td>
<td>108.73</td>
<td>226.72</td>
<td>800.81</td>
</tr>
<tr>
<td>A1 282.ast</td>
<td>1.1</td>
<td>186.34</td>
<td>497.66</td>
<td>78.41</td>
<td>-111.91</td>
<td>224.1</td>
<td>789.12</td>
</tr>
</tbody>
</table>

Part of the reason why eight sample measurements are made per sample is that the potentiometric sensors require time to become “conditioned” to the sample media. It is a well-known concept in the electronic tongue field that sensors are prone to drift, i.e. the sensor output is not linear unless the sensors are fully conditioned to the sample media (Legin et al., 2002). Frequently the first few average potentiometric voltage values for a particular sensor are different to the rest of the values because of sensor drift. Data treatment is, therefore, often carried out on the data library to identify drift. Drift is identified by creating bar graphs of each replicate measurement per sensor (known as a taste print) (Figure 4.8).

Figure 4.8: A bar graph taste print for sensor DA created during ASTREE electronic tongue taste masking analysis with all eight replicates present per sample.
Standard deviation (SD) and relative standard deviation (RSD) values are calculated across each set of sample measurement per sensor to monitor drift and reproducibility. The recommended criterion for an accurate and precise data library is that the SD across the whole data set should be no greater than 30 mV and the RSD should be within ± 10 mV (Alpha M.O.S, 2006). Using both the taste prints and SD/RSD values, drifting replicates can be omitted from the data library (Figure 4.9). It should be noted, however, that if the first three replicates of a particular sample for a particular sensor, require omission from the data library then the first three replicates for all the samples and all the sensors are also omitted. Any replicate omission has to be ubiquitous across the data library. If more than three replicates need omission then the entire sensor measurements can be omitted from the data library. A minimum of five replicates is required for each sample (Alpha M.O.S, 2006). If sensors are extremely well conditioned to the sample media then replicates will not have to be omitted from the data library. Typically, however, the first three replicates from the data library have to be omitted and perhaps one sensor.

Figure 4.9: A bar graph taste print for sensor DA created during ASTREE electronic tongue taste masking analysis with the first three replicates omitted per sample

Statistics and data interpretation - Once replicate and sensor selection has been applied respectively, multivariate statistics are carried out on the amended data library. Partial least squares regression analysis (PLS) and PCA is conducted on the quantification analysis data libraries, whilst PCA is conducted on the taste masking
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

analysis data libraries. Both methods have been used extensively in electronic tongue analysis (Vlasov et al., 2002; Legin et al., 2002; 2004).

PLS is an extension of the multiple linear regression model that enables the prediction of certain variables (in this instance drug concentration), from the measurement of observable variables (in this instance potentiometric voltage measurements) (de Jong, 1993). In this particular research, a PLS graph, and calculated correlation coefficient, is used to illustrate the ability of the ASTREE to predict the drug concentrations of the samples (x-axis), against the actual drug concentrations based on the potentiometric voltage measurements of the samples (y-axis). When the correlation coefficient is low ($r^2 < 0.80$), it indicates typically that the drug concentration range is either near the sensor’s limit of detection or the drug concentration range is too high for accurate concentration prediction. A PLS graph that has a high correlation coefficient ($r^2 > 0.80$), indicates that the ASTREE can predict accurately the drug concentration throughout the whole range and that the concentration range can be used to determine the drug concentration of samples with “unknown” drug concentration.

PCA is an unsupervised multivariate technique primarily used to reduce the dimensionality of multiple inputs to just two or three dimensions (Jolliffe, 2002). PCA consists of the calculation of a set of new variables, principal components, which are orthogonal and non-correlated. The first principal component (C1) corresponds to the direction of the largest variance in the data and the second principal component (C2) corresponds to the direction of the next largest variance in the data. Usually the first two or three principal components contain about 80 % (or even more), of the variance of the data. Thus, the objective of PCA is to simplify and reduce dimensionality by extracting the smallest number of components that account for most of the variation in the original multivariate data and to summarise the data with little loss of information.

In electronic tongue studies, PCA is used to reduce the multivariate sensor data into 2-dimensional components that can be subsequently used for the recognition, identification, and discrimination of tastants within analysed samples (Legin et al., 2002). This is achieved by plotting C1 on the x-axis and C2 on the y-axis to form a PCA map. Data points are expressed on the PCA map in sample clusters. The PCA DI represents the overall discrimination between the sample clusters. The maximum DI is 100 and illustrates that all samples are discriminated from one another by the ASTREE. A negative DI illustrates sample clusters are overlapping and that samples are not
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies
discriminated from one another. Discrimination between the sample clusters is directly
related to the PCA distances, which are also calculated during PCA. If PCA distances
between sample clusters are < 30 then the samples can not be fully discriminated from
one another and are, therefore, deemed to taste the same (Alpha M.O.S, 2006).

There are two main methods to interpret PCA distances. One method, termed **Pn-An PCA Distances**, is used to determine the taste discrimination between corresponding active and placebo sample clusters; the greater the distance (discrimination), the less efficient the taste masking. Corresponding active and placebo samples that taste similar will be discriminated less than samples that taste different. This particular method of PCA distances interpretation was used previously by researchers using the ASTREE for taste masking analysis (Sadrieh et al., 2005; Zheng and Keeney, 2006; Li et al., 2007; Lorenz et al., 2009). Another method, termed **A0-An PCA Distances**, is used to determine the taste discrimination from the A0 sample and a range of active samples (An). For taste masking analysis, the greater distance between the A0 sample (sample with just drug), and the An samples (sample with drug and taste masking agent), the greater the difference in taste from the A0 sample and, therefore, the more efficient the taste masking agent. For the quantification analysis data the A0-An PCA Distances method is used to gauge the ASTREE discrimination between the samples of increasing drug concentration starting from the A0 sample (water), and A1, A2...An samples. Theoretically, the A0-An PCA Distance will increase as a function of increasing drug concentration where the greatest distance indicates the greatest intensity in taste. When a plateau of A0-An PCA Distance is reached the taste intensity of the samples has reached a maximum. In this particular ASTREE research the taste intensity of the Pd, HC and Qu refers to bitterness intensity.

4.1.3 Aims and objectives

**Overall goals of taste assessment studies:** Part of this thesis was to investigate through taste masking assessment *in vivo* and *in vitro* whether, and how, CDs taste mask corticosteroids in an oral liquid formulation and compare the taste masking efficiency with sweeteners and/or flavouring agents. It was also decided to examine whether excess concentrations of CD were better at taste masking bitter drugs than molar equivalent concentrations of CD, as well as determine whether a formulation containing CD and a clinically relevant concentration of solubilised corticosteroid (e.g. 2.0 mg/mL HC), was bitterer than a formulation containing the same concentration of CD and a
concentration of solubilised corticosteroid below the intrinsic solubility concentration (e.g. 0.20 mg/mL HC).

Before these studies were conducted it was necessary to carry out some preliminary ASTREE experiments. The aims of this chapter were to:

- Investigate whether the ASTREE was capable of analysing the bitter drugs of choice by quantifying accurately a series of Pd, HC, and Qu standards, and ultimately establishing drug concentrations that can be used for preliminary ASTREE taste masking analysis. This was achieved by using the ASTREE to analyse a series of Pd, HC, and Qu standards and interpreting the potentiometric voltage data via PCA and PLS analysis.
- Carry out preliminary taste masking analysis of HC and Qu with CDs, sweeteners and/or flavouring agents to screen and reduce the number of formulations to be assessed by the human taste panel. This was achieved by analysing active and placebo samples of HC and Qu with increasing concentrations of CDs, sweeteners and/or flavouring agents with the ASTREE.
- Investigate whether the sensor cleaning influences ASTREE taste assessment. This was achieved by varying the number of cleaning dips incorporated into the analysis sequences during the quantification of Pd standards.
- Investigate whether the analysis sequence (S1 and S2), influences ASTREE taste assessment. This was achieved by conducting the preliminary taste masking analysis with both analysis sequences.
- Investigate whether the method of PCA interpretation (Pn-An PCA Distances and A0-An PCA Distances), influences ASTREE taste assessment. This was achieved by interpreting the PCA of the preliminary taste masking analysis with Pn-An and A0-An PCA Distances.

4.2 Materials and methods

4.2.1 Materials

As per Section 2.2.3.1, as well as Erythritol (Ph. Eur) (PharmEridex™ 16956) kindly donated by Cargill Inc., (Cedar Rapids IA, USA), acesulpham K purchased from Nutrinova Nutrition Specialists & Food Ingredients (Frankfurt, Germany), sucrose (BP, EP) and hydrochloric acid (1 M) acquired from Fisher Scientific (Loughborough, UK), all analytical grade, sodium chloride (1 M) purchased from Fluka Biochemika (Buchs, Switzerland), cherry flavour (96475-31), tangerine flavour (10888-71), and strawberry
flavour (11030-31) kindly donated by Givaudan (Vernier, Switzerland), and mono-
sodium glutamate (MSG) obtained from Chemlab Scientific Products (Laindon, UK).
All materials were used as received and solutions were prepared using distilled water
produced in the laboratory.

4.2.2 Preliminary analysis - prednisolone, hydrocortisone and quinine
quantification by the ASTREE electronic tongue

Part 1a: Before the start of the quantification analysis the “start-up” procedure was
carried out successfully and the ASTREE sensors were conditioned and calibrated in 80
mL of 0.20 mg/mL Pd, under the standard calibration parameters mentioned previously
(Section 4.1.2.3). Pd standards (0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16,
0.18, and 0.20 mg/mL), were quantified with ASTREE method parameters
incorporating: 120 s of sample measurement by all sensors, 1 cleaning dip for 10 s in
80 mL distilled water after every sample measurement, 8 repeats (8 measurements per
sample), and a data library created by acquiring mean sensor recordings of the last 20 s
for each sample measurement. Pd standards were termed P0 - P11 in order of ascending
concentration.

Part 1b: Pd standards (0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, and
0.20 mg/mL), were analysed under the same conditioning/calibration procedures and
ASTREE method parameters as Part 1a, except 2 cleaning dips in 80 mL distilled water
were made after every sample measurement. These Pd standards were termed A1 - A12
in order of ascending concentration.

Part 1c: HC standards (0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, and
0.20 mg/mL), were analysed under the same ASTREE method parameters as Part 1a,
except sensors were firstly conditioned and calibrated in 80 mL of 0.20 mg/mL HC, and
2 cleaning dips in 80 mL distilled water were made after every sample measurement.
HC standards were termed A1 - A12 in order of ascending concentration to coincide
with new sample terminology.

Part 1d: Qu standards (0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, and 0.16
mg/mL), were analysed under the same ASTREE methods parameters as Part 1a, except
sensors were firstly conditioned and calibrated in 80 mL of 0.16 mg/mL Qu, and 2
cleaning dips in 80 mL distilled water were made after every sample measurement. Qu
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

standards were termed Q1 - Q9 in order of ascending concentration to coincide with new sample terminology.

### 4.2.3 Preliminary taste masking analysis of hydrocortisone and quinine with cyclodextrins, sweeteners, and flavouring agents

As per the quantification analysis, the “start-up” procedure was carried out successfully and the sensors were conditioned and calibrated in 80 mL of 0.20 mg/mL HC or 80 mL of 0.16 mg/mL Qu, under the standard calibration parameters. The analysis sequence for each experiment was conducted under both S1 and S2 sequences, with fresh samples being used for each sequence (Section 4.1.2.3 - Analysis Sequence). ASTREE method parameters used for each analysis incorporated: 120 s of sample measurement by all sensors, 2 cleaning dips for 10 s in 80 mL distilled water after every sample measurement, 8 repeats (8 measurements per sample), and a data library created by acquiring mean sensor recordings of the last 20 s for each sample measurement. Taste masking analysis of 0.20 mg/mL HC and 0.16 mg/mL Qu was conducted with 80 mL solutions with several types of taste masking agent (Table 4.2). Active samples were termed A0, A1,...An and placebo samples P0, P1,...Pn in order of ascending concentration of taste masking agent.

**Table 4.2: Sample concentrations used during preliminary taste masking analysis**

<table>
<thead>
<tr>
<th>Taste masking agent</th>
<th>Concentrations (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sweeteners</strong></td>
<td></td>
</tr>
<tr>
<td>Erythritol</td>
<td>0, 5, 10, 15, 20, 25</td>
</tr>
<tr>
<td>Acesulpham K</td>
<td>0, 0.02, 0.04, 0.06, 0.08, 0.10</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0, 3, 6, 9, 12, 15</td>
</tr>
<tr>
<td><strong>Flavouring Agent</strong></td>
<td></td>
</tr>
<tr>
<td>Tangerine</td>
<td>0, 0.4, 0.8, 1.2, 1.6, 2.0</td>
</tr>
<tr>
<td>Strawberry</td>
<td>0, 0.4, 0.8, 1.2, 1.6, 2.0</td>
</tr>
<tr>
<td>Cherry</td>
<td>0, 0.4, 0.8, 1.2, 1.6, 2.0</td>
</tr>
<tr>
<td><strong>Cyclodextrin</strong></td>
<td></td>
</tr>
<tr>
<td>HP-β-CD (HC analysis)</td>
<td>0, 1.12, 2.24, 5.60, 8.96, 11.20</td>
</tr>
<tr>
<td>Me-β-CD (HC analysis)</td>
<td>0, 0.53, 1.06, 2.65, 4.24, 5.30</td>
</tr>
<tr>
<td>HP-β-CD (Qu analysis)</td>
<td>0, 2.89, 5.78, 14.45, 23.12, 28.90</td>
</tr>
<tr>
<td>Me-β-CD (Qu analysis)</td>
<td>0, 1.04, 2.08, 5.20, 8.32, 10.40</td>
</tr>
</tbody>
</table>

Taste masking analysis of 0.20 mg/mL HC and 0.16 mg/mL Qu was also performed with flavouring agents/ sweetener combinations at selected taste masking concentrations determined from the individual analysis of the agents. In addition, taste masking analysis of 2.0 mg/mL HC and 1.6 mg/mL Qu with CD concentrations as per the analysis at lower drug concentrations was performed by the ASTREE. CD
concentrations were deduced from the minimum amount of CD required to obtain a solution of 2.0 mg/mL HC or 1.6 mg/mL Qu determined from phase solubility studies (Chapter 2). The corresponding molar ratios of CD to HC or Qu are listed in Table 4.3.

Table 4.3: Molar ratio of CD used during the preliminary taste masking analysis

<table>
<thead>
<tr>
<th>Molar ratio in relation to 0.20 mg/mL HC</th>
<th>Molar ratio in relation to 2.0 mg/mL HC</th>
<th>Molar ratio in relation to 0.16 mg/mL Qu</th>
<th>Molar ratio in relation to 1.6 mg/mL Qu</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-β-CD (% w/v)</td>
<td>HP-β-CD (% w/v)</td>
<td>Me-β-CD (% w/v)</td>
<td>Me-β-CD (% w/v)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.12</td>
<td>1:14</td>
<td>2.89</td>
<td>1:41</td>
</tr>
<tr>
<td>5.60</td>
<td>1:70</td>
<td>14.45</td>
<td>1:204</td>
</tr>
<tr>
<td>8.96</td>
<td>1:113</td>
<td>23.12</td>
<td>1:326</td>
</tr>
<tr>
<td>11.20</td>
<td>1:141</td>
<td>28.90</td>
<td>1:408</td>
</tr>
</tbody>
</table>

4.2.4 Data processing, and replicate and sensor selection

Data processing, and replicate and sensor selection was carried out as described in Section 4.1.2.3. Once the quantification and preliminary taste masking analysis was completed the sensor measurements of each experiment was checked for linearity. A data library of each analysis was built with the ASTREE software, consisting of average potentiometric readings of each sample over the last 20 s of sample measurement, per sensor. The dispersion of the eight sample replicates was assessed by SD and RSD, as well as the sensor response to the varying samples. Outlying measurements that were ubiquitous to the analysis were removed from the data library, as were poorly performing sensors.

4.2.5 Data analysis

PCA and PLS analysis was conducted on the processed data libraries of the Pd, HC, and Qu standards. PCA DI, A0-An PCA Distances, and PLS correlation coefficients generated between the actual drug concentrations and the predicted drug concentrations were used to interpret the multivariate data. PCA was conducted on the processed data.
libraries of the preliminary taste masking analysis. PCA distances were deduced and taste masking conclusions were made from both Pn-An and A0-An PCA Distances.

4.3 Results

4.3.1 Preliminary analysis - prednisolone, hydrocortisone and quinine quantification by the ASTREE electronic tongue

Part 1a and 1b: Pd standards were analysed with the ASTREE. The discrimination of the Pd standards analysed with one cleaning dip (Figure 4.10), and two cleaning dips (Figure 4.11), was calculated via PCA. The A0-An PCA Distances were calculated between the data clusters from each PCA (Table 4.4). The ability of the ASTREE to quantify the Pd standards from both analysis were carried out by PLS analysis (Figure 4.12).

Figure 4.10: A PCA map of the ASTREE electronic tongue discrimination of prednisolone standards analysed with one cleaning dip after every sample measurement. Key: P00-water (blue), P01-0.01 mg/mL Pd (red), P02-0.02 mg/mL Pd (orange), P03-0.04 mg/mL Pd (yellow), P04-0.06 mg/mL Pd (light green), P05-0.08 mg/mL Pd (turquoise), P06-0.10 mg/mL Pd (violet), P07-0.12 mg/mL Pd (pink), P08-0.14 mg/mL Pd (peach), P09-0.16 mg/mL Pd (dark green), P10-0.18 mg/mL Pd (black), P11-0.20 mg/mL Pd (purple)
Figure 4.11: A PCA map of the ASTREE electronic tongue discrimination of prednisolone standards analysed with two cleaning dips after every sample measurement. Key: A1-water (blue), A2-0.01 mg/mL Pd (red), A3-0.02 mg/mL Pd (orange), A4-0.04 mg/mL Pd (yellow), A5-0.06 mg/mL Pd (light green), A6-0.08 mg/mL Pd (turquoise), A7-0.10 mg/mL Pd (violet), A8-0.12 mg/mL Pd (pink), A9-0.14 mg/mL Pd (peach), A10-0.16 mg/mL Pd (dark green), A11-0.18 mg/mL Pd (black), A12-0.20 mg/mL Pd (purple).

Table 4.4: A0-An PCA Distances calculated during ASTREE electronic tongue analysis of prednisolone standards.

<table>
<thead>
<tr>
<th>Prednisolone (mg/mL)</th>
<th>Prednisolone A0-An PCA Distance (one cleaning dip)</th>
<th>Prednisolone A0-An PCA Distance (two cleaning dips)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>429</td>
<td>310</td>
</tr>
<tr>
<td>0.02</td>
<td>504</td>
<td>278</td>
</tr>
<tr>
<td>0.04</td>
<td>559</td>
<td>133</td>
</tr>
<tr>
<td>0.06</td>
<td>623</td>
<td>552</td>
</tr>
<tr>
<td>0.08</td>
<td>900</td>
<td>229</td>
</tr>
<tr>
<td>0.10</td>
<td>679</td>
<td>186</td>
</tr>
<tr>
<td>0.12</td>
<td>598</td>
<td>144</td>
</tr>
<tr>
<td>0.14</td>
<td>782</td>
<td>140</td>
</tr>
<tr>
<td>0.16</td>
<td>760</td>
<td>172</td>
</tr>
<tr>
<td>0.18</td>
<td>1020</td>
<td>211</td>
</tr>
<tr>
<td>0.20</td>
<td>345</td>
<td>47</td>
</tr>
</tbody>
</table>
Figure 4.12: PLS of prednisolone standards analysed by the ASTREE electronic tongue with one cleaning dip (left) and two cleaning dips (right) after every sample measurement. Key: 0.01 mg/mL Pd (red), 0.02 mg/mL Pd (orange), 0.04 mg/mL Pd (yellow), 0.06 mg/mL Pd (light green), 0.08 mg/mL Pd (turquoise), 0.10 mg/mL Pd (violet), 0.12 mg/mL Pd (pink), 0.14 mg/mL Pd (peach), 0.16 mg/mL Pd (dark green), 0.18 mg/mL Pd (black), 0.20 mg/mL Pd (purple)
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

When the Pd standards were analysed with one cleaning dip after every sample measurement the majority of the Pd standards were discriminated from one another, indicating that some of the Pd standards had differing tastes (Figure 4.10). The cluster of each Pd standard was, however, slightly dispersed across the PCA map, indicating the ASTREE was not measuring reproducible potentiometric voltages and signifying slight sensor drift was occurring. The negative PCA DI of -6 demonstrated that some of the Pd standards were considered to be similar in taste. Nevertheless, none of the Pd standards was within a PCA distance of 30 from one another, indicating the ASTREE did recognise that each Pd standard tasted different.

When the Pd standards were analysed with two cleaning dips after every sample measurement, the cluster of each Pd standard was less dispersed compared to the analysis with one cleaning dip, demonstrating that the reproducibility was enhanced and sensor drift reduced with the extra cleaning dip (Figure 4.11). This was likely to have resulted from the extra cleaning dip reducing cross contamination between samples. Nonetheless, the PCA DI of -5 was only slightly better than the analysis with one cleaning dip, signifying the overall discrimination between the standards was not influenced by the extra cleaning dip.

The A0-An PCA Distances of the Pd standards analysed with one cleaning dip after every sample measurement increased in correlation to the increasing Pd concentration up until 0.08 mg/mL (Table 4.4). The A0-An PCA Distances of 0.10 to 0.16 mg/mL Pd were below the A0-An PCA Distance of 0.08 mg/mL Pd, indicating that they were deemed less bitter than 0.08 mg/mL Pd by the ASTREE. The greatest A0-An PCA Distance for the Pd standards analysed with one cleaning dip was 1020 and was produced by the 0.18 mg/mL standard. This sample was, therefore, considered the bitterest Pd standard for this particular analysis. The least bitter Pd standard with the smallest A0-An PCA Distance was 0.20 mg/mL Pd. The A0-An PCA Distances of the Pd standards analysed with two cleaning dips demonstrated that 0.06 mg/mL Pd was the bitterest standard and the 0.20 mg/mL Pd was the least bitter standard.

These results show that the ASTREE was not producing reproducible assessments even though the only difference in the two sequences was the extra cleaning dip of 10 seconds after every sample measurement. The extra cleaning dip improved sample measurement reproducibility, reduced sensor drift, and influenced the final conclusions. It is believed, however, that the differing assessments should not be fully attributed to
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

The reduction of cross contamination between the samples. One cleaning dip was found to be sufficient for experiments where more complex formulations were tested, even formulations containing chocolate milk (Sadrieh et al., 2005). Thus, one cleaning dip should have been sufficient for samples as simple as the ones tested in this research. These results might have been caused by the extra cleaning dip altering sensor sensitivity or affecting the conditioning of the sensors to the Pd.

The A0-An PCA Distances of the Pd standards analysed with two cleaning dips indicated no trend in bitterness. Since both analyses concluded that the 0.20 mg/mL Pd standard was the least bitter standard the 0.20 mg/mL Pd standard may have been outside the ASTREE limit of quantification for Pd. This is demonstrated further by the poor correlation coefficients from the PLS analysis. Figure 4.12 shows that when the Pd standards were analysed with one cleaning dip the correlation coefficient was $R^2$: 0.69. This signified that the ASTREE was not capable of predicting accurately the concentration of the Pd standards across the whole concentration range. The correlation coefficients of the Pd standards analysed with two cleaning dips was $R^2$: 0.79, indicating that the extra cleaning dip improved the ability of the ASTREE to predict the Pd concentration but because the correlation coefficient was < 0.80, the ASTREE was not capable of predicting accurately the Pd standards. It was, therefore, concluded that the ASTREE was unable to quantify successfully Pd standards up to across the concentration range tested.

Part 1c: HC standards were analysed with the ASTREE. The ASTREE discrimination of the HC standards was calculated via PCA (Figure 4.13), and the A0-An PCA Distances were calculated between the data clusters from each PCA (Table 4.5). The ability of the ASTREE to quantify the HC standards were carried out by PLS analysis (Figure 4.14).
Figure 4.13: A PCA map of the ASTREE electronic tongue discrimination of hydrocortisone standards analysed with two cleaning dips after every sample measurement. Key: A1-water (blue), A2-0.01 mg/mL HC (red), A3-0.02 mg/mL HC (orange), A4-0.04 mg/mL HC (yellow), A5-0.06 mg/mL HC (light green), A6-0.08 mg/mL HC (turquoise), A7-0.10 mg/mL HC (violet), A8-0.12 mg/mL HC (pink), A9-0.14 mg/mL HC (peach), A10-0.16 mg/mL HC (dark green), A11-0.18 mg/mL HC (black), A12-0.20 mg/mL HC (purple)

Table 4.5: A0-An PCA Distances calculated during ASTREE electronic tongue analysis of hydrocortisone standards

<table>
<thead>
<tr>
<th>Hydrocortisone (mg/mL)</th>
<th>Hydrocortisone A0-An PCA Distance (two cleaning dips)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>56</td>
</tr>
<tr>
<td>0.02</td>
<td>72</td>
</tr>
<tr>
<td>0.04</td>
<td>100</td>
</tr>
<tr>
<td>0.06</td>
<td>113</td>
</tr>
<tr>
<td>0.08</td>
<td>157</td>
</tr>
<tr>
<td>0.10</td>
<td>149</td>
</tr>
<tr>
<td>0.12</td>
<td>179</td>
</tr>
<tr>
<td>0.14</td>
<td>163</td>
</tr>
<tr>
<td>0.16</td>
<td>220</td>
</tr>
<tr>
<td>0.18</td>
<td>240</td>
</tr>
<tr>
<td>0.20</td>
<td>310</td>
</tr>
</tbody>
</table>
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

**Figure 4.14:** PLS of hydrocortisone standards analysed by the ASTREE electronic tongue with two cleaning dips after every sample measurement. Key: A2-0.01 mg/mL HC (red), A3-0.02 mg/mL HC (orange), A4-0.04 mg/mL HC (yellow), A5-0.06 mg/mL HC (light green), A6-0.08 mg/mL HC (turquoise), A7-0.10 mg/mL HC (violet), A8-0.12 mg/mL HC (pink), A9-0.14 mg/mL HC (peach), A10-0.16 mg/mL HC (dark green), A11-0.18 mg/mL HC (black), A12-0.20 mg/mL HC (purple).

Because the ASTREE assessment of Pd standards analysed with two cleaning dips after every sample measurement produced more reproducible potentiometric measurements with the less sensor drift compared to the analysed with one cleaning dip, the remaining ASTREE experiments were conducted with two cleaning dips. The PCA map, representing the ASTREE discrimination of the HC standards, showed that the ASTREE did not fully discriminate all of the HC standards as some of the sample clusters were overlapping (Figure 4.13). This was highlighted by the DI of -118. These results indicated that the ASTREE was better at discriminating the Pd standards than the HC standards since the DI of the Pd PCA was less negative than the DI of the HC PCA. Furthermore, the ASTREE could not discriminate between the 0.01 - 0.02 mg/mL HC, the 0.04 - 0.06 mg/mL HC, and the 0.16 - 0.18 mg/mL HC, as these sample clusters had PCA distances less than 30 between them, unlike the Pd standard. These particular samples were, therefore, deemed to taste the same.
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

As depicted by the smallest A0-An PCA Distance, the least bitter HC standard was the 0.01 mg/mL HC standard (Table 4.5). The A0-An PCA Distance (and bitterness) increased as a function of increasing HC concentration. The 0.20 mg/mL HC standard was deemed the bitterest HC standard with the largest A0-An PCA Distance. These results emulate the expected theoretical results where the A0-An PCA Distance increases as a function of increasing drug concentration and indicated that the ASTREE was better at perceiving the bitterness of the HC than the Pd. Moreover, the ASTREE appeared not to have reached its limit of quantification with the 0.20 mg/mL HC, unlike the 0.20 mg/mL Pd. This was further illustrated by the PLS correlation coefficient of $R^2$: 0.89, signifying that the ASTREE was capable of predicting accurately the concentration of the HC standards from 0.01 to 0.20 mg/mL (Figure 4.14). These results signified that the ASTREE would be able to perform taste masking assessment of HC formulations, although, the maximum A0-An PCA Distance of 300 is not considered a particularly large PCA distance (Alpha M.O.S, 2006).

Part Id: Qu standards were analysed with the ASTREE. The ASTREE discrimination of the Qu standards was calculated via PCA (Figure 4.15), and the A0-An PCA Distances were calculated between the data clusters (Table 4.6). The ability of the ASTREE to quantify the Qu standards were carried out by PLS analysis (Figure 4.16).
Figure 4.15: A PCA map of the ASTREE electronic tongue discrimination of quinine standards analysed with two cleaning dips after every sample measurement. Key: Q1-water (blue), Q2-0.01 mg/mL Qu (red), Q3-0.02 mg/mL Qu (orange), Q4-0.04 mg/mL Qu (yellow), Q5-0.08 mg/mL Qu (light green), Q6-0.10 mg/mL Qu (turquoise), Q7-0.12 mg/mL Qu (violet), Q8-0.14 mg/mL Qu (pink), Q9-0.16 mg/mL Qu (peach)

Table 4.6: A0-An PCA Distances calculated during ASTREE electronic tongue analysis of quinine standards

<table>
<thead>
<tr>
<th>Quinine (mg/mL)</th>
<th>Quinine A0-An PCA Distance (two cleaning dips)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>295</td>
</tr>
<tr>
<td>0.02</td>
<td>459</td>
</tr>
<tr>
<td>0.04</td>
<td>476</td>
</tr>
<tr>
<td>0.08</td>
<td>421</td>
</tr>
<tr>
<td>0.10</td>
<td>365</td>
</tr>
<tr>
<td>0.12</td>
<td>298</td>
</tr>
<tr>
<td>0.14</td>
<td>271</td>
</tr>
<tr>
<td>0.16</td>
<td>307</td>
</tr>
</tbody>
</table>
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

Figure 4.16: PLS of quinine standards analysed by the ASTREE electronic tongue with two cleaning dips after every sample measurement. Key: Q1-0.01 mg/mL Qu (red), Q2-0.02 mg/mL Qu (orange), Q3-0.04 mg/mL Qu (yellow), Q4-0.08 mg/mL Qu (light green), Q5-0.10 mg/mL Qu (turquoise), Q6-0.12 mg/mL Qu (violet), Q7-0.14 mg/mL Qu (pink), Q8-0.16 mg/mL Qu (peach)

The ASTREE produced good discrimination of the various Qu standards. This was highlighted by the separated sample clusters and the PCA DI of 89 (Figure 4.15). The ASTREE, therefore, discriminated the Qu standards more efficiently than the Pd and HC standards, which produced larger overlapping sample clusters and negative DIs from the PCA. The size of the sample clusters also signified that the ASTREE measured the Qu standards with reproducibly, with little sensor drift. These results compare well with the results of Zheng and Keeney (2006), who reported that the ASTREE detected changes in Qu concentration from 0.08 to 0.32 mg/mL.

The smallest A0-An PCA Distances was 271 from the 0.14 mg/mL Qu standard, and the biggest A0-An PCA Distances was 476 from the 0.04 mg/mL Qu standard (Table 4.6). These standards were, therefore, considered the least bitter and bitterest Qu standards,
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

respectively. These results indicated that the ASTREE was unable to ascertain accurately the bitterness of the Qu standards and signified that the bitterness of the Qu may have reached a limit at 0.04 mg/mL. The PLS analysis indicated that the ASTREE was capable of predicting the concentration of the Qu standards based on their potentiometric voltage measurements as illustrated by the correlation coefficient of $R^2$: 0.96 (Figure 4.16). Moreover, these results indicate that the ASTREE was better at predicting the concentration of Qu than either Pd or HC as the correlation coefficient was higher for the Qu compared to the Pd and HC. Based on these results, it was concluded that the ASTREE was capable of assessing Qu formulations, thus demonstrating that Qu would be a suitable model bitter drug to analyse along side the corticosteroids throughout the remainder of the taste assessment.

4.3.2 Preliminary taste masking analysis of hydrocortisone and quinine with cyclodextrins, sweeteners and flavouring agents

The taste masking analysis of 0.20 mg/mL HC (and 2.0 mg/mL HC for the CDs), and 0.16 mg/mL Qu (and 1.6 mg/mL Qu for the CDs), with various concentrations of sweeteners, flavouring agents, CDs, and sweetener/flavouring agent combinations was carried out by the ASTREE to help screen and reduce the number of formulations to be assessed by a human taste panel. The experiments were conducted using analysis sequences S1 and S2. Discrimination of the analysis samples was performed via PCA and PCA distances were interpreted by two methods (See Appendix 1, were PCA distance graphs for all formulations are presented). Taste masking concentrations were selected from the lowest P0-A0 PCA Distances and highest A0-An PCA Distances, whilst ranges of selected concentration were determined if the PCA Distances were similar ($\leq 30$), for HC and Qu, respectively (Table 4.7 and Table 4.8).

The results show that the ASTREE was capable of screening and discriminating optimal taste masking concentrations for some of the taste masking agents that were assessed. Nevertheless, the two PCA distance interpretation methods and the two sequences of sample analysis produced varying selected concentrations for the majority of experiments, indicating that varying sequences influenced the outcome of the analysis and that the PCA distances interpretation methods altered the way in which the data was interpreted. These results showed the analysis method was not robust and the data was easily construed in a different way by the differing PCA distances. Possible reasons for these results are listed in Table 4.9.
### Table 4.7: Selected taste masking concentrations determined by Pn-An PCA Distances and AO-An PCA Distances from preliminary taste masking analysis of 0.20 mg/mL hydrocortisone (and 2.0 mg/mL hydrocortisone for the cyclodextrins)

<table>
<thead>
<tr>
<th>Taste Masking Agent</th>
<th>Analysis sequence 1</th>
<th>Analysis sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Selected taste masking concentration (% w/v) Pn-An Distances</td>
<td>Selected taste masking concentration (% w/v) AO-An Distances</td>
</tr>
<tr>
<td>Acesulpham K</td>
<td>0.02 - 0.06</td>
<td>0.08 - 0.10</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erythritol</td>
<td>5 - 25</td>
<td>15 - 25</td>
</tr>
<tr>
<td>Cherry</td>
<td>0.4 - 2.0</td>
<td>1.6 - 2.0</td>
</tr>
<tr>
<td>Strawberry</td>
<td>0.8 - 2.0</td>
<td>1.6 - 2.0</td>
</tr>
<tr>
<td>Tangerine</td>
<td>0.4 - 0.8</td>
<td>1.6 - 2.0</td>
</tr>
<tr>
<td>Cyclodextrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP-β-CD (0.2 mg/mL HC)</td>
<td>1.12 - 11.2</td>
<td>8.96 - 11.2</td>
</tr>
<tr>
<td>HP-β-CD (2.0 mg/mL HC)</td>
<td>1.12 - 2.24</td>
<td>8.96 - 11.2</td>
</tr>
<tr>
<td>Me-β-CD (2.0 mg/mL HC)</td>
<td>2.65</td>
<td>0.53, 4.24 - 5.3</td>
</tr>
<tr>
<td>Flavouring agent/sweetener combination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 % Cherry + Sweetener</td>
<td>20 % Erythritol</td>
<td>0.08 % Ace K</td>
</tr>
<tr>
<td>2 % Strawberry + Sweetener</td>
<td>15 % Sucrose</td>
<td>0.08 % Ace K</td>
</tr>
<tr>
<td>2 % Tangerine + Sweetener</td>
<td>0.08 % Ace K</td>
<td>0.08 % Ace K</td>
</tr>
</tbody>
</table>

### Table 4.8: Selected taste masking concentrations determined by P0-A0 PCA Distances and A0-An PCA Distances from preliminary taste masking analysis of 0.16 mg/mL quinine (and 1.6 mg/mL quinine for the cyclodextrins)

<table>
<thead>
<tr>
<th>Taste Masking Agent</th>
<th>Analysis sequence 1</th>
<th>Analysis sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Selected taste masking concentration (% w/v) Pn-An Distances</td>
<td>Selected taste masking concentration (% w/v) AO-An Distances</td>
</tr>
<tr>
<td>Acesulpham K</td>
<td>0.02</td>
<td>0.08 - 0.10</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3 - 6</td>
<td>9 - 15</td>
</tr>
<tr>
<td>Erythritol</td>
<td>5</td>
<td>10 - 20</td>
</tr>
<tr>
<td>Cherry</td>
<td>0.4</td>
<td>0.8 - 1.2</td>
</tr>
<tr>
<td>Strawberry</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Tangerine</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Cyclodextrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP-β-CD (0.16 mg/mL Qu)</td>
<td>28.9</td>
<td>23.12 - 28.9</td>
</tr>
<tr>
<td>HP-β-CD (1.6 mg/mL Qu)</td>
<td>2.89</td>
<td>14.45</td>
</tr>
<tr>
<td>Me-β-CD (0.16 mg/mL Qu)</td>
<td>1.04</td>
<td>5.2 - 10.4</td>
</tr>
<tr>
<td>Me-β-CD (1.6 mg/mL Qu)</td>
<td>5.2</td>
<td>1.04</td>
</tr>
<tr>
<td>Flavouring agent/sweetener combination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 % Cherry + Sweetener</td>
<td>0.08 % Ace K</td>
<td>0.02 % Ace K</td>
</tr>
<tr>
<td>2 % Strawberry + Sweetener</td>
<td>15 % Sucrose</td>
<td>0.02 % Ace K</td>
</tr>
<tr>
<td>2 % Tangerine + Sweetener</td>
<td>15 % Sucrose</td>
<td>15 % Sucrose</td>
</tr>
</tbody>
</table>
Table 4.9: Possible reasons for the differing preliminary taste masking results

<table>
<thead>
<tr>
<th>Possible reasons for differing results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pn-An &amp; A0-An PCA Distances</strong></td>
</tr>
<tr>
<td>The difference in magnitude between the two types of PCA distance calculated, i.e. the Pn-An PCA Distances tended to be small and the A0-An PCA Distances tended to be large</td>
</tr>
<tr>
<td>A lack of sensitivity to the drug compared to the taste masking agents; the concentration of drug in the samples was significantly smaller than the taste masking agent</td>
</tr>
<tr>
<td>A possible tendency for the sensors to respond better to samples with a higher concentration of taste masking agent, i.e. the sweetener/flavouring agent combination formulations tended to produce larger distances</td>
</tr>
<tr>
<td><strong>S1 &amp; S2 analysis sequences</strong></td>
</tr>
<tr>
<td>The different number of samples incorporated in each sequence affected the sensor sensitivity and conditioning; S1 had six active and placebo samples, whilst S2 had five active and placebo samples</td>
</tr>
<tr>
<td>The conditioning of the sensors may have favoured one particular sequence over another. The sensors were conditioned in 0.20 mg/mL HC or 0.16 mg/mL Qu creating a situation where the sensors were accustomed to the active samples but not the placebo</td>
</tr>
</tbody>
</table>

Although the exact reasoning behind the differing results was unknown, what remained important to determine was which PCA interpretation method and analysis sequence was the most accurate so that taste masking agent concentrations could be selected for the human taste panel study. This, however, could have only been achieved by comparing all these results with a human taste panel and was beyond the scope of this research. According to the manufacturers of the ASTREE, the S2 sequence with A0-An PCA Distances produces the most accurate taste assessment (Alpha M.O.S, 2006). It was, therefore, decided that the selected concentrations put forward for the human taste panel were the results determined in this fashion. In light of this, the best taste masking concentration of sucrose (12 % w/v), was the same for the taste masking of 0.20 mg/mL HC and 0.16 mg/mL Qu, indicating the two drugs had the same degree of bitterness. Nevertheless, more acesulpham K and erythritol was necessary to achieve an optimal HC taste masking concentration than the Qu, implying that the HC might be bitterer than the Qu or the sweet taste of the acesulpham K and erythritol was better at taste masking the Qu than the HC. Furthermore, selected taste masking concentrations were achieved below the maximum sweetener concentrations analysed, indicating that the highest sweetener concentration was not better at taste masking than the lower concentrations.

There is little published literature of ASTREE taste assessment to compare these results to. It was, however, concluded that 5.0 mM (0.1 % w/v) acesulpham K was more efficient at taste masking 0.2 mM (0.065 mg/mL) Qu HCl than 1.0 mM (0.02 % w/v) and 0.1 mM (0.002 % w/v) acesulpham K (Zheng and Keeney, 2006). These results differ from the results presented here, although the Pn-An PCA Distances from the
published results indicated that 1.0 mM acesulpham K was as efficient as 5.0 mM acesulpham K because the PCA distances were within 30. This signifies that these results were similar and confirms 0.02 % w/v acesulpham K was as efficient at taste masking Qu than higher concentrations.

In contrast to the sweetener results, the highest concentrations of flavouring agent (2 % w/v) provided the optimal taste masking concentrations to taste mask 0.20 mg/mL HC and 0.16 mg/mL Qu. These results demonstrated that the flavouring agents could elicit a taste masking response in vitro despite considered to mainly provide taste masking in vivo by smell. Utilising the selected taste masking concentrations of the flavouring agents and sweeteners, combination formulations were assessed for taste masking of the HC and Qu. The best sweetener to be combined with the flavouring agents was acesulpham K, illustrating that this particular sweetener had the best synergy with the various flavouring agents than the erythritol and sucrose.

Concerning the capabilities of the CDs, the maximum concentration of HP-β-CD achieved the best taste masking of 0.20 and 2.0 mg/mL HC, as well as 0.16 and 1.60 mg/mL Qu, signifying that according to the ASTREE taste assessment a large excess amount of HP-β-CD did enhance the taste masking efficiency compared to the lower HP-β-CD concentrations and that the solutions with more HP-β-CD were less bitter than the solutions less HP-β-CD. This coincides with the preferred use of excess amounts of CD to produce bitterness taste masking by previous researchers (Szejtli and Szente, 2005). These HP-β-CD results indicated that the HC and Qu molecules in the 0.20 mg/mL HC and 0.16 mg/mL Qu solutions already solubilised without any CDs formed inclusion complexes with the CD molecules and that the degree of complexation was greatest when the largest molar excesses of HP-β-CD was present. The solutions containing HP-β-CD and 2.0 mg/mL HC and 1.60 mg/mL Qu contained smaller molar ratios of HP-β-CD to drug than the 0.20 mg/mL HC and 0.16 mg/mL Qu solutions, since a larger drug concentration was present. Nonetheless, the molar excess in these solutions was sufficient enough to produce taste masking and the larger molar excess of HP-β-CD had more efficient taste masking capabilities than the smaller excess quantities of CD. This implies that the excess HP-β-CD that was not required to solubilise the extra drug was likely to be complexing with the drug molecules which were present in the solution uncomplexed.
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

In comparison to the HP-β-CD results, the maximum concentration of Me-β-CD achieved the optimal taste masking of 0.20 and 2.0 mg/mL HC. Thus, similar complexation dynamics that may have occurred within the formulations of the HP-β-CD were likely to have occurred in the Me-β-CD/0.20 or 2.0 mg/mL HC formulations. These results showed that excess quantities of CD are more efficient at taste masking bitter drugs than smaller quantities. The Me-β-CD taste masking of the Qu, however, produced contradictory results. Whilst the maximum concentration of Me-β-CD achieved the optimum taste masking of 0.16 mg/mL Qu, only two times the molar ratio concentration of Qu was required for the Me-β-CD to achieve optimal taste masking of 1.60 mg/mL Qu. Larger concentrations of Me-β-CD did not improve the bitterness taste masking and, therefore, larger excess amount of Me-β-CD were not necessary. Since large excess concentrations of Me-β-CD was capable of complexing the intrinsic concentration of Qu (0.16 mg/mL), the results of the Me-β-CD taste masking of 1.60 mg/mL Qu indicated that the excess amounts of Me-β-CD was not ample enough to improve taste masking by complex any extra Qu that could have existed in solution uncomplexed and were capable of eliciting a bitter taste.

4.4 Discussion

The ASTREE is a commercially available analytical instrument marketed as providing fast and efficient qualitative and quantitative taste assessment of liquid samples. The preliminary ASTREE experiments conducted during this research demonstrated that the apparatus can perform quantitative assessment of bitter drugs and can be used to screen and reduce the number of formulations to be tested during future taste assessment. This compares well with the published literature concerning the ASTREE (Kataoka et al., 2005; Sadrieh et al., 2005; Zheng and Keeney, 2006; Kayumba et al., 2007; Li et al., 2007; Lorenz et al., 2009). Overall the analysis took less time and preparation being conducted by with the ASTREE than if it was conducted using a human taste panel, highlighting one of the main attributes of taste assessment in vitro and why it has great appeal to the pharmaceutical industry.

Nevertheless, it was found during this research that the instrument had some limitations: 1) In some instances the conditioning and calibration steps during the “start-up” procedure took far longer than anticipated, delaying the start of each experiment and, therefore, causing the overall analysis to take longer than expected. From an analysist’s
2) The results were sequence and data analysis dependant. The differing analysis sequences and methods of PCA interpretation created a scenario where four sets of results were produced for one assessment. The majority of these results were contradictory with no rational method to decipher which were correct. This demonstrated that the ASTREE was not a robust instrument when the sequence was altered slightly or when differing PCA interpreted methods were used, which were supposed to produce the same conclusions. This indicated that the ASTREE studies published in the literature could have been conducted differently to produce differing results. Because there is a lack of ambiguity in the way ASTREE experiments can be conducted and the way the data can be interpreted, set procedures of the most accurate analysis and data processing techniques need to be implemented so all future results produced from the ASTREE are from the same methodology. If ubiquitous standard operating procedures are implemented then at least all experiments conducted on the ASTREE and data analysis performed will be the same.

3) The sensors were drug particular. By comparing the results of the HC standards analysed with two cleaning dips and the Pd standards analyse with the same method parameters it was clear to see that the ASTREE produced differing results from the two structurally similar corticosteroids. For instance, the results from the final HC quantification analysis was as predicted - the 0.01 mg/mL HC was the least bitter standard and the 0.20 mg/mL HC was the bitterest standard (Table 4.5). The results from the final Pd quantification, however, were not expected and followed no rational explanation regarding sample bitterness. The ASTREE was analysing the two structurally similar corticosteroids very differently.

It was apparent after checking all the raw data of each standard that the sensor response produced from the Pd standards was often sinusoidal (Figure 4.17), and not a linear sensor response that was produced from the HC standards (Figure 4.3). The data libraries produced from the sinusoidal measurements were, therefore, average values from inaccurate potentiometric voltage measurements. The subsequent PCA and data interpretations via A0-An PCA Distances, and PLS analysis was consequently deemed inaccurate for the Pd standards despite sufficient sensor and replicate selection. This
was attributed to the sinusoidal response, although inaccurate, being reproducible for each sample replicate.

Figure 4.17: Sinusoidal raw data response of the ASTREE electronic tongue sensors when analyzing prednisolone standards

The cause of the sinusoidal sensor response remains unknown after numerous repeat experiments with fresh Pd standards, new sensor arrays and reference electrodes, alternative method parameters, and extensive sensor conditioning. Nevertheless, it seems that the ASTREE has successfully analysed Pd samples without any mention of the sinusoidal sensor response (Zheng and Keeney, 2006). In this research it was reported that prednisolone Na was used at a concentration of 10 mM. Perhaps using a salt of Pd and a higher concentration of drug (a maximum of 0.55 mM Pd was used in the research of this thesis), enabled Zheng and Keeney to obtain satisfactory sensor responses to Pd. Moreover, the low concentration of Pd used in this research may have been below the limit of quantification for the ASTREE, although this does not fully explain why HC and Qu produced satisfactory sensor outputs. Perhaps the sensors were simply unable to be used with Pd. It has been reported that other electronic tongue systems may struggle with assessment of particular compounds because of the very nature of their cross-sensitive sensors (Legin et al., 2002), although it has not been reported that two compounds so close in structure can elicit such a different sensor response. For instance, caffeine is a non-detectable substance on the INSENT taste sensing system, whilst other substances, such as acesulpham K, are considered as interfering (INSENT, 2006). It was for this reason that HC was used for the remainder of the taste masking assessment studies and not Pd.
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

4) It was not possible to achieve true AO samples for 2.0 mg/mL HC or 1.6 mg/mL Qu, because these concentrations were not achievable in water without CD. The AO samples only contained 0.20 mg/mL HC or 0.16 mg/mL Qu because this was the maximum intrinsic concentration. The AO-An PCA Distances were, therefore, not a true representation of taste masking of 2.0 mg/mL HC or 1.6 mg/mL Qu. Furthermore, the PO-AO PCA Distances was also not a true representation of the taste discrimination of water from a 2.0 mg/mL HC or 1.6 mg/mL Qu sample. This highlights that not all types of taste masking analysis can be conducted with the ASTREE and that compromises have to be made.

5) As all of the excipients were analysed by the ASTREE in individual sequences only intra-experimental comparisons could be made from the data and no inter-experimental comparisons. For example, the results could not compare which taste masking agent was the most efficient at taste masking the 0.20 mg/mL HC and 0.16 mg/mL Qu solutions. This could have only been conducted from merged data libraries, which were mathematically calibrated, as well as chemically calibrated. (See Chapter 6 for an explanation of mathematical calibration of data libraries). Without mathematical calibration, only intra-experimental comparisons could be made, i.e. comparisons of taste masking efficiency from the different concentrations of the same taste masking agent.

6) The replicate and sensor selection may have had more of an influence than first perceived. The raw potentiometric voltage measurements were processed to rid the data library of sensor drift. Whilst the majority of experiments had the same replicates omitted, the omitted sensors were not always the same. It was impossible to keep enough same sensors across all experiments. This may have prevented the true conclusions being generated.

7) The ASTREE analytical methods could not be validated as per typical ICH method validation protocol (e.g. accuracy, precision, specificity, linearity and range), to demonstrate that the ASTREE procedures were suitable for their intended purpose. If the methods were validated fully then greater confidence in the results would have been achieved. It was found during this research that the ASTREE methods were not robust/rugged and the results were not reproducible. It may be likely, therefore, that other parameters of the analytical methods will affect the outcome of the assessment and produce contradictory results. For instance, the sensors were conditioned and
Chapter 4 - Preliminary ASTREE Electronic Tongue Studies

calibrated in a sample containing just the drug to enable the sensors to become accustomed to the drug and to ensure the potentiometric voltage measurements were within the measurements limits of the ASTREE. It would be of interest to see whether altering the conditioning/calibration sample at the beginning of each experiment would affect the results of the analysis.

It has been suggested that the ASTREE can be used for screening the taste attributes of formulations in a rapid timeframe, when used in conjunction with human taste assessment data (Figure 4.18) (Lorenz et al., 2009). This raises the question, however, of the legitimacy of the ASTREE, since taste panel data will always be required.

Figure 4.18: Proposed application of the ASTREE electronic tongue and sensory panel in formulation development (Lorenz et al., 2009)
4.5 Conclusions

Preliminary taste analysis of Pd, HC and Qu standards, and taste masking analysis of 0.20 mg/mL HC and 0.16 mg/mL Qu was carried out using an ASTREE electronic tongue. For reasons unbeknown the Pd standards evoked sinusoidal sensor responses, thus rendering the preliminary taste analysis data of Pd inaccurate and, therefore, indicating that the ASTREE could not be used for Pd taste masking analysis studies. Nevertheless, the preliminary taste analysis indicated that the ASTREE could quantify HC and Qu and, therefore, provide a taste masking assessment method for formulations containing these drugs. The preliminary taste masking analysis of HC and Qu was carried out with sweeteners and/or flavouring agents, HP-β-CD and Me-β-CD. These results showed that the analysis sequence and method of PCA distance interpretation influenced the outcome of the analysis. Nevertheless, from these results taste masking concentrations were selected, effectively reducing the number of formulations to be assessed during the human taste panel studies (Chapter 5), and the final ASTREE/human taste panel correlation studies (Chapter 6).
Chapter 5 - Adult Human Taste Panel Study
Chapter 5 - Adult Human Taste Panel Study

5.1 Introduction

5.1.1 Taste masking with cyclodextrins

As previously discussed in Chapter 1 the majority of pharmaceutical active ingredients, such as corticosteroids, elicit a bitter taste. The palatable taste of paediatric oral medications is a vital element for therapeutic adherence and successful treatment (Matsui, 2007). Although oral solid formulations achieve successful taste masking and are taken typically without concern by older children and adolescents, solid dosage forms are unacceptable for younger children, infants and neonates (EMEA, 2006). Thus, liquid formulations are more appropriate for children under six - eight years of age, especially those who cannot swallow tablets/capsules. Taste masking of bitter drugs in liquid formulations is, however, more challenging than in solid formulations and patients cease taking their medication frequently, because of the aversive taste (Matsui, 2007). Taste masking via taste deception is the most common taste masking technique for oral liquid formulations. Sweeteners and/or flavouring agents are used typically to overcome the bitterness of drugs. For instance, a Pd oral solution containing sorbitol and grape flavour was better tolerated by a group of children aged between two to 10 years of age than a generic Pd formulation that was not taste masked (Kim et al., 2006). Nevertheless, when the bitterness is very intense, masking efficiency can be compromised, even when the drug is formulated as a suspension (Cram et al., 2009). Moreover, numerous sweeteners and flavouring agents are absorbed in the gut and can cause toxicity and hypersensitivity, especially in children, thus raising concern for their use in certain populations of paediatric patients (Pawar and Kumar, 2002). An alternative taste masking technique may provide enhanced taste masking efficiency than typical sweetener and/or flavouring agents.

CDs provide taste masking of poorly soluble bitter drugs by taste obstruction - molecular encapsulation of drug molecules via inclusion complexation has been successful in eliminating the bitter taste of drugs in various formulations (Szejtli and Szente, 2005). The slight sweet taste of CDs may also help reduce bitter taste sensations since it was found that a 2.5 % β-CD solution was as sweet as a 1.71 % solution of sucrose (Toda et al., 1985). Furthermore, all toxicity studies have demonstrated that orally administered CDs are practically nontoxic, because their bulky and hydrophilic nature causes a lack of absorption through the gastrointestinal tract (Irie and Uekama, 1997). Thus, CDs may prove to be more efficient taste masking agents than sweeteners and/or flavouring agents at taste masking corticosteroids in oral liquid formulations.
Chapter 5 - Adult Human Taste Panel Study

When CDs were used in a taste masking capacity the molar ratio of guest to CD is typically greater than 1:1. For instance, 1:11 and 1:15 complexes of ibuprofen and HP-β-CD were used to improve the palatability of ibuprofen solutions (Motola et al., 1991). This supposedly creates a system where more than 90% (or frequently more than 99%) of the guest molecules are present in CD complexed form (Szejtli and Szente, 2005). The degree of taste masking is considered to be dependant on the concentration of free uncomplexed drug in solution (Funasaki et al., 1999). Nevertheless, it is hypothesised that using molar excess CD may be unnecessary and may not improve taste masking efficiency, especially if the guest molecules are only partially complexed and capable of interacting with the taste receptors of the tongue. In addition, excess CD may not complex all of the drug molecules within a solution since a small quantity of drug (equal to the intrinsic solubility concentration or below), is capable of existing in solution uncomplexed and capable of interacting with the taste receptors of the tongue.

5.1.2 Taste assessment in vivo

During the development of oral liquid paediatric medicines, children, as a target population, are regarded as the most suitable panel to evaluate the palatability of the formulations (EMEA, 2006), since taste preferences and perceptions differ between adults and children (James et al. 1997). Taste assessment studies with children should be suited to the age and ability of the panel. For instance, taste trials in children should be short in duration to maintain concentration and attention span, have an exciting and motivational approach, consist of easy to follow procedures, and contain a minimal number of variants (EMEA, 2006). Nevertheless, taste assessment of oral liquid paediatric medicines by adult human taste panels may be the only option available, especially when ethical and safety dilemmas prevent taste assessment by children. Furthermore, adult taste panels are more robust and suffer less fatigue, generate a greater volume of taste information, are able to carry out more complex procedures and are more likely to complete the study than paediatric taste panels.

Taste assessment of pharmaceutical products in vivo, as carried out by an adult human taste panel, is a well-established and standardised method of taste assessment. The protocol for a human adult taste panel study typically follows standardised guidelines developed by the International Organization for Standardization and is preapproved commonly by an ethical committee.
Chapter 5 - Adult Human Taste Panel Study

Before a study commences it is important to define clearly the aims and objectives of the study, as they will influence many aspects of the study, such as the design, the cohort, the environment, the measurement scale, and the statistical analysis. Once the objectives of the study are outlined, the study protocol can be designed. The first process of a taste panel study is the recruitment and screening of taste assessors. During this process planning, advertising, application procedures, interviews, screening tests, and recruitment criteria need to be established. The number of taste assessors to be recruited into the study needs to be determined. This is dependent typically on the desired size of the final panel, the number of samples tested, the statistical analysis to be used, and the ability of the taste assessors (trained or untrained). Once a sufficient number of potential taste assessors are recruited and screened the analysis location requires evaluating to minimise bias, enhance panellist sensitivity and eliminate variables unconnected with the products being analysed. Important issues concerning the presentation of samples, such as sample coding and randomisation require consideration.

Taste panel selection and/or training takes place to enhance the ability of the taste panel. Training sessions are typically ongoing, held frequently and utilise training references for the five basic tastes. Selection processes include basic taste recognition and ranking of basic taste, and eliminates recruited taste assessors who lack reliable sensory acuity for the tastes being evaluated. Finally, the test analysis is conducted with the test formulations by the selected and/or trained taste panel, preferably in a blind, or double blind fashion. The scale used to quantify or the process used to qualitatively assess the parameter(s) of taste is an important and crucial part of the study and depends commonly on the three main categories of sensory test i.e. discrimination, descriptive, and preference/acceptance. Evaluation of pharmaceutical products involves typically the discrimination of formulations based on one of the five basic tastes, commonly bitterness. This assessment is best performed using a validated and balanced scale, such as a hedonic scale or visual analogue scale (VAS). Statistical analysis of the recorded data depends on numerous factors, such as the overall objectives of the study, the number of taste assessors, the number of samples tested, the type of test performed, and the type of data recorded.

5.1.3 Aims and objectives

The preliminary ASTREE taste masking analysis (Chapter 4), helped screen and reduce the number of formulations to be assessed by an adult human taste panel by selecting
Chapter 5 - Adult Human Taste Panel Study

0.20 mg/ml HC and 0.16 mg/mL Qu formulations containing optimal concentrations of sweetener and/or flavouring agent. Formulations containing 0.20 and 2.0 mg/mL HC, and 0.16 and 1.60 mg/mL Qu with various concentrations of HP-β-CD and Me-β-CD were also selected for the adult taste panel assessment (Chapter 2). Utilising these formulations the aims of this chapter were to:

• Examine whether CDs could provide bitterness taste masking of corticosteroids in an oral liquid paediatric formulation and compare the taste masking efficiency with sweeteners and/or flavouring agents.
• Investigate whether excess concentrations of CD were better at taste masking corticosteroids than moderate concentrations of CD.
• Determine whether a formulation containing CD and a clinically relevant concentration of solubilised corticosteroid (e.g. 2.0 mg/mL HC), was bitterer than a formulation containing the same concentration of CD and corticosteroids at a concentration below the intrinsic solubility (e.g. 0.20 mg/mL HC).

These aims were fulfilled by bitterness taste masking assessment in vivo with an adult human taste panel on selected HC formulations. To provide comparative data, the adult human taste panel also assessed selected Qu formulations. This particular research has yet to be published in the literature and represents a novel aspect of this thesis.

5.2 Materials and methods

5.2.1 Materials

As per Section 4.2.1

5.2.2 Adult human taste panel study - taste assessor selection (study day 1)

The University College London Research Ethics Committee approved the adult taste masking assessment protocol and volunteer consent form for both parts of the study (UCL Ethics Project ID # 0851/001) (Appendix 2).

Subjects: Healthy adult volunteers were recruited for the taste masking selection. The number of volunteers was based on literature recommendations (ISO 6658:2005) and experimental design considerations to enable adequate power to detect equivalence among the formulations. Age was not studied as a covariant, although all the taste assessors were in between 18 and 65 years of age and the assessors were a racially
Chapter 5 - Adult Human Taste Panel Study
mixed group. No taste assessor was excluded or dropped out from the study after the initial recruitment. To assure balance, volunteers were tested in three separate groups of nine.

Taste assessors were asked to refrain from eating, drinking (except water), smoking and chewing gum 1 h before the study commenced. Each assessor was provided with a “Taste Station” (Figure 5.1), where they remained throughout the duration of the study. Each taste station consisted of a desk and stool, taste assessment score sheets, a spittle bowl, a bottle of distilled water and some tissues. Samples for tasting and assessment were presented to the assessors at their taste station, which provided an enclosed neutral environment from the other assessors.

**Figure 5.1:** An example of taste station used during the adult taste panel study. Key: A) desk, B) stool, C) taste assessment score sheet, D) spittle bowl, E) distilled water, F) tissues, G) divisional screen

**Samples:** Each assessor evaluated and recorded in triplicate and in a randomised order the initial bitterness intensity of 3 blinded HC samples (0.05, 0.10, and 0.20 mg/mL) and 3 blinded Qu samples (0.04, 0.08, and 0.16 mg/mL) on a 100 mm VAS (Figure 5.2). In total, the taste assessors evaluated 18 standard samples.
Chapter 5 - Adult Human Taste Panel Study

**Initial Bitterness Intensity**

| No Bitter Taste | Extremely Strong Bitter Taste |

**Figure 5.2:** Initial bitterness intensity 100 mm visual analogue scale used during the adult taste panel study (not to scale)

**Procedure:** At the beginning of the study the taste assessors were given an unsalted cracker and some mineral water to cleanse their palate and rinse their mouth. Ten min after ingesting the cracker the assessors were presented with 15 mL of each sample contained in needleless syringes and identified only by random three digit codes. Upon administration each sample was rinsed and gargled in the oral cavity for 15 sec and spat out - the initial bitterness intensity was scored on the VAS located on a score sheet that corresponded to the sample. After scoring the bitterness intensity the assessors rinsed their mouths with distilled water and waited approximately nine min until the next sample was tasted so that there was a 10 min washout between samples.

**Data analysis:** Bitterness intensity scores were quantified by measuring the distances marked on the VAS from the “No bitter taste” side. The scores for each taste assessor were tabulated and the mean bitterness score calculated. Taste assessors who could not assess accurately and reproducibly either model drug in the correct order of bitterness were eliminated from the selection process. PCA was applied on the mean bitterness scores of the remaining taste assessors to select the final assessors based on their homogeneity. The bitterness scores of the selected taste panel were analysed by repeated measures ANOVA to determine whether the bitterness scores of the three HC and three Qu standards were significantly increased as a function of increasing HC/Qu concentration or significantly increased as a result of the timing of sample \( p = 0.05 \).

**5.2.3 Adult human taste panel study - bitterness assessment of hydrocortisone and quinine formulations (study day 2 & 3)**

**Subjects:** Based on the statistical analysis of the sensory scores from study day 1, 12 taste assessors out of the original recruitment were selected to take part in the bitterness
Chapter 5 - Adult Human Taste Panel Study

taste assessment of the HC and Qu formulations that were selected with the help of the results of the preliminary ASTREE taste masking analysis (Chapter 4).

**Samples:** Each assessor evaluated and recorded the initial bitterness intensity of 17 HC formulations (study day 2), and 17 Qu formulations (study day 3), on a VAS, each in duplicate and in a randomised order (Table 5.1 and Table 5.2).

**Table 5.1:** Hydrocortisone formulations assessed during the adult taste panel study

<table>
<thead>
<tr>
<th>Formulation No.</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distilled water (no bitter taste reference)</td>
</tr>
<tr>
<td>2</td>
<td>0.20 mg/mL hydrocortisone (extremely strong bitter taste reference)</td>
</tr>
<tr>
<td>3</td>
<td>2 mg/mL hydrocortisone + 1.12 % w/v HP-β-CD (1:1)</td>
</tr>
<tr>
<td>4</td>
<td>2 mg/mL hydrocortisone + 11.20 % w/v HP-β-CD (1:14)</td>
</tr>
<tr>
<td>5</td>
<td>2 mg/mL hydrocortisone + 0.53 % w/v Me-β-CD (1:1)</td>
</tr>
<tr>
<td>6</td>
<td>2 mg/mL hydrocortisone + 2.65 % w/v Me-β-CD (1:4)</td>
</tr>
<tr>
<td>7</td>
<td>0.20 mg/mL hydrocortisone + 1.12 % w/v HP-β-CD (1:14)</td>
</tr>
<tr>
<td>8</td>
<td>0.20 mg/mL hydrocortisone + 11.20 % w/v HP-β-CD (1:141)</td>
</tr>
<tr>
<td>9</td>
<td>0.20 mg/mL hydrocortisone + 0.53 % w/v Me-β-CD (1:7)</td>
</tr>
<tr>
<td>10</td>
<td>0.20 mg/mL hydrocortisone + 2.65 % w/v Me-β-CD (1:37)</td>
</tr>
<tr>
<td>11</td>
<td>0.20 mg/mL hydrocortisone + 12 % w/v Sucrose</td>
</tr>
<tr>
<td>12</td>
<td>0.20 mg/mL hydrocortisone + 20 % w/v Erythritol</td>
</tr>
<tr>
<td>13</td>
<td>0.20 mg/mL hydrocortisone + 0.08 % w/v Acesulpham K</td>
</tr>
<tr>
<td>14</td>
<td>0.20 mg/mL hydrocortisone + 2 % w/v Cherry</td>
</tr>
<tr>
<td>15</td>
<td>0.20 mg/mL hydrocortisone + 2 % w/v Strawberry</td>
</tr>
<tr>
<td>16</td>
<td>0.20 mg/mL hydrocortisone + 2 % w/v Strawberry + 0.08 % w/v Acesulpham K</td>
</tr>
<tr>
<td>17</td>
<td>0.20 mg/mL hydrocortisone + 2 % w/v Tangerine + 0.08 % w/v Acesulpham K</td>
</tr>
</tbody>
</table>

The concentration of HP-β-CD within the formulations was based on the minimum concentration of HP-β-CD that was required to solubilise 2.0 mg/mL HC or 1.6 mg/mL Qu, which was determined from phase solubility studies (Chapter 2), as well as 10 times the minimum concentration to give formulations containing excess HP-β-CD. For example, a minimum of 2.89 % w/v HP-β-CD was required to solubilise 1.6 mg/mL Qu and 28.9 % w/v HP-β-CD is ten times this minimum concentration. In contrast, the concentration of Me-β-CD within the formulations was based on the minimum concentration of Me-β-CD that was required to solubilise 2.0 mg/mL HC or 1.6 mg/mL Qu, as well as 5 times the minimum concentration to give formulations containing excess Me-β-CD. This allowed for examination of whether differing degrees of CD concentration provided differing bitterness taste masking. The concentrations of sweetener, flavouring agent, and sweetener/flavouring agent combination were selected with the help of the preliminary ASTREE taste masking analysis (Chapter 4).
Table 5.2: Quinine formulations assessed during the adult taste panel study

<table>
<thead>
<tr>
<th>Formulation No.</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distilled water (no bitter taste reference)</td>
</tr>
<tr>
<td>2</td>
<td>0.16 mg/mL quinine (extremely strong bitter taste reference)</td>
</tr>
<tr>
<td>3</td>
<td>1.6 mg/mL quinine + 2.89 % w/v HP-β-CD (1:4)</td>
</tr>
<tr>
<td>4</td>
<td>1.6 mg/mL quinine + 28.9 % w/v HP-β-CD (1:41)</td>
</tr>
<tr>
<td>5</td>
<td>1.6 mg/mL quinine + 1.04 % w/v Me-β-CD (1:2)</td>
</tr>
<tr>
<td>6</td>
<td>1.6 mg/mL quinine + 5.2 % w/v Me-β-CD (1:8)</td>
</tr>
<tr>
<td>7</td>
<td>0.16 mg/mL quinine + 2.89 % w/v HP-β-CD (1:41)</td>
</tr>
<tr>
<td>8</td>
<td>0.16 mg/mL quinine + 28.9 % w/v HP-β-CD (1:408)</td>
</tr>
<tr>
<td>9</td>
<td>0.16 mg/mL quinine + 1.04 % w/v Me-β-CD (1:16)</td>
</tr>
<tr>
<td>10</td>
<td>0.16 mg/mL quinine + 5.2 % w/v Me-β-CD (1:81)</td>
</tr>
<tr>
<td>11</td>
<td>0.16 mg/mL quinine + 12 % w/v w/v Sucrose</td>
</tr>
<tr>
<td>12</td>
<td>0.16 mg/mL quinine + 15 % w/v Erythritol</td>
</tr>
<tr>
<td>13</td>
<td>0.16 mg/mL quinine + 0.08 % w/v Acesulpham K</td>
</tr>
<tr>
<td>14</td>
<td>0.16 mg/mL quinine + 2 % w/v Tangerine</td>
</tr>
<tr>
<td>15</td>
<td>0.16 mg/mL quinine + 2 % w/v Cherry</td>
</tr>
<tr>
<td>16</td>
<td>0.16 mg/mL quinine + 2 % w/v Cherry + 0.02 % w/v Acesulpham K</td>
</tr>
<tr>
<td>17</td>
<td>0.16 mg/mL quinine + 2 % w/v Tangerine + 0.02 % w/v Acesulpham K</td>
</tr>
</tbody>
</table>

Procedure: The bitterness assessment of the formulations by the taste assessors was as per the assessment procedure of study day 1. On this occasion samples were presented four at a time for logistics reasons, with CD formulations being assessed in the morning session and sweetener and flavouring agent formulations being assessed in the afternoon. This minimised assessor bias against the formulations containing CDs, which were considered to have a more neutral taste than the formulations containing sweetener and/or flavouring agent. The morning session consisted of two 90 min sessions and a 15 min break, where formulations 1 to 10 were tasted in duplicate and in a randomised order, whilst the afternoon session consisted of two 80 min sessions and a 15 min break, where formulations 11 to 17 were tasted in duplicate and in a randomised order. Each session commenced with reference formulations 1 and 2, which were identified to the panel to enable assessors to gauge the two regions of bitterness; the remaining formulations were assessed in a blinded randomised order. A standardised non-spicy and lightly salted lunch was provided for the assessors between the morning and afternoon sessions. The afternoon session commenced 1 h after taste assessors stopped eating, drinking (except water), smoking and chewing gum. Both sessions commenced after the assessors had cleansed their palate and rinse their mouths with an unsalted cracker and some mineral water.
Chapter 5 - Adult Human Taste Panel Study

**Data analysis:** Bitterness intensity scores were measured as per study day 1 and were analysed by general ranking, variation statistics, PCA, and significance testing by parametric t-test. If a formulation containing a particular taste masking agent was found to produce a low bitterness intensity, the taste masking agent was considered to have high taste masking efficiency, and vice versa. Equivalence testing was also carried out on the adult taste panel data to test for equivalence in bitterness between certain formulations. Equivalence testing is not just the opposite of significance testing - testing for a significant difference in bitterness between two formulations and illustrating no significant difference at a confidence level of $p = 0.05$ does not prove that the formulations are equivalent in terms of bitter taste. Absence of evidence does not mean evidence of absence (Altman and Bland, 1995).

As the adult taste panel study was designed with distilled water and 0.20 mg/mL HC or 0.16 mg/mL Qu as reference samples - whereby they were not randomly tasted with the other formulations and were identified to the panel as being reference samples, it was not applicable to directly compare the test formulations to the reference samples during the equivalence testing. The individual reference scores were used to create a personal scale for each taste assessor and equate the scale between 0 and 100, i.e. the bitterness scores of formulations 3 - 17 were corrected based on formulation 1 being 0 mm (no bitter taste), on the VAS and formulation 2 being 100 mm (extremely bitter taste) (Equation 5.1). This directly involved the reference samples into the equivalency testing and united the morning and afternoon tasting sessions.

**Corrected mean bitterness score** = \[(\text{Formulation VAS score} - F_1 \text{ VAS scale}) \times \frac{100}{F_2 - F_1}\]

**Equation 5.1**

Before any equivalence testing was conducted it was essential to set out equivalence limits - that is to say a VAS score range where the bitterness was perceived to be the same, i.e. although the VAS bitterness scores maybe different, the perceived bitterness was equivalent (the same). As yet no literature suggests a suitable equivalence limit for VAS bitterness scoring. It was, therefore, decided to choose an equivalence interval of +/- 15 ($\delta = 15$). Equivalence was accepted when there was evidence to reject the Null hypothesis.

To determine whether: excess concentrations of CD were better at taste masking HC or Qu than moderate concentrations of CD, and whether a formulation containing CD and 2.0 mg/mL HC or 1.6 mg/mL Qu was bitterer than a formulation containing the same
concentration of CD and 0.20 mg/mL HC or 0.16 mg/mL Qu, equivalency testing was conducted between:

- Formulation 3 and Formulation 4, Formulation 7 and Formulation 8, Formulation 5 and Formulation 6, and Formulation 9 and Formulation 10 to determine the effect of CD concentration on bitter intensity.
- Formulation 3 and Formulation 7, Formulation 4 and Formulation 8, Formulation 5 and Formulation 9, and Formulation 6 and Formulation 10 to determine the effect of HC/Qu concentration on bitterness intensity.

Further equivalency testing was conducted on HC formulations between: Formulation 11 and Formulation 4, and Formulation 11 and Formulation 9 to determine whether certain CD formulations were equivalent to the best traditional taste masking agent. In addition, equivalency testing was conducted on Qu formulations between: Formulation 12 and Formulation 4, and Formulation 12 and Formulation 7 to determine whether certain CD formulations were equivalent to the best traditional taste masking agent.

5.3 Results

5.3.1 Adult human taste panel study - taste assessor selection (study day 1)

There were 27 healthy adult volunteers recruited for the taste masking selection (17 female, 10 male; aged 18 to 57). The number of volunteers was based on literature recommendations (ISO 6658:2005), and experimental design considerations to enable adequate power to detect equivalence among the formulations. The bitterness intensities of the HC and Qu standards from each assessor were measured and tabulated (Appendix 3). To begin the taste assessor selection assessors who could not place either model drug in the correct order of bitterness were eliminated from the taste panel. This reduced the panel of 27 taste assessors to 16. From the 16 taste assessors that were remaining the mean bitterness intensity scores were analysed by PCA to investigate the homogeneity of the sample population (Figure 5.3). Although there are no set criteria relating to homogeneity parameters, the taste assessors who produced sensory scores that fell outside the main sample population were omitted from the taste panel. In this instance four taste assessors were omitted for not being homologous among the bitterness scores of the panel. This resulted in 12 taste assessors being selected for the remainder of the adult taste panel based on their ability to rank the HC and Qu samples in the correct order and on their homogeneity (Figure 5.4).
Chapter 5 - Adult Human Taste Panel Study

**Figure 5.3:** PCA map of the hydrocortisone bitterness intensity scores from the 16 taste assessors who scored the all standards in the correct order. Taste assessors selected for omission were vol 1, vol 6, vol 7 and vol 15.

**Figure 5.4:** Mean bitterness taste intensities (+/- SEM) of hydrocortisone and quinine standards assessed by the selected 12 adult taste assessors. Each point: mean ± SEM, $n = 3$. Note that some error bars are within data points.

After taste assessor selection was carried out repeated measures ANOVA on the mean bitterness scores of the HC and Qu standards determined that the bitterness of the three HC and three Qu standards were significantly different ($p = 0.05$), and the bitterness of the standards increased as a function of increasing concentration. Repeated measures ANOVA also determined there was no significant difference ($p = 0.05$), in the mean bitterness scores from each assessor in relation to the timing of the tastings. The results of the repeated measures ANOVA provided assurance that the concentration of drug
5.3.2 Adult human taste panel study - bitterness assessment of hydrocortisone and quinine formulations (study day 2 & 3)

The bitterness intensities of the selected HC and Qu formulations were assessed by a selected adult taste panel (12 taste assessors (10 female, 2 male; aged 18 to 30)), and the mean bitterness intensities of the formulations were calculated (Figure 5.5 and 5.6).

![Figure 5.5: Mean bitterness intensity (+/- SEM) of 17 hydrocortisone formulations assessed by a selected adult human taste panel. Each point: mean ± SEM, n = 3. Note that some error bars are within data points. * Significant difference between F1 & F2, # significant difference between F3 & F4, ** significant difference between F5 & F6, ## significant difference between F7 & F8, ### significant difference between F9 & F10, #### significant difference between F14 & F15, ####* significant difference between F16 & F17. P = 0.05.](image-url)
**Figure 5.6:** Mean bitterness intensity (+/- SEM) of 17 quinine formulations assessed by a selected adult taste panel. Each point: mean ± SEM, $n = 3$. Note that some error bars are within data points. * Significant difference between F1 & F2, # significant difference between F3 & F4, ** significant difference between F5 & F6, ## significant difference between F7 & F8, ### significant difference between F9 & F10, #### significant difference between F14 & F15, ##### significant difference between F16 & F17. \( P = 0.05. \)

**Reference samples:** The distilled water reference sample received a mean bitterness intensity score of 5 (± 2) and was, therefore, deemed to have no bitter taste by the panel. The 0.20 mg/mL HC and 0.16 mg/mL Qu received mean bitterness intensity scores of 87 (± 3) and 83 (± 2), respectively, and were not significantly different \( (p = 0.05). \) This indicated that the drugs were both extremely bitter substances.

**Sweeteners:** The results demonstrated that the sweeteners provided ~ 50% bitterness reduction of 0.20 mg/mL HC and 0.16 mg/mL Qu. The bitterness intensities of these formulations indicated that no sweetener was considered to have the best taste masking efficiency, since none of the formulations were significantly less bitter than both the other formulations containing sweetener \( (p = 0.05). \) This was attributed to the sweeteners all having similar sweetness intensities close to the maximum perceived
Chapter 5 - Adult Human Taste Panel Study

limit of sweetness (near the sweetness perception plateau for humans (Schiffman et al., 1995)), as well as similar sweetness profiles. It is known that viscosity plays an important role in taste masking by sweeteners. Nevertheless, viscosity was not a parameter in this study, since the concentrations of sweetener were too low and the viscosity of the samples was similar to water. The use of higher concentrations of sweetener to create more viscous samples was outside the scope of the taste assessment study, because the ASTREE cannot be used with viscous liquids.

Flavouring agents: The results illustrated that the flavouring agents reduced the bitterness intensity of 0.20 mg/mL HC and 0.16 mg/mL Qu, demonstrating that flavouring agents without the presence of a sweetener could provide bitterness taste masking of bitter drugs. Strawberry was considered significantly more efficient at taste masking 0.20 mg/mL HC than cherry, whilst cherry was significantly more efficient at taste masking 0.16 mg/mL Qu than tangerine ($p = 0.05$).

Flavouring agent/sweetener combinations: All combination formulations demonstrated bitterness taste masking of 0.20 mg/mL HC and 0.16 mg/mL Qu. The results also illustrated that flavouring agent/sweetener combinations were able to enhance the bitterness taste masking for some of individual taste masking agents. For instance, the combination of tangerine/acesulpham K significantly enhanced the HC taste masking of acesulpham K, and the combination of tangerine/acesulpham K significantly enhanced the Qu taste masking of tangerine ($p = 0.05$). Nevertheless, not all combination formulations reduced the bitterness intensity of the drugs compared to the individual taste masking agents, therefore, indicating that compatibility of acesulpham K varies from flavour to flavour.

Cyclodextrins: The results found that the moderate concentrations of CD (e.g. 1:1), provided no bitterness taste masking for the clinically relevant concentrations of HC and Qu, with bitterness intensities not significantly different from the extremely bitter reference sample ($p = 0.05$). On the other hand, excess CD (e.g. 1:14), produced bitterness taste masking of the clinically relevant concentrations of HC and Qu, with a three to five fold decrease in bitterness intensity, as well as bitterness taste masking of the lower concentrations of HC and Qu. Moreover, the largest molar excess of CD (e.g. 1:141), produced bitterness intensities not significantly different from the non-bitter reference sample, indicating total bitterness taste masking of the HC and Qu ($p = 0.05$). The results also found that the formulations containing CD and a clinically relevant
concentration of drug (i.e. 2.0 mg/mL HC or 1.6 mg/mL Qu), had greater bitterness intensities than the formulations containing the same concentration of CD and drug at a concentration below the intrinsic solubility (i.e. 0.20 mg/mL HC and 0.16 mg/ml Qu). Overall, the results demonstrated that the greater the molar excess of CD, the greater the bitterness taste masking.

In light of these results it was hypothesised that the formulations containing moderate concentrations of CD had insufficient free CD to complex the drug molecules already dissolved in solution, which were, therefore, able to interact with the taste receptors of the tongue and elicit a bitter taste. Formulations that contained molar ratios of drug to CD greater than 1:1 were able to form inclusion complexes with the drug molecules already dissolved in solution and, therefore, reduced the bitterness of the drug and provided taste masking. When the molar excess was extremely large predominantly all of the drug molecules already dissolved in solution were complexed with the CD molecules. These particular results demonstrated that the HC and Qu molecules were complexed fully by the CDs, leaving no portion of the drug molecules exposed to the taste receptors after complexation (Figure 5.7).

Figure 5.7: An illustration of the proposed hypothesis of cyclodextrin taste masking
Chapter 5 - Adult Human Taste Panel Study

Directly comparing the two types of CD, Me-β-CD was found to be a more efficient taste masking agent than HP-β-CD at taste masking HC and Qu, enabling formulations to have equivalent bitterness intensities to the formulations containing HP-β-CD but with less CD present (i.e. less molar ratio of drug to CD). For example, the 0.16 mg/mL Qu formulation containing 2.89 % w/v HP-β-CD (1:41), had a mean bitterness intensity of 37 (± 7), whilst the 0.16 mg/mL Qu formulation containing 1.04 % w/v Me-β-CD (1:16), had a mean bitterness intensity of 36 (± 8). This was attributed to Me-β-CD having a greater association constant for the drugs than the HP-β-CD and, therefore, a greater capacity to complex more drug molecules (i.e. the uncomplexed drug already dissolved in solution) (Chapter 2).

By comparing the mean bitterness intensities of the formulations containing CD with the formulations containing traditional taste masking agent, these results demonstrated that CDs were better taste masking agents than the traditional taste masking agents, especially when used in excess. It was also found that CDs were able to taste mask 2.0 mg/mL HC or 1.6 mg/mL Qu with the same degree of taste masking efficiency as the best traditional taste masking agent, which was only taste masking 0.20 mg/mL HC or 0.16 mg/mL Qu. This signifies that CDs were capable of taste masking higher concentrations of drug than the other taste masking agents. Overall this would be beneficial for a formulator of an oral liquid paediatric formulation because if CDs were used instead of traditional taste masking agents the dose volume would be lower and less taste masking excipient would be used.

HP-β-CD and Me-β-CD were both able to provide bitterness taste masking of 0.20 and 2.0 mg/mL HC, and 0.16 and 1.6 mg/mL Qu, demonstrating that the CDs were capable of taste masking drug molecules that were already dissolved in solution, as well as drug molecules that were present in solution with the aid of CD encapsulation. These results coincide with the results from Chapters 2 and 3, which also showed that CDs were able to form inclusion complexes with drug molecules already solubilised. Furthermore, these results highlighted that CDs were able to provide bitterness taste masking for drug molecules of differing chemical structure and at a varied concentration.

5.4. Discussion

Association constant as an indication for taste masking efficiency

It may be possible to predict how efficient a CD will be at taste masking a particular drug based on the association constant between the CD and drug. The results from this
Chapter 5 - Adult Human Taste Panel Study

Research found that Me-β-CD was a more efficient taste masking agent than HP-β-CD and this was attributed to Me-β-CD having a greater binding affinity (association constant), to the bitter drug molecules than HP-β-CD (Chapter 2). This, therefore, enabled Me-β-CD to have a greater ability to complex the uncomplexed drug molecules already in solution compared to HP-β-CD. Nevertheless, there is a lack of published research detailing the taste masking of bitter drugs by CDs to compare these results and most documented literature are patent application with limited information. These findings do, however, compare well with Patel and Vavia, (2008) who attributed the increased taste masking efficiency of CDs to an increase in association complex caused by the addition of hydroxy propyl methyl cellulose (HPMC) to the system. These researchers found that the association constant of the CD to famotidine was increased from 538 M\(^{-1}\) to 15096 M\(^{-1}\) with the addition of HPMC, which resulted in an increase in bitterness taste masking of the famotidine. This was also demonstrated by Funasaki et al., (1999; 2006), who determined that an increase in association constant was correlated with the decrease in the bitterness intensity of the formulation. These researchers attributed these findings to the increased association constant reducing the concentration of free uncomplexed dmg in solution and proposed an equation to determine the concentration of free uncomplexed drug in the formulation.

Using this methodology, therefore, it is proposed that it may be possible to predict the CD taste masking efficiency of a drug formulation by determining the association constant between the drug and CD, and subsequently the concentration of free uncomplexed drug in the formulation. This concentration could then be used to assess whether the concentration of CD within the formulation is sufficient to enable bitterness taste masking by fitting it to a calibration curve of bitterness intensity v free drug concentration already determined from a taste panel (Funasaki et al., 1999; 2006). This method would also enable a formulator to predict the bitterness intensity of numerous formulations containing varying concentrations of CD and drug, without the need for extensive bitterness assessment by a taste panel. An additional benefit of this method would be to allow direct comparison of the taste masking efficiency of other CDs, providing the association constants were determined. Nonetheless, it is uncertain the accuracy of the equation used to calculate the concentration of free uncomplexed drug in solution, which has been a continuing dilemma for scientists researching CD.
Chapter 5 - Adult Human Taste Panel Study

Optimising the excess CD

It was determined during this study that excess CD was more efficient at taste masking bitter drugs than moderate concentrations of CD. To continue this study it would be of interest to optimise the concentrations of CD so that a precise molar ratio of drug to CD for an acceptable taste could be achieved. For example, the 2.0 mg/mL HC formulation with 1.12 % HP-β-CD (1:1), was found to have extreme bitterness intensity and the 2.0 mg/mL HC formulation with 11.20 % HP-β-CD (1:14), was found to have a mean bitterness intensity of 22 (± 4). It would be of interest to determine the mean bitterness intensities of 2.0 mg/mL HC formulations with molar ratios between 1:1 and 1:14, or even greater, by further taste assessment with a taste panel so that optimal concentrations could be selected.

Although the use of excess CD is beneficial for taste masking, utilising too much CD within a formulation may raise safety concerns and encroach the limit of daily CD intake for a patient. The World Health Organisation has appointed a 5 mg/kg/day ADI for β-CD whilst no other ADI has been established for the remaining CDs. Without there being an established ADI for HP-β-CD and Me-β-CD it is difficult to assess how much excess HP-β-CD and Me-β-CD would remain a feasible option. If both of these CDs were imposed an ADI of 5 mg/kg/day then this would limit the use of excess CDs in very young/small paediatric patients and greater optimisation of the excess CD would be required.

Less bitter but more preferred? (Acceptability versus preference)

Although the taste panel found that the CDs were more efficient at taste masking HC and Qu compared to the traditional taste masking agents, it remains uncertain whether the formulations containing CDs would be preferred by patients over the formulations containing traditional taste masking agents. The assessors were selected based on their ability to perform accurate bitterness assessment and the assessors were asked to assess the bitterness of the test formulations. Preference was not assessed. The results found that some of the formulations containing traditional taste masking agents had low bitterness intensities. Although the bitterness intensities of these formulations were not as low as some of the formulations containing CDs, the bitterness intensities were close; a patient may, therefore, prefer a formulation containing traditional taste masking agents than CDs.
Chapter 5 - Adult Human Taste Panel Study

To develop the taste of the formulations containing CDs and make them more preferable, it would be of interest to investigate whether the addition of flavouring agents to a drug formulation containing CDs enhances the bitterness taste masking and makes the formulation more preferable. The formulations may benefit from the taste masking potential of the CD and the preferable taste of the flavouring agent. A consideration, however, would be the influence of the flavouring agent on the inclusion complexation of the drug, since it was demonstrated in Chapter 3 that CDs can complex other excipients in solution. Thus, with the use of phase solubility studies and taste assessment studies it may be possible to develop a drug formulation that contains CDs and flavouring agents, which would have a pleasurable taste.

Taste assessment in vivo methodology

The adult taste panel was conducted with the minimum number of taste assessors for valid results in the context of this research. Ideally, however, more taste assessors should have been recruited to enhance the statistical power of the results. Nevertheless, the running of the adult taste panel still took a great amount of time. Firstly, the whole taste assessment in vivo required a large amount of planning. The ethical approval, by an appropriate ethical committee, required large amounts of paper work. The taste assessor recruitment process took numerous months until adequate numbers were achieved, even with a time reimbursement incentive. The logistical running of the taste panel was a laborious process and required continued concentration throughout to avoid errors that could have easily occurred. The preparation required to set up each study day was long and repetitive, especially for one invigilator. Running a taste panel in an academic environment proved to be surprisingly difficult, especially regarding the correct study location.

The protocol of each study day was simple but required great attention from both taste assessor and invigilator. The number of formulations assessed by the panel was just up to its upper limit. The number of tastings per session made the study day duration long. Taste assessors commented that near the end of the afternoon session, concentration dropped and fatigue started to set in. This may have affected the assessment of the formulations containing traditional flavouring agent since they were assessed in the afternoon. The results may, therefore, be biased unknowingly towards the CDs. In an ideal scenario less formulations would have been assessed during each session but more study days would have been included. This would have helped to boost assessor moral, improve concentration and reduce assessor fatigue and potential bias. Furthermore,
formulations were only tasted in duplicate; in an ideal scenario tasting would have been conducted in triplicate to provide more accurate results. For logistical reasons (space, time, organisation), only a maximum of 12 taste assessors were able to carry out the study at any one time. Because of the taste assessor selection process, data analysis time, and the necessary washout period between each study day the whole *in vivo* study (3 days), was spread over a minimum of a fortnight.

**Adult taste assessment relating to paediatric taste assessment**

This research was based on the bitterness assessment of adult taste assessors and not a paediatric taste panel. Although this research found that CDs provided taste masking of bitter drug molecules, it is uncertain whether these results can be extrapolated to paediatric taste. Taste preferences and perceptions have been found to differ between adults and children (James *et al* 1997), and results in adults may not be applicable in children (Matsui, 2007). Thus, to take this research further it would be of interest to investigate whether paediatric patients considered CDs to be efficient taste masking agents. To conduct this research, however, the whole methodology would have to change to suit the needs of paediatric taste assessors, such as fewer formulation tested to avoid taste fatigue and avoid confusion, and perhaps a VAS with facial hedonic scale to enable accurate taste assessment.

## 5.5 Conclusion

By utilising a selected adult taste panel it was illustrated in this chapter that HP-β-CD and Me-β-CD (depending on concentration), were able to taste mask the bitter drugs studied as efficiently, and often better, than traditional taste masking agents. It was also determined that the CDs were able to taste mask clinically relevant concentrations of drug with the same bitterness intensities as formulations containing ten times less drug. Finally, excess concentrations of HP-β-CD and Me-β-CD were found to be more efficient at taste masking the bitter drugs than molar equivalent concentrations, and that total bitterness taste masking could be achieved with a large excess concentration of CD.
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison
6.1 Introduction

6.1.1 Taste masking assessment: *In Vitro* and *In Vivo* comparison

An aim of this thesis was to assess a range of selected taste masking agents for their ability to taste mask bitter corticosteroids. It was subsequently demonstrated during part of the taste assessment studies, that adult human taste panels provide successful bitter taste assessment for various selected formulations (Chapter 5). Taste panels are, however, laborious and expensive. Volunteers typically require training and selection, whilst studies commonly involve complex protocols. Ethical issues also hinder taste panels, especially in the absence of toxicological data or regarding potent/toxic compounds. The ASTREE is a novel analytical instrument developed to assess the taste of liquid samples and was used to aid the selection of various taste masking formulations (Chapter 4). *In vivo* taste analysers, like the ASTREE, have been described as having the advantage of providing fast, reproducible data without exposing taste panels to unnecessary amounts of chemicals. The ASTREE could, therefore, be an advantageous tool during formulation research and development, such as helping to select the optimum ingredients needed for a palatable medicine and by reducing the workload undertaken by human taste panels. Nevertheless, before electronic tongues can be implemented successfully into formulation research and development, positive correlation between human taste and *in vivo* taste analyser needs to be demonstrated.

The published research detailing positive correlation between human taste and the ASTREE is minimal. Sadrieh *et al.*, (2005), showed that the ASTREE and a human taste panel (*n* = 30), correlated well when providing taste masking assessment of various antibiotics. The correlation, however, was not comprehensive and differences were observed between the taste masking assessment from the human taste panel and the ASTREE. Kataoka *et al.*, (2005), showed positive correlation between the ASTREE and a human taste panel (*n* = 7), during the assessment of 15 sports drinks. Nonetheless, the correlation was not comprehensive, since the correlation was determined from individual sensors and not from all seven of the ASTREE sensors. Another study found that the bitterness intensity of various formulations, as determined by the PCA distances between placebo and active formulations (Pn-An PCA Distances), from the ASTREE, correlated well with the bitterness intensity determined from a small taste panel (*n* = 5) (Li *et al.*, 2007). It seems, however, that the authors did not take into account the fact that the majority of the PCA distances determined during this particular analysis were small (e.g. < 37), resulting in the bitterness interpretation being impaired. The PCA
distances actually indicated that the majority of formulations had the same degree of bitterness intensity, and in light of this, the correlation between the ASTREE and the human taste panel was not exhaustive.

A recent study concluded that the ASTREE bitterness assessment of various formulations had a very good correlation with a human taste panel (Lorenz et al., 2009). In this particular study, bitterness intensities of formulations containing acetaminophen, diphenhydramine HCL, and oxybutynin chloride were measured by a trained pharmaceutical sensory panel (number of assessors not published), as well as mean potentiometric voltage measurements from an ASTREE. The two sets of data were analysed by PLS analysis to enable the prediction of bitterness intensities by the ASTREE. The results showed that the ASTREE prediction of the bitterness intensities correlated well with the human taste panel with a PLS analysis correlation coefficient of $r^2 = 0.99$.

**6.1.2 Aims and objectives**

Continuing on from the preliminary ASTREE taste analysis, the aim of this chapter was to correlate the taste masking assessment of selected HC and Qu formulation from the adult taste panel (assessment *in vivo*) (Chapter 5), with the taste assessment from the ASTREE (assessment *in vitro*) (Chapter 4). These aims were fulfilled by carrying out ASTREE analysis on the same selected HC and Qu formulations that were analysed in Chapter 5, plus placebo samples, and comparing the bitterness taste masking assessment *in vitro* with the previously made assessment *in vivo*. This particular research has yet to be published in the literature and represents a novel aspect of this thesis.

**6.2 Materials and methods**

**6.2.1 Materials**

As per Section 4.2.1

**6.2.2 Taste masking assessment *in vitro*: ASTREE electronic tongue analysis of preselected hydrocortisone and quinine formulations**

**6.2.2.1 Experimental procedure**

Before the ASTREE analysis of the preselected formulations, the ASTREE “start-up” procedure was carried out as discussed in Section 4.1.2.3. The ASTREE analysis was
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

subsequently carried out in four analysis stages to accommodate the 17 test samples (Table 6.1 and Table 6.2).

**Table 6.1**: Preselected hydrocortisone formulations analysed during ASTREE electronic tongue analysis

<table>
<thead>
<tr>
<th>Formulation No.</th>
<th>Active</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2 mg/mL hydrocortisone + 1.12 % w/v HP-β-CD</td>
<td>1.12 % w/v HP-β-CD</td>
</tr>
<tr>
<td>4</td>
<td>2 mg/mL hydrocortisone + 11.20 % w/v HP-β-CD</td>
<td>11.20 % w/v HP-β-CD</td>
</tr>
<tr>
<td>5</td>
<td>2 mg/mL hydrocortisone + 5.53 % w/v Me-β-CD</td>
<td>5.53 % w/v Me-β-CD</td>
</tr>
<tr>
<td>6</td>
<td>2 mg/mL hydrocortisone + 2.65 % w/v Me-β-CD</td>
<td>2.65 % w/v Me-β-CD</td>
</tr>
<tr>
<td><strong>Stage 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.20 mg/mL hydrocortisone + 1.12 % w/v HP-β-CD</td>
<td>1.12 % w/v HP-β-CD</td>
</tr>
<tr>
<td>8</td>
<td>0.20 mg/mL hydrocortisone + 11.20 % w/v HP-β-CD</td>
<td>11.20 % w/v HP-β-CD</td>
</tr>
<tr>
<td>9</td>
<td>0.20 mg/mL hydrocortisone + 0.53 % w/v Me-β-CD</td>
<td>0.53 % w/v Me-β-CD</td>
</tr>
<tr>
<td>10</td>
<td>0.20 mg/mL hydrocortisone + 2.65 % w/v Me-β-CD</td>
<td>2.65 % w/v Me-β-CD</td>
</tr>
<tr>
<td><strong>Stage 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.20 mg/mL hydrocortisone + 12 % w/v Sucrose</td>
<td>12 % w/v Sucrose</td>
</tr>
<tr>
<td>12 (Ref 1)</td>
<td>0.20 mg/mL hydrocortisone + 20 % w/v Erythritol</td>
<td>20 % w/v Erythritol</td>
</tr>
<tr>
<td>13</td>
<td>0.20 mg/mL hydrocortisone + 0.08 % w/v Ace K</td>
<td>0.08 % w/v Ace K</td>
</tr>
<tr>
<td>1 &amp; 2 (Ref 2)</td>
<td>0.20 mg/mL hydrocortisone</td>
<td>Distilled water</td>
</tr>
<tr>
<td><strong>Stage 4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.20 mg/mL hydrocortisone + 2 % w/v Cherry</td>
<td>2 % w/v Cherry</td>
</tr>
<tr>
<td>15 (Ref 2)</td>
<td>0.20 mg/mL hydrocortisone + 2 % w/v Strawberry</td>
<td>2 % w/v Strawberry</td>
</tr>
<tr>
<td>16</td>
<td>0.20 mg/mL hydrocortisone + 2 % w/v Strawberry + 0.08 % w/v Ace K</td>
<td>2 % w/v Strawberry + 0.08 % w/v Ace K</td>
</tr>
<tr>
<td>17</td>
<td>0.20 mg/mL hydrocortisone + 2 % w/v Tangerine + 0.08 % w/v Ace K</td>
<td>2 % w/v Tangerine + 0.08 % w/v Ace K</td>
</tr>
</tbody>
</table>

**Table 6.2**: Preselected quinine formulations analysed during ASTREE electronic tongue analysis

<table>
<thead>
<tr>
<th>Formulation No.</th>
<th>Active</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.6 mg/mL quinine + 2.89 % w/v HP-β-CD</td>
<td>2.89 % w/v HP-β-CD</td>
</tr>
<tr>
<td>4</td>
<td>1.6 mg/mL quinine + 28.9 % w/v HP-β-CD</td>
<td>28.9 % w/v HP-β-CD</td>
</tr>
<tr>
<td>5</td>
<td>1.6 mg/mL quinine + 1.04 % w/v Me-β-CD</td>
<td>1.04 % w/v Me-β-CD</td>
</tr>
<tr>
<td>6</td>
<td>1.6 mg/mL quinine + 5.2 % w/v Me-β-CD</td>
<td>5.2 % w/v Me-β-CD</td>
</tr>
<tr>
<td><strong>Stage 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 (Ref 1)</td>
<td>0.16 mg/mL quinine + 2.89 % w/v HP-β-CD</td>
<td>2.89 % w/v HP-β-CD</td>
</tr>
<tr>
<td>8</td>
<td>0.16 mg/mL quinine + 28.9 % w/v HP-β-CD</td>
<td>28.9 % w/v HP-β-CD</td>
</tr>
<tr>
<td>9</td>
<td>0.16 mg/mL quinine + 1.04 % w/v Me-β-CD</td>
<td>1.04 % w/v Me-β-CD</td>
</tr>
<tr>
<td>10</td>
<td>0.16 mg/mL quinine + 5.2 % w/v Me-β-CD</td>
<td>5.2 % w/v Me-β-CD</td>
</tr>
<tr>
<td><strong>Stage 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.16 mg/mL quinine + 12 % w/v w/v Sucrose</td>
<td>12 % w/v w/v Sucrose</td>
</tr>
<tr>
<td>12</td>
<td>0.16 mg/mL quinine + 15 % w/v Erythritol</td>
<td>15 % w/v Erythritol</td>
</tr>
<tr>
<td>13</td>
<td>0.16 mg/mL quinine + 0.08 % w/v Acesulpham K</td>
<td>0.08 % w/v Acesulpham K</td>
</tr>
<tr>
<td>1 &amp; 2 (Ref 2)</td>
<td>0.16 mg/mL quinine</td>
<td>Distilled water</td>
</tr>
<tr>
<td><strong>Stage 4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.16 mg/mL quinine + 2 % w/v Tangerine</td>
<td>2 % w/v Tangerine</td>
</tr>
<tr>
<td>15</td>
<td>0.16 mg/mL quinine + 2 % w/v Cherry</td>
<td>2 % w/v Cherry</td>
</tr>
<tr>
<td>16</td>
<td>0.16 mg/mL quinine + 2 % w/v Cherry + 0.02 % w/v Acesulpham K</td>
<td>2 % w/v Cherry + 0.02 % w/v Ace K</td>
</tr>
<tr>
<td>17</td>
<td>0.16 mg/mL quinine + 2 % w/v Tangerine + 0.02 % w/v Acesulpham K</td>
<td>2 % w/v Tangerine + 0.02 % w/v Ace K</td>
</tr>
</tbody>
</table>
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

Before each analysis stage, the sensors were conditioned and calibrated in 80 mL of 0.20 mg/mL HC or 0.16 mg/mL Qu, under the standard calibration parameters (Section 4.1.2.3). Analysis samples consisted of 80 mL of the active and placebo formulations. Two reference formulations were also included in each analysis to allow merging of all data into one library for overall analysis. Formulation 12 & 15 were reference formulations for the ASTREE analysis of the HC formulations and formulations 2 & 7 were reference formulations for the ASTREE analysis of the Qu formulations. Each analysis sequence incorporated six cleaning samples and ASTREE method parameters incorporated: 120 s of sample measurement by all sensors, 2 cleaning dip for 10 s in 80 mL distilled water after every measurement made, 8 repeats (8 measurements per sample), and a data library created by acquiring mean sensor recordings of the last 20 s for each sample measurement.

Four data libraries from the four stages were produced initially for both the HC formulations and Qu formulations. The samples in each analysis sequences were analysed via the S2 sequence (See Section 4.1.2.3) with the two reference samples being the first two samples tested followed by the various placebo and active samples (Figure 6.1). The prefix P was given to placebo samples and the prefix F were assigned to the active formulations.

**Figure 6.1:** An example of the analysis sequence used during the ASTREE electronic tongue analysis of preselected quinine formulations. Key: W - Water/cleaning, P - Placebo, F - Formulation (Active)
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

6.2.2.2 Data analysis

**Replicate and sensor selection:** From each data library the first three replicates of each sample measurement were deleted. For each drug, a mathematical calibration was performed so that all four analysis stages could be analysed together. The mathematical calibration was performed on each of the four data libraries, utilising the mean potentiometric voltage measurements of one of the reference samples that was incorporated into the analysis: formulation 12 for the HC data libraries, formulation 7 for the Qu data libraries. The mathematically calibrated individual libraries were merged together to form one data library for the HC formulations and one data library for the Qu formulations. Global sensor selection could then be carried out by monitoring the dispersion between the repeated measurements of the reference samples and by monitoring the sensor discrimination between the two references across all four analysis stages. Normalisation of the potentiometric voltage measurements from the reference samples aided the monitoring of sensor dispersion. Sensors that did not discriminate the two reference samples across the four analysis stages, or did not produce reproducible sample measurements across the five replicates were omitted from the data library.

**Data analysis:** Data analysis on the mathematically calibrated data libraries was conducted via PCA, with Pn-An and A0-An PCA Distances being employed to interpret the results of the PCA.

6.2.3 Taste masking assessment *in vivo:* Selected adult taste panel assessment of preselected hydrocortisone and quinine formulations

The methods used for the selected adult taste panel assessment of preselected HC and Qu formulations are presented in Chapter 5.

6.2.4 *In vitro* and *in vivo* correlation

Correlation between the ASTREE and taste panel data was examined by comparing the bitterness intensities determined by the two taste assessment methods, including grouping formulations according to bitterness intensity variation (95 % CI for the ASTREE results and 90 % CI for the taste panel results). Comparisons were drawn also from trends seen within the differing classes of taste masking agent (CD, sweetener, and flavouring agent). To enable the bitterness assessment of each test formulation to be analysed according to each taste assessor’s own personal definition of “No bitterness intensity” and “Extreme bitterness intensity”, and for successful comparison on the two
Chapter 6 - Taste Masking Assessment: \textit{In Vitro} and \textit{In Vivo} Comparison

taste assessment methods, the individual VAS scores produced during the \textit{in vivo} bitterness assessment were corrected using the VAS scores of the two reference samples, distilled water (F1) and 0.20 mg/mL HC or 0.16 mg/mL Qu (F2). This was carried out using the following equation:

\[
\text{Corrected mean bitterness score} = (\text{Formulation VAS score - F1 VAS scale}) \times (100/F2 - F1).
\]

\textbf{Equation 6.1}

\section*{6.3 Results and discussion}

\subsection*{6.3.1 Taste masking assessment \textit{in vitro}: ASTREE electronic tongue analysis of preselected hydrocortisone formulations}

ASTREE discrimination of the selected HC formulations and respective placebo formulations was calculated via PCA (Figure 6.2). The majority of the individual sample clusters within the PCA map were tight and compact, indicating that the ASTREE potentiometric voltage measurements of each sample was analysed reproducibly throughout each analysis stage. The clusters of formulation 12 (R1) were represented as one cluster because the mathematical calibration was carried out using this particular reference formulation, resulting in similar potentiometric voltage measurements. In contrast, the clusters of formulation 15 (R2) were dispersed slightly more with a mean PCA distance of 245 (+/- 73 (95 \% CI), between the R2 clusters, illustrating the variation in potentiometric voltage measurements made by the ASTREE across the four analysis stages. \emph{This variation (+/- 73 (95 \% CI)) was applied subsequently to every Pn-An and A0-An PCA Distance calculated.}
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

**Figure 6.2:** A PCA map of the ASTREE electronic tongue discrimination of hydrocortisone formulations (F1-F17), and their respective placebo formulations (P3-P17), including reference formulations 12 (R1) and 15 (R2). Key: See Table 6.1.

The PCA discrimination index of -168 showed that the ASTREE was not discriminating some of the active and placebo formulations, thus indicating that perhaps taste masking was occurring between some of the samples. The dispersion of sample clusters within the PCA map shows clearly that the ASTREE discriminated various types of sample. For instance, the samples that contained flavouring agent (bottom right corner (F14, P14, F15, P15, F16, P16, F17, and P17)), are separated from all the other samples. The active and placebo samples of acesulpham K (F13 and P13), are also discriminated from all the other samples, as well as the active and placebo samples that contained 11.2 % w/v HP-β-CD (F4, P4, F8, and P8). The ASTREE, therefore, measured differing potentiometric voltages for these particular samples as opposed to the remaining CD
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

formulations, sweetener formulations, water sample, and 0.20 mg/mL HC sample, which were grouped together on the PCA map (bottom left corner).

The **Pn-An PCA Distances** were calculated between the data clusters from the PCA (Figure 6.3). The variation error bars applied to the Pn-An PCA Distances (a PCA distance of +/- 73 calculated from the PCA distance variation of R²), illustrated the Pn-An PCA Distances from the various formulations were not significantly different ($p = 0.05$). Thus, all taste masking formulations had the same degree of bitterness intensity and taste masking of the HC. According to the PCA interpretation via Pn-An PCA Distances, no trends in bitterness taste masking of the HC from the various taste masking agents were observed. These results were not attributed to the size of the variation but, however, the small Pn-An PCA Distances that were recorded between the samples. These Pn-An PCA Distances correspond to the small Pn-An PCA Distances determined between the placebo and active HC formulations in Chapter 4 and strengthens the hypothesis that the ASTREE sensors were poor at detecting the HC. In both cases the distances being determined may have only been representative of the minimal discrimination between the taste of the taste masking agent in the active and placebo sample, which the sensors were sensitive to, and not the presence of HC, which the sensors were not sensitive to.

![Figure 6.3: Pn-An PCA Distances (+ 73 (95 % CI)), of selected hydrocortisone formulations analysed by ASTREE electronic tongue](image.png)
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

**A0-An PCA Distances** were calculated from the data clusters from the PCA (Figure 6.4). Unlike the Pn-An PCA Distances, the variation error bars did not group together all the formulations in terms of bitterness intensity. This was attributed to the large distances seen by A0-An PCA Distances. The large A0-An PCA Distances provided further evidence that the sensors had greater sensitivity to the taste masking agents than the HC because greater PCA distances were seen between the A0 sample (containing HC and no taste masking agent), and the active samples that contained the various taste masking agents (An) than the active and placebo sample, which both contained the taste masking agent but only one contained the drug. Directly putting this into context, it is believed that the Pn-An PCA Distances were a direct measure of the sensitivity of the sensors to the HC and the A0-An PCA Distances were a direct measure of the sensitivity to the taste masking agent.

The formulations with the greatest A0-An PCA Distances and subsequently the most efficient taste masking agents were the combination formulations: 2 % w/v strawberry + 0.08 w/v % acesulpham K and 2 % w/v tangerine + 0.08 % w/v acesulpham K with A0-An PCA Distances of 1511 (+/- 73 (95 % CI)), and 1592 (+/- 73 (95 % CI)), respectively. The most efficient CD taste masking agent was 11.20 % w/v HP-β-CD, with either 0.20 mg/mL or 2.0 mg/mL HC present. Based on these results the CDs were not considered to be superior at taste masking HC than traditional taste masking agents. The most efficient sweetener was 0.08 % w/v acesulpham K, whilst the most efficient flavouring agent was 2 % w/v strawberry. Both of these traditional taste masking agents were considered more efficient at taste masking 0.20 mg/mL HC than HP-β-CD or Me-β-CD. Flavouring agent/sweetener combination formulations were deemed more efficient taste masking agents than the individual flavouring agents and sweeteners, with greater A0-An PCA Distances. These results indicated that taste masking synergy occurred between the two types of taste masking agent enabling enhanced taste masking efficiency.
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

Figure 6.4: A0-An PCA Distances (+/- 73 (95 % Cl)), of selected hydrocortisone formulations analysed by ASTREE electronic tongue. * Significant difference between F3 & F4, # significant difference between F7 & F8, ** significant difference between F11 & F12 and F11 & F13, ### significant difference between F12 & F13, ** significant difference between F14 & F15. *p = 0.05.

According to the A0-An PCA Distances excess concentrations of HP-β-CD was more efficient at taste masking 0.20 mg/mL HC or 2.0 mg/mL HC than molar equivalent concentrations of HP-β-CD. For instance, formulation 3 had an A0-An PCA Distance of 154 (+/- 73 (95 % Cl)), whilst formulation 4, with ten times more HP-β-CD, had an A0-An PCA Distance of 511 (+/- 73 (95 % Cl)). In contrast, the A0-An PCA Distances produced from excess Me-β-CD and molar equivalent Me-β-CD were not significantly different (*p = 0.05*), whether taste masking 0.20 mg/mL HC or 2.0 mg/mL HC, indicating indistinguishable taste masking efficiencies between the two concentrations of Me-β-CD. Furthermore, the A0-An Distances indicated that the taste masking efficiency of the CDs were not significantly different (*p = 0.05*), when ten times more HC was added. For example, formulation 9 had an A0-An Distance of 295 (+/- 73 (95 % Cl)), whilst formulation 5, with ten times more HC, had an A0-An PCA Distance of 270 (+/- 73 (95 % Cl)).
These results may provide evidence that because Me-β-CD had such a large stability constant (Kc) with the HC and because such a high percentage of CD molecules were complexing with the HC (Chapter 2), adding more Me-β-CD did not enhance its taste masking capabilities. In comparison, because HP-β-CD had a smaller association constant with the HC and less CD molecules were complexing, adding more HP-β-CD enabled more HC to be complexed and, therefore taste masked.

### 6.3.2 Taste masking assessment *in vitro*: ASTREE electronic tongue analysis of preselected quinine formulations

ASTREE discrimination of the selected Qu formulations and respective placebo formulations was calculated via PCA (Figure 6.5). Individual sample clusters within the PCA map were dispersed slightly compared to the sample clusters from the PCA of the HC formulations. Nonetheless, the dispersion of the individual clusters was ubiquitous throughout the PCA map indicating the slight dispersion did not affect the reproducibility of the potentiometric voltage measurements of each sample. As per the HC analysis, the clusters of the reference sample (R1) used for the mathematical calibration (F7) were grouped together having similar potentiometric voltage measurements. The R2 reference sample (F2) had sample clusters with a mean PCA distance of 318 (+/− 73 (95 % CI)), indicating that the variation in potentiometric voltage measurement across the four analysis stages was greater than the variation seen in the HC analysis, although the 95 % CI remained the same. The fact that both the HC and Qu taste masking analysis produced the same degree of variation illustrated that the ASTREE produces robust and reproducible results, although the similar variation may be considered coincidence.

The discrimination index of -21 from the PCA of the Qu formulations illustrates that taste masking between the active and placebo samples had occurred but to less of an extent than the HC formulations, which had a more negative discrimination index. Furthermore, the discrimination of the various classes of taste masking agent (CD, sweetener, and flavouring agent), was less evident from the PCA of the Qu formulations than the HC formulations with no distinct regions observed.
Figure 6.5: A PCA map of the ASTREE electronic tongue discrimination of quinine formulations (F1-F17) and their respective placebo formulations (P3-P17), including reference formulations 7 (R1) and 2 (R2). Key: See Table 6.2

The Pn-An PCA Distances were calculated between the data clusters from the PCA (Figure 6.6). As per the PCA distances from the HC formulations, a PCA distance variation of +/- 73 was applied to each Pn-An PCA Distance originating from the 95 % CI determined from the mean PCA distances calculated between the various R2 samples. Unlike the Pn-An PCA Distances calculated from the PCA of the HC formulations the PCA distance variation of +/- 73 did not encompass the majority of the samples in terms of bitterness intensity. This implied that the ASTREE was likely to have been more sensitive to the Qu than the HC much like the results from the preliminary ASTREE analysis.

The greatest Pn-An PCA Distance from the Qu formulations was 1956 (+/- 73 (95 % CI)), from the formulation containing 1.6 mg/mL Qu + 28.9 % w/v HP-β-CD. This
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

formulation was, therefore, deemed to be most discriminated from its placebo formulation and tasted consequently the most different from its placebo. As such, no taste masking of 1.6 mg/mL Qu occurred from the 28.9 % w/v HP-β-CD. Furthermore, the 1.6 mg/mL Qu + 28.9 % w/v HP-β-CD formulation and its placebo had a far greater Pn-An PCA Distance than the 0.16 mg/mL Qu formulation at its placebo (distilled water), which had a Pn-An PCA Distance of 326 (+/- 73 (95 % CI)). In theory this formulation should have had the greatest Pn-An PCA Distance, since the preconceived assumption was that the 0.16 mg/mL quinine and distilled water had the greatest difference in taste and subsequently the greatest Pn-An PCA Distance. This particular result may have been caused by the ASTREE not being successful at analysing fully the discrimination between 0.16 mg/mL Qu and distilled water or that the 28.9 % HP-β-CD, and the majority of the other formulations that had Pn-An PCA Distances greater then 326 (+/- 73 (95 % CI)), were not able to taste mask the Qu.

![Graph](image)

**Figure 6.6:** Pn-An PCA Distances (+/- 73 (95 % CI)), of selected quinine formulations analysed by ASTREE electronic tongue. * Significant difference between F3 & F4, # significant difference between F7 & F8, ** significant difference between F9 & F10, ## significant difference between F14 & F15, ### significant difference between F16 & F17, $p = 0.05$. 

206
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

The smallest Pn-An PCA Distance from a formulation containing CD was 241 (+/- 73 (95 % Cl)), from the 0.16 mg/mL Qu + 2.89 % HP-β-CD formulation, whilst the smallest Pn-An PCA Distance from a formulation containing traditional taste masking agent was 340 (+/- 73 (95 % Cl)), from the 0.16 mg/mL Qu + 15 % erythritol formulation. Since these Pn-An PCA Distance were not significantly different ($p = 0.05$), it cannot be concluded that CDs were more efficient at taste masking Qu than traditional taste masking agents.

There was no significant difference ($p = 0.05$), in taste masking efficiencies produced from the sweeteners, whilst 2 % w/v tangerine was more efficient than 2 % w/v cherry. The addition of sweetener to the flavouring agents had no significant effect on taste masking efficiency for the flavouring agent but reduced significantly the taste masking efficiency of the sweetener, acesulpham K ($p = 0.05$). The Pn-An PCA Distances produced mixed results as to whether excess CD was more efficient than molar equivalent concentrations of CD. For example, molar equivalent concentrations of HP-β-CD was significantly more efficient at taste masking 0.16 and 1.6 mg/mL Qu than excess concentrations of HP-β-CD, whilst there was no significant difference between excess Me-β-CD and molar equivalent concentrations of Me-β-CD at taste masking 1.6 mg/mL Qu ($p = 0.05$). On the other hand, excess Me-β-CD was significantly more efficient at taste masking 0.16 mg/mL Qu than molar equivalent concentrations of Me-β-CD ($p = 0.05$). The Pn-An PCA Distances also signified that adding ten times more Qu did reduce significantly the taste masking efficiencies of 2.89 and 28.9 % w/v HP-β-CD and 5.2 % w/v Me-β-CD ($p = 0.05$). Yet adding ten times more Qu did not reduce significantly the taste masking efficiencies of 1.04 % w/v Me-β-CD ($p = 0.05$).

The **A0-An PCA Distances** were calculated between the data clusters from the PCA (Figure 6.7). The formulation with the greatest A0-An PCA Distances and subsequently the most efficient taste masking agent was 2131 (+/- 73 (95 % Cl)), from the formulation containing 0.16 mg/mL Qu + 28.9 % w/v HP-β-CD. The greatest A0-An PCA Distances from a traditional taste masking agent was 1599 (+/- 73 (95 % Cl)), from the combination formulation containing 0.16 mg/mL Qu + 2 % w/v tangerine + 0.02 % w/v acesulpham K. These results indicate clearly that HP-β-CD was more efficient at taste masking 0.16 mg/mL Qu than traditional taste masking agents. Nonetheless, 2 % w/v tangerine + 0.02 % w/v acesulpham K, 2 % w/v tangerine, 2 % w/v cherry + 0.02 % w/v acesulpham K and 2 % w/v cherry were all deemed to be
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

significantly more efficient at taste masking 0.16 mg/mL than 1.04 and 5.2 % w/v Me-β-CD, and 2.89 % w/v HP-β-CD, as well as all the CD formulations containing 1.6 mg/mL Qu ($p = 0.05$). The most efficient sweetener was 0.08 % w/v acesulpham K and the most efficient flavouring agent was 2 % w/v tangerine. The addition of acesulpham K did not significantly improve the taste masking efficiencies of the flavouring agents but did enhance significantly the taste masking efficiencies of the acesulpham K ($p = 0.05$).

![Figure 6.7: A0-An PCA Distances (+/- 73 (95 % CI)), of selected quinine formulations analysed by ASTREE electronic tongue. * Significant difference between F3 & F4, # significant difference between F5 & F6, ** significant difference between F7 & F8, ## significant difference between F11 & F13 and F12 & F13, ### significant difference between F14 & F15, #### significant difference F16 & F17. $p = 0.05$.](image)

The A0-An PCA Distances illustrated excess concentrations of HP-β-CD were more efficient at taste masking 0.16 and 1.6 mg/mL Qu than molar equivalent concentrations. Yet molar equivalent concentration of Me-β-CD was more efficient at taste masking 1.60 mg/mL Qu than excess concentrations and there was no significant difference in taste masking efficiency between 1.04 and 5.2 % w/v Me-β-CD at taste masking 0.16
mg/mL Qu ($p = 0.05$). The A0-An PCA Distances highlighted also that adding ten times more Qu to the formulation reduced significantly the taste masking efficiency of the CD apart from the 2.89 % w/v HP-β-CD which had significantly the same taste masking efficiency at taste masking 0.16 and 1.60 mg/mL Qu ($p = 0.05$).

### 6.3.3 Taste masking assessment: in vitro and in vivo comparison of hydrocortisone and quinine formulations

**Hydrocortisone:** The corrected mean bitterness scores of the HC formulations determined by the selected adult taste panel results in Chapter 5 were calculated and compared against the results of the ASTREE analysis (Figure 6.8).

To visualise the grouping (overlapping of variation error bars), and ranking of the *in vitro* and corrected *in vivo* data, linear graphs of the corrected mean bitterness scores and ASTREE PCA Distances were produced for the HC formulations (Figure 6.9). *Please note the number assigned to each taste masking agent represents the ranking of most efficient taste masking agent where 1 equals best and 11 equals worst.*
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

**Figure 6.8:** Pn-An and A0-An PCA Distances (+/- 73 (95% CI)) from the ASTREE electronic tongue and corrected mean bitterness intensity (90% CI) from the human adult taste panel from assessment of selected hydrocortisone formulations (F3 - F17).

Key: See Table 6.1. $p = 0.05$
Figure 6.9: Linear Pn-An and A0-An PCA Distances (+/- 73 (95 % CI)), from the ASTREE electronic tongue and corrected mean bitterness scores (+/- 90 % CI), from the human adult taste panel of the assessment of selected hydrocortisone formulations (A- 2 mg/mL, B- 0.2 mg/mL)
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

**Quinine**: The corrected mean bitterness scores of the Qu formulations determined by the selected adult taste panel results in Chapter 5 were calculated and compared against the results of the ASTREE analysis (Figure 6.10).

**Figure 6.10**: Pn-An and A0-An PCA Distances (+/- 73 (95 % CI)) from the ASTREE electronic tongue and corrected mean bitterness intensity (90 % CI) from the human adult taste panel from assessment of selected quinine formulations (F3 - F17). Key: See Table 6.2. $p = 0.05$
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

To visualise the grouping and ranking of the *in vitro* and corrected *in vivo* data, linear graphs of the corrected mean bitterness scores and ASTREE PCA Distances were produced for the HC formulations (Figure 6.11). *Please note the number assigned to each taste masking agent represents the ranking of most efficient taste masking agent where 1 equals best and 11 equals worst.*

<table>
<thead>
<tr>
<th>Taste Masking Agent</th>
<th>Corrected Mean Bitterness Score</th>
<th>ASTREE PCA Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>284 mg/mL quinine</td>
<td>1.6 mg/mL quinine</td>
</tr>
<tr>
<td>B</td>
<td>16 mg/mL quinine</td>
<td>0.16 mg/mL quinine</td>
</tr>
</tbody>
</table>

**Figure 6.11:** Linear Pn-An PCA Distances (+/- 73 (95% CI)), A0-An PCA Distances (+/- 73 (95% CI)), and corrected mean bitterness scores (+/- 90% CI), with grouping and ranking of selected quinine formulations (A- 1.6 mg/mL, B- 0.16 mg/mL), analysed by the ASTREE electronic tongue and selected adult taste panel.
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

The aim of this chapter was to examine whether the taste masking assessment of 17 HC and 17 Qu formulations using the ASTREE would correlate successfully with the taste masking assessment obtained from a selected adult taste panel. Pn-An PCA Distances and A0-An PCA Distances were both used to interpret the PCA data so full potential of the ASTREE data analysis would be used.

**Pn-An PCA Distances:** The Pn-An PCA Distances method has been used previously to interpret the PCA data produced during ASTREE data analysis (Sadrieh *et al.*, 2005; Zheng and Keeney, 2006; Li *et al.* 2007; Lorenz *et al.*, 2009). Nevertheless, the results from this particular research indicated that PCA interpretation by Pn-An PCA Distances was unable to correlate fully with the results of the selected adult taste panel. As seen in Figure 6.8 the small Pn-An PCA Distances for the HC formulations did not allow for any taste assessment conclusions to be made, apart from the formulations all having the same magnitude of bitterness and taste masking efficiency, and, therefore, were all grouped and ranked together. Because of this, the results of the Pn-An PCA Distances correlated with the selected adult taste panel by indicating that there was no significant difference in taste masking efficiency between the sweeteners, between the flavouring agents, and between the combination formulations and individual agents ($p = 0.05$). As discussed previously, it is suspected, however, that Pn-An PCA Distances from the HC formulations were too small due to a lack of sensitivity by the sensors to the HC compared to the taste masking agents.

The Pn-An PCA Distances for the Qu formulation showed similar mixed correlation with the selected adult taste panel. The Pn-An PCA Distances correlated with the selected adult taste panel by demonstrating 5.2 % w/v Me-β-CD was significantly more efficient at taste masking 0.16 mg/mL Qu than 1.04 % w/v Me-β-CD, as well as indicating that no significant difference in taste masking efficiency existed between the sweeteners, and no significant difference between combination formulations and individual agent ($p = 0.05$). The grouping and ranking of the Qu formulations according to Pn-An PCA Distances was also different to the grouping and ranking of the Qu formulations according to the selected adult taste panel (Figure 6.11). These mixed results produced a lack of confidence in the Pn-An PCA Distances method of PCA interpretation, since there was no means of determining which Pn-An PCA Distances results will correlate with a taste panel.
AO-An PCA Distances: The A0-An PCA Distances method provided an alternative method of interpreting the PCA from the ASTREE analysis. Favoured by the manufacturers of the ASTREE (Alpha M.O.S), the fundamentals of the A0-An PCA Distances method are based on the most efficient taste masking agent being the most discriminated active formulation from the formulation that contains just the drug, i.e. the A0 formulation. If an active formulation containing taste masking agent is not discriminated from the A0 formulation then the taste will be discriminated poorly from the A0 formulation, i.e. the formulation will taste bitter. Based on this method and illustrated by Figure 6.8 the ASTREE HC formulation analysis correlated with the selected adult taste panel by concluding that excess concentrations of HP-β-CD was more efficient at taste masking 0.20 mg/mL HC or 2.0 mg/mL HC than molar equivalent concentrations of HP-β-CD. It did not correlate, however, with the panel by signifying that excess concentrations of Me-β-CD was more efficient at taste masking HC than molar equivalent concentrations of Me-β-CD. This indicated that the ASTREE might have had good sensitivity with one CD but not the other. This illustrated further that the ASTREE was molecule particular even when chemical composition between the molecules was very similar, much like when the ASTREE analysed Pd and HC in Chapter 4.

The A0-An PCA Distances from the PCA of the HC formulations, failed also to demonstrate that the presence of ten times more HC enhanced significantly the bitterness intensity of the formulations and more importantly, that both CDs were more efficient at taste masking the HC than the traditional formulations. The A0-An PCA Distances did, however, correlate with the selected adult taste panel by indicating that there was no significant difference in taste masking efficiency between sweeteners and flavouring agents. Nevertheless, the A0-An PCA Distances did indicate sweetener & flavouring agent combinations were more efficient at taste masking 0.20 mg/mL HC than individual agents, unlike the selected adult taste panel. The grouping and ranking of the formulations was varied also between the A0-An PCA Distances results and the selected human taste panel results. As per the Pn-An PCA Distances results, it is not clear, without the help of a human taste panel, which of the A0-An PCA Distances conclusions can be relied on.

The A0-An PCA Distances of the Qu formulations correlated partially with the selected adult taste panel (Figure 6.10). For instance, the A0-An PCA Distances concluded that
Chapter 6 - Taste Masking Assessment: *In Vitro* and *In Vivo* Comparison

The 28.9 % w/v HP-β-CD was the most efficient taste masking agent, thus correlating with the taste panel by signifying that CDs were more efficient than traditional taste masking agents. Yet the remaining CD formulations that were assessed as being more efficient than sweetener and/or flavouring agent by the taste panel were seen to be less efficient by the A0-An PCA Distances. In addition, as per the A0-An PCA Distances conclusions from the HC formulations, correlation with the taste panel was produced with regard to excess concentrations of HP-β-CD being more efficient at taste masking 0.20 mg/mL HC or 2.0 mg/mL HC than molar equivalent concentrations of HP-β-CD but not with the Me-β-CD. Aside from the A0-An PCA Distances correctly ranking the 28.9 % w/v HP-β-CD as the best taste masking agent, in comparison with the selected adult taste panel, the ranking of the formulations in terms of taste masking efficiency was varied between the A0-An PCA Distances and taste panel.

In addition to comparing human taste panel data with PCA distances, other researchers have utilised PLS analysis to determine the correlation between ASTREE and human taste panel assessment (Kataoka *et al*., 2005; Kayumba *et al*., 2007; Lorenz *et al*., 2009). Nonetheless, the ASTREE data from this particular analysis was unable to be correlated with the adult taste panel data by PLS analysis, since the ASTREE experiments were conducted in four different experiments and with placebo formulations, which meant that PLS analysis was not possible. To be able to perform PLS analysis, only active formulations would have to have been analysed and in only one experiment. This was not possible since there were too many active formulations to be assessed. The ASTREE analysis presented here does not, therefore, have a numerical value (i.e. correlation coefficient), to represent how well it correlated with a human taste panel.

Apart from the taste assessment correlation between the methods *in vivo* and *in vitro* it was important to consider the experimental aspect of both techniques. A discussion regarding the methodology of the human taste panel is given in Section 5.4. Meanwhile, compared to the human taste panel the ASTREE taste assessment experiments took only eight days to complete and required no ethical approval. Nevertheless, this does not take into consideration the training required to become efficient at using the ASTREE, especially handling the data processing and data analysis. The experimental procedures were easy to set up and placebo formulations could be prepared easily - a process that was shown to hinder previous ASTREE analysis (Lorenz *et al*., 2009). The start up
procedure, which prepares the ASTREE for formulation analysis, however, took longer than expected. Because the analysis of the formulations was automated by the ASTREE, analysing the formulations overnight reduced the overall analysis time. The most complex process regarding the use of the ASTREE was the intricate and laborious data analysis, especially the replicate and sensor selection, and the merging of data libraries to allow for inter library analysis. Newer ASTREE software, however, has now been developed to improve this types of complex data analysis.

**Correlation summary**

Hydrocortisone formulations:
- Pn-An PCA Distances did not show sample discrimination. ASTREE correlated with the human taste panel by showing that:
  - All sweeteners were equivalent in bitterness taste masking.
- A0-An PCA Distances showed sample discrimination. ASTREE correlated with the human panel by showing that:
  - Excess HP-β-CD was better at taste masking 2.0 mg/mL HC than moderate concentration of HP-β-CD.
  - Excess Me-β-CD was better at taste masking 0.20 mg/mL HC than moderate concentration of Me-β-CD.
  - Strawberry was better at taste masking 0.20 mg/mL HC than cherry.

Quinine formulations:
- Pn-An PCA Distances showed sample discrimination. ASTREE correlated with the human panel by showing that:
  - Excess Me-β-CD was better at taste masking 0.16 mg/mL Qu than moderate concentration of Me-β-CD.
  - All sweeteners were equivalent in bitterness taste masking.
- A0-An PCA Distances showed sample discrimination. ASTREE correlated with the human panel by showing that:
  - Excess HP-β-CD was better at taste masking 1.6 mg/mL Qu than moderate concentration of HP-β-CD.
  - Excess HP-β-CD was better at taste masking 0.16 mg/mL Qu than moderate concentration of HP-β-CD.
  - Tangerine/acesulpham K combination was better at taste masking 0.16 mg/mL Qu than K Cherry/acesulpham combination.
6.4 Conclusion

It was demonstrated in this chapter that the results from the Pn-An PCA Distances provided partial correlation with the results from the selected adult taste panel, especially with relation to the HC formulations. The results from the A0-An PCA Distances provided slightly improved correlation but the results were not fully conclusive. Uncertainty was cast over what conclusions can be drawn from both sets of ASTREE results as only certain aspects correlated with the selected adult taste panel without any logical scientific rational. The adult taste panel showed that if HC or Qu was complexed with a HP-β-CD or Me-β-CD, its bitter taste was masked and that the taste masking by the CDs was better than commonly used bitter-taste masking agents, such as sweeteners and flavouring agents, especially when in excess. Unfortunately the ASTREE data did not correlate consistently with the sensory panel results in this respect. Thus, from a formulator’s perspective the correlation between the selected adult taste panel and the ASTREE was insufficient. Confidence cannot be achieved in the conclusions produced from the ASTREE without the help of a human taste panel. This, therefore, limits the advantages of the ASTREE if there is no rational or scientific means of determining which conclusions will or will not correlate with an adult taste panel, let alone a paediatric taste panel. Regarding the experimental process of both taste assessment techniques, the ASTREE measurement was found to be much quicker and easier than the selected adult taste panel but the data produced less reliable.
Chapter 7 - General Discussion and Future Work
Chapter 7 - General Discussion and Future Work

7.1 Introduction

During the research and development of new drug products, clinical trials are conducted to ensure patients receive medicines with sufficient safety, efficacy, and quality. Drug products are, however, developed typically for adult patients and clinical studies are carried out on adult populations. The paediatric population is, therefore, neglected - paediatric patients are administered formulations that are not age-appropriate or developed for their needs, as well as lacking in evidence based studies in areas such as bioavailability. Recent EU legislation has been implemented to address the issues of medicinal products for paediatric use and certify that new drugs, when indicated, are tested suitably in children and supplied in an age-appropriate medicine. The challenges, however, surrounding the development of paediatric medicine is vast. An ideal paediatric formulation requires many attributes. These include easy and safe administration; minimal dosage and frequency; adequate bioavailability; palatability; and minimal and safe excipients. Thus far, there has been a lack of published research, detailing the use, safety and acceptability of different types of formulations in paediatric patients.

Oral liquid solutions are considered a good avenue for formulation development. They provide acceptable age-appropriate drug administration for all paediatric patients and the dose can be adjusted easily by volume adaptation. Nevertheless, to maintain adherence, oral liquid solutions should ideally ensure dose uniformity; a low dose volume (≤ 5 mL for children under 5 years and ≤ 10 mL for children of 5 years or older); be stable physically, chemically, and microbiologically, and have good palatability. To obtain these characteristics oral liquid formulations may require excipients, such as solubilising agents, preservatives or sweetener/flavouring agents, which can be harmful to some paediatric patients.

Cyclodextrins (CDs) are considered nontoxic excipients used in drug products for their ability to form inclusion complexes with drug molecules. In the previous chapters the potential of CDs as solubility enhancers and bitterness taste masking agents for poorly soluble bitter drugs in oral liquid paediatric formulations was investigated. The focus of research had a particular application to corticosteroids, whilst quinine was used as a model bitter drug, for comparison purposes, where necessary. These investigations were conducted in attempt to provide data on the viability of CDs as excipients in paediatric liquid formulations, as well as to gain an understanding on the mechanism of CD taste...
Chapter 7 - General Discussion and Future Work

masking, to build or refute a rationale towards using CDs in future paediatric drug development.

7.2 Methodology considerations

7.2.1 Complex characterisation techniques

The phase solubility experiments conducted in Chapter 2 were the simplest and most common method for determination of the inclusion complex characteristics between CD and drug, and were considered an effective approach for the initial experiments of this research. Phase solubility experiments also allowed for the influence of other excipients and aqueous media on inclusion complexation to be investigated, as well as determination of the minimum concentration of CD required to achieve a certain drug concentration. Nevertheless, the phase solubility studies had limitations. For instance, inclusion complex formation was not specifically determined and the results of the experiment were interpreted based on the assumption that inclusion complexes were formed. Furthermore, phase solubility studies were unable to elude the mechanisms of inclusion complex formation and the processes that drove complexation. These attributes of inclusion complexation were determined by the ITC and $^1$H NMR spectroscopy studies carried out in Chapter 2 and enabled continued characterisation of inclusion complexation.

The thermodynamic parameters determined from the ITC experiments enabled elucidation of the possible mechanisms of the molecular interaction between the CD and corticosteroid. As such it was found that the inclusion complex formation between HP-β-CD and Me-β-CD with HC was a spontaneous process. The release of water molecules from the CD cavity was found to be the likely driving force for inclusion complexation. These results also determined that van der Waal’s forces were likely to be involved in complexation. In addition, both classical and nonclassical hydrophobic interactions were found to be involved in complexation. From a formulators view point the ITC provided confirmation of the results obtained from the phase solubility studies. Combining both sets of data would help select a lead CD for the solubilisation and taste masking of corticosteroids in an oral liquid paediatric formulation.

The $^1$H NMR spectroscopy results confirmed that HC entered the cavity of the molecules and, therefore, indicated that corticosteroids are likely to form inclusion complexes with CDs. These results provided assurance that inclusion complexes were being formed during all the other experiments and not any other form of interaction.
Chapter 7 - General Discussion and Future Work

The results also found that the HC was likely to have entered the HP-β-CD cavity from the more accessible wider side of the cavity, where the secondary hydroxyl groups are located. The $^1$H NMR spectroscopy experiments were unable to provide sufficient data to determine the exact configuration of the inclusion complex nor sufficient data to relate to excess CD being able to perform bitterness taste masking. To enable these queries to be determined 2D ROESY spectroscopy should be performed. With enhanced NMR data, computer generated molecular modeling could perhaps be performed to provide a visual representation of CD/drug inclusion complex configuration. This would help with various queries, such as whether a portion (and if so which portion), of the drug was exposed from the CD cavity and potentially be exposed to the taste receptors of the tongue.

7.2.2 Efficacy of antimicrobial preservation

Determining whether a preservative has sufficient antimicrobial efficacy within a formulation is an important exercise. In Chapter 3 the British Pharmacopoeia test for efficacy of antimicrobial preservation was carried out successfully and determined that the concentrations of preservative used in HC formulations containing the necessary concentrations of HP-β-CD to ascertain a clinically relevant dose volume of drug failed to meet the necessary requirement for sufficient antimicrobial efficacy. From a formulator’s perspective, however, the efficacy of antimicrobial preservation test was laborious, costly and required a great deal of logistical planning. Alternative screening methods for microbial preservation, such as the use of reverse transcriptase-mediated polymerase chain reaction to measure the number of viable microorganisms, could enable an easier determination of the antimicrobial efficacy of a preservative within a formulation (Long et al., 2006).

7.2.3 Taste assessment in vitro

An in vitro taste analyser (electronic tongue), which correlates fully with human taste, would be an advantageous tool during formulation taste assessment. For instance, taste assessment could be conducted on new chemical entities/drugs yet to have their safety/toxicological profiles assessed fully; screening of taste masking agents with an electronic tongue would greatly reduce the number of formulations to be tested by a human taste panel; quality assurance could also be assessed during any stage of formulation manufacturing. For paediatric drug development, electronic tongue studies
could be implemented during the paediatric investigation plan to provide taste data that could be used to provide evidence of taste masking and palatability of a formulation.

The preliminary ASTREE taste assessment conducted in Chapter 4 screened various concentrations of taste masking agents to help reduce the number of formulations to be assessed by a human taste panel. The number of samples (> 100), tested during these preliminary experiments would have taken an unrealistic amount of time to be tested by a human taste panel. The results indicated that the ASTREE provided fast sample throughput, good intra-measurement repeatability, good sensitivity, and a decent capability to discriminate samples. Furthermore, from the results, optimal concentrations of various taste masking agent were determined. Nevertheless, during the preliminary analysis, the instrument was found to have some limitations that a formulator/analyst should be made aware of. These include:

- **Interfering drug** - When analysing very similar drug molecules (Pd and HC), the sensor response was greatly affected. The instrument was able to analyse HC and not Pd for any rational reason. Thus, taste assessment of Pd formulations could not be conducted with the ASTREE. It could be considered, therefore, that the subsequent ASTREE experiments were biased because the drugs used during the remaining taste assessment were the ones that the instrument was capable of analysing successfully like in most of the published literature using the ASTREE.

- **Delayed analysis** - The duration of the "start-up" procedure varied greatly; frequently the chemical calibration was extremely long and resulted in delayed analysis.

- **Lack of robustness/ruggedness** - The results were 'analysis sequence dependant'; alterations in the analysis sequence affected the outcome of the results, even though the sequences analysed the same samples. Variations in sensors cleaning were also able to affect the outcome of the results.

- **Variable data interpretation** - The results were 'data analysis dependant'; the results of the analysis were not the same if the PCA was interpreted via Pn-An or A0-An PCA Distances.

To enable *in vitro* taste analysers to be implemented fully into formulation assessment, there must first be assurance that the analytical instrument correlates with human taste. In Chapter 6 the correlation between the ASTREE and a human taste panel was
investigated. The results showed that the ASTREE correlated partially with the results of the human taste panel from Chapter 5. These results are encouraging; an analytical instrument that can provide high-throughput sensory evaluation would be a major achievement. Nevertheless, because the correlation was only partial, the ASTREE was unable to provide accurate taste assessment for the formulations tested.

Aside from the ASTREE, other electronic tongues have been developed with a varying numbers of sensors and reference electrodes, differing sensor technologies, alternative cleaning methods, longer measurement times and more taste-specific sensors. These systems have demonstrated varying degrees of correlation, yet no particular electronic tongue has demonstrated full correlation with a human taste panel. This raises the question as to whether an analytical instrument could ever be optimised to provide taste assessment as per a human (young or old), which took thousands of years to evolve.

7.2.4 Taste assessment in vivo

Human taste panels remain the preferred method of formulation taste assessment. In Chapter 5 an adult human taste panel was conducted to provide taste assessment of various formulations. The taste panel was conducted successfully and enabled taste masking evaluations to be made for the various taste masking agents. A concern is raised, however, as to whether the adult taste assessment could be extrapolated to paediatric taste. The published literature is inconclusive, whilst the EMEA recommends that paediatric taste panels should be used for taste assessment of paediatric formulations as these are the ultimate target population for most of the taste masked drugs or formulations. In addition, the use of adult taste panels may have led to poorly palatable formulations for paediatric patients in the past. In light of this, it is apparent that further taste assessment with a paediatric taste panel should be conducted to confirm that CDs provide acceptable bitterness taste masking in paediatric patients. Nevertheless, paediatric taste panels are limited compared to adult taste panels, e.g. fewer formulations can be tested, major ethical/safety issues, restricted measurement scale, and limited ability to understand and follow guidance. Moreover, it is uncertain whether taste assessment should be conducted with healthy or disease state paediatric assessors. An alternative means of taste assessment that correlates to paediatric taste may be a more viable option. For instance, a professionally trained adult panel, similar to the expert tasters used mainly in the wine, coffee, beer, and tea industries that correlates fully with paediatric taste could possibly be developed.
Chapter 7 - General Discussion and Future Work

7.3 Formulation considerations

7.3.1 Drug solubility

There are various types of CD, including natural and synthetic derivatives, having the potential to solubilise a diverse range of guest molecule via inclusion complexation. The ability of natural CDs and CD derivatives to solubilise poorly soluble corticosteroids in an aqueous environment was investigated in Chapter 2. This was achieved by validated phase solubility studies. To achieve microbial preservation, preservatives are also added to an oral liquid formulation. A formulation will be exposed to different pH and biological media after ingestion. Following from the initial phase solubility studies, the influence of the preservative, pH, and simulated media in vivo, on the ability of CDs to solubilise corticosteroids was investigated.

Chapter 2 demonstrated that the solubility of Pd and HC was greatly enhanced by the likely formation of 1:1 water-soluble inclusion complexes, ensuring dose uniformity. The CD derivatives (HP-β-CD and Me-β-CD), were found to provide the greatest drug solubility, across the CD concentrations used, compared to the natural CDs (β-CD and γ-CD). Furthermore, Me-β-CD was found to have the strongest affinity to the corticosteroids with larger association constants, complexation efficiencies, and higher percentage complexation compared to the other CDs. Despite Me-β-CD being the best solubilising agent in this study, other CDs exist that may have a greater affinity to corticosteroids than Me-β-CD. It would, therefore, be of interest to examine whether other CD, particularly other β-CD derivatives such as sulphobutyl ether β-CD or heptakis-di-O-methyl-β-CD, could provide further solubility enhancement of corticosteroids because using less CD would expose the patient to less excipient.

Inclusion complex characteristics were found to vary between the two corticosteroids, indicating that slight differences in drug structure played an important role in complexation and implied that not all corticosteroids will form CD inclusion complexes with the same affinity. This is an important finding considering that other corticosteroids are even more different. Some contain nitrogen or fluorine (e.g. triamcinolone and deflazacort), and are administered orally to paediatric patients, instead of Pd and HC. A formulator should, therefore, be aware that corticosteroids can have significantly differing binding affinities, despite being analogues. Thus, the taste masking approach with CDs might not be applicable across the range of corticosteroids.
Chapter 7 - General Discussion and Future Work

It is essential for oral liquid paediatric solutions to be formulated with drug dose volumes that are satisfactory for paediatric patients. The phase solubility studies in Chapter 2 showed that the CDs were capable of achieving solubilised drug concentrations that would enable a wide range of drug dose volumes. For instance, a 5 mg/mL Pd or 2 mg/mL HC solution provides a clinically relevant paediatric dose volume. This was achieved with a minimum concentration of 6.0 % w/v HP-β-CD and 2.8 % w/v Me-β-CD for the Pd solutions and 1.1, 0.5 and 1.2 % w/v of HP-β-CD, Me-β-CD and γ-CD, respectively, for the HC solutions. At these concentrations the corticosteroids would be completely solubilised in the formulation media, allowing for accurate and reproducible administration of drug at a low dose volume. Nonetheless, oral liquid formulations may contain other excipients to facilitate the drug delivery, such as flavouring agents, sweeteners, colouring agent, preservatives, antioxidants, pH modifiers, and buffering agents. These excipients may influence inclusion complex formation by competing with the drug for the CD cavity. The phase solubility studies demonstrated that the CDs formed inclusion complexes with preservative, thus altering drug solubility enhancement and the concentration of CD required to achieve a particular drug concentration. In this instance, however, CDs were still capable of achieving satisfactory drug dose volumes, although more CD was required compared to when no preservative was present. These results showed that the incorporation of other excipients into a formulation should be considered as early as possible in formulation development. Experiments, such as phase solubility studies, can be conducted during early development to ascertain whether the CD can still provide adequate drug solubility enhancement and drug dose volumes when in the presence of competing guest molecules.

Oral liquid formulations are exposed to a range of biological fluids (e.g. saliva, stomach acid, intestinal fluid), with various pH after administration. This may affect the inclusion complexes and consequently the drug bioavailability upon ingestion, especially if the drug or CD molecules are ionisable, or if compounds within the biological media compete with the drug for the CD cavity. The phase solubility studies in Chapter 2 showed that pH and simulated media in vivo had no major detriment on inclusion complexation, indicating that the pH or compounds within the simulated media in vivo would not influence the solubility of the tested corticosteroids. Furthermore, these results showed that inclusion complex formation was likely to be stable in the mouth, especially since liquid formulations have a fast transit time in the
Chapter 7 - General Discussion and Future Work

oral cavity. These results, however, may not relate to other compounds that are ionisable, such as sulphobutyl ether β-CD. Furthermore, the use of more realistic simulated media containing the same components as actual biological media would be preferable. Genuine biological media contains components such as enzymes, glycoproteins and food that were not present in the simulated media in vivo.

7.3.2 Preservative efficacy

Microbial preservation is an important attribute of oral liquid formulations. The phase solubility studies demonstrated that parabens competed with corticosteroids for the cavity of CDs, reducing the uncomplexed parabens concentration in solution and, therefore, potentially influencing the parabens antimicrobial efficacy. In Chapter 3 the antimicrobial efficacy of parabens in corticosteroid formulations, containing CD as solubilising agent, was investigated. The British Pharmacopoeia test for efficacy of antimicrobial preservation found that CDs reduced the antimicrobial efficacy of parabens at the concentration tested (0.05 to 0.20 % w/v). These results indicated that a concentration of parabens greater then 0.20 % w/v would be required to achieve microbial preservation, although microbial preservation would not be guaranteed unless the unbound parabens concentration was sufficient. To overcome this, perhaps an alternative, more-soluble preservative could be used with an acceptable toxicological/safety profile. A more soluble preservative may not complex with the CDs molecules in a formulation as much as a preservative with low aqueous solubility, thus enabling a formulation containing CDs to be adequately preserved.

7.3.3 Taste masking

The taste of an oral formulation is an important factor concerning patient adherence. Obtaining a non-bitter taste is considered one of the hardest challenges in developing an oral liquid formulation. To obtain a non-bitter oral liquid solution various methods of taste masking have been used. The typical method to taste mask the bitterness of drugs within a formulation is to add sweeteners and/or flavouring agents. Some sweeteners and/or flavouring agents are, however, required to be in high concentration, especially when the bitter drug is particularly soluble. Moreover, various sweeteners and/or flavouring agents are not appropriate because they can be unsafe to certain paediatric populations. In Chapter 5 it was found by an adult human taste panel that CDs were able to provide bitterness taste masking. The CDs also had greater taste masking efficiencies compared to the sweeteners and/or flavouring agents and still maintained a
Chapter 7 - General Discussion and Future Work

satisfactory paediatric dose volume. These results indicated that they could be a viable option, instead of sweeteners and/or flavouring agents, for bitterness taste making in oral liquid paediatric formulations.

CDs provide bitterness taste masking predominantly through taste obstruction - bitter drug molecules are encapsulated by the CDs where they are consequently unable to interact with the taste receptors of the tongue. Nevertheless, there are only few published studies where CDs are used to provide bitterness taste masking in oral liquid formulations with the majority of information provided from patent applications, where details are limited and involving mainly solid dosage forms. Of the studies that detail the bitterness taste masking of CDs, an excess concentration/quantity of CD is used typically. For an oral liquid formulation it is believed that this will enable a high percentage of drug (> 90 %), to be in CD complexed form. Excess CD may, however, be unnecessary since a low percentage of drug (< 10 %), can exist in solution uncomplexed, which can still interact with the receptors of the tongue and evoke a bitter taste. Furthermore, not all drugs are complexed fully by CDs, with a small portion of the drug still potentially exposed to the taste receptors of the tongue. This also raises the question of how much excess will provide bitterness taste masking and how much excess is considered too much? If a 1:5 molar ratio of drug to CD provides optimum bitterness taste masking then adding 1:10 would not be needed. The results from the adult human taste panel in Chapter 5 demonstrated that the HC and Qu were fully taste masked by excess CD and that excess CD was more efficient at taste masking bitter drugs than moderate concentrations of CD. Although these results showed excess CD enabled total bitterness taste masking, the concentration of CD could still be optimised to determine whether less excess CD can still provide the same degree of bitterness taste masking.

Despite the fact that the results of the human taste panel found CDs to be more efficient taste masking agents than sweeteners and/or flavouring agents, the overall palatability of the formulation to a paediatric patient may not greatly improved over traditional taste masking agents. It was discussed in Chapter 5 that perhaps the addition of sweeteners and/or flavouring agents to a formulation containing CDs may improve the overall taste of the formulation to make it more palatable to a paediatric patient. If the bitterness can be reduced by CDs and a pleasant flavour is provided by a flavouring agent then perhaps patient compliance would be improved. It is important to bear in mind, however, that CDs are capable of complexing other excipients within a formulation, as
shown in Chapters 2 and 3, and that flavour is not elicited by just one compound. A particular flavour may be the result of a wide range of compounds; the complexation of flavouring agents with CDs would need to be investigated. This leads to the proposal that perhaps CDs may be able to provide a pleasant taste via its sweetness, as well as bitterness taste masking via its cavity, thus reducing the need for other excipients to provide a pleasurable taste. A 2.5 % w/v β-CD solution was found to be as sweet as a 1.71 % sucrose solution, demonstrating that CDs can provide a slightly sweet taste. This level of sweetness is not expected to provide an overwhelmingly sweet taste. Nevertheless, other CDs may have a sweet taste. For instance, there are glucosyl and maltosyl branched CD derivatives for all natural CDs, which may provide a sweeter taste to the parent CD.

7.3.4 Industrial application

It was beyond the scope of this thesis but certain industrial applications of CDs should be mentioned when considering CDs for a paediatric formulation. Production volumes and cost were major issues when CDs were first implemented in pharmaceutical products. Advances in manufacturing technology and the reduction in price of raw materials has led to CDs being produced in bulk and at an affordable price (£1.5 per gram for β-CD). Furthermore, because CDs are multifunctional, providing solubility enhancement and bitterness taste masking, less excipients would be required, which would be more cost effective. Moreover, because CDs are a multifunctional excipient less processing steps would be required to produce the formulation, saving further time and money. Manufacturing scale-up is not considered to be a problem when using CDs, because process extrapolation from laboratory to large scale should not change dramatically, although some CDs are hygroscopic and may be more difficult to handle when in large amounts.

7.3.5 Drug dissociation

The experiments conducted in this Chapters 2 and 5 demonstrated that CDs were able to solubilise and taste mask corticosteroids via inclusion complex formation. Aside from these attributes, it is important to consider the dissociation of the drug from the CD so that sufficient drug absorption can be achieved. The main location of drug dissociation from CDs from oral liquid formulations is in the GI tract. Because oral solutions transit very fast in the mouth, the dissociation of drug in the oral cavity (and saliva) is minimal. When located in the GI tract, poorly soluble drugs dissociate from
Chapter 7 - General Discussion and Future Work

the CDs and are absorbed into the systemic system, typically because the drugs have greater affinity for the lipid membranes of the GI tract than the cavity of the CD. Competitive displacement by endogenous materials, complex dilution by biological media, rapid elimination of the cyclodextrin and possibly pH and temperature effects, may also affect drug dissociation after ingestion. Furthermore, just because CDs are able to solubilise and taste mask poorly soluble drugs it does not mean that dissociation and absorption is guaranteed. If the molar excess of CD is too great then the equilibrium could be driven towards complexation and the amount of free drug available for absorption in the GI tract might be too small to provide a therapeutic effect. Similarly, if the association constant is too great then the drug may not dissociate from the complex appreciably. In light of this, the CD concentrations and association constants determined from this research may not be applicable for adequate bioavailability and a desired therapeutic effect. It would, therefore, be of interest to investigate whether the excess concentrations of CD used in the taste masking assessment affected bioavailability of the drug by conducting a bioequivalence study in vivo using formulations with moderate concentrations of CD for comparison.

7.3.6 Safety

Despite the results from this research demonstrating that CDs were capable of achieving age-appropriate dose volumes and providing bitterness taste masking for poorly soluble bitter drugs, a major impeding aspect as to whether CDs are able to be used as an excipient in oral liquid paediatric formulations is the limited evidence-based data regarding its safety, especially the paediatric population. CDs are generally considered to be safe when administered orally, whilst concerns are raised regarding the safety of some CDs when administered intravenously. As discussed in Chapter 5, the World Health Organisation has appointed a 5 mg/kg/day ADI for oral consumption of β-CD, whilst no other oral ADI has been established for the remaining CDs. Although CDs are used in marketed pharmaceutical formulations it is unknown whether the concentrations used are safe for daily repetitive paediatric consumption. It is, therefore, vital to establish CD safety profiles and ADIs in children so that the potential of these excipients can be exploited in paediatric formulations.
Appendix 1

Pn-An and A0-An PCA Distances for hydrocortisone formulations

Figure 1: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with acesulpham K. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Appendix 1

Figure 2: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg mL hydrocortisone with sucrose. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 3: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with erythritol. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 4: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with HP-β-CD. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 5: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 2.0 mg/mL hydrocortisone with HP-β-CD. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 6: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with Me-β-CD. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 7: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 2.0 mg/mL hydrocortisone with Me-β-CD. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 8: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with cherry. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 9: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with strawberry. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 10: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with tangerine. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 11: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with cherry + sweetener. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
**Figure 12:** Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with strawberry + sweetener. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 13: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.20 mg/mL hydrocortisone with tangerine + sweeteners. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Appendix 1

**Pn-An and A0-An PCA Distances for quinine formulations**

Figure 14: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with acesulpham K. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 15: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with sucrose. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 16: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with erythritol. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 17: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with HP-β-CD. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Appendix 1

Figure 18: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 1.60 mg/mL quinine with HP-β-CD. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 19: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with Me-β-CD. Analysis carried out via sequence 1 (S1) and sequence 2 (S2).
Appendix 1

Figure 20: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 1.60 mg/mL quinine with Me-β-CD. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 21: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with cherry. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 22: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with strawberry. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 23: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with tangerine.
Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 24: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with cherry + sweetener. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 25: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with strawberry + sweetener. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Figure 26: Pn-An and A0-An PCA Distances calculated during ASTREE electronic tongue preliminary taste masking analysis of 0.16 mg/mL quinine with tangerine + sweeteners. Analysis carried out via sequence 1 (S1) and sequence 2 (S2)
Informed Consent Form for Participants in Research Studies
(This form is to be completed independently by the participant after reading the Information Sheet and/or having listened to an explanation about the research.)

Title of Project: Bitterness-masking Evaluation of Pharmaceutical Excipients
This study has been approved by the UCL Research Ethics Committee [Project ID Number]: 0851/001

Participant's Statement
I .................................................................
agree that I have
• Read the information sheet and/or the project has been explained to me orally;
• Had the opportunity to ask questions and discuss the study;
•Received satisfactory answers to all my questions about the research and my rights as a participant and whom to contact in the event of a research-related injury.
• Understood that I must not take part if I have antecedent of deterioration of the taste or of the sense of smell, if I had recent dental care, if I take a regular medicine treatment (except contraceptives) or took a treatment during 15 days before the tests
• Understood that I am being paid for my assistance in this research and that some of my personal details will be passed to The School of Pharmacy Finance for administration purposes.
• Understood that the data produced will be published but that confidentiality and anonymity will be maintained and it will not be possible to identify me from any publications.

I understand that I am free to withdraw from the study without penalty if I so wish and I consent to the processing of my personal information for the purposes of this study only and that it will not be used for any other purpose. I understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.

Signed: Date:

Investigator's Statement
I .................................................................
confirm that I have carefully explained the purpose of the study to the participant and outlined any reasonably foreseeable risks or benefits (where applicable).

Signed: Date:
Do you want to be part of a Human Taste Panel?

We are carrying out research to evaluate the use of cyclodextrins as potential bitterness-masking excipients. We are looking for healthy volunteers to help in a clinical trial as part of a human taste panel.

*If you are a healthy adult 18 years old or older and not on medication, it could be you we are looking for.*

The study will involve tasting a range of liquid formulations and evaluating their bitterness over a maximum 3 day period, commencing as soon as possible.

*If you are a smoker you must be prepared to cease smoking for the duration of the tasting. You will be compensated for participating in this study.*

If you would like to participate and help us with our study please email roy.turner@pharmacy.ac.uk or catherine.tuleu@pharmacy.ac.uk, Department of Pharmaceutics, The School of Pharmacy, University of London to receive a participant information sheet.

Studies have been approved by an ethics committee.
Appendix 2

Information Sheet for Participants in Human Taste Panel

You will be given a copy of this information sheet.

Title of Project: Bitterness-Masking Evaluation of Pharmaceutical Excipients

This study has been approved by the UCL Research Ethics Committee [Project ID Number]: 0851/001

Name, Address and Contact Details of Investigators:

Dr Catherine TULEU and Roy Turner
Department of Pharmaceutics
The School of Pharmacy, University of London
29/39 Brunswick square, London WC1N 1AX
Tel: 02077535857 Fax: 02077535942
catherine.tuleu@pharmacy.ac.uk & roy.turner@pharmacy.ac.uk

We would like to invite you to participate in this research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or you would like more information.

Details of Study

Our project has two main research objectives:

The first is to assess the use of cyclodextrins as innovative taste masking agents to improve palatability of medicines and compare their efficiency to commonly used bitter taste-masking agents. Cyclodextrin, a sugar derivative compound, forms a cavity where drugs can hide; it will be compared with classical taste masking agents such as sweeteners (sucrose, erythritol, acesulpham K), flavouring agents (strawberry, cherry, Tangerine) and a combination of both.

The second objective of our study is to find out if the data you will provide correlates with the data obtained from an electronic tongue (ASTREE, Alpha MOS). If so, the electronic tongue could replace the need for extensive human taste panels and avoid in the future exposing volunteers to unnecessary levels of chemicals during medicines development.

You role in the study will be to taste but not to swallow various formulations and to rank them on a non-structured scale of bitterness. The model drugs of bitterness you will be in contact with are well known (hydrocortisone, quinine).

During the 1st day there is a selection phase that will last a maximum of 3 hours. You will taste 6 formulations of known and increasing bitterness intensity, 3 times each.

If you are among the top 12 participants to discriminate and rank bitterness, you will take part in 2 further research days (day 2 and day 3) composed of a maximum of 3 hours in the morning and 3 hours in the afternoon, where you will taste a water sample and 16 formulations of hydrocortisone on day 2 and quinine on day 3.

If the solutions you taste are actually bitter there is a potential to suffer from temporary oral discomfort. For sensitive participants this may evoke a gag reflex and induce vomiting (although this is an extreme). There is a potential for buccal absorption or accidental swallowing of the formulation tasted. Nevertheless, the time of rinsing has been minimised to 15 seconds, which minimizes the potential for adverse effects, risks or hazards. A delay of 10 minutes will be respected between each tested solution and subjects will have to rinse their mouth with mineral water before and after each test. Moreover major side effects of the well established drugs we plan to use usually occur if overdosed or if their use is prolonged and this is highly unlikely to occur as the cumulative daily dose will be low.

We will make sure that you know the outcomes of the study. If the study is published or presented to a wider audience, your anonymity will be respected through anonymisation procedures. All data will be collected and stored in accordance with the Data Protection Act 1998.

You will receive compensation for your participation:

Day 1 - max 4 hours - £15
Day 2 - max 6 hours - £50
Day 3 - max 6 hours - £50

No further payment such as travel expenses, child-care expenses, demonstrable loss of earnings etc will be reimbursed.

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason and without any penalty at any stage.
Appendix 2

**Bitterness-Masking Evaluation of Pharmaceutical Excipients**

**Health Questionnaire and Demographic Information**

**CONFIDENTIAL**

**DEMOGRAPHIC DATA**

Date of birth (dd/mm/yy): ____________________________

Ethnic Group: ____________________________

Height (cm): ____________________________

Weight (kg): ____________________________

**HEALTH DATA**

Please complete the following health check as accurately as possible. It will only be used for purposes of ensuring that it is appropriate for you to participate in the study.

Have you experienced or still experience problems from the following conditions:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Delete as appropriate</th>
<th>If yes, please give date and state if still a problem/ please give details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart condition</td>
<td>Yes/No</td>
<td></td>
</tr>
<tr>
<td>Respiratory disease</td>
<td>Yes/No</td>
<td></td>
</tr>
<tr>
<td>Allergies (inc asthma)</td>
<td>Yes/No</td>
<td></td>
</tr>
<tr>
<td>Skin condition</td>
<td>Yes/No</td>
<td></td>
</tr>
<tr>
<td>Kidney disease</td>
<td>Yes/No</td>
<td></td>
</tr>
<tr>
<td>Liver disease</td>
<td>Yes/No</td>
<td></td>
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<tr>
<td>Gastrointestinal disease</td>
<td>Yes/No</td>
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<tr>
<td>Diabetes</td>
<td>Yes/No</td>
<td></td>
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<tr>
<td>Eye disease</td>
<td>Yes/No</td>
<td></td>
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<tr>
<td>Ear trouble</td>
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<tr>
<td>Any other illness</td>
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<tr>
<td>Have you ever been admitted to hospital?</td>
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<tr>
<td>Do you have any disability or handicap?</td>
<td>Yes/No</td>
<td></td>
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<tr>
<td>Do you take any medicines or medicinal supplements?</td>
<td>Yes/No</td>
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<tr>
<td>Do you smoke?</td>
<td>Yes/No</td>
<td>per day</td>
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<td>Do you drink alcohol?</td>
<td>Yes/No</td>
<td>units per week</td>
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<tr>
<td>Have you had any recent dental work?</td>
<td>Yes/No</td>
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</table>

Volunteers signature ____________________________ Date ____________________________

Investigators signature ____________________________ Date ____________________________
### Appendix 3

#### Table 1: Bitterness intensity for hydrocortisone standards assessed during the taste assessor selection

<table>
<thead>
<tr>
<th>Taste assessor</th>
<th>0.05 mg/mL hydrocortisone</th>
<th>Bitterness intensity</th>
<th>0.10 mg/mL hydrocortisone</th>
<th>0.20 mg/mL hydrocortisone</th>
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#### Table 2: Bitterness intensity for quinine standards assessed during the taste assessor selection

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<th>Bitterness intensity</th>
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Publications and Communications

Publications and Communications

2007
Poster presentation at the Pharmaceutical Science World Conference (PSWC), Amsterdam, Netherlands. R.M. TURNER, M-A. AKIOYAME, K. KEENE, P. LONG and C. TULEU. *The Effect of Parabens on Cyclodextrin Inclusion Complex Formation and the Subsequent Influence on their Antimicrobial Properties.*

2008

2009
Manuscripts in preparation:

R.M. TURNER & C. TULEU. *Bitterness taste masking assessment of cyclodextrins in oral liquid paediatric formulations using a human taste panel.*

R.M. TURNER & C. TULEU. *Bitterness taste masking assessment of cyclodextrins in oral liquid paediatric formulations using an electronic tongue: In vivo and in vitro comparison.*
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Norinsuisansho, Kyushu, Nogyo, Shikenjyo. Soybean milk manufacturing method for dessert preparation - involves using lipooxygenase deleted soybean as raw material, adding specified amount of cyclodextrin to the soybean milk and increasing amount of milk to specific level by hydrolysis, JP 11332496. 2000.


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